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Rôle de la voie PGD_2/L-PGDS dans la physiopathologie de l'arthrose

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Rôle de la voie PGD_2/L-PGDS dans la physiopathologie de l'arthrose

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RÉSUMÉ

L'arthrose (OA) est la maladie articulaire la plus répandue dans le monde faisant l'objet de nombreux travaux de recherche en raison de son lourd impact socioéconomique. Plusieurs travaux dans ce domaine ont pour objectif de déterminer les mécanismes moléculaires impliqués dans sa physiopathologie. Plusieurs travaux ont appuyés l'implication de la prostaglandine (E_2) PGE_2 dans sa physiopathologie, contrairement à la prostaglandine ($D_{2)}$ (PGD_2) dont le rôle reste à déterminer. C'est pourquoi, nous nous sommes penchés dans cette thèse à l'étude de cette dernière molécule.

Dans la première partie de nos travaux, nous avons montré que la PGD_2 diminue au niveau du cartilage articulaire et au niveau niveau des explants de cartilage humains, la production des métalloprotéases-1(MMP-1) et MMP-13 induites par (Interleukine-1β) l'IL-1β. Cette diminution de la production protéique est accompagnée d'une diminution de l'expression au niveau de l'ARNm, et d'une diminution de l'activité du promoteur de MMP-1 et MMP-13. Cet effet est exercé via le récepteur D prostanoïde (DP1), bien que le *Chemoattractant receptor expressed on Th2 cells* (CRTH2) soit également exprimé chez les chondrocytes humains, mais ne semble pas être impliqué dans l'effet observé. Cette action inhibitrice se fait via la voie DP1/AMPc/protéine kinase A (AMPc/PKA).

Dans la suite de nos travaux, nous avons montré pour la première fois l'expression des prostaglandines D-synthases responsables de la biosynthèse de la PGD_2 au niveau des chondrocytes humains par immunohistochimie, avec des niveaux d'expression de l'ARNm plus élevés de la L-PGDS au niveau du cartilage OA comparativement au cartilage normal. L'IL-1β pourrait être responsable de cette augmentation via l'activation de la voie JNK et p38 MAPK, ainsi que par la voie NF-κB.

L'ensemble de ces données indiquent que la modulation des niveaux de la PGD_2 au niveau de l'articulation pourrait être pourvue d'un important potentiel thérapeutique. La L-PGDS pour sa part semble avoir un rôle important dans la physiopathologie de l'OA.

Mots-clés : Arthrose, cartilage, chondrocytes, catabolisme, IL-1β, MMP1, MMP-13, prostaglandines, PGD_2, H-PGDS, L-PGDS.

ABSTRACT

Osteoarthritis (OA) is the most common joint disease world wide, because of its higher socioeconomic impact it is one of the most studied joint diseases. The aims of these studies was to determine the molecular mechanisms involved in the pathophysiology of osteaarthritis. Previous studies have mainly focused on the involvement of prostaglandin (E2) PGE_2 in contrast to PGD2 in the pathogenesis osteoarthritis as such the role of PGD_2 remains unclear. In this thesis we examined the involvment of PGD_2 in the pathogenesis of OA.

In the first part of our work, we showed that in a dose dependent manner PGD_2 decreased the interleukin-1β (IL-1β)–induced mettalloproteases (MMP-1) and MMP-13 expression both at protein and mRNA levels by supression of their promoter activity. The inhibitory effect was exerted via the D prostanoid receptor (DP1) and mediated through the cAMP/protein kinase A (PKA) signalling pathway. Although human chondrocytes do express the Chemoattractant Receptor Expressed on Th2 cells (CRTH2) the latter were not implicated in the inhibiton of MMP-1 and MMP-13.

In the second part of our work, we showed the expression of prostaglandin D synthases (PGDS) responsible for the biosynthesis of PGD_2 in human chondrocytes, with higher levels of mRNA expression of lipocaline type prostaglandin D-synthase (L-PGDS) in OA cartilage compared to normal cartilage. IL-1β may be responsible for this increase via the activation of Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK), as well as the nuclear factor-κB (NF-κB).

Together, these data indicate that modulation of the levels of PGD_2 at the joint may be provided with an important therapeutic potential. L-PGDS in turn seems to have an important role in the pathogenesis of OA.

Keywords : osteoarthritis, cartilage, chondrocyte, catabolism, IL-1β, MMP-1, MMP-13, prostaglandins, PGD_2, H-PGDS, L-PGDS.

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LISTE DES ABBRÉVIATIONS

AA : Acide arachidonique

ACLT : *Anterior cruciate ligament transection*

ADAMTS : *A desintegrin and metalloproteinase with thrombospondin motif*

AINS : Anti-inflammatoires non stéroidiens

ARNm : Acide ribonucléique messager

COX2 : Cyclooxygénase-2

ColII : Collagène II

CRTH2 : *Chemoattractant Receptor expressed on Th2 cells*

CS : Chondroitine sulphate

DP1 : *D Prostanoid Receptor* 1

DS : Dermatane Sulfate

ERK : *Extracellular signa regulated kinase*

FGF : *Fibroblast growth factor*

GAG : Glycosaminoglycanes

GDF5 : Facteur de différenciation de croissance

GPCR : Récepteur couplé aux protéines G

H-PGDS : Hématopoiétique Prostaglandine D-synthase

IL : Interleukine

IL-1Ra : Antagoniste du récepteur à l'IL-1

iNOS : *Inducible nitric oxide synthase*

JNK : Jun N-terminal kinase

KS : Kératane ulfate

LNO2: *Litrolenic acid* (Acide litrolénique)

L-PGDS: Lipocaline Prostaglandine D-synthase

LT: Leukotriène

LTA_4 : Leukotriène A4

LOX : Lipoxygénases

LXA4: Lipoxine A4

MAPK: *Mitogen-activated protein kinase*

MEC : Matrice ExtraCellulaire

MIA : MonoIodoAcétate

MMP : Métalloprotéase matricielle

mPGES : microsomal prostaglandin E synthase

MT-MMP: *Membrane Type-Matricial MetalloProteinase*

NF-kB : *Nuclear Factor kappa B*

NO : Nitric Oxide (acide nitrique)

OA : Arthrose

OPG : Ostéoprotégrine

PGs : Prostaglandines

PGD_2 : Prostaglandine (D2)

PGI_2 : Prostacycline

PGE_2 : Prostaglandine (E2)

PKC : Protéine kinase C

PLA2 : Phospholipase A2

PLC : Phospholipase C

PPARγ: *Peroxisome proliferator receptor γ*

RA: Arthrite rhumatoide

RANK : *Receptor activator of nuclear factor kappa B*

RANKL: *Receptor activator of nuclear factor kappa B ligand*

RANTES: *Regulated upon Activation, Normal T-cell Expressed, and Secreted*

ROS : *Reactive oxygen species*

TGF-β : *Transforming growth factor-β*

TIMP : *Tissu inhibitor of metalloproteinase*

TNF-α : *Tumor necrosis factor-α*

TXA_2 : Thromboxane

VDR : Recepteur de la vitamine D

VEGF: *Vascular Endothelial Growth Factor*

13-HODE : *13(S)-Hhydroxy octaecadienoic acid*

15-HETE : 15(S)-hydroxyicosatetraenoic

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INTRODUCTION

L'arthrose (OA) est une maladie articulaire qui résulte d'un système complexe d'interactions d'ordre mécanique, biologique, biochimique ou encore moléculaire [1]. La dégénérescence du cartilage articulaire ayant pour origine la destruction de la matrice extracellulaire a lieu malgré les nombreuses tentatives de réparation du chondrocyte, qui ciblent le rétablissement de l'équilibre homéostatique, entre la synthèse et la dégradation des composants matriciels. Malheureusement, ces tentatives s'avèrent infructueuses [2].

Cette dégénérescence est à l'origine de l'apparition de fibrillations, de fissures, et d'ulcérations [3, 4]. Bien que la dégradation du cartilage articulaire soit une caractéristique de l'arthrose, l'inflammation de la membrane synoviale participe aussi significativement à la pathologie de la maladie [5]. Celle-ci peut probablement être la cause primaire ou plutôt un phénomène secondaire lié à de multiples facteurs [6]. Cependant, l'OA n'affecte pas seulement les tissus cités précédemment mais plutôt l'articulation entière, incluant l'os sous chondral, les ligaments, la capsule et les muscles périarticulaires [7-9]. L'atteinte de l'un de ces composants ou des tissus de l'articulation, conduit à l'atteinte de l'organe en entier, en raison de la complémentarité structurale et fonctionnelle entre les différents constituants.

Cependant, malgré les recherches intensives ayant permis une meilleure compréhension de la maladie, il n'existe aucun traitement capable de l'arrêter à ce jour. Les traitements actuels sont des traitements symptomatiques n'affectant en rien sa progression, ils ont pour effet seulement d'atténuer la douleur.

CHAPITRE I: REVUE DE LA LITTÉRATURE

I.A Arthrose et cartilage articulaire

I.A.1 Localisation de l'arthrose:

Comme il a été précisé précédemment, l'OA est une maladie articulaire pouvant affecter n'importe quelle articulation du corps humain. Cependant, certaines articulations comme celles du genou, et de la hanche sont plus vulnérables à la maladie comparativement à d'autres, à savoir celles de l'épaule, du coude et de la cheville qui sont moins souvent atteintes. Cette différence d'incidence, a pour raison la différence de la charge pondérale portée pour chaque type d'articulation. De plus, bien que l'OA du genou soit la forme la plus répandue, elle est encore plus fréquente au niveau de la main et plus particulièrement au niveau des articulations interphalangiennes distales et proximales. La colonne vertébrale à son tour peut également être touchée par l'OA. Cela conduit à une perte totale du mouvement aboutissant à l'incapacité physique [1, 10].

I.A.2 Épidémiologie de l'arthrose:

Des études épidémiologiques visant à décrire ″l'occurrence, la distribution de la maladie chez différentes populations, ainsi que les facteurs de risque à l'origine de son apparition″ ont été réalisées. Cependant, à ces fins l'OA peut être définie d'un point de vue pathologique, radiologique ou encore clinique [11].

Toutefois, différentes études ont montré que la prévalence de l'OA varie selon plusieurs critères, entre autres la définition adaptée, l'articulation en question, ainsi que les caractéristiques de la population étudiée [12].

Une étude publiée par *Nevitt et al*, a rapporté des signes radiographiques de l'OA survenant chez la majorité des personnes de 65 ans, et dans environ 80% chez les personnes âgées de plus de 75 ans [13].

La prévalence de l'OA de la hanche est d'environ 9% chez la population blanche [14]. En revanche, des études réalisées sur d'autres populations indiquent sa très faible

incidence estimée à 4% comparativement à la race caucasienne [15]. Cette faible incidence peut être attribuée aux faibles taux d'anomalies congénitales ou développementales chez ces groupes. L'accroissement de l'incidence de l'OA chez certains groupes culturels est lié à l'adoption de certaines postures, telles que l'accroupissement appliquant une forte pression sur la hanche [16].

Ces données indiquent que la variabilité de la prévalence de l'OA dépend d'un nombre de facteurs, entre autres la définition, l'articulation atteinte, la population, mais aussi de facteurs prédisposant à son apparition: les facteurs de risque [12].

I.A.3 Facteurs de risque

Les facteurs prédisposant à l'OA ont été divisés en deux grands groupes, les facteurs de risque systémiques et les facteurs de risque locaux.

I.A.3.1 Facteurs de risque systémiques

En sus de l'âge, le sexe, les hormones, ainsi que d'autres facteurs prédisposent à l'OA.

I. A.3.1.1 Âge

L'âge, représente le facteur de risque majeur lié à l'apparition de la maladie. Dans la plupart des études, la prévalence de l'OA augmente avec ce dernier [17, 18] quelque soit l'articulation [14, 19, 20]. Ceci peut être expliqué d'une part, par la diminution du nombre de chondrocytes à cause de la mort cellulaire, mais aussi par la diminution de leur capacité de produire et de répondre aux facteurs anaboliques. Le phénotype des chondrocytes est dévié vers un phénotype producteur de médiateurs inflammatoires et cataboliques conduisant à la dégradation plutôt qu'à la réparation du cartilage articulaire. À ces changements cellulaires s'ajoute l'exposition prolongée aux nombreux facteurs de risque [11].

I. A.3.1.2 Sexe et hormones:

Les femmes d'une manière générale sont plus susceptibles à développer l'OA comparativement aux hommes [21]. Les femmes américaines plus précisément sont les plus atteintes comparativement aux femmes indiennes et chinoises [22, 23], l'atteinte augmente chez elles après la ménopause.

Dans l'objectif de déterminer la relation existant entre l'apparition de l'OA et les changements hormonaux plusieurs investigations ont eu lieu [11].

Cependant, des études ont montré que la substitution hormonale est associée à une diminution de la prévalence de l'OA du genou et de la hanche, mais sans aucun effet protecteur notable [24-26]. Toutefois, l'administration des œstrogènes diminue le risque d'atteinte de l'OA de la hanche de 50% et de 30% les manifestations sévères [24]. Ces résultats ont été confirmés par une autre étude réalisée par *Nevitt et al*, qui avait montré que les femmes ayant subi un traitement aux œstrogènes présenteraient une réduction de 15% dans le nombre de prothèses de remplacement de la hanche ou du genou comparativement aux femmes n'ayant reçu aucun traitement [26], et donc le rôle des oestrogènes dans l'OA reste encore controversé.

I. A.3.1.3 Facteurs géographiques et prédisposition génétique:

La prévalence de l'OA et l'articulation affectée varient d'une population à l'autre. Toutefois, l'OA de la hanche et de la main sont moins fréquentes chez la population chinoise comparativement à d'autres populations. Bien au contraire, l'OA du genou est plus fréquente chez cette population. Des différences raciales sont à l'origine de cette variation, notamment des différences anatomiques [27-30]. En plus des différences géo-éthniques qui peuvent exister d'une population à l'autre, plusieurs évidences révèlent l'importance de facteurs génétiques dans l'OA [24]. À titre d'exemple, une partie du facteur de différenciation de croissance 5 (GDF5) est associée à la susceptibilité de l'OA chez certaines populations comme la population chinoise [31, 32]. Il existe toutefois une association entre le polymorphisme de ce gène et l'apparition de la maladie à travers le monde. Tout comme le GDF5, les gènes de l'*Asporin* [33, 34], le *cartilage oligomeric*

matrix protein COMP [35, 36], le gène de *COL2A1* et le récepteur à la vitamine D (VDR) sont également liés à l'OA. Le gène *COL2A1* est associé au pincement de l'interligne articulaire, tandis que le polymorphisme du gène *VDR* est associé au développement d'ostéophytes [37]. Un effet combiné d'un faible apport en calcium et des polymorphismes du VDR a été observé dans le cas de l'OA symétrique [38].

I. A.3.1.4 Régime alimentaire:

Une relation étroite pourrait exister entre l'alimentation et l'incidence ou encore la progression de l'OA. Une étude réalisée par *Wluka et al*, [39] avait montré que la progression de l'OA symptomatique et radiologique du genou était associée à un faible apport en vitamine C. Toutefois, une relation inverse existerait entre l'apport en vitamine E et la progression de l'OA. De plus, un essai clinique a montré qu'un apport contrôlé en vitamine E, n'a pas eu d'effet sur la progression de l'OA du genou tel que mesuré par le volume du cartilage à l'imagerie par raisonance magnétique (IRM). De plus, une autre étude réalisée par *Neogi et al*, a montré que des niveaux élevés en vitamine K étaient associés à une faible prévalence de l'OA radiologique de la main [40].

I.A.3.2 Facteurs de risque locaux

I.A.3.2.1 Obésité et activité professionnelle:

L'obésité est considérée comme un facteur de risque [5], plus particulièrement à l'OA du genou [19], sa relation avec l'OA de la hanche n'est toujours pas évidente, mais si cette dernière existait son impact serait sans doute inférieur à celui de l'OA du genou [41, 42]. L'augmentation de la charge sur l'articulation en raison d'une forte masse corporelle est probablement le mécanisme principal par lequel l'obésité est à l'origine de l'OA du genou: par l'induction de la dégradation du cartilage articulaire et par l'atteinte des structures ligamentaires et des autres composantes de l'articulation [11]. Toutefois, la charge pondérale ne semble pas être le seul mécanisme responsable de l'apparition de l'OA chez

les personnes obèses dans la mesure où une augmentation de l'OA de la main est notée également chez ces personnes. Chez ces personnes, le tissu adipeux blanc est responsable de la production de nombreux médiateurs inflammatoires dont l'IL-1β, l'IL-6, TNF-α. S'ajoute à ces médiateurs, les adipokines connus pour leurs effets pléiotropiques et qui sont impliqués dans un large éventail d'activités, notamment dans le métabolisme des lipides et la modulation de l'inflammation [43, 44], parmi ces adipokines on retrouve la leptine.

La leptine est une hormone ayant un rôle anabolique au niveau du cartilage. Paradoxalement, il a été démontré dans les travaux réalisés par *Toussirot et al*, [45] que cette hormone possède un rôle dans la physiopathologie de l'OA, entre autres par l'induction de la production des MMPs, dont la MMP-9 et la MMP- 13 [45]. Cependant, elle peut agir en synergie avec l'IL-1β en induisant la production de MMP-1 et MMP-3 [46]. En résumé elle est responsable de l'augmentation de médiateurs cataboliques et de la diminution de la production des médiateurs anaboliques. L'induction de la production de l'IL-1β, MMP-9 et MMP-13 au niveau des chondrocytes indique son rôle pro-inflammatoire et catabolique sur le métabolisme du cartilage [47].

Le niveau d'expression de la leptine et de son recepteur est augmenté au niveau du cartilage OA, ainsi qu'au niveau du liquide synovial au stade avancé de la maladie. Ku et al, [48] ont montré qu'il existe une relation entre la concentration en leptine au niveau du liquide synovial et la sévérité radiologique de l'OA, ce qui confirme son implication dans la physiopathologie de l'OA. L'ensemble de ces données peuvent nous mener à penser que les adipokines d'une manière générale et la leptine plus spécifiquement peut être le lien entre l'obesité et l'OA. Ce qui pourrait expliquer l'apparition de l'OA au niveau de certaines articulations du corps comme la main ne supportant pas d'importantes charges pondérales.

De plus *Coggon et al*, ont indiqué que les risques de l'OA du genou associés à la position d'accroupissement étaient plus élevé chez les personnes en surpoids ou dont le travail nécessitait des prises de charge [49]. En outre, une autre étude réalisée par *Zhang et al*, a montré que le risque de développement de l'OA du genou est deux fois plus grand

chez les personnes dont l'emploi nécessite à la fois le transport et l'agenouillement que chez celles dont l'emploi ne nécessite pas ces activités physiques [11].

I.A.3.2.2 Facteurs mécaniques et alignement:

Pendant longtemps, il a été suggéré que la faiblesse et l'atrophie musculaire observées lors de l'OA du genou étaient la conséquence de la douleur. Or, au fil du temps il s'est avéré que les personnes présentant une OA radiologique asymptomatique présentaient également ces symptômes. Ces résultats sont confirmés par une étude réalisée par *Zhang et al*, qui a montré que la faiblesse des quadriceps n'est pas uniquement la conséquence de la douleur reliée à l'OA, mais plutôt constitue en elle-même un facteur de risque pour des dommages structuraux de l'articulation [11].

L'alignement du genou constitue à son tour un facteur de risque pour l'OA. On définit par l'alignement du genou; l'angle de la hanche-genou-cheville qui est un déterminant clé de l'application de la charge sur l'articulation telle qu'elle soit la hanche, le genou, ou encore la cheville. Ce qui laisse supposer que les genoux mal alignés contribuent au développement et à la progression de l'OA [50]. Néanmoins, un défaut d'alignement varus ou valgus augmenterait le risque de progression de l'OA de 4 fois de plus ou d'une prédisposition de 2 à 5 fois de plus au niveau des compartiments médiaux et latéraux respectivement [11, 51, 52]. *Hunter et al*, avaient émis l'hypothèse qu'un défaut d'alignement du genou pourrait ne pas être un facteur de risque principal pour l'apparition de l'OA du genou radiographique, mais plutôt un marqueur de gravité de la maladie et /ou de son évolution [50].

Ainsi, comprendre le rôle de l'alignement est important dans la mesure où celui-ci module les facteurs de risque standards de l'OA y compris l'obésité [53], la force du quadriceps [54], la laxité [54], et le stade de la maladie [51, 52].

I.A.3.2.3 Laxité et contribution du ménisque:

La laxité du genou peut être considérée comme un facteur de risque. Cependant, une étude transversale a montré que la laxité du genou est plus importante chez les patients non

arthritiques et ayant une maladie idiopathique que dans les genoux contrôles, ce qui laisse supposer que la laxité du genou pourrait précéder l'apparition de l'OA et par conséquent, représenter un facteur de risque [55]. Des études réalisées par *Biswal et al*, et *Berthiaume et al*, ont montré qu'il existait une association entre la position du ménisque, la présence de dommages à son niveau et la perte cartilagineuse. Cependant, une atteinte du ménisque médial augmenterait le ratio d'atteinte du compartiment médial de 6.3 sur une échelle allant de 3.1-12.6 [56-58].

L'ensemble de ces données indiquent que l'OA est une maladie multifactorielle qui dépend aussi bien de facteurs intrinsèques liés à l'individu lui-même comme la génétique, la laxité ligamentaire, la position du ménisque ou encore des facteurs extrinsèques comme le régime alimentaire ou l'activité professionnelle.

I.A.4 Les principaux composants de l'articulation:

Une articulation est formée de deux os arrondis et supperposés, le glissement de l'un par rapport à l'autre permet un mouvement. Ces os sont recouverts d'un tissu cartilagineux qui permet d'éviter tout frottement mécanique entre eux et aussi de faciliter le mouvement, par conséquent préserver l'os de l'usure. Une articulation n'est pas uniquement formée du cartilage articulaire, mais aussi de structures annexes comprenant le ménisque, les ligaments, et la membrane synoviale qui fait partie intégrante de l'articulation.

I.A.4.1 Le cartilage articulaire:

Tissu sans vascularisation dont la nutrition est assurée principalement par diffusion à partir de l'os et du liquide synovial. Le cartilage articulaire possède des propriétées biomécaniques lui permettant de supporter, d'amortir, et de distribuer les charges appliquées à l'articulation. Ces propriétés sont dûes à la composition de la matrice extracellulaire formée principalement d'eau à une proportion allant de 70 à 80% du poids

humide, de protéoglycanes et du collagène. Les cellules ne représentent qu'environs 1% du volume du cartilage adulte. L'ensemble constitue le cartilage articulaire [59-63].

I.A.4.1.1 La matrice extracellulaire (MEC):

Elle est composée du collagène de type II (ColII), de protéoglycanes et d'eau à une proportion de 80% [63]. Cependant, ces protéoglycanes avec un fort pouvoir hydrophile [64] sont responsables de l'hydratation du tissu grâce à leur charge négative qui leur permet d'attirer les molécules d'eau [65] et ainsi de conférer au cartilage articulaire ses propriétées biomécaniques. Une augmentation du volume tissulaire d'une proportion de 30-50% est observée suite à l'absorption de l'eau [63, 66, 67].

Les protéoglycanes, sont des biomolécules ubiquitaires composées d'une protéine axiale et d'une ou de plusieurs chaînes de glycosaminoglycanes sulfatés (GAG) attachées de façon covalente [63, 68], représentés principalement par le chondroïtine sulfate (CS), le kératane sulfate (KS), et le dermatane sulfate (DS) [61] qui au niveau du cartilage articulaire se lient de façon non covalente à l'acide hyaluronique formant ainsi des aggrégats, dont les plus gros sont formés d'environs 300 molécules [63]. L'aggrécane est le protéoglycane majeur du cartilage selon sa masse moléculaire il peut être répertorié en deux classes; aggrécane de haut poids moléculaire et aggrécane à faible poids moléculaire comme le biglycan et la fibromoduline [63, 66, 67].

La structure rigide du cartilage est conférée par le collagène fibrillaire [63], constitué principalement du ColII et représentant 90-95% du collagène articulaire. Sa structure est d'une importance majeure dans la mesure où elle permet de maintenir aussi bien le volume que l'architecture tissulaires. D'autres types de collagène comme le collagène IX et XI sont également présents avec une proportion minime [63, 65, 66].

Cependant, lors d'une pathologie le ColII est dégradé, celui-ci est substitué de manière aberrante par le collagène de type I n'ayant pas les mêmes propriétées que le ColII. La synthèse et le renouvellement des composants de la MEC sont assurés par les chondrocytes [67] qui à leur tour sont bordés par le collagène type VI leur permettant de s'adhérer à la MEC [63, 69]. Les deux types de collagène IX et XI semblent être impliqués dans la

stabilisation de la structure en fibre du ColII. Quant au collagène type X, il joue un rôle dans la minéralisation; il entoure le chondrocyte au niveau de la zone du cartilage calcifiée et au niveau de la plaque de croissance [63].

En sus des types du collagène cités d'autres sont également présents au niveau de l'articulation constituant les autres structures articulaires, tel que le ménisque composé principalement du collagène de type I avec le collagène III, V, VI et une contribution mineure du ColII au niveau de cette structure [70].

I. A.4.1.2 Les chondrocytes et évolution:

Les étapes de maturation suivant l'embryogénèse ont pour finalité la formation de deux populations chondrocytaires. L'une conduisant à la formation du cartilage articulaire et la deuxième subissant une maturation en chondrocytes hypertrophiques, nommés ainsi en fonction de leur taille et en raison de l'expression de certaines molécules caractéristiques, plus précisément le collagène type X, la phosphatase alcaline, la Runx2, l'osteopontine et la MMP-13. En contre partie, une diminution de l'expression du ColII est observée chez cette population qui finit par disparaître par apoptose. Les zones d'apoptose chondrocytaire seront entravées par des vaisseaux sanguins et la synthèse de la matrice osseuse a lieu à ce niveau sous l'action des ostéoblastes; il s'agit de l'ossification enchondrale **(figure1)**. La vitesse de maturation des chondrocytes est sous le contrôle de facteurs qui peuvent soit l'accélérer ou la décélérer [71, 72].

Quant à la première population, elle est à l'origine des chondrocytes articulaires qui possèdent la capacité d'exprimer les constituants de la MEC comme le ColII et l'aggrécane. La chondrogénèse est régulée par des facteurs de croissance et de différenciation via la régulation des protéines kinases PKA, PKC et MAPK [73, 74]. La signalisation pour la régulation de la différenciation et de la prolifération chondrocytaire se fait via des intégrines et des récepteurs au collagène, dont le *discoidin domain-containing receptor 2* (DDR2) [75].

La taille, la forme et l'activité métabolique des chondrocytes articulaires varient dans les diverses couches du cartilage. Ce type cellulaire est constitué de tous les organelles nécessaires pour la synthèse de la matrice sous l'effet de facteurs anaboliques, entre autres les protéoglycanes, l'aggrécane et le ColII. Ces facteurs s'opposent aux facteurs cataboliques dont l'IL-1β et les MMPs [76, 77].

Chez l'adulte, la nutrition des chondrocytes se fait par diffusion au travers de la cavité synoviale pouvant atteindre les différentes couches du cartilage. Les canaux existant au niveau de l'os sous chondral permettent la nutrition de la couche calcifiée en contact avec ce dernier [66, 78]. Après la maturation du squelette, les chondrocytes deviennent probablement incapables de se diviser et la synthèse de la matrice diminue aussi fortement. Mais malgré cela elle persiste. Contrairement aux croyances propagées, les chondrocytes ont une activité comparable à celle des autres cellules [78]. Bien qu'ils ont été considérés comme des cellules quiescentes pendant longtemps, ils ont la capacité d'assurer l'homéostasie cartilagineuse dans les conditions physiologiques. Cette homéostasie est assurée par de nombreux facteurs, contraintes mécaniques, facteurs de croissance; transforming growth factor (TGF) β, fibroblast growth factor FGFβ, insulin-like growth factor (IGF). L'activité métabolique du chondrocyte est fonction des informations qu'il reçoit par l'intermédiaire de ses intégrines [60, 78].

Avec l'âge, le nombre de chondrocyte diminue en raison de la mort cellulaire souvent accompagnée de changements morphologiques, notamment la présentation d'un phénotype semblable à celui des chondrocytes hypertrophiques produisant la MMP-13, et du collagène de type X. Ces chondrocytes ont subit un phénomène de dédifférenciation. De plus, un raccourcissement des télomères résultant des dommages au niveau de l'ADN sous l'effet des *reactive oxygen species* (ROS) augmentés avec l'âge est noté. Ce raccourcissement est caractéristique de la sénescence cellulaire observée chez les chondrocytes et conséquence de changements phénotypiques appelés phénotype sécrétoire sénescent qui contribue au vieillissement tissulaire par stimulation de la dégradation de la MEC via la favorisation du catabolisme sous l'effet des cytokines et facteurs de croissance.

À ces changements, s'ajoutent d'autres changements ultrastructuraux au niveau de la MEC [79-81].

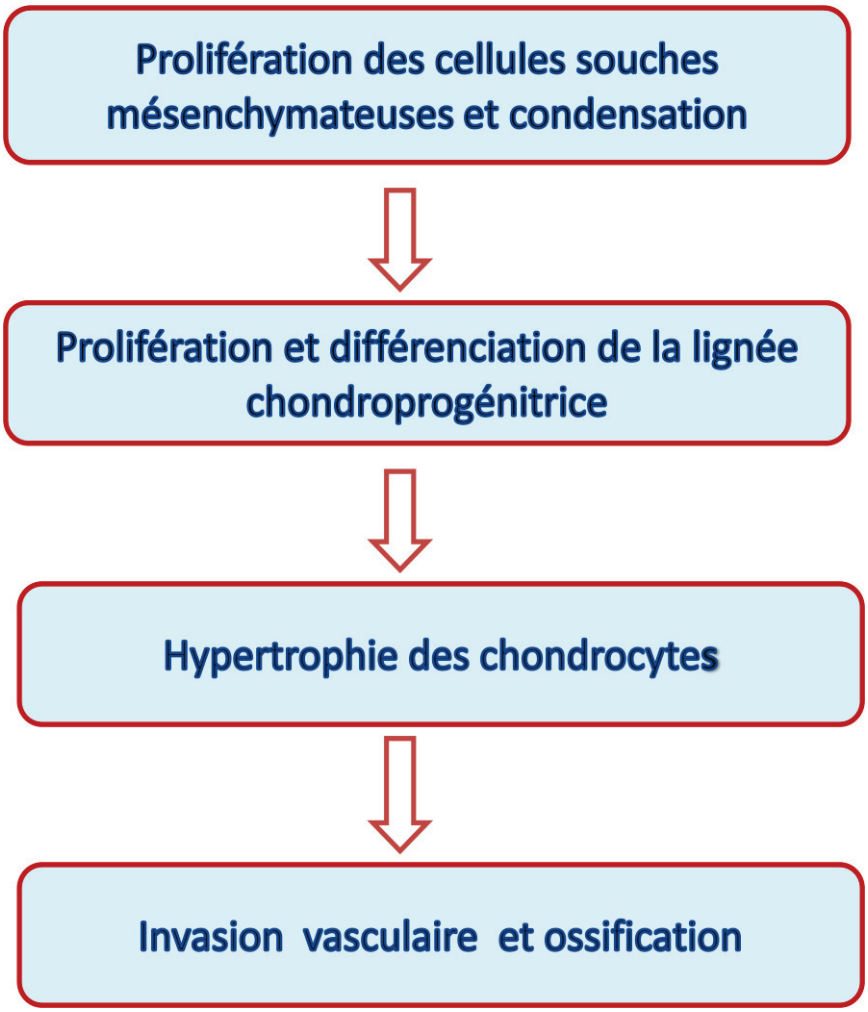

Figure 1: Schéma récapitulatif des principales étapes de l'évolution du cartilage articulaire et de l'ossification enchondrale (adapté de [72]).

I.A.5 Cartilage normal et cartilage arthrosique:

I.A.5.1 Caractéristiques du cartilage normal:

Le cartilage articulaire normal est un tissu blanc et brillant. Microscopiquement, il est d'anisotropie et d'entropie distinctes, formé de trois zones différentes selon la disposition cellulaire et les molécules sécrétées (**Figure 2)**. Cependant, la zone la plus superficielle est constituée de cellules aplaties sécrétant une protéine lubricine, la zone intermédiaire est constituée par des cellules arrondies qui ont la capacité de produire le ColII et le protéoglycane, quant à la zone profonde, elle est constituée de cellules en colonne avec une MEC minéralisée. À l'état physiologique, lorsque ce dernier est soumis à un stress mécanique, le cartilage articulaire est capable de rétablir l'équilibre entre l'anabolisme et le catabolisme. Les chondrocytes sont à l'origine du maintien de l'homéostasie de la matrice grâce à leurs capacités de synthèse [62, 66, 82-84].

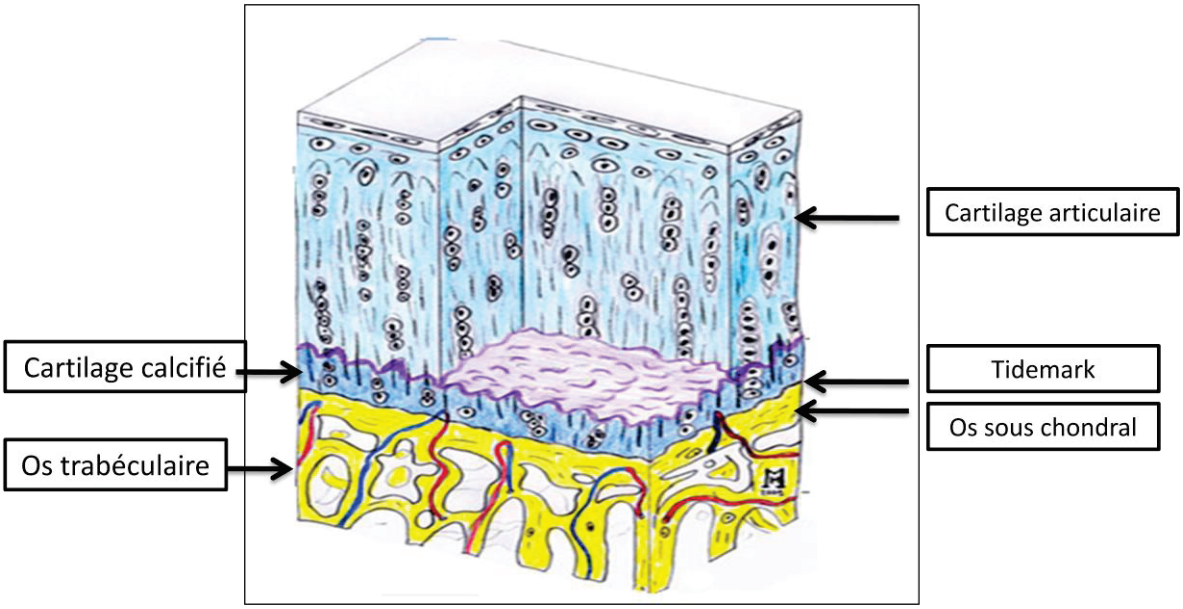

Figure 2: Organisation et architecture des différentes couches du cartilage articulaire. (Adapté de [66])

I.A.5.2 Caractéristiques du cartilage arthrosique:

Le début de l'OA est caractérisé par la perte de la coloration blanchâtre du cartilage articulaire avec ramollissement [85, 86]. La dégradation des protéoglycanes par des protéinases spécifiques augmente l'accéssibilité des MMPs aux fibres de collagène [87] dont la dégradation a pour conséquence un changement de l'architecture matricielle. Les contraintes biomécaniques à l'origine de ces changements affectent également la différenciation des chondrocytes qui s'hypertrophient et commencent à produire en excès des médiateurs inflammatoires et cataboliques, mais aussi un anabolisme dévié via la production de molécules qui ne deveraient pas être exprimées dans un cartilage normal. Ce déséquilibre du comportement chondrocytaire est à l'origine de la dégradation des composants matriciels [87] conduisant à l'altération de ses propriétées biomécaniques [82, 83] (**Tableau 1).**

Au même temps, une prolifération cellulaire se manifestant par la formation de clones est visible à proximité des régions endommagées avec augmentation du métabolisme cellulaire. Ces cellules expriment des niveaux élevés de Smad2/3 et Smad1/5/8 phosphorylés. En fonction du niveau de la phosphorylation des Smads, les cellules peuvent être différenciées en deux catégories; présentant un haut niveau de phoshorylation et localisées aux alentours des dommages, ou des cellules ne présentant pas de Smads phosphorylés et situées loin des zones affectées [87-89].

Tableau 1: Principales caractéristiques des chondrocytes OA. (Adapté de [90])

Les principales caractéristiques des chondrocytes OA
↓ Diminution de l'anabolisme par: La synthèse du ColII La production de l'antagoniste du récepteur à l'IL-1β (IL-1Ra) ↑ Augmentation du catabolisme par: La production des cytokines inflammatoires La production des protéases La production des enzymes inflammatoires : Cyclooxygénase-2 (COX-2), (microsomal Prostaglandin E synthase) mPGES, *inducible nitric oxide synthase* (iNOS) L'apoptose cellulaire Augmentation de la synthèse du ColIV, ColIII, ColX

L'ensemble de ces changements conduit à l'apparition de la fibrillation à la surface du cartilage qui va de plus en plus s'approfondir avec la progression de la maladie [91] sans toutefois porter atteinte à la zone du cartilage calcifiée, la tidemark est toujours visible au début de la maladie [92]. Au fil du temps le cartilage peut disparaître mettant à nu l'os sous chondral [60]. En marge articulaire, des ostéophytes calcifiés prennent place et le tissu synovial est également affecté avec infiltration des cellules inflammatoires [60] (**Figure3).**

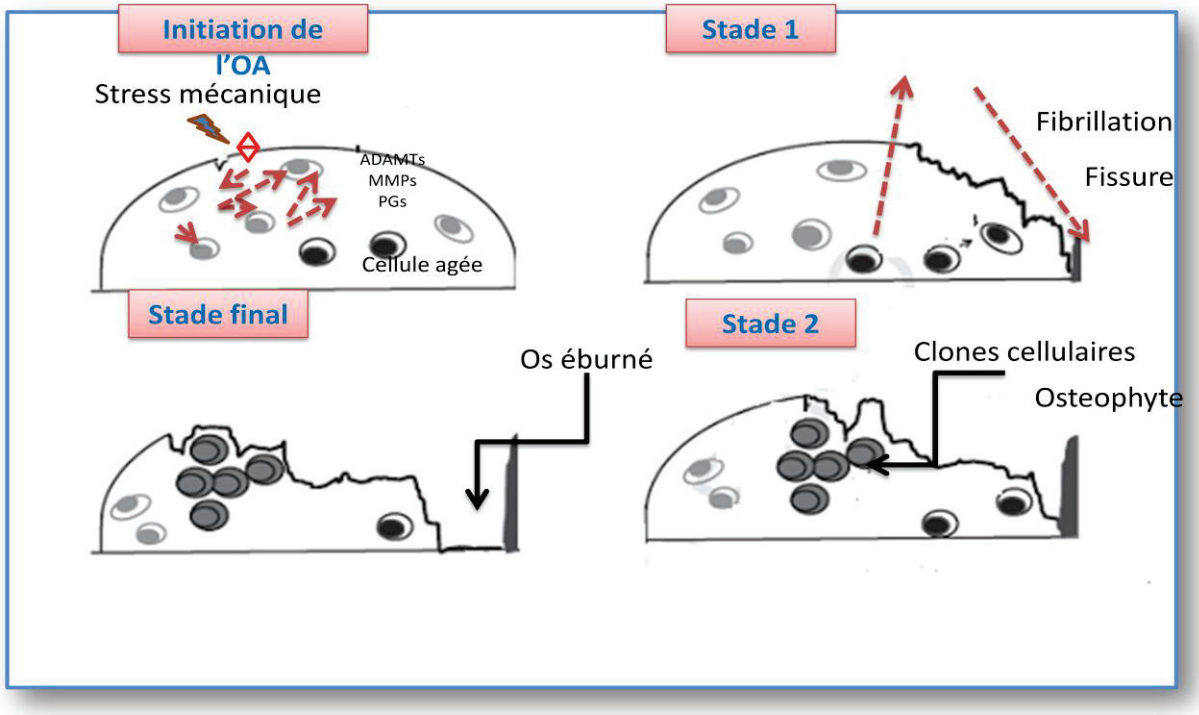

Figure 3: Schéma récapitulatif des différents stades caractéristiques de l'OA (Adapté de [87])

I.B Inflammation et facteurs intervenant dans la dégradation du cartilage articulaire

En réponse aux perturbations mécaniques appliquées au chondrocyte, ce dernier répond par des tentatives de réparation. Mais malgré cela, l'état d'équilibre au cours de l'OA est rompu en faveur du catabolisme par la production de médiateurs inflammatoires principalement des cytokines produites par les chondrocytes et les synoviocytes lors de l'OA [93].

I.B.1 Cytokines inflammatoires:

L'IL-1β, le TNF-α et l'IL-6 représentent les cytokines majeures impliquées au cours de l'OA en raison de leur rôle dans l'induction de la dégradation du cartilage articulaire [94, 95]. En plus de ces cytokine, d'autres médiateurs comme le, *monocyte chemoattractant protein* 1 (MCP-1), *Leukemia Inhibitory Factor* (LIF), ainsi que d'autres dérivés d'oxygène ROS, GRO, oncostatin M et les composés lipidiques dont les prostaglandines (PGs) et les leukotriènes (LTs) augmentent l'activité catabolique, ce qui conduit à la destruction du cartilage articulaire qui n'est autre que la résultante de son inflammation au niveau moléculaire [93].

Toutefois, et grâce à des récepteurs localisés sur les surfaces cellulaires, les chondrocytes deviennent sensibles aux stimulations mécaniques. La plupart de ces récepteurs sont des récepteurs des constituants de MEC [96]. Il s'agit principalement d'intégrines reconnaissant la fibronectine (FN) et les fragments du ColII. Ces produits de dégradation contribuent à l'activation des chondrocytes via ces intégrines et conduisent à la production de protéinases dégradant la matrice en outre des médiateurs inflammatoires [97].

I.B.1.1 Interleukin-1β et TNF-α:

En plus des autres médiateurs inflammatoires contribuant à la perpétuation de l'inflammation, l'IL-1β semble jouer un rôle pivot [98-100]. La famille de l'IL-1, renferme 11 membres dont l'IL-1β [94] qui joue un rôle central dans la physiopathologie des maladies inflammatoires comme la polyarthrite rhumatoïde (RA), tout comme l'OA, considérée initialement comme maladie non inflammatoire. L'IL-1β est synthétisée sous forme d'un précurseur de 31 kDa, forme inactive nécessitant l'intervention de caspase 1 connue également sous le nom d'*IL-1β converting enzyme* (ICE) [101], ayant la capacité de générer l'IL-1β sous sa forme active de 17.5 kDa [102, 103]. Cette forme est retrouvée au niveau de la membrane synoviale, du liquide synovial et au niveau de la zone superficielle

du cartilage articulaire où d'importants changements dégénératifs sont notés lors de l'examen histologique. De même, l'expression d'ICE est augmentée au niveau des tissus OA et est co-localisée avec IL-1β [104]. Les différents effets de l'IL-1 sont exercés via des récepteurs à l'IL-1 appartenant à une famille composée de neuf gènes distincts [7]. Contrairement au récepteur à l'IL-1 type II (IL-1RII) possédant plus d'affinité à l'IL-1α qu'à l'IL-1β et connu par son incapacité à transduire un signal [105, 106]. L'IL-1 type-1 (IL-1RI) est le récepteur ayant le plus d'affinité pour l'IL-1β. Son expression est augmentée au niveau des chondrocytes et fibroblastes synoviaux chez les sujets humains OA comparativement aux sujets normaux. La liaison de l'IL-1RI à l'IL-1β conduit à une série de réactions sensibilisant la cellule aux stimulations [107]. Les effets de l'IL-1β via ce type de récepteur peuvent être inhibés par des inhibiteurs naturels comme l'antagoniste du récepteur à l'IL-1 (IL-1Ra) [105, 108] dont l'expression est diminuée au niveau des tissus OA avec une diminution du ratio IL-1Ra/IL-1ß et même IL-1Ra/IL-1α avec les grades croissants de la sévérité de l'OA [109]. Ceci pourrait expliquer l'accentuation des effets cataboliques de l'IL-1β au niveau des tissus articulaires OA [105]. De plus, les souris déficientes en IL-1β sont protégées contre l'inflammation induite par la perte cartilagineuse [110].

En plus de ces différents effets de l'IL-1β, cette dernière agit également par l'induction de l'apoptose chondrocytaire via la dépolarisation mitochandriale et l'augmentation de la production de protéines proapoptotiques [111] ou encore par l'induction de l'acide nitrique (NO). Une étude réalisée par *Tenor et al,* a montré que la production du NO augmente au niveau des chondrocytes humains sous l'effet de l'IL-1β de manière temps et dose dépendante via la stimulation de la NO synthase inductible (iNOS) [112].

Bien que l'IL-1β soit le principal acteur du catabolisme au cours de l'OA, ceci n'exclue pas son importance sur le plan physiologique où sa délétion entraîne une accélération des lésions OA chez des souris déficientes au gène de l'IL-1β [7, 113].

Tout comme l'IL-1β, le TNF-α semble également être l'une des cytokines majeures impliquées dans l'OA, l'IL-1β serait responsable de la destruction du cartilage articulaire et le TNF-α de la cascade inflammatoire [113]. Le TNF-α est synthétisé sous la forme d'un précurseur protéique de 76 acides aminés activé sous l'action d'une enzyme *TNF-alpha converting enzyme* (TACE) présente à la surface cellulaire et dont l'expression est augmentée au niveau du cartilage OA. La protéine générée est d'un poids moléculaire de 17 kDa. Le TNF-α exerce ses effets via deux récepteurs nommés en fonction de leur poids moléculaire le TNF-R55 et TNF-R75 [114].

Dans les tissus articulaires le TNF-R55 est le récepteur responsable de la médiation de l'activité du TNF-α. De même, une augmentation d'expression de ce récepteur est observée chez les chondrocytes et fibroblastes synoviaux [115-118]. Au cours de l'OA, des niveaux élevés d'expression et de production du TNF-α sont retrouvés au niveau du liquide synovial, de l'os sous chondral et du cartilage articulaire. Le TNF-α exerce des effets anti-anaboliques par l'induction de l'inhibition de la synthèse des constituants de la MEC [119]et des effets cataboliques par la stimulation de la libération des MMPs [120].

Voies de signalisations activées par l'IL-1β:

L'IL-1β, une fois liée à son récepteur type I peut déclencher différentes voies à l'origine des différents effets pathologiques. Il s'agit principalement des voies JNK, p38 MAPK et NF-κB avec la contribution de la voie Wnt/β-catenin dont l'activation conduit aux effets mentionnés ci-dessus. En plus de l'activation des *A desintegrin and metalloproteinase with thrombospondin motif* (ADAMTS), l'IL-1β peut également induire une augmentation du Ca^{2+} intracellulaire [113, 121, 122].

I.B.1.2 Autres cytokines:

En plus de l'IL-1β et du TNF-α, d'autres cytokines contribuent de façon conséquente à la progression de la maladie, les principales IL-6, l'IL-8, IL-11, IL-15, IL-17, IL-18, IL-21 et LIF9 [98-100, 102-104]. L'IL-6 est une cytokine pleiotypique pro-

inflammatoire [123] produite par différents types cellulaires, entre autres les chondrocytes et les ostéoblastes [90, 124]. Dans les conditions physiologiques, l'IL-6 est retrouvée à une faible concentration qui augmente sous l'effet des autres cytokines inflammatoires, facteurs de croissance ou encore sous l'effet de prostanoïdes représentés principalement par la PGE_2 [114, 125, 126], qui entraîne une augmentation de sa production aussi bien au niveau des chondrocytes qu'au niveau des ostéoblastes humains [127, 128]. De plus, des niveaux élevés d'IL-6 sont également retrouvés au niveau des ostéophytes. Une étude réalisée par *Stannus et al*, a montré que son niveau est corrélé positivement avec le pincement de l'interligne articulaire au niveau de la hanche [129].

Cependant, les patients OA sont subdivisés en deux principaux groupes selon le niveau d'IL-6 produit par les ostéoblastes de l'os sous chondral. Le premier groupe montre de bas niveaux d'IL-6 et de PGE_2 [128] avec une augmentation de la résorption osseuse [130]. Tandis que le deuxième groupe montre des niveaux élevés d'IL-6 et de PGE_2 associés à une diminution de la résorption osseuse [130].

L'IL-6 possède un double rôle au cours de l'OA; un rôle anticatabolique et un rôle pro-inflammatoire. Le rôle anticatabolique revient à la capacité du chondrocyte à diminuer la production des facteurs cataboliques sous l'effet d'IL-6 impliqués dans la dégaradtion du cartilage articulaire via la production des TIMPs [128] et plus particulièrement de TIMP-1 [7]. Les TIMPs sont impliqués dans les mécanismes de rétroaction qui limitent les dommages protéolytiques [107]. En plus de la production des TIMPs, l'IL-6 peut également induire la production de l'IL-1Ra, diminuant de cette manière les effets médiés par l'IL-1β, comme elle induit également le TNF-R55 [7, 129].

Paradoxalement, l'IL-6 pourrait être impliquée dans le processus inflammatoire par l'augmentation du nombre des cellules inflammatoires au niveau du tissu [131], l'amplification de la production de MMP-1 et MMP-13 en combinaison avec l'IL-1β et l'oncostatine au niveau d'explants de cartilage humains et bovin [107, 132, 133], et par l'inhibition de la production des protéoglycanes suite à des blessures mécaniques [134, 135].

Ces different effets de l'IL-6 sont la conséquence de sa liaison à son récepteur multimérique constitué du récepteur membranaire à l'IL-6 (IL-6R), de la forme soluble de l'IL-6R (sIL-6R) et de la sous unité bêta gp130 du récepteur. La combinaison de ces trois entités entraîne une cascade de signalisation aboutissant à la transcription des gènes cibles de l'IL-6 [113]. De plus, des études ont démontré que le rôle inflammatoire et dégénératif de l'IL-6 au cours de l'OA est plus prononcé en présence de la sous unité soluble sIL-6R qui augmente son affinité au récepteur. De même, des niveaux élevés d'IL-6 et de sIL-6R sont présents dans le liquide synovial et le sérum des patients OA [136].

Un autre membre de la famille de l'IL-6, l'OSM exerce des effets procataboliques au niveau des tissus articulaires en induisant la protéolyse des protéoglycanes et du collagène du cartilage articulaire, et pro-inflammatoires en agissant en synergie avec d'autres cytokines inflammatoires comme l'IL-1β, le TNF-α et l'IL-17. Ce qui se traduit par l'augmentation de la production de la MMP-3, MT1-MMP et des aggrécanases [7].

L'IL-11, contribue également au développement de l'OA. Il s'agit d'une cytokine partageant le domaine gp130 du récepteur à l'IL-6 et du facteur inhibiteur de leucémie LIF, sa production est induite par des cytokines inflammatoires. Elle peut également à son tour induire ces mêmes cytokines, l'IL-1β et le TNF-α au niveau des fibroblastes synoviaux et l'IL-1β et l'IL-8 au niveau des chondrocytes [137]. Du fait que l'IL-11 partage le domaine gp130 avec des médiateurs impliqués dans la physiopathologie de l'OA peut amener à penser que cette dernière exerce des effets similaires. Tout de même, cette cytokine peut être induite au niveau des chondrocytes articulaires et des fibroblastes synoviaux en culture [138, 139]. Néanmoins, elle peut à son tour diminuer la production de la PGE_2 au niveau des fibroblastes synoviaux [140].

Le LIF est également induit au cours de l'OA induit l'expression des collagénases et des stromélysines au niveau des tissus articulaires, stimule la résorption des protéoglycanes du cartilage [141], et enfin la production du NO [107].

Outre ces médiateurs, d'autres cytokines, telles que l'IL-17 et l'IL-18 sont exprimées au niveau du cartilage et de la membrane synoviale OA [7, 107] et partageant certaines propriétés avec l'IL-1β semblent jouer un rôle important dans la physiopathologie de l'OA et plus précisément dans la phase précoce de l'inflammation. Ces cytokines augmentent certains gènes comme celui au TNF-α, IL-6, NO et COX-2 impliqués dans l'inflammation. Une augmentation de l'affinité de l'IL-17 à son récepteur spécifique et celle de l'IL-18 au récepteur à l'IL-1RI est notée [7, 107]. (**Tableau 2**)

Tableau 2: Tableau récapitulatif des principaux effets induits par les médiateurs inflammatoires au cours de l'OA.

Médiateurs inflammatoires	Expression et effets induits
○ Cytokines inflammatoires	
IL-1β	➢ Expression élevée au niveau du cartilage, os, membrane synoviale et liquide synovial OA. ➢ Induit l'apoptose chondrocytaire ➢ Induit de la production de NO ➢ Induit la destruction de cartilage articulaire par la production des MMPs
TNF-α	➢ Expression élevée au niveau du cartilage, os, membrane synoviale et liquide synovial OA. ➢ Induit la cascade inflammatoire ➢ Inhibe la synthèse des constituants de la MEC

	↓ Stimule la libération des MMPs
IL-6	↓ Expression au niveau du liquide synovial OA. ↓ Induit la production des TIMPs ↓ Augmente le nombre des cellules inflammatoires au niveau du tissu ↓ Amplification de la production de MMP-1 et MMP-13 ↓ Inhibition de la production des protéoglycanes
OSM	↓ Induit la protéolyse des protéoglycanes et du Collagène ↓ Induit la production de MT1-MMP et aggrécanases en synergie avec d'autres cytokines
IL-11	↓ Induit l'IL-1β, TNF-α, IL-8
LIF	↓ Expression au niveau du liquide et membrane synoviale OA. ↓ Induit l'expression des collagénases et stromélysines ↓ Stimule la résorption des protéoglycanes ↓ Stimule la production du NO

IL-17 et IL-18	↓ Expression au niveau du cartilage et membrane synoviale OA ↓ Augmentent la production du TNF-α, IL-6, NO et COX2.

I.B.1.3 Chemokines:

En plus des cytokines, les chemokines impliquées dans de nombreux processus physiologiques et pathologiques [142, 143] semblent également être impliquées au cours de l'OA. Il s'agit d'une famille de protéines de faible poids moléculaire allant de 6-14 kDa réparties en quatre classes principales [142, 144] et exerçant leurs effets via des récepteurs couplés aux protéines G [143].

Les chondrocytes humains normaux expriment deux classes de chemokines et leurs récepteurs respectifs indiquant leurs implications dans l'homéostasie du cartilage articulaire et probablement leur incrimination dans la maladie. L'implication des chemokines dans l'OA a été démontrée dans des travaux réalisés par *Silvestri, et al* et *Yuan et al*, qui ont montré leur action catabolique de part l'induction de la libération des MMPs, mais aussi par la perturbation du métabolisme des protéoglycanes [145-147]. De plus, des travaux réalisés par *Alaaedine et al*, ont montré que les chondrocytes humains normaux n'expriment pas une chemokine RANTES. Cette dernière est par contre induite au cours de l'OA où elle est stimulée par l'IL-1β et l'IL-18. La protéine ainsi que son ARNm sont localisés principalement au niveau de la zone superficielle et au niveau de la zone intermédiaire du cartilage articulaire où elle aura pour fonction de stimuler iNOS, IL-6 et MMP-1. Le traitement du cartilage articulaire avec RANTES augmente la libération des glycosaminoglycanes GAGs [143, 148]. Une autre chemokine pro-inflammatoire l'IL-8 intervient également au cours de l'OA. Elle est synthétisée par les chondrocytes et les

synoviocytes [149], et est retrouvée au niveau du liquide synovial avec d'autres médiateurs inflammatoires comme l'IL-1β, l'IL-6 et le TNF-α au niveau du liquide synovial de patients OA. Cette chemokine a pour fonction d'augmenter la production des produits du stress oxydatif au niveau du chondrocyte [149-151].

I.B.1.4 L'acide nitrique:

L'oxidation de la L-Arginine sous l'action de synthases spécifiques appelées oxide nitrique synthase (NOS) aboutit à la formation de l'acide nitrique (NO). Ces synthases existent sous trois isoformes dont deux exprimées constitutivement. NOS-1 est exprimée au niveau neuronal et NOS-3 au niveau de l'endothélium. Ces deux synthases produisent de faibles taux de NO. Quant à la troisième isoforme inductible iNOS produit des quantités soutenues de NO à la suite d'un stimulus par des cytokines inflammatoires chez certains types cellulaires comme les hépatocytes et les chondrocytes [152, 153]. Le NO ainsi produit exerce de multiples effets biologiques et physiopathologiques [7, 154].

À l'inverse d'un cartilage normal où aucune production de NO n'est notée sauf à la suite d'une stimulation par l'IL-1β, le cartilage OA produit des quantités de NO de l'ordre du micromole générées sous l'effet de iNOS lors de cultures cellulaires *ex-vivo* [155]. Cependant, lors de l'OA, le NO généré par iNOS possède des effets régulateurs pro-inflammatoires et destructeurs [7]. Il exerce ses effets principalement au niveau du cartilage articulaire où il promeut de nombreux effets cataboliques aboutissant à l'altération de l'activité du chondrocyte et à la perte de la matrice cartilagineuse [156], par inhibition de la synthèse du collagène et des protéoglycanes [157], par l'activation des MMPs : MMP-1,-2, -3, et -9 [154, 158], par la stimulation de la production du peroxynitrie [159], et par l'induction de l'apoptose [154]. Le NO généré sous l'action de iNOS contribue de manière considérable aux effets cataboliques de l'IL-1β non seulement par la diminution de la synthèse et de la réponse aux facteurs anaboliques de la matrice cartilagineuse tels que l'IGF [160] et le TGFβ [156], mais aussi par la diminution de la synthèse de l'antagoniste

IL-1Ra [7]. Le NO peut agir également par augmentation de la synthèse de la COX-2 et de la PGE_2, et par induction de l'apoptose chondrocytaire [7, 161, 162].

Outre ces effets obtenus *in vitro*, des expériences réalisées *in-vivo* sur un modèle canin, ont montré que la stimulation des chondrocytes par le NO est responsable de l'augmentation de la synthèse de l'IL-18 et de l'ICE. L'ICE est requise aussi bien pour la maturation de l'IL-1β que l'IL-18 [163]. L'immunohistochimie réalisée sur des tissus OA et RA a montré une co-localisation de iNOS et des cellules apoptotiques aussi bien au niveau de cartilage articulaire qu'au niveau de la membrane synoviale [164]. Ce qui laisse supposer l'implication du produit de iNOS dans l'apoptose chondrocytaire. Ceci est confirmé par la diminution des facteurs apoptotiques sous l'effet de l'inhibiteur de NOS, le L-NMMA [165-167] (**Tableau 3**).

Chemokine /Acide nitrique	Effets induits
○ Chemokines	↓ Induisent la libération des MMPs ↓ Perturbent le métabolisme des protéoglycanes
RANTES	↓ Stimule iNOS, IL-6 et MMP-1 ↓ Augmente la libération des GAGs
IL-8	↓ Augmente la génération des produits du stress oxidatif
○ L'acide nitrique	↓ Effets proinflammatoires et destructeurs ↓ Inhibe la synthèse du Collagène et des protéoglycanes ↓ Active la MMP-1, -2, -3 et -9 ↓ Stimule la production du peroxynitrite ↓ Induit l'apoptose chondrocytaire ↓ Diminue la synthèse de l'IL-1Ra ↓ Augmente la synthèse de COX2 et PGE_2

Tableau 3: Tableau récapitulatif des principaux effets induits par les chemokines et l'acide nitrique

I.B.2 Facteurs intervenant dans la dégradation du cartilage articulaire:

I.B.2.1 Les métalloprotéases (MMPs):

Les MMPs ont été découvertes pour la première fois par Gross et Lapière en 1962 [168]. Ces MMPs appartiennent à la superfamille des MMPs zinc-dépendantes, qui renferme plus de 26 endopeptidases dont les collagénases (MMP-1,-8 et -13) responsables de la destruction du collagène natif [169], les gélatinases (MMP-2 et MMP-9), les stromélysines (MMP-3,-7,-10 et-11), et les MMP de type membranaire dont MT-MMP1-4 et différents autres sous groupes. Ces MMPs se trouvant de façon majoritaire sous forme soluble avec quelques exceptions sous forme membranaire ont pour fonction de dégrader les protéines matricielles. Leur activité est optimale à pH neutre [170, 171], et requiert le calcium et le zinc. Structuralement, les MMPs sont constitués de trois domaines; **i)** un prodomaine au niveau de l'extrémité N terminale qui permet le maintien de l'enzyme sous sa forme latente et dont le clivage conduit à son activation, **ii)** un domaine catalytique reliant les atomes de zinc et de calcium et enfin **iii)** un domaine hemopexine au niveau de l'extrémité C terminale [172].

1- La MMP-13 fortement impliquée au cours de l'OA est constituée de 471 acides aminés répartis sur les domaines principaux. Comme les autres MMPs, elle est sécrétée sous sa forme latente, son activation se fait sous l'action de la MMP-2 (gélatinase A) et MMP-14 (MT1-MMP). Pour ce faire, cette dernière requiert le domaine structural N terminal [173]. Le domaine catalytique de la MMP-13 humaine présente une grande homologie structurale avec la MMP-13 de rongeurs, de souris et du rat [174]. L'action physiologique de la MMP-13 est contrôlée par des inhibiteurs naturels dont: L' α2-microglobuline, d'un poids moléculaire de 750 kDa, sécrétée par le foie chez les personnes normales ou

atteintes, retrouvée principalement au niveau du sérum et du liquide synovial. En raison de son haut poids moléculaire, elle ne peut pénétrer au niveau du cartilage articulaire et agit sur les MMPs en empêchant leur accès au substrat [172].

2- Les *Tissue Inhibitor of Metalloproteases* (TIMP) sont également capables d'inhiber les MMPs. Cette famille d'inhibiteurs est constituée de 4 membres, mais seuls TIMP-1 et TIMP-2 sont synthétisés par les chondrocytes et donc exprimés par le cartilage articulaire au cours de l'OA et en raison de la forte présence des MMPs dans les tissus pathologiques, l'équilibre avec les TIMPs est rompu [172, 175].

Dans les conditions physiologiques, la MMP-13 est exprimée par les chondrocytes hypertrophiques et les ostéoblastes humains lors du développement fœtal. Au niveau des tissus normaux son expression est très faible ou même inexistante [176].

Sous l'effet de médiateurs inflammatoires comme l'IL-1β et le TNFα, une augmentation de l'expression des MMPs est notée au niveau du cartilage humain, ainsi que chez des modèles animaux induits [177]. Les MMPs ainsi produites par les chondrocytes sous l'action de ces cytokines [178-181], sont connues par leur implication dans la dégradation des composants de la MEC au cours de l'OA.

La MMP-13 de façon plus précise intervient dans la dégradation du ColII avec une activité cinq à 10 fois plus importante sur ce type de collagène que la MMP-1 également fortement impliquée au cours de l'OA [169, 182, 183]. En plus de son action sur le ColII, la MMP-13 intervient aussi dans la destruction d'autres composants matriciels. Elle est 5 fois moins active sur le collagène de type I et III en comparaison à la MMP-1 [175]. Malgré qu'elle puisse intervenir dans la dégradation de ces types de cartilage, mais son action reste inférieure à celle de MMP-1**.** De plus, des études réalisées *in-vivo* ont montré qu'une expression transgénique postnatale de la MMP-13 chez des souris conduit à une pathologie locale du cartilage articulaire au niveau des zones portantes [184, 185].

De même, le rôle de la MMP-13 a été démontré suite à la génération de souris MMP-13 déficientes, chez qui une diminution de l'érosion du cartilage articulaire a été observée à quatre mois et à huit mois post-induction. La diminution est comparable à des souris normales. Cette déplétion par contre n'affectait en rien le développement des ostéophytes [186].

En outre, l'expression constitutive de la MMP-13 au niveau de cartilage articulaire et au niveau d'articulation de souris transgéniques conduit à des changements semblables à ceux observés lors de l'OA [185]. En plus de l'implication de la MMP-13 au cours de l'OA, cette dernière est également exprimée lors de maladies inflammatoires [187] d'où son éventuel implication dans l'aspect inflammatoire lors de l'OA.

Ces nombreux résultats indiquant le rôle fonctionnel de la MMP-13 dans l'OA ont soulevé la possibilité d'utiliser des interventions thérapeutiques dirigées contre sa production [169]. Or, en raison de la forte ressemblance structurale entre les différents membres de la famille et par conséquent le risque d'inhibition de formes non impliquées dans la maladie, ayant même des fonctions physiologiques, complique la conception de nouvelles thérapies contre les MMPs.

En plus de la MMP-13, les autres collagénases MMP-1 et MMP-8 sont pareillement impliquées lors de l'OA et présentent une spécificité pour le collagène de types I et III respectivement. L'action de ces MMPs sur le collagène fibrillaire génère des fragments de dimensions 3 sur 4 et 1 sur 4 de la même manière que pour la MMP-2 et MMP-14 [171].
La MMP-1 à son tour n'est pas détectée au niveau du cartilage normal, *in vitro* elle est produite par une variété de cellules normales comme les fibroblastes, les macrophages, les cellules endothéliales et épithéliales, et est également impliquée dans le remodelage tissulaire physiologique et patholohique [188]. Les gènes des MMPs est régulés au niveau transcriptionnel par des facteurs de croissance, hormones, cytokines et facteurs de transcription e.g. AP-1, NF-κB [187].

L'action de ces MMPs conduit à une destruction irréversible du cartilage articulaire qui ne peut être restauré une fois perdu. Ces nombreux résultats appuyent le rôle des MMPs dans la physiopathologie de l'OA aboutissant tous à la conclusion que les MMPs contribuent significativement à la progression de la maladie par la dégradation de la MEC, mais aussi à l'inflammation et plus particulièrement la MMP-13 en raison de sa spécificité au collagène majeur du cartilage articulaire qui fait d'elle un acteur principal dans l'OA. Bien que certains membres de la famille puissent dégrader l'aggrécane matricielle, le collagène reste leur molécule préviligiée [182, 189].

I.B.2.2 Les ADAMTs

Tout comme le collagène, l'aggrécane est également dégradé lors de l'OA. Cependant, la séquence dans laquelle les constituants de la MEC sont dégradés n'est pas connu, mais plusieurs études réalisées *in vitro* sur des explants de cartilage ont montré que le collagène est protégé par l'aggrécane et que celui ci ne peut être dégradé qu'après la dégradation de l'aggrécane [190-193].

La destruction de l'aggrécane comme celle du collagène représente une caractéristique de l'OA. Cependant, en plus de la dégradation des protéoglycanes par les MMPs, ces derniers peuvent être également dégradés par les aggrécanases découvertes pour la première fois en 1991 [194]. Les aggrécanases ont pour propriété d'agir au niveau de la liaison reliant deux acides aminés en position Glu373 et Ala374. Tandis que les MMPs, notamment la MMP-3 agit au niveau de la liaison Asn341et Phe342 localisés entre les domaines globulaires G1 et G2 [183]. Les fragments générés de cette rupture sont retrouvés au niveau du liquide synovial de patients OA [195]. L'ADAMTS4 et l'ADAMTS5 sont les aggrécanases qui attirent le plus d'attention car elles sont les plus actives *in vitro* [196, 197]. De plus, l'ADAMTS5 est la plus active dans la dégradation du cartilage [197-199], tel qu'observé *in vivo* chez des souris ADAMTS-5 déficientes chez qui l'atteinte du cartilage articulaire est réduite, sa régulation se fait soit par contact direct soit via la voie MAPK [200].

De plus, le traitement d'explants de cartilage avec l'IL-1β conduit à la génération de fragments résultant de la dégradation de l'aggrécane au niveau de la liaison Glu173-Ala374. Les liquides synoviaux de patients OA indiquent des taux élevés de rupture de ces liaisons [201] (**Tableau 4).**

Tableau 4: Tableau englobant les principaux médiateurs inflammatoires et cataboliques impliqués au cours de l'OA (Adapté de [177])

Médiateurs inflammatoires	MMPs/ ADAMTS
○ Interleukines: IL-1α IL-1β IL-17 IL-18 ○ Tumor Necrosis Factor TNF-α ○ Oncostatine\IL-1β Ou ○ Oncostatine \TNF-α	Métalloprotéases (MMPs): ○ Collagénases: MMP-1 MMP-8 MMP-13 ○ Stromélysines MMP-3 MMP-10 MMP-11 ○ Gélatinases: MMP-2 MMP-9 ADAMTS: ADAMTS-4 ADAMTS-5 Autres: Cathepsines

En plus de l'action des différentes cytokines inflammatoires et des MMPs, les composés lipidiques désignés par eicosanoïdes ont fait l'objet de nombreux travaux de recherche en raison de leur forte implication dans la maladie.

I.B.3 Les eicosanoïdes:

Eicosanoïdes, constituent une famille de produits dérivant d'un acide gras polyinsaturé de 20 atomes de carbone (eicosa-), de l'acide arachidonique (AA) [202]. Il s'agit d'une famille complexe de médiateurs lipidiques intervenant dans la régulation d'une variété de processus physiologiques et pathologiques. Toutefois, malgré leur implication dans la régulation de l'homéostasie cellulaire, entre autres par la protection de la muqueuse gastrique et l'agrégation plaquettaire, ils sont aujourd'hui connus pour leurs rôles dans la régulation de processus immunopathologiques, notamment dans le cas du cancer, l'asthme, RA et les maladies autoimmunes [203]. Les acides gras desquels dérivent les eicosanoïdes sont divisés en deux classes; n-6 l'acide linoléique et n-3 l'acide linolénique connus également sous le nom d'oméga 3 et oméga 6, respectivement. L'acide linoléique sert de substrat pour la synthèse de l'acide arachidonique et d'autres eicosanoïdes dans les cellules humaines. Ces acides gras sont uniquement apportés par l'alimentation car ils ne peuvent être synthétisés par les cellules, [204].De ce fait, la biosynthèse des eicosanoïdes dépend de la biodisponiblité de l'AA libre. Lorsque les tissus sont exposés à divers stimuli physiologiques et pathologiques tels que facteurs de croissance, hormones et cytokines, l'AA est libéré des phospholipides membranaires sous l'action de la phospholipase A2 (PLA2) agissant au niveau de la liaison sn-2, il sera oxygéné par la suite par les cyclooxygénases (COX) afin de former la PGG2, qui sera par la suite réduite pour générer un métabolite instable la PGH2 qui fera l'objet de conversion par d'autres enzymes dont les COXs, lipoxygénases (LOXs) et cytochrome P450 (Cyt p450).

I.B.3.1 Biosynthèse des eicosanoïdes:

I.B.3.1.1 La Phospholipase A2:

Il existe une quinzaine de gènes reponsables de la synthèse des différentes formes de PLA2 existant chez les mammifères. Quatre classes de PLA2 ont été identifiées: **i)** *PLA2 sécrétée* (sPLA2) avec un faible poids moléculaire (14-19 kDa) et regroupant 10 enzymes distinctes identifiées. Cette enzyme requiert le calcium pour hydrolyser l'AA au niveau de la position sn-2 et induit sa libération de manière stimulus indépendante [205, 206]; **ii)** la deuxième classe représentée par *PLA2 Calcium indépendante*, groupe intracellulaire VI (GVI iPLA2) avec un poids moléculaire de 85 kDa, son activité est maximale même en absence du calcium, **iii)** *PLA2 cytosolique PLA2 groupe IV* (GIV cPLA2) [170], et le groupe PAF acetylhydrolase [207, 208].

L'AA est la molécule cible de ces isozymes, la quantité libérée de la membrane détermine le niveau d'eicosanoïdes produit. Autrement dit la PLA2, l'enzyme impliquée de sa libération est la responsable de la détermination de ce niveau [209, 210], son activité est régulée par des hormones et des cytokines, notamment des cytokines pro-inflammatoires. Ceci explique l'augmentation de la production des eicosanoïdes lors d'une inflammation [211].

Bien que les différentes formes de PLA2 possédent la capacité de libérer l'AA des phospholipides membranaires, la cPLA2 semble être la plus impliquée dans sa libération pour la synthèse des eicosanoïdes, en raison de sa spécificité pour la position sn-2 [205, 212-214], pour ce faire son activation est étroitement contrôlée par la fixation d'ions calcium et par la phosphorylation en réponse à des stimuli externes [215]. Ces deux facteurs augmenteraient la translocation de la cPLA2 du cytosol vers l'appareil de Golgi, le réticulum endoplasmique et l'enveloppe nucléaire. Le calcium agit par sa liaison au niveau de l'extrémité N terminale du domaine C2 permettant ainsi au domaine catalytique de l'enzyme d'interagir avec l'AA, sa phosphorylation par la MAPK au niveau des résidus Ser 505, Ser727 ou Ser515 conduit à une augmentation du niveau d'expression [215-220].

I.B.3.1.2 Les cyclooxygénases (COX):

La cyclooxygénase appelée également (PGG/H synthase (PGHS), PG endoperoxidase synthase, ou PG synthase), est une enzyme membranaire d'un poids moléculaire de 72 kDa. Ces deux isoformes COX1 et COX2 sont du point de vue structural très semblables présentant une homologie de 63% [221]. Toutefois, malgré les similitudes entre les deux isoformes du point de vue structural, mécanismes catalytiques, produits et cinétiques, elles présentent certaines différences au niveau de leur site catalytique plus grand chez la COX2 que chez la COX1. Cette propriété a été utilisée pour une approche thérapeutique visant à produire des molécules ayant une action ciblée contre la COX2 [222]
.

Outre la différence au niveau de leur site actif, une autre différence au niveau de la région promotrice, est à l'origine de la différence d'expression entre les deux isoformes. Cependant, COX1 localisée sur le chromosome 9q32—q33.3 est responsable de la synthèse basale des prostanoïdes impliqués dans des fonctions physiologiques et est exprimée constitutivement dans la majorité des tissus. Son promoteur est dépourvu de la TATA box et contient deux sites Sp1 qui contribuent à ce type d'expression. La COX2 inductible localisée sur le chromosme 1q25.2—q25.3 renferme au niveau de son promoteur des séquences transcriptionnelles régulatrices inductibles dont le motif NF-IL6, deux sites AP-2, trois sites Sp1, deux sites NF-κB, un motif CRE et E-box. Les inducteurs de la COX2 sont représentés principalement par les cytokines inflammatoires d'où son implication dans plusieurs pathologies [223-226].

Paradoxalement, la COX2 peut être exprimée de manière constitutive dans certains types cellulaires comme l'endothélium vasculaire et l'épithélium respiratoire où elle est responsable de l'élaboration de l'homéostasie de certaines fonctions cellulaires. L'une comme l'autre de ces enzymes sont responsables de la biosynthèse des prostaglandines (PGs), et thromboxane à partir de l'AA converti en PGH2 [203, 224].

De plus, bien que la cinétique des deux isoformes soit très proche l'une de l'autre, mais une différence d'affinité pour l'AA est notée entre les deux isoformes. La COX1 possèdant un

allostérisme négatif à faibles concentrations d'AA n'est capable de l'utiliser qu'à des concentrations plus importantes de l'ordre de 10 μM provenant d'une source exogène principalement apportée par l'alimentation ou générée lors d'une l'inflammation. Quant à la COX-2, elle peut l'utiliser à des concentrations plus faibles de l'ordre de 2.5 μM, il s'agit généralement de l'AA endogène [223, 227].

Contrairement, à la COX1 exprimée de manière constitutive, une augmentation d'expression et de production de COX2 sont notées lors d'épisodes inflammatoires, notamment au niveau de la membrane synoviale lors de l'OA [228, 229]. Une étude menée par *Geng et al*, a montré que contrairement aux chondrocytes articulaires de lapin exprimant de manière constitutive la COX2, et dont l'expression n'est affectée que par de très fortes concentrations d'IL-1β, les chondrocytes humains à leur tour montrent une augmentation d'expression de la COX2 sous l'effet de médiateurs inflammatoires [230, 231]. Son expression est également augmentée sous l'action d'hormones, facteurs de croissance et lipopolysaccharides. Elle est toutefois responsable de l'augmentation de la synthèse des prostanoïdes responsables des effets pathobiologiques. Cependant, au moment où la COX2 produit préférentiellemt la PGE_2 et la prostacycline (PGI2), la COX1 assure le maintien de l'équilibre entre les différents prostanoïdes [232].

Des résultats contradictoires ont été rapportés quant à la localisation de la COX1 et de la COX2. Des travaux réalisés par *Morita et al*, ont montré que la COX2 était associée à la membrane nucléaire, tandis que la COX1 était confinée au réticulum endoplasmique. D'autres travaux réalisés par *Spencer et al*, ont conclu que la COX1 et la COX2 étaient présentes aussi bien au niveau du réticulum endoplasmique qu'au niveau de la phase interne et externe de l'enveloppe nucléaire [233, 234]. L'analyse du produit des deux isoformes par l'utilisation d'inhibiteurs spécifiques a montré qu'elles génèrent le même produit au niveau des cellules NIH 3T3 murines. Ceci indique que la localisation n'affecterait en rien leur accès à l'acide arachidonique, ni la fonction des isoformes [234].

En plus de COX1 et COX2, une autre isoforme de COX (COX3), dérivant du même gène que COX1, est exprimée au niveau du cortex cérébral et au niveau du cœur. Cette isoforme partage les mêmes propriétés catalytiques que COX1 et COX2 [235].

A- *Les prostanoïdes:*

Le PGH2 en lui-même ne joue pas un rôle important comme médiateur inflammatoire, il sert cependant de substrat pour des enzymes spécifiques responsables de la production de prostanoïdes plus stables. Ce terme regroupe la PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, et enfin les thromboxanes (TXA2). La synthèse des prostanoïdes s'effectue sous l'action des enzymes PGE_2, PGD_2, $PGF2_{\alpha}$, PGI_2 et TXA_2 synthases, respectivement, ou appelées également synthases terminales [236]. Les prostaglandines (PGs) PGE_2 et PGD_2 sont rencontrées dans une variété de tissus humains ainsi que chez des animaux primitifs. Elles ont été découvertes pour la première fois en 1930 par *Von Euler en Suède* et *Goldblatt au Royaume-Uni* [237]. Elles sont synthétisées de manière constitutive ou libérées à la suite d'un stimulus à l'extérieur de la cellule immédiatement après leur synthèse [238, 239].

La demi-vie des prostanoïdes est courte en raison de leur instabilité, car elles font l'objet de dégradation par de nombreuses enzymes dans le microenvironnement où elles sont générées [240, 241]. Les PGs sont impliquées dans de nombreuses fonctions biologiques telles que la douleur, l'inflammation, l'agrégation plaquettaire et le maintien de l'homéostasie gastro-intestinale et rénale [237]. Le mécanisme par lequel le PGH2 est converti en une PG ou à une autre n'est toujours pas connu (**Figure 4).**

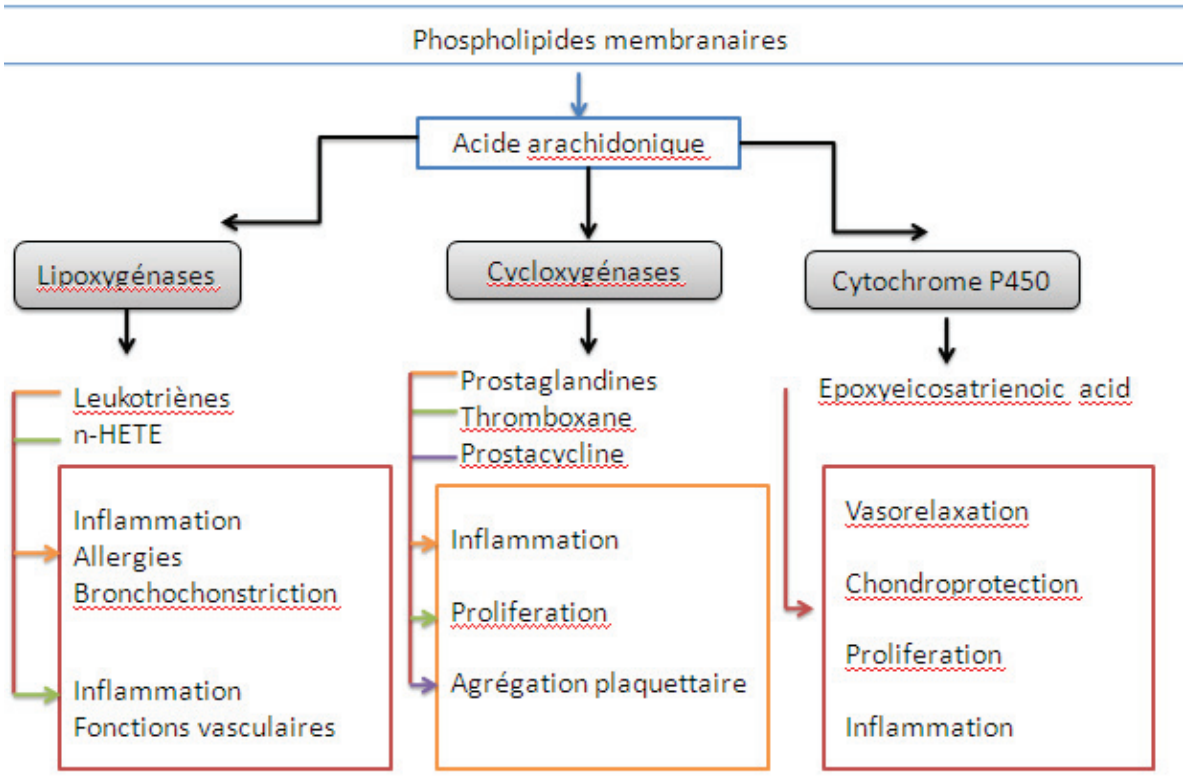

Figure 4: Biosynthèse des eicosanoïdes (Adapté de [242]).

B- Récepteurs des prostanoïdes:

Les PGs exercent leur action via des récepteurs couplés à la protéine G. La PGE_2 possède quatre récepteurs EP1-EP4 codés par des gènes différents et dont l'expression est spécifique au type cellulaire [213]. Quant à la PGD_2, elle exerce ses effets via deux types de récepteurs DP et CRTH2, exprimés par les cellules Th2 [240]. Les prostanoïdes PGF2α, PGI2 et TXA2 exercent leur action via les récepteurs, FP, IP et TP respectivement [243]. En plus des récepteurs membranaires, les prostanoïdes peuvent exercer leurs effets via des récepteurs hormonaux nucléaires, appelés également *peroxisome proliferator activator* (PPARs). Il existe cependant, trois isoformes de PPAR, PPARα, β/δ, et γ, qui ont été isolés et caractérisés [244]. Le métabolite de la PGD_2 formé par hydratation, le 15d-PGJ2 se lie de façon spécifique à PPARγ, alors qu'un analogue stable de PGI_2 et carbaprostacyclin (cPGI) se lie à PPAR α et δ [245].

C- Prostaglandine E_2 (PGE_2):

Le prostanoïde est le plus abondant au niveau du corps humain, son implication varie entre ses fonctions physiologiques et pathologiques, il peut cependant avoir un effet homéostasique, inflammatoire ou encore anti-inflammatoire, son inhibition est une cible thérapeutique depuis une centaine d'années [213]. La PGE_2 reste le prostanoïde majeur généré lors d'une inflammation. L'isoforme COX2 est l'enzyme principale responsable de sa biosynthèse [223, 246]. La PGE synthase (PGES), l'enzyme terminale responsable de sa génération existe sous trois isoformes incluant la **i)** PGES cytosolique (cPGES), exprimée dans une variété de tissus et capable de convertir le PGH2 issu de COX1 et non de COX2 en PGE_2 [247]. **ii)** La mPGES-1 localisée sur le chromosome 9q34.4 est purifiée à partir de fractions microsomales des glandes vésiculaires des bovins et des moutons. Cette molécule est instable et perd son activité dans les 30 minutes suivant son isolement et requiert le groupe gluthation pour son activité qui peut être remplacé par un autre composé dans

certains organes comme le cœur et la rate. L'IL1-β induit la mPGES-1 par la stimulation des voies *Extracellular signal-regulated kinase* (ERK) et p38 [223, 248]. L'expression de mPGES1 est augmentée à la suite d'un stimulus inflammatoire, augmentation couplée à l'amplification de l'expression de COX2 responsable de la biosynthèse de la PGE_2 [249, 250]. **iii)** La troisième isoforme est mPGES2**,** qui assure différentes fonctions physiologiques et pathologiques *in vivo*, son ARNm est exprimé dans différentes régions du cœur et du cerveau, aucune production n'a été rapportée au niveau des organes génitaux [248].

La PGE_2 générée sous l'action de synthases spécifiques joue un rôle important dans la physiopathologie de nombreuses maladies dont l'OA. Cependant, la PGE_2 est le prostanoïde le plus abondant dans l'articulation des patients atteints ou encore chez des modèles animaux induits où une production excessive de PGE_2 a été rapportée au niveau du sérum, du liquide synovial et du cartilage. La PGE_2 est principalement synthétisée par les chondrocytes, synoviocytes, et macrophages [250, 251]. Cette augmentation contribue à l'inflammation et au catabolisme au niveau du cartilage articulaire [251]. De plus, la PGE_2 est fortement impliquée dans la résorption du cartilage. Ses effets sont exercés via la stimulation de la dégradation des composantes de la MEC dont le ColII et les GAGs sulphatés [252] sous l'action de protéinases spécifiques libérées par les chondrocytes [253], synoviocytes [254], explants de cartilage OA humains [252, 255], aussi par l'induction de l'apoptose chondrocytaire via la voie AMPc/PKA conduisant à l'élevation de l'AMPc intracellulaire suivie par l'activation de la PKA [252, 256-258]. La PGE_2 augmente également le dépôt de la matrice cartilagineuse autour des chondrocytes hypertrophiques lors de l'apoptose au niveau de la plaque de croissance [258, 259]. En plus de ces effets, elle promeut l'expression de l'IL-1β. Ce qui conduit à l'amplification du processus inflammatoire, sa production *in vivo* et *in vitro* a lieu au cours de la phase retardée où la COX2 (mais pas la COX1) joue un rôle important [247]. L'implication de la PGE_2 dans l'inflammation peut être liée également à son action vasodilatatrice et à l'augmentation de la perméabilité vasculaire [252].

Outre ces effets, la PGE_2 promeut la différenciation et la proliferation des chondrocytes de la plaque de croissance [260], ainsi que la résorption osseuse via la stimulation de la formation d'ostéoclastes à partir des cellules souches [257]. En plus de ces effets, la PGE_2 stimule l'apoptose chondrocytaire par des effets directs médiés par l'AMPc, suggérant l'implication des récepteurs EP2 ou EP4 [258], ou indirectement à travers le NO libéré induit par iNOS [261]. Enfin elle contribue à la néoangiogénèse par l'induction de la production de facteurs proangiogéniques [262] **(Tableau 5).**

Tableau 5: Résumé des principaux effets exercés par la PGE_2 au niveau des tissus articulaires OA(Adapté de [257])

Effets induits par la PGE_2
Diminue la synthèse du collagène
Augmente l'apoptose chondrocytaire
Augmente la production des MMPs
Augmente la production de médiateurs inflammatoires
Augmente la production de facteurs angiogéniques
Augmente la production et l'apoptose des cellules endothéliales

Tous ces événements sont responsables des effets cataboliques de la PGE_2, ainsi sa neutralisation empêche la dégradation du cartilage induite par les cytokines [263], augmente la synthèse des protéoglycanes dans le cartilage OA [264], et prévient le développement d'œdème et d'hyperalgésie dans un modèle d'arthrite induite, *Adjuvent-Induced Arthritis (AIA)* [265]. De plus, la PGE_2 est responsable de la douleur comme elle potentialise les effets des autres médiateurs inflammatoires [257]. La génération de l'AMPc au niveau des chondrocytes articulaires de lapin est liée à la stimulation des

protéoglycanes [258] par opposition aux effets de l'IL-1β via la stimulation de l'expression des gènes du ColII.

Ces différents effets antagonistes de la PGE_2 sur le cartilage dépendent de sa liaison à des récepteurs différents dont chacun est couplé à des réponses distinctes. Une étude menée par *Mitsui et al*, utilisant un agoniste spécifique a révélée que la PGE_2 agit comme anti-inflammatoire via EP2 [266]. En ce qui a trait aux effets anti-cataboliques de la PGE_2, d'autres réultats *in vitro* obtenus par S*ato et al*, montrent que la PGE_2 via EP2 exerce des effets protecteurs sur le cartilage articulaire; par la diminution de l'expression de MMP-13. Ceci concorde avec les effets obtenus *in vivo* chez le lapin [267, 268]. À l'opposé, la liaison de la PGE_2 à son recepteur EP4 conduit à une réponse catabolique, contrairement à sa liaison à EP2 où elle supprime cet effet [252]. Une étude réalisée par *Alvarez-Soria et al*, a montré que les recepteurs EP sont exprimés au niveau des chondrocytes humains OA et que l'expression de EP2 et de EP4 est augmentée en présence d'IL-1β [269]. Toutefois, le mécanisme exact de la régulation de ces récepteurs durant l'OA est encore obscure. Néanmoins, la PGE_2 présente au niveau du liquide synovial est impliquée dans la destruction de l'articulation via ces récepteurs [266]. La stimulation de EP2 ou de EP4 par des agonistes spécifiques augmente l'activité des ostéoblastes de souris, et induit la transcription du ligand NF-κB (RANKL) [270] produit par les ostéoblastes. Ce dernier est essentiel à la résorption osseuse via la régulation de l'ostéoclastogénèse en se liant au récepteur RANK situé sur les précursseurs d'ostéoclastes et sur les ostéoclastes matures [271-276]. L'ostéoprotégrine (OPG) est sécrétée par les cellules stromales et d'autres types cellulaires comme les ostéoclastes, sa liaison à RANKL inhibe l'interaction RANK-RANKL prévenant ainsi l'activation de RANK et par conséquent l'ostéoclastogénèse [277]. L'analyse chez des souris EP-déficientes a montré que la PGE_2 stimule la résorption osseuse via la voie de signalisation EP4-cAMP [278]. De ce fait, la PGE_2 stimule la production de RANKL [270, 279] et inhibe la production de l'OPG au niveau des ostéoblastes de manière cAMP dépendante [215, 280] **(figure 5).**

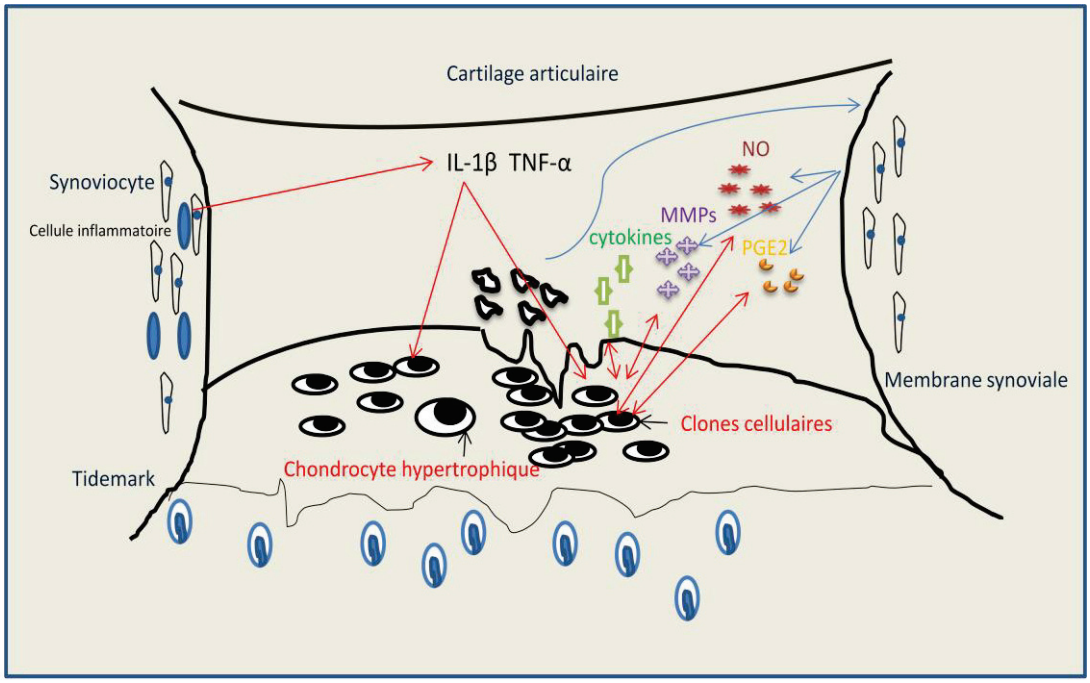

Figure 5: Schéma récapitulatif de la physiopathologie de l'OA (Adapté de [106]).

La PGE_2 est la molécule la plus étudiée au cours de l'OA. Peu de travaux ont été consacrés à l'étude des autres prostanoïdes, notamment la PGD_2, qui fait l'objet de cette thèse. Cependant, des travaux réalisés par *Gallant et al*, ont montré la production de la PGD_2 par les ostéoblastes humains. Les deux types de récepteurs DP1 et CRTH2, sont également exprimés par ces cellules et sont fonctionnels [371]. Les effets de la PGD_2 au cours de l'OA seront dicutés un peu plus loin. Concernant, les prostanoïdes PGI_2 et TXA_2, à leur tour, ont fait l'objet de peu de travaux dans le cas de l'OA.

D- Prostaglandine I_2:

Prostanoïde majeur, produit par l'endothélium vasculaire exerçant son effet via le récepteur IP, il agit comme vasodilatateur et inhibiteur de l'agrégation plaquettaire. Tout comme la PGE_2, la PGI_2 est également impliquée dans la réponse à la douleur [281]. Cependant, les liquides synoviaux de patients atteints OA et de RA contiennent un taux élevé de PGI_2 [281]. Plusieurs travaux ont montré l'implication de la PGI_2 dans la douleur aigue [282]. Des résultats obtenus de modèles animaux d'OA induite par le MonoIodoAcétate (MIA) et d'arthrite induite par le *Collagen Antibody Induced Arthritis* (CAIA), ont montré des taux élevés de PGI_2 au niveau des tissus enflammés. L'utilisation d'antagoniste du récepteur IP a montré des effets anti-inflammatoires supportant la contribution de la PGI_2 au développement d'arthrite chronique [283]

E- Thromboxane A2:

Contrairement à la PGI_2, TXA_2 est un vasoconstricteur majeur et un agent potentiel induisant l'agrégation plaquettaire. Il exerce ses effets via un récepteur appelé TP [241]. Le TXA_2 induit la synthèse des cytokines pro-inflammatoires telles que l'IL-1β et TNF-α [282], son implication dans les maladies arthritiques n'a pas été démontrée.

I.B.3.1.3 Voie des lipoxygénases (LOXs) :

Cette voie donne lieu principalement à la biosynthèse des leukotriènes (LTs) et des hépoxillines. Composés impliqués dans plusieurs réponses biologiques importantes telles que l'inflammation et les réactions d'hypersensibilité. Les LOXs constituent une famille d'enzymes incorporant l'oxygène moléculaire à une position bien déterminée au niveau des acides gras polyinsaturés. Les différentes oxygénases sont la 5-, 8-, -12 et -15 LOXs, nommées selon la position du carbone de l'AA subissant l'oxygénation. Les plus étudiés

sont la 5-LOX, la 12-LOX exprimées chez les leucocytes et les plaquettes et la 15-LOX1 exprimée au niveau des réticulocytes [284, 285].

Dans les tissus humains trois types de LOX ont été caractérisées, il s'agit de la 5-LOX, 12-LOX et 15-LOX. Cette dernière existe sous deux isoformes différant par rapport à la distribution cellulaire et le substrat utilisé. La 15-LOX1 est exprimée au niveau des réticulocytes, éosinophiles, la peau et macrophage. Elle convertit l'AA en 15(S)-hydroxyeicosatetraenoic acid (15-HETE), tandis que la 15-LOX2 retrouvée au niveau de la prostate, les poumons, la peau et la cornée [284, 286].

L'AA est métabolisé par les LOXs en hydroperoxy–eicosatetraenoic acids (HPETEs) qui après une réaction de réduction conduit à la formation du HETEs, tandis que la conversion de l'acide linoléique conduit préférentiellement à la formation de hydroxy–octadecadienoic acids (HODEs) [287].

A- La 5-lipoxygénase (5-LOX):

La 5-LOX est une enzyme qui fait partie de la famille des LOXs ayant pour rôle de catalyser l'ajout d'une molécule d'oxygène à l'AA. Il s'agit de l'enzyme initiale intervenant dans la biosynthèse des leukotriènes (LTs) et est impliquée dans la régulation de l'inflammation [288]. Des études réalisées par *Abramovitz et al*, ont montré la présence d'une protéine FLAP qui en plus de permettre une utilisation efficace de l'AA par la 5-LO chez une lignée cellulaire Sf9, elle augmente la capacité de conversion du 5-HPETE en LTA4 [289]. Dans le cas de la 5-LOX, le HPETE est métabolisé pour former un composé instable LTA4, qui sera convertit en 5(S)-hydroxy-6-trans8,11,14-cis-eicosatetranoic acid (5-HETE) ou hydrolysé en LTB4 par la LTA4 hydrolase ou encore en cysteinyl leukotrienes (LT), LTC4, LTD4 and LTE4 [285]

B- La 15-lipoxygénase (15-LOX) :

Comme il a été précisé précédemment, la 15-LOX est subdivisé en deux isoformes 15-LOX1 et 15-LOX2 dont la régulation a lieu au niveau transcriptionnelle, traductionnelle et post-traductionnelle. La 15-LOX1 est exprimée par les réticulocytes, éosinophiles et les cellules

épithéliales. Cette isoforme joue un rôle important dans la différenciation cellulaire, l'asthme et l'inflammation [285]. La 15-LOX1 intervient dans la synthèse des LOXs dont les principales sont la lipoxine A4 (LXA4), LXB4 et la 15-epi lipoxin. Les lipoxines diminuent le chimiotactisme des polynucléaires et leur adhésion aux cellules endothéliales. Leur rôle anti-inflammatoire s'oppose donc à celui des LTs pro-inflammatoires [290].

Plusieurs études ont montré que la 15-LOX et ses métabolites ont des propriétés anti-inflammatoires et immunomodulatrices. Les métabolites de la 15-LOX inhibent la production du TNF-α, cytokine clé impliquée dans RA [291, 292]. De plus, les métabolites 15-HETE et 13-HODE sont des ligands du récepteur PPARγ [293, 294] dont l'activation supprime la production des MMP dans plusieurs types cellulaires, notamment les chondrocytes et les synoviocytes. Des travaux de notre laboratoire, [287] ont montré que les chondrocytes arthrosiques expriment 15-LOX1 et 15-LOX2. Ils ont également montré que le traitement avec les métabolites 13-HODE et 15-HETE produits par 15-LOX1 et 15-LOX2 respectivement, induisent la diminution de l'expression de MMP-1 et MMP-13 induites par l'IL1-β, ainsi que la dégradation du ColII. Ces effets suggèrent fortement l'effet chondroprotecteur des 15-LOX par diminution de la production de MMP-1 et MMP-13. L'ensemble de ces données suggèrent que 13-HODE et 15-HETE inhibent la production de MMP-1 et MMP-13 via PPARγ. En plus de ces métabolites, les 15-LOXs produisent des molécules anti-inflammatoires telles que les lipoxines, resolvins et les protectins [295]. Par conséquents, les 15-LOX peuvent contrer l'inflammation par la production de classes distinctes de métabolites anti-inflammatoires.

I.B.3.1.4 Voie du cytochrome P450:

Les familles COX et LOX sont responsables du métabolisme de l'AA qui aboutit à la formation de PGs et de LTs, respectivement. Elles représentent la cible pour des traitements contrant la douleur, l'inflammation, l'asthme et les allergies. L'AA peut également être métabolisé par une autre voie enzymatique, le cytochrome P450, qui aboutit à la formation d'hydroxyeicosatetraenoic (HETEs) et epoxyeicosatrienoic acids (EETs).

Cette voie est plus présente dans les tissus présentant de faibles activités de COX et de LOX, la voie du cytochrome P450 semble être impliquée dans les maladies inflammatoires et cardiovasculaires, elle est également impliquée dans la croissance tumorale [242, 296].

CHAPITRE II : TRAVAUX RÉALISÉS

Rationnelle de l'étude:

L'OA, longtemps considérée comme une maladie résultante du processus naturel du vieillisement à l'origine des failles au niveau du cartilage articulaire n'attirait guère l'attention. Mais en regard de la souffrance des patients qui en sont atteints, de l'absence de toute médication efficace auxquels s'ajoutent les retombées socioéconomiques, tous ces éléments ont conduit à amplifier les recherches sur la maladie.

L'objectif de cette thèse est de contribuer à l'avancement des connaissances sur la maladie en vue de concevoir une médication appropriée, non seulement capable d'atténuer la douleur, mais aussi de ralentir la maladie et pourquoi pas de l'arrêter. Bien que l'OA touche l'organe (l'articulation) avec ses différents tissus et structures annexes, nous nous interesserons dans cette thèse de façon particulière au cartilage articulaire, vu son rôle central dans la maladie, et du fait de son influence sur l'ensemble des composants.

Pour ce faire, des cultures primaires de chondrocytes provenant de sujets atteints sont l'outil de base dans nos expériences. L'utilisation de ces cultures ont permis auparavant de faire la lumière sur les différentes molécules impliquées dans l'OA qu'elles soient anaboliques ou cataboliques. Dans notre laboratoire, plusieurs travaux ont mis en évidence la prostaglandine 15d-PGJ_2, avec ses effets anti-inflammatoires au cours de l'OA via le récepteur PPARγ, ce qui sera détaillé dans la discussion. Comme le 15dPGJ_2 résulte de la conversion de la PGD_2 et que cette dernière n'a pas fait l'objet de travaux auparavant, j'évaluerais dans **cette thèse le rôle de la PGD_2 au cours de l'OA.**

Article I: Inhibition de la production de MMP-1 et MMP-13 induites par l'IL-1β sous l'effet de la PGD_2 au niveau des chondrocytes OA humains.

Hypothèse:

La PGD_2 de laquelle dérive le 15d-PGJ_2, ligand naturel de PPARγ connu par ses effets anti-inflammatoires pourrait être impliquée dans l'OA par la diminution de l'expression de médiateurs cataboliques impliqués dans la dégradation de la MEC, entre autres les MMPs.

Buts :

1- Etudier l'effet de la PGD_2 sur MMP-1 et MMP-13 connus pour leurs effets dans la dégradation de la MEC
   - a- La production de MMP-1 et MMP-13
   - b- L'expression ARNm et l'activité du promoteur de MMP-1 et MMP-13

2- Définir quel récepteur de la PGD_2 est responsable des effets obtenus

3- Définir la voie de signalisation mise en œuvre par le récepteur.

Résumé:

Le rôle de la PGD_2 reste très peu connu au cours de l'OA. Afin de mieux connaître son rôle, des chondrocytes dérivés de genoux de patients OA et des explants de cartilage ont été traitées par l'IL-1β et des concentrations croissantes de la PGD_2. Le niveau de production de MMP-1 et MMP-13 sous l'effet de la PGD_2 est évalué par ELISA et le niveau d'expression de l'ARNm et l'activité du promoteur sont évalués par la RT-PCR et des expériences de transfection transitoire, respectivement. Ces expériences ont montré une diminution de MMP-1 et MMP-13 de manière dose dépendante avec les concentrations croissantes de la PGD_2, aussi bien au niveau des cultures de chondrocytes qu'au niveau d'explants de cartilage. L'utilisation d'agonistes spécifiques aux récepteurs DP1, BW245C et au CRTH2, DK-PGD_2, via lesquels la PGD_2 exerce ses effets a permis de mettre en évidence que la PGD_2 diminue la production de MMP-1 et MMP-13 via DP1 et non via CRTH2, et plus précisément par l'activation de la voie AMPc/PKA, tel que révélé par des agents capables d'augmenter le niveau de l'AMPc et d'inhiber la PKA. En conclusion, la PGD_2 possède des effets anticataboliques au cours de l'OA par la diminution de la production et de l'expression de MMP-1 et MMP-13 via la voie DP1/AMPc/PKA.

Prostaglandin D_2 inhibits interleukin-1β-induced matrix metalloproteinase-1 and -13 production by human osteoarthritic chondrocytes.

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Contribution:

Hassan Afif a participé au design de l'étude, à l'acquisition, à l'analyse et à l'interprétation des données. Prs. Martel-Pelletier et Pelletier nous ont permis d'obtenir les tissus, comme ils ont contribué au design de l'étude et à l'acquisition des données. Dr. Nicolas Duval nous a également fourni des tissus. Dr. Mohamed Trebak a contribué au design de l'étude, avec Motiani Rajender ont aussi participé à l'acquisition, à l'analyse et à l'interprétation des données. Dr. Benderdour, Chabane et Mfuna-léondra ont contribué à l'acquisition, à l'analyse et à l'interprétation des données. Dr. Fahmi et moi avons contribué au design de l'étude, à l'acquisition, l'analyse, l'interprétation des données, et à la préparation du manuscrit.

ABSTRACT

Objective. To investigate the effects of prostaglandin (PG) D_2 on interleukin-1β (IL-1)-induced matrix metalloproteinase (MMP)-1 and MMP-13 expression in human chondrocytes and the signalling pathways involved in the effects of PGD_2.

Methods. Chondrocytes were stimulated with IL-1 ± PGD_2 and MMP-1 and MMP-13 protein expression was evaluated by ELISA. mRNA expression and promoter activity were analyzed by real-time RT-PCR and transient transfections, respectively. The role of the PGD_2 receptors, D prostanoid receptor 1 (DP1) and chemoattractant-receptor-like molecule expressed on Th2 cells (CRTH2), was evaluated using specific agonists and antibody blocking experiments. The contribution of the cAMP/PKA pathway was determined using cAMP elevating agents and PKA inhibitors.

Results. PGD_2 dose-dependently decreased IL-1-induced MMP-1 and MMP-13 protein and mRNA expression as well as their promoter activation. DP1 and CRTH2 are expressed and functional in chondrocytes. The effect of PGD_2 was mimicked by the selective DP1 agonist BW245C, but not by the CRTH2 selective agonist DK-PGD_2. Furthermore, treatment with an anti-DP1 antibody reversed the effect of PGD_2, indicating that the inhibitory effect of PGD_2 is mediated by DP1. The cAMP elevating agents, 8-Br-cAMP and forskolin, suppressed IL-1-induced MMP-1 and MMP-13 expression, and the PKA inhibitors, KT5720 and H-89, reversed the inhibitory effect of PGD_2, suggesting that the effect of PGD_2 is mediated by the cAMP/PKA pathway.

Conclusion. PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 production by chondrocytes through the DP1/cAMP/PKA signalling pathway. These data also suggest that modulation of PGD_2 levels in the joint may have therapeutic potential in the prevention of cartilage degradation.

INTRODUCTION

The destruction of articular cartilage is a typical pathological characteristic of arthritic diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). The degradative process is believed to be largely mediated by proteases belonging to the metalloproteinase (MMP) class of enzymes (1). Among these, MMP-1 and MMP-13 are considered to be of particular interest since they directly degrade the components of the cartilage matrix including aggrecan and collagen (2-4). Proinflammatory cytokines such as interleukin-1β (IL-1), are known to strongly induce the production of MMP-1 and MMP-13 by articular joint cells, including chondrocytes (4; 5).

Many inhibitors of MMP activity have been developed over the past 25 years and have been shown to inhibit cartilage and bone destruction in several animal models of OA (6; 7) and RA (8; 9). However, results from clinical trials failed to demonstrate a beneficial effect of MMP activity inhibition on the progression of joint damage in arthritis (10-12). Therefore, an understanding of the factors and pathways that regulate MMP-1 and -13 expression is of major importance to understand and possibly to prevent cartilage damage in arthritis.

In addition to MMPs, prostaglandins (PGs) also play an important role in cartilage metabolism and inflammation associated with arthritis (13) and inhibitors of PG production are widely used in the treatment of OA and RA. PGs are formed from arachidonic acid through the action of cyclooxygenase (COX). COX converts arachidonic acid to an intermediate substrate PGH_2, which is further metabolized by specific terminal synthases to generate PGE_2, PGD_2, $PGF_2\alpha$, PGI_2 and thromboxane (13). PGD_2 is involved in the regulation of multiple physiological and pathological processes, including sleep, nociception, vasodilatation, bronchoconstriction, and bone metabolism. In addition, PGD_2 has been shown to display anti-inflammatory effects in several models of inflammation (14-17). PGD_2 elicits its downstream effects by activating two plasma membrane receptors, the D prostanoid receptor (DP) 1 (18) and chemoattractant-receptor-like molecule expressed on Th2 cells (CRTH2), also known as DP2 (19). DP1 stimulation results in an increase in

intracellular levels of cAMP and subsequent activation of protein kinase A (PKA) (20), while CRTH2 activation leads to calcium mobilization (19).

Most studies to date addressing the role of PGs in chondrocyte metabolism have focused on PGE_2, while much less is known about the role of PGD_2 in this process. In the present study we investigated the effect of PGD_2 on MMP-1 and MMP-13 production by human chondrocytes. We demonstrated that PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 production. We also showed that this effect is mediated through the DP1/cAMP/PKA signalling pathway.

MATERIALS AND METHODS

Reagents. Recombinant human (rh) IL-1 was obtained from Genzyme (Cambridge, MA). PGD_2, BW245C, 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2), and anti-DP1 antibody were from Cayman Chemical Co. (Ann Arbor, MI). Anti-CRTH2 antibody was from BD Pharmingen (Mississauga, ON, Canada). 8-Bromo-cAMP (8-Br-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and the PKA inhibitors H-89 and KT5720 were from Calbiochem, EMD Biosciences (San Diego, CA). Cycloheximide, ionomycin and forskolin were from Sigma-Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from either Sigma-Aldrich Canada or Bio-Rad (Mississauga, ON, Canada).

Chondrocyte isolation and treatment. Articular cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement (n = 46, mean ± SD age: 67 ± 17 years). All OA patients were diagnosed according to the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (21). At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX2 inhibitors. Patients who had received intraarticular injection of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as previously described (22). Briefly, small pieces of cartilage were incubated with 2 mg/ml pronase for 1 hour followed by 1 mg/ml collagenase (type IV; Sigma-Aldrich) for 6 hours at 37°C in DMEM and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS.

Confluent chondrocytes were detached by trypsinization, seeded at $3.5x10^5$ cells per well in 12-well culture plates (Costar, Corning, NY) or at $7x10^5$ cells per well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 hours. Cells were washed and incubated for an additional 24 hours in DMEM containing 0.5% FCS, before stimulation with either IL-1 alone or in combination with PGD_2, BW-245C, DK-PGD_2, 8Br-cAMP or forskolin (the drugs were added at the same time as IL-1). In another set of experiments chondrocytes were pretreated for 30 minutes with cycloheximide, IBMX, PKA inhibitors (H89, KT-5720), anti-DP1 or anti-CRTH2 antibody before stimulation with IL-1 or PGD_2. The level of MMP proteins released in supernatants was determined 24 hours after stimulation, whereas the level of MMP mRNA was determined at 8 hours. Only first passaged chondrocytes were used.

Cartilage explants were placed in the wells of a 24-well plate (80-100 mg/well) and maintained in DMEM with 0.5% FCS for 72 hours. The explants were washed and incubated for an additional 24 hours in the same medium before stimulation with IL-1 alone or co-stimulation with IL-1 and PGD_2 (IL-1 and PGD_2 were added simultaneously) for 72 hours. Conditioned media were harvested and the levels of MMP proteins were determined by ELISA.

MMP-1, MMP-13 and cAMP determinations. The levels of MMP-1 and MMP-13 in conditioned media were determined by specific enzyme-linked immunosorbent assays (ELISA, R&D Systems Inc, Minneapolis, MN). The intracellular levels of cAMP were measured using a cAMP enzyme immunoassay (EIA) from R&D systems Inc. All measurements were performed in duplicate.

Western blot analysis. Chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12 000 rpm for 15 minutes. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL). Twenty µg of total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and

electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a Tris buffer (Tris-buffered saline (TBS) pH 7.5, with 0.1% Tween 20). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak Ltd, Rochester, NY).

RNA extraction and real-time quantitative PCR. Total RNA was isolated using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR), dissolved in diethylpyrocarbonate (DEPC)-treated-H2O and stored at -80°C until use. One µg of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as detailed in the manufacturer's guidelines. One fiftieth of the reverse transcriptase reaction was analyzed by traditional PCR or real-time quantitative PCR. The following primers were used: MMP-1, sense 5'-CTGAAAGTGACTGGGAAACC-3' and antisense 5'-AGAGTTGTCCCGATGATCTC-3'; MMP-13, sense 5'-CTT AGA GGT GAC TGG CAA AC-3' and antisense 5'-GCC CAT CAA ATG GGT AGA AG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-CAGAACATCATCCCTGCCTCT-3' and antisense 5'-GCTTGACAAAGTGGTCGTTGAG -3'; DP1, sense 5'-GCAACCTCTATGCGATGCAC and antisense 5'-CAAGGCTCGGAGGTC TTCT-3', and CRTH2, sense 5'-CCTCTGTGCCCAGAGCCCCACGATGTCGGC-3' and antisense 5'-CACGGCCAAGAAGTAGGTGAAGAAG-3'

Quantitative PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM of sense and antisense primers, 25 µl of SYBR® Green master mix (QIAGEN, Mississauga, ON, Canada) and uracil-N-glycosylase (UNG, 0.5 Unit, Epicentre Technologies, Madison, WI). After incubation at 50°C for 2 minutes (UNG reaction), and

at 95°C for 10 minutes (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 sec at 95°C for denaturation and 1 minutes for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative mRNA expression in chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising 2 to the $-\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

Plasmids and transient transfection. The human MMP-1 promoter/luciferase reporter plasmid (pMMP1-0.5kb-Luc) was kindly provided by Dr. C.E. Brinckerhoff (Dartmouth Medical School, Hanover, NH). The human MMP-13 promoter (pMMP-13-1.6kb-Luc) has been previously described (22). β-galactosidase reporter vector under the control of SV40 promoter (pSV40-β-galactosidase) was from Promega (Madison, WI). Transient transfection experiments were performed using FuGene-6 (1 µg DNA for 4 µl FuGene 6) (Roche Applied Science, Laval, QC, Canada) according to the manufacturer's recommended protocol. Briefly, chondrocytes were seeded 24 hours prior to transfection at a density of $6x10^5$ cells/well in 6-well plates and transiently transfected with 1 µg of the

reporter construct and 0.5 μg of the internal control pSV40-β-galactosidase. Six hours later, the cells were rinsed in phosphate-buffered saline and changed to medium containing 0.5% FCS for an additional 18 hours. The cells were then treated with IL-1 in the absence or presence of PGD_2 for 18 hours. In these conditions, transfection efficiency typically ranges between 40% and 50%. After harvesting, luciferase activity was determined and normalized to β-galactosidase activity (22). All of the transfection experiments were repeated at least three times in duplicate.

Calcium mobilization assay. Ca^{2+} mobilization assays were performed as previously described (23). Briefly, coveslips with attached chondrocytes were mounted in a Teflon chamber and incubated at 37°C for 50 minutes in culture media containing 5 μM Fura-2 AM (Molecular Probes, Eugene, OR). Cells were then washed 3 times and bathed in HEPES-buffered saline solution (140 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.13 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) for at least 10 minutes before Ca^{2+} measurements were made. Baseline was established a few minutes in the presence of extracellular Ca^{2+}, before addition of PGD_2 where indicated. Fura-2 fluorescence at an emission wavelength of 510 nm was induced by exciting Fura-2 alternately at 340 and 380 nm. Fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging Inc., Cincinnati, OH). The data are expressed as the ratio of Fura-2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm.

Statistical analysis. Data are expressed as the mean ± SEM. Statistical significance was assessed by the 2-tailed Student's t-test. P values less than 0.05 were considered statistically significant.

Results

PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 protein production in human chondrocytes and cartilage explants. To examine the effects of PGD_2 on MMP-1 and MMP-13 protein release, cultured human chondrocytes were stimulated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of PGD_2 (1-10 μM), and the amount of MMP-1 and MMP-13 proteins in conditioned media were measured by ELISA. As expected, IL-1 strongly enhanced the production of both MMP-1 and MMP13. Treatment with PGD_2 inhibited IL-1-induced MMP-1 and MMP-13 production in a dose-dependent manner (Fig. 1A). The utilized concentrations of PGD_2 did not affect chondrocyte viability as judged by the trypan blue exclusion assay and the MTT salt assay (data not shown). Next, we evaluated the effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 protein production in cartilage explants. As shown in Fig. 1B, PGD_2 dose-dependently suppressed IL-1-induced MMP-1 and MMP-13 release from cartilage explants.

PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 expression at the transcriptional level. To further characterize the effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 expression, we measured the steady-state level of MMP-1 and MMP-13 mRNAs by real-time PCR. As shown in Fig. 2A, IL-1 dramatically increased the expression of MMP-1 and MMP-13 mRNAs (32-fold and 26-fold respectively). Consistent with its effects on protein expression, PGD_2 dose-dependently suppressed the induction of MMP-1 and MMP-13 mRNAs by IL-1. Next, we evaluated the effect of PGD_2 on IL-1-mediated activation of MMP-1 and MMP-13 promoters. Chondrocytes were transiently transfected with the human MMP-1 or MMP-13 promoter-luciferase reporter gene and stimulated with IL-1 in the absence or presence of PGD_2. As shown in Fig. 2B, IL-1 activated MMP-1 and MMP-13 promoters and this activation was decreased in a dose-dependent manner by PGD_2. Together, these data suggest that PGD_2 suppress IL-1-induced MMP-1 and MMP-13 expression at the transcriptional level.

Role of protein synthesis in PGD_2-mediated inhibition of IL-1-induced MMP-1 and MMP-13 expression. To determine whether the effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 expression is direct or indirect, we tested the impact of the protein synthesis inhibitor cycloheximide (CHX). Chondrocytes were pretreated with CHX for 30 minutes and stimulated with IL-1 alone or in combination with PGD_2 for 8 hours. The levels of MMP-1 and MMP-13 mRNAs were analyzed by real-time PCR. Pretreatment with CHX did not affect PGD_2-mediated inhibition of IL-1-induced MMP-1 and MMP-13 expression (Fig. 2C), suggesting that the suppressive effect of PGD_2 was a direct primary effect through pre-existing factors and was not dependent on *de novo* protein synthesis.

DP1 and CRTH-2 are expressed and functional in human chondrocytes. To determine the mechanisms involved in PGD_2-mediated down-regulation of IL-1-induced MMP-1 and -13 expression, we first examined the expression of mRNA corresponding to the two plasma membrane PGD_2 receptors DP1 and CRTH2. RT-PCR analysis demonstrated the presence of both DP1 and CRTH-2 mRNAs in three chondrocyte populations derived from three different donors (Fig. 3A). These results were confirmed by Western blot analysis using specific anti-DP1 and anti-CRTH2 antibodies (Fig. 3B). Human osteoblasts express both DP1 and CRTH2 and were used as positive controls.

Activation of DP1 by PGD_2 leads to increased intracellular cAMP levels (20), whereas activation of CRTH2 by PGD_2 results in intracellular Ca^{2+} mobilization (19). To determine whether DP1 and CRTH2 are functional in chondrocytes, we examined the capacity of PGD_2 to modulate the intracellular levels of cAMP and to induce Ca^{2+} mobilization. As shown in Fig. 3C, treatment with PGD_2 enhanced intracellular levels of cAMP (Fig. 3C) in a time dependent-manner, suggesting that DP1 is functional in chondrocytes. To test whether PGD2 can induce Ca^{2+} mobilization in chondrocytes, cells were first stimulated with PGD2 in a nominally Ca^{2+} free solution to assess Ca^{2+} release from internal stores, followed by replenishment of Ca^{2+} (2 mM) to the extracellular milieu, to evaluate calcium entry. Ionomycin was added as a control at the end of Ca2+ imaging experiment to assess

the maximum Fura-2 signal in the cells. The results shown in Fig. 3D indicate that CRTH2 is functional in chondrocytes and are consistent with the activation of a receptor coupled to a phosphoinositide-specific phospholipase, inducing Ca^{2+} release from the internal stores and Ca^{2+} entry through plasma membrane channels.

As shown in Fig. 3C and D, treatment with PGD_2 enhanced intracellular levels of cAMP (Fig. 3C) and induced Ca^{2+} mobilization (Fig. 3D) in chondrocytes. These results indicate that chondrocytes express functional DP1 and CRTH2 and suggest that PGD_2 might modulate IL-1-induced MMP-1 and MMP-13 production through activation of either PGD2 receptor.

Inhibition of IL-1-induced MMP1 and MMP-13 expression by PGD_2 is mediated by the DP1, but not the CRTH2, receptor. To determine which of these receptors is responsible for the observed effect of PGD_2, we examined the effects of BW245C, a specific DP1 agonist (24; 25), and DK-PGD_2, a specific CRTH2 agonist (26), on IL-1-induced MMP-1 and MMP-13 production. The results show that BW245C dose-dependently prevented IL-1-induced MMP-1 and MMP-13 production (Fig. 4). In contrast, DK-PGD_2 was without effect on IL-1-induced MMP-1 and MMP-13 expression (Fig. 4). These data suggest that PGD_2 modulates IL-1-induced MMP-1 and MMP-13 gene expression through activating the DP1 receptor.

To confirm the involvement of DP1 in the inhibitory effect of PGD_2, chondrocytes were treated with different dilutions of a DP1 polyclonal antibody and then stimulated with IL-1 in the absence or presence of PGD_2 and the levels of MMP-1 and MMP-13 were evaluated in conditioned media. As shown in Fig 5A, treatment with the DP1 polyclonal antibody reversed the inhibitory effect of PGD_2 in a dose-dependent manner. In contrast, treatment with an anti-CRTH2 antibody had no effect on PGD_2-mediated down-regulation of IL-1-induced MMP-1 and MMP-13 expression.

The ineffectiveness of the anti-CRTH2 antibody to prevent the effect of PGD_2 on MMP-1 and MMP-13 release was not due to its inability to block CRTH2 since it completely

blocked PGD_2-induced Ca^{2+} mobilization (Fig. 5B). Taken together these data strongly suggest that the inhibitory effect of PGD_2 on MMP-1 and MMP-13 production is mediated by DP1, but not CRTH2, activation

The inhibitory effect of PGD_2 is mediated by the cAMP/protein kinase A (PKA) pathway. To investigate the role of the cAMP/PKA pathway in DP1-mediated inhibition of MMP-1 and MMP-13 expression by PGD_2, we first examined whether elevating intracellular levels of cAMP would affect IL-1-induced MMP-1 and MMP-13 production. Chondrocytes were stimulated with IL-1 in the absence or presence of two cAMP elevating agents, 8-Br-cAMP, a membrane-permeable cAMP analog, and forskolin, an adenylate cyclase activator. Twenty-four hours later, the MMP-1 and MMP-13 content in conditioned media were analyzed by ELISA. Interestingly, both 8-Br-cAMP and forskolin prevented IL-1-induced MMP-1 and MMP-13 production (Fig. 6A). 8-Br-cAMP and forskolin did not have any significant effect on cell viability as judged by the trypan blue exclusion assay (data not shown).

Next, we evaluated the effect of inhibitors of PKA, a major effector of cAMP actions, on PGD_2-mediated suppression of IL-1-induced MMP-1 and MMP-13 production. Treatment with H89 (1 μM) or KT5720 (1 μM), two PKA inhibitors, dramatically antagonized the inhibitory effect of PGD_2 on IL-1-induced MMP1 and MMP-13 protein production (Fig. 6B). Together, these data indicate that PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 production by activating the cAMP/PKA pathway.

DISCUSSION

In this study we demonstrate for the first time that PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 expression in human chondrocytes. We also provide evidence that the inhibitory effect of PGD_2 is mediated by the DP1 receptor through the cAMP-PKA signalling pathway. Thus, PGD_2 has the potential to prevent cartilage damage by blocking the expression of MMP-1 and MMP-13.

PGD_2 elicits its effects through two plasma membrane receptors, the DP1 and CRTH2 (also named DP2) (18; 19). To determine which receptor mediates the inhibitory effect of PGD_2 on IL-1-induced MMP-1 and -13 expression, we first investigated the expression of DP1 and CRTH-2 in chondrocytes. We found that DP1 and CRTH2 are expressed at both the mRNA and protein levels. We also showed that treatment with PGD_2 increased intracellular levels of cAMP and induced Ca^{2+} mobilization thereby confirming the presence of functional DP1 and CRTH2 receptors in chondrocytes. To our knowledge this is the first report showing that DP1 and CRTH2 are expressed and functinal in chondrocytes. We next investigated the effect of specific agonists of DP1 and CRTH2 on IL-1-induced MMP-1 and MMP-13 expression. Importantly, we found that the DP1 agonist BW245C, but not the CRTH2 agonist DK-PGD_2, reduced IL-1-induced MMP-1 and MMP-13 expression. In line with this finding, treatment with a DP1 polyclonal antibody blocked the inhibitory effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 expression. Taken together these findings suggest that PGD_2 down-regulates IL-1-induced MMP1 and MMP-13 expression through activation of the DP1 receptor. Although the inhibitory effect of BW245C was as potent as that of PGD_2, we cannot rule out the possibility that PGD_2 downregulates IL-1-induced MMP-1 and MMP-13 expression through DP1-independent mechanisms. Indeed, PGD_2 can be non-enzymatically converted to 15-deoxy-$\Delta^{12,14}$-PGJ2 (15d-PGJ2) (27), which has been shown to inhibit the induction of MMP-1 and MMP-13 expression in several cell types including chondrocytes (22; 28). Further studies are clearly warranted to define whether DP1-independent mechanisms are involved in the inhibitory effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 expression in chondrocytes.

In general, occupancy of DP1 activates adenylate cyclases and enhances intracellular cAMP levels leading to activation of PKA (20). Our results suggest that the intracellular mechanisms that mediate the inhibitory effect of PGD_2 on IL-1-induced MMP-1 and MMP-13 expression involve the cAMP-PKA pathway. This is supported by the fact that stimulation of chondrocytes with PGD_2 increases the intracellular levels of cAMP. Moreover, cAMP elevating agents inhibited IL-1-induced MMP-1 and MMP-13 expression. Finally, the inhibitory effect of PGD_2 was blocked by inhibitors of PKA (H89 or KT5720). These data thus identified the DP1/cAMP/PKA signalling pathway as the intracellular mechanism by which PGD_2 prevents MMP-1 and MMP-13 production in IL-1-stimulated chondrocytes.

PGD_2 is among the most abundantly produced prostaglandin in synovial fluid (29) and can be released by several cell types present within the joint including chondrocytes (30), osteoblasts (31), synovial fibroblasts (32), and synovial mast cells (33), suggesting that PGD_2 can contribute to the maintenance of cartilage homeostasis. Recently, PGD_2 was shown to prevent chondrocyte apoptosis (34) and to enhance chondrogenic differentiation and hyaline cartilage matrix deposition (35). PGD_2 was also reported to stimulate collagen synthesis (36), indicating that PGD_2 may protect cartilage integrity not only through inhibition of MMP-1 and MMP-13 expression, but also by enhancing chondrocyte anabolic events. Furthermore, several lines of evidence suggest that PGD_2 has anti-inflammatory properties. For instance, the initiation of inflammation in a number of animal models of inflammation, was shown to be associated with reduced production of PGD_2, while the resolution phase was associated with increased levels of PGD_2 (14; 15; 17). Recently, Trivedi et al (37) analyzed the duration and severity of delayed type hypersensitivity reaction in PGD_2 synthase knockout and transgenic mice and found that knockout mice exhibit a more severe inflammatory response that failed to resolve, whereas transgenic mice had little detectable inflammation. Moreover, retrovirally mediated ectopic expression of PGD_2 synthase reduces several inflammatory responses and cellular infiltration in a murine air-pouch model of monosodium urate monohydrate crystal-induced inflammation (16).

Thus, in addition to its chondroprotective effects, PGD_2 can also reduce inflammatory responses, suggesting that PGD_2 may be of therapeutic value in arthritis.

Nonsteroidal anti-inflammatory drugs (NSAIDs) which act through COX inhibition are widely used to relieve pain and inflammation in arthritis, but NSAIDs might also have deleterious effects on cartilage. Indeed, indomethacin and diclofenac were shown to display chondrotoxic effects in animal models of OA (38; 39) and to accelerate radiographic OA progression in patients with hip and knee OA (40; 41). This is consistent with in vitro studies showing that several NSAIDs (such as indomethacin, nimesulide, ibuprofen and naproxen) inhibit the synthesis of proteoglycans and collagens by articular cartilage explants (42). These data together with the demonstration that PGD_2 may have chondroprotective effects suggest that inhibition of endogenous PGD_2 biosynthesis by NSAIDs may be related to their deleterious effect on cartilage and underline the limits of therapeutic approaches for the treatment of inflammatory joint diseases by inhibition of all prostaglandin biosynthesis

In conclusion, our data show that PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 expression by chondrocytes. This effect is mediated through DP1 and involves the cAMP/PKA pathway. These results suggest that modulation of PGD_2 levels within the joint might be of therapeutic interest in arthritis.

List of abbreviations

cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; DP1, PGD_2 receptor 1; IL, interleukin; MMP, matrix metalloproteinase; OA, osteoarthritis; PGD_2, prostaglandin D_2; PKA, protein kinase A; RA, rheumatoid arthritis.

Acknowledgements

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Figure legends

Figure 1. Inhibition by PGD_2 of IL-1-induced MMP-1 and MMP-13 protein release in cultured chondrocytes and cartilage explants. A, Chondrocytes were stimulated with either IL-1 alone (100 pg/ml) or co-stimulated with IL-1 and increasing concentrations of PGD_2 for 24 h. **B**, Cartilage explants were stimulated with IL-1 alone (1 ng/ml) or co-stimulated with IL-1 and increasing concentrations of PGD_2 for 72 h. The levels of MMP-1 and MMP-13 proteins in conditioned media were measured by ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells or explants treated with IL-1β alone and represent the mean ± SEM of four independent experiments. * $p<0.05$ compared with cells or explants treated with IL-1 alone.

Figure 2. PGD_2 down-regulates IL-1-induced MMP-1 and MMP-13 expression at the transcriptional level and does not involve *de novo* protein synthesis. A, Chondrocytes were treated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of PGD_2 for 8 h. Total RNA was isolated, reverse transcribed into cDNA, and MMP-1 and MMP-13 mRNAs were quantified using real-time PCR. The housekeeping gene GAPDH was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. **B,** Chondrocytes were co-transfected with 1 μg/well of either the MMP-1 (pMMP1-0.5kb-Luc) or the MMP-13 (pMMP-13-1.6kb-Luc) promoter and 0.5 μg/well of the internal control pSV40-β-galactosidase, using FuGene 6 transfection reagent. Six hours later, the cells were washed and changed to medium containing 0.5% FCS for an additional 18 hours. Transfected cells were then treated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of PGD_2 for 18 h. Luciferase activity values were determined and normalized to β-galactosidase activity. All experiments were repeated at least three times in duplicate. **C.** Chondrocytes were incubated with cycloheximide (10 μg/ml) for 30 minutes prior to stimulation with 100 pg/ml IL-1 in the absence or presence of 10 μM PGD_2 for 8 hours. Total RNA was isolated, reverse transcribed into cDNA, and MMP-1 and MMP-13 mRNAs were quantified using real-time PCR. Results are expressed as -fold changes,

considering 1 as the value of untreated cells, and represent the mean ± SEM of 4 independent experiments. *p<0.05 compared with cells treated with IL-1 alone.

Figure 3. DP1 and CRTH2 are expressed and functional in chondrocytes.

A, Total RNA of chondrocytes from three different donors was reverse transcribed and amplified by PCR using specific primers for DP1, CRTH2, and GAPDH. PCR in the absence of RT and with RNA from human osteoblasts were used as negative and positive controls, respectively. **B,** Cell lysates from chondrocytes obtained from three different donors were analyzed for DP1 and CRTH2 protein expression by Western blotting using specific anti-DP1 and anti-CRTH2 antibodies. Protein extracts from human osteoblasts were used as positive control. **C.** Effect of PGD_2 on intracellular levels of cAMP. Chondrocytes were pre-treated with 10 μM IBMX for 30 minutes followed by incubation with PGD_2 (10 μM) for the indicated time periods. cAMP levels were measured by EIA. Results are expressed as pmol/10^6 cells and represent the mean ± SEM of four independent experiments. *p<0.05 compared with untreated cells. **D.** Effect of PGD_2 on Ca^{2+} mobilization in chondrocytes. Chondrocytes were loaded with Fura-2 and stimulated with 10 μM PGD_2 in a nominally Ca2+ free solution, followed by replenishment of calcium (2 mM) to the extracellular milieu where indicated. Ionomycin was added as a control at the end of the experiment to assess the maximum Fura-2 signal in the cells. The changes in intracellular levels of Ca^{2+} were monitored as described in Materials and Methods. Traces obtained with individuals cells (upper panel) and the average (lower panel) are presented. The data shown are representative of four independent analyses from separate donors, each showing similar results.

Figure 4. Effects of DP1 and CRTH2 agonists on IL-1-induced MMP-1 and MMP-13 production. Chondrocytes were treated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of BW245C or DK-PGD_2 for 24 h. The levels of MMP-1 and MMP-13 in conditioned media were evaluated by ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells treated with IL-1 alone, and

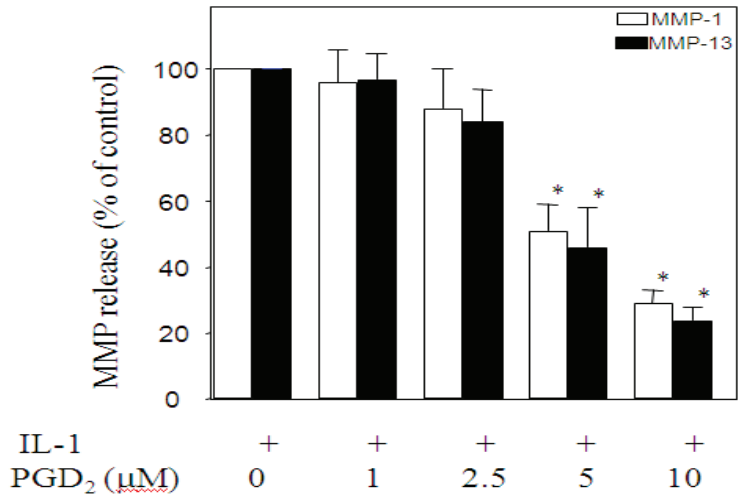
represent the mean ± SEM of four independent experiments. *p<0.05 compared with cells treated with IL-1 alone.

Figure 5. PGD_2 inhibits IL-1-induced MMP-1 and MMP-13 production via DP1. A. Chondrocytes were pre-treated with different dilutions (1:1000, 1:500 and 1:100) of anti-DP1 or anti-CRTH2 antibodies for 30 minutes, then stimulated with IL-1 (100 pg/ml) in the absence or presence of PGD_2 (10 μM) for 24 h. The levels of MMP-1 and MMP-13 in conditioned media were evaluated by ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells treated with IL-1 alone, and represent the mean ± SEM of four independent experiments. *p<0.05 compared with cells treated with IL-1 + PGD_2. **B.** Inhibition of PGD_2-induced Ca^{2+} mobilization by the anti-CRTH2 antibody. Fura-2-loaded chondrocytes were either directly subjected to Ca^{2+} mobilization assay (upper panel) or pretreated with the anti-CRTH2 antibody (final 1/100) at room temperature for 20 minutes, then subjected to the assay (lower panel). These measurements were performed in continuous presence of extracellular Ca2+ (2 mM). Arrows indicate the time of addition of PGD_2 (10 μM) or inomycin (10 μM). Changes in intracellular levels of Ca^{2+} were monitored as described in Materials and Methods. Traces represent average from (6-14) cells per condition and are representative of at least four independent experiments.

Figure 6. Role of the cAMP-PKA pathway in the inhibition of IL-1-induced MMP-1 and -13 by PGD_2. A, Effect of cAMP elevating agents on IL-1-induced MMP-1 and MMP-13 production. Chondrocytes were treated with 100 pg/ml IL-1 in the absence or presence of 8-Br-cAMP (0.5 mM) or forskolin (10 μM) for 24 h. **B,** Effect of PKA inhibitors on PGD_2-mediated down-regulation of IL-1-induced MMP-1 and -13 production. Chondrocytes were pre-treated with the PKA inhibitors H89 (1 μM) or KT5720 (1 μM) for 30 min before co-stimulation with IL-1 (100 pg/ml) and PGD_2 (10 μM) for 24 h. The levels of MMP-1 and MMP-13 in conditioned media were evaluated by ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells treated with IL-1 alone and represent the mean ± SEM of four independent experiments. * p<0.05 compared with cells treated with IL-1 alone (A) or IL-1 + PGD_2 (B).

Figure 1:

A


MMP-1
MMP-13
MMP release (% of control)
100
80
60
40
20
0
*
IL-1 + + + + +
PGD2 (μM) 0 1 2.5 5 10

B

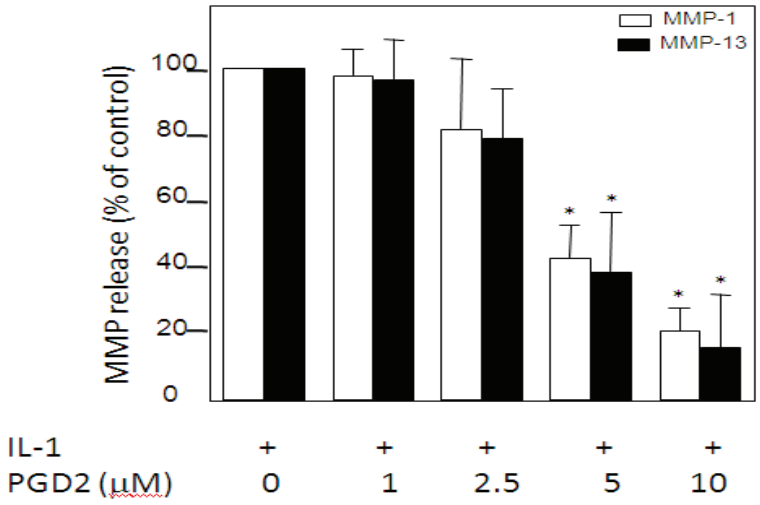
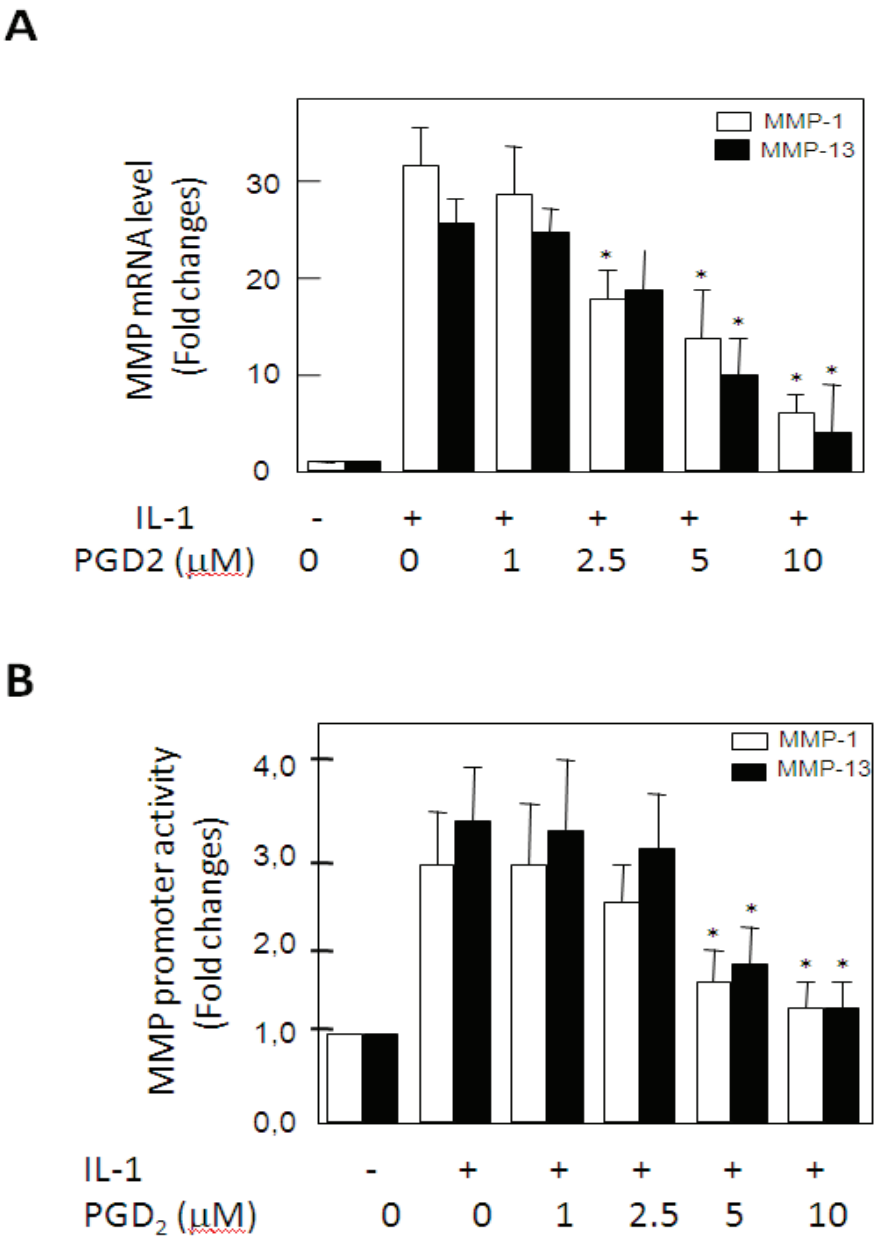

MMP-1
MMP-13
MMP release (% of control)
100
80
60
40
20
0
*
IL-1 + + + + +
PGD2 (μM) 0 1 2.5 5 10

Figure 2:


A
MMP-1
MMP-13
MMP mRNA level
(Fold changes)
30
20
10
0
IL-1 - + + + + +
PGD2 (µM) 0 0 1 2.5 5 10
B
MMP-1
MMP-13
MMP promoter activity
(Fold changes)
4,0
3,0
2,0
1,0
0,0
IL-1 - + + + + +
PGD2 (µM) 0 0 1 2.5 5 10

C

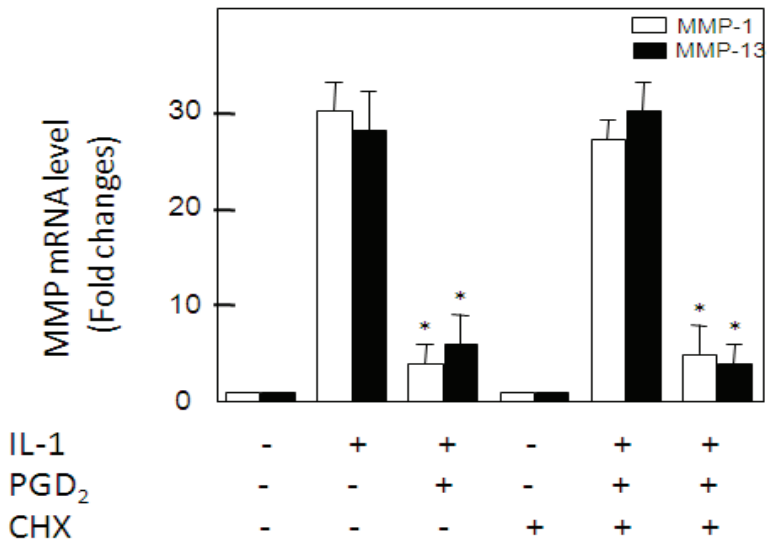


Figure 3:

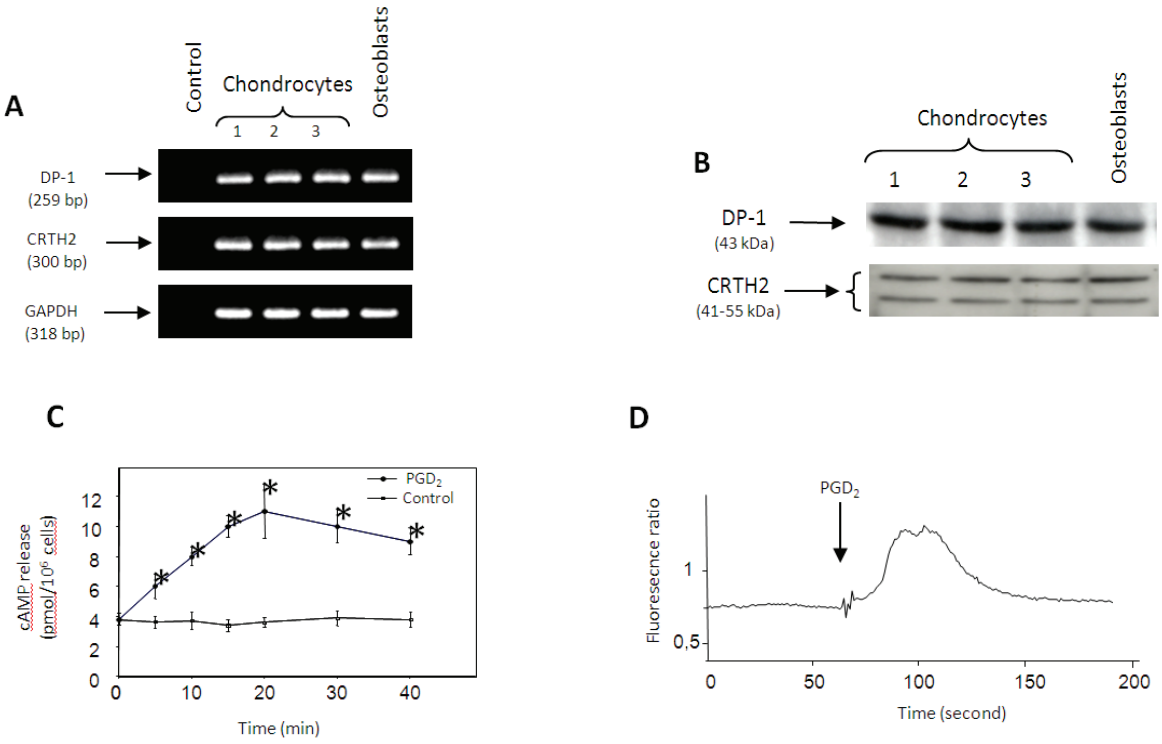

A
Control
Chondrocytes
1 2 3
Osteoblasts
DP-1
(259 bp)
CRTH2
(300 bp)
GAPDH
(318 bp)
B
Chondrocytes
1 2 3
Osteoblasts
DP-1
(43 kDa)
CRTH2
(41-55 kDa)
C
PGD2
Control
cAMP release
(pmol/10^6 cells)
0 2 4 6 8 10 12
0 10 20 30 40
Time (min)
D
PGD2
Fluoresecnce ratio
1
0,5
0 50 100 150 200
Time (second)

Figure 4:

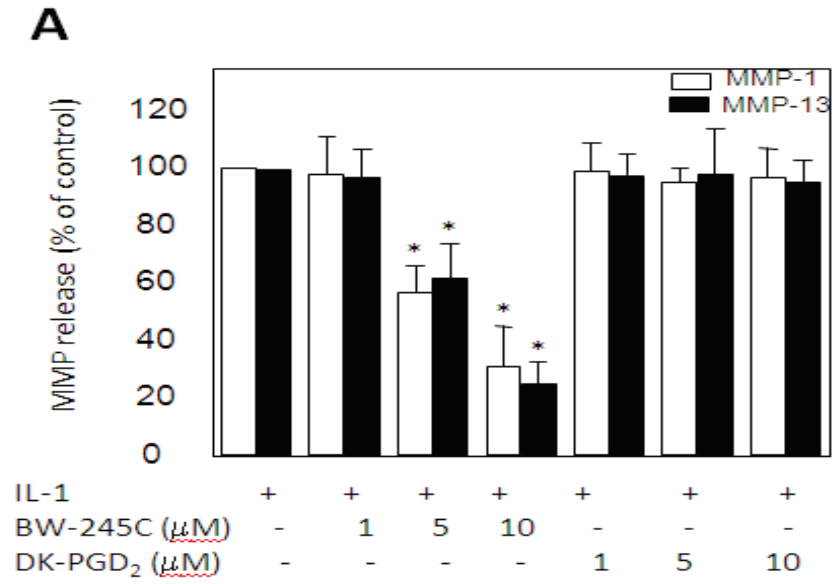

A
MMP release (% of control)
120
100
80
60
40
20
0
MMP-1
MMP-13
*
*
*
*
IL-1 + + + + + + +
BW-245C (μM) - 1 5 10 - - -
DK-PGD2 (μM) - - - - 1 5 10

Figure 5 :

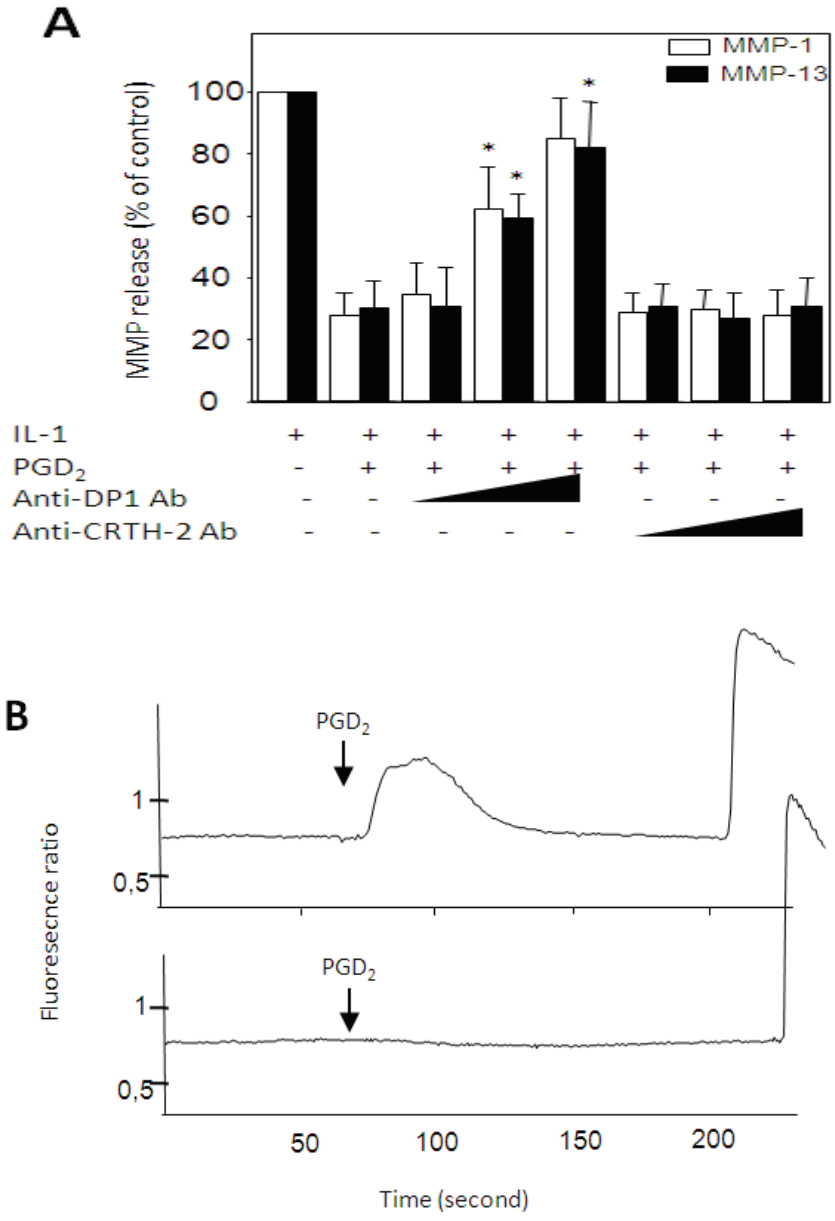
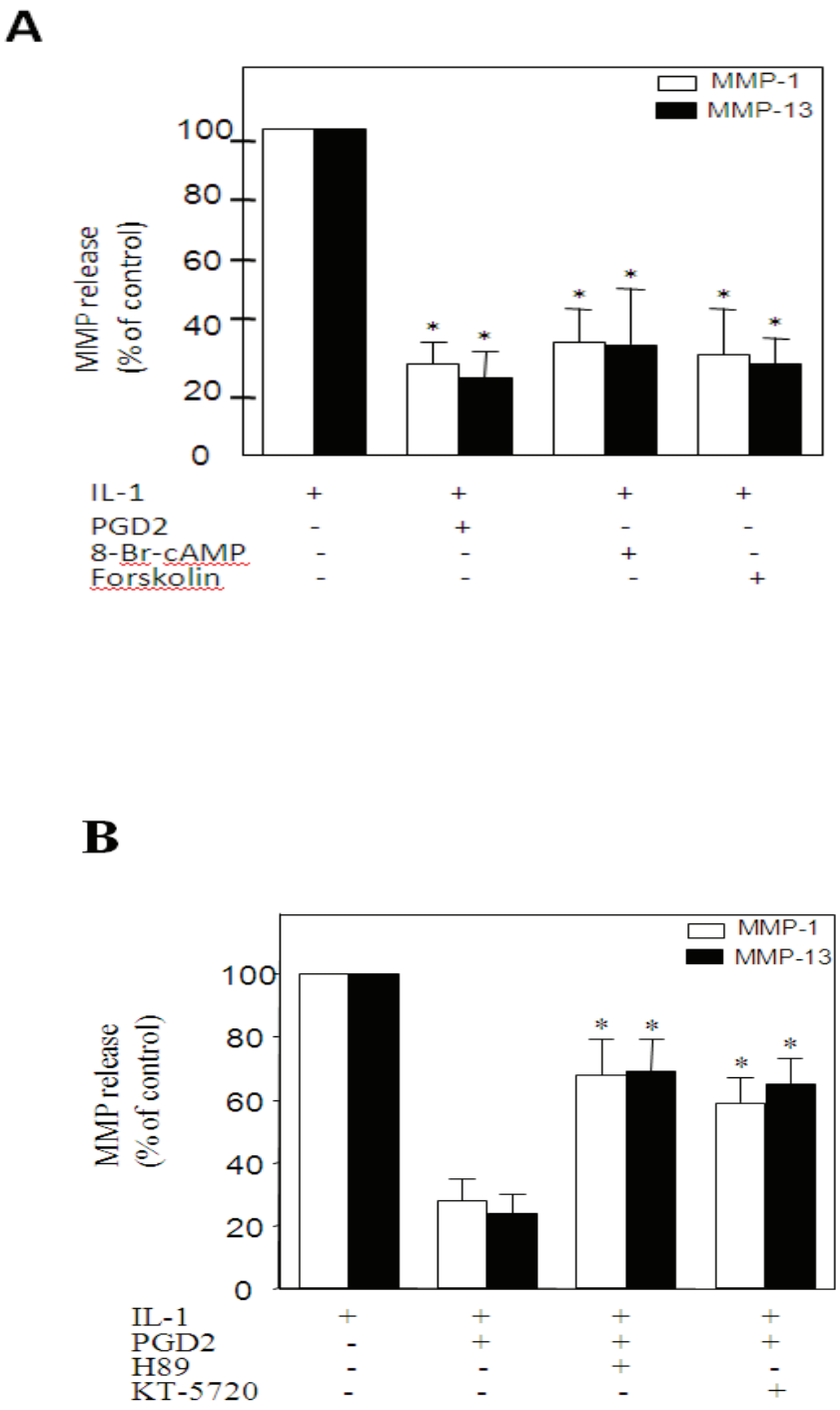

A
MMP-1
MMP-13
MMP release (% of control)
100
80
60
40
20
0
*
*
*
IL-1
PGD2
Anti-DP1 Ab
Anti-CRTH-2 Ab
B
PGD2
PGD2
1
0,5
1
0,5
Fluoresecnce ratio
50
100
150
200
Time (second)

Figure 6:


A
MMP-1
MMP-13
MMP release
(% of control)
100
80
60
40
20
0
IL-1 + + + +
PGD2 - + - -
8-Br-cAMP - - + -
Forskolin - - - +
B
MMP-1
MMP-13
MMP release
(% of control)
100
80
60
40
20
0
IL-1 + + + +
PGD2 - + + +
H89 - - + -
KT-5720 - - - +

Article II: **Augmentation de l'expression de la L-PGDS au niveau du cartilage articulaire OA**

Hypothèse:

La PGD_2 est synthétisée par des prostaglandines D-synthases (PGDS) qui existent sous deux isoformes H-PGDS et L-PGDS. Notre hypothèse de travail est que la forme responsable de la génération de la PGD_2 pourrait être modulée au niveau du cartilage OA, sous l'action de médiateurs inflammatoires.

Buts :

Déterminer laquelle des GPDS, est exprimée au niveau du cartilage articulaire aussi bien normal que OA ;

1- Étudier l'expression de la L-PGDS dans différentes conditions expérimentales et évaluer le niveau de production de la PGD_2 ;

2- Étudier la régulation de la L-PGDS

Résumé:

La biosynthèse de la PGD_2 est catalysée par des PGDS qui existent sous deux isoformes H-PGDS et L-PGDS. À ce jour, très peu de données sont disponibles quant à l'expression et à la régulation de ces PGDS au niveau du cartilage articulaire. Dans un premier temps, nous avons utilisé la RT-PCR afin de determiner le niveau d'expression de l'H-PGDS et L-PGDS au niveau du cartilage normal issu de genoux de personnes décédées, et de cartilage OA. Dans le même objectif, l'immunohistochimie a été réalisée pour évaluer aussi bien l'expression que la localisation des PGDS. Les résultats récoltés de ces expériences ont montré que la L-PGDS paraît plutôt être la forme exprimée par le cartilage articulaire normal et OA, mais que son expression est augmentée chez le cartilage OA. Par la suite, les chondrocytes OA sont traités par l'IL-β, le niveau d'expression et de production de L-PGDS est évalué par RT-PCR et western blot respectivement. Ces expériences ont montré que le niveau de production et d'expression de L-PGDS augmentent sous l'effet de l'IL-1 de manière temps et dose dépendante, de même que pour la production de la PGD_2 au niveau du liquide synovial. L'utilisation de cycloheximide ayant pour pouvoir d'inhiber la synthèse de toute nouvelle protéine n'a montré aucune augmentation de L-PGDS sous l'effet de l'IL-1, son expression requiert donc de *novo* des protéines. Les différents inhibiteurs des voies MAPK p38, JNK, NF-kB et Notch ont été utilisés et ont montré que la régulation de L-PGDS par l'IL-1 est médiée par l'une des voies JNK, p38 MAPK ou encore NF-κB. En conclusion, une augmentation de l'expression de L-PGDS est notée au niveau du cartilage OA comparativement au cartilage normal. L'IL-1 est en partie responsable de cette augmentation via l'activation des voies JNK, p38MAPK et NF-κB.

Increased expression of lipocalin-type prostaglandin D_2 synthase in osteoarthritic cartilage

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Contribution:

Chabane a contribué au design de l'étude, comme il a participé à la préparation et à la stimulation de quelques cultures de cellules primaires. Li a contribué aussi à la préparation de cultures primaires et à l'analyse des données. Dr. Benderdour a participé au design de l'étude et à l'analyse des données. Prs. Martel-Pelletier, Pelletier, et Dr. Duval, nous ont permis d'obtenir les tissus, comme ils ont contribué au design de l'étude et ont réalisé quelques expériences d'immunohistochimie. Dr. Fahmi a contribué au design, à la conception, et à la coordination de l'étude, et aussi à la rédaction du manuscrit. De ma part, j'ai partcicipé au design et à la conception de l'étude, comme j'ai réalisé des RT-PCR et quelques expériences d'immunohistochimie.

ABSTRACT

INTRODUCTION: Prostaglandin D synthase (PGDS) is responsible for the biosynthesis of prostaglandin (PG) D and J series, which have been shown to exhibit anti-inflammatory and anti-catabolic effects. Two isoforms have been identified: hematopoietic- and lipocalin-type PGDS (H-PGDS and L-PGDS, respectively). The aim of this study was to investigate the expression of H- and L-PGDS in cartilage from normal donors and from patients with osteoarthritis (OA), and to characterize their regulation by interleukin-1β (IL-1) in cultured OA chondrocytes. METHODS: The expression of H- and L-PGDS mRNA and protein in cartilage was analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. Chondrocytes were stimulated with IL-1 and the expression of L-PGDS was evaluated by real-time RT-PCR and Western blotting. The role of de novo protein synthesis, and of the signalling pathways mitogen-activated protein kinases (MAPKs), NF-kB, and Notch were evaluated using specific pharmacological inhibitors. RESULTS: L-PGDS and H-PGDS mRNAs were present in both normal and OA cartilage, with higher levels of L-PGDS than H-PGDS (> 20-fold). The levels of L-PGDS mRNA and protein were increased in OA compared with normal cartilage. Treatment of chondrocytes with IL-1 up-regulated L-PGDS mRNA and protein expression as well as PGD_2 production in a dose- and time-dependent manner. The up-regulation of L-PGDS by IL-1 was blocked by the translational inhibitor cycloheximide, indicating that this effect is indirect, requiring *de novo* protein synthesis. Specific inhibitors of the MAPK p38 (SB 203580) and c-jun N-terminal kinase (JNK) (SP600125), and of the NF-κB (SN-50) and Notch (DAPT) signalling pathways suppressed IL-1-induced up-regulation of L-PGDS expression. In contrast, an inhibitor of the extracellular signal-regulated kinase (ERK/MAPK) (PD98059) demonstrated no significant influence. We also found that PGD_2 prevented IL-1-induced up-regulation of L-PGDS expression. CONCLUSION: This is the first report demonstrating increased levels of L-PGDS in OA cartilage. IL-1 may be responsible for this up-regulation through activation of the JNK and

p38 MAPK and NF-κB signalling pathways. These data suggest that L-PGDS might have an important role in the pathophysiology of OA.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder and is a leading cause of disability throughout the world [1]. It can cause pain, stiffness, swelling and loss of function in the joints. Pathologically, OA is characterized by progressive degeneration of articular cartilage, synovial inflammation, and subchondral bone remodeling. These processes are thought to be largely mediated through excess production of proinflammatory and catabolic mediators. Among these mediators, interleukin-1β (IL-1) has been demonstrated to be predominantly involved in the initiation and progression of the disease [2-4]. One mechanism through which IL-1 exerts its effects is by inducing connective tissue cells, including chondrocytes, to produce matrix metalloproteinases (MMPs), aggrecanases, reactive oxygen species, and prostaglandins (PGs) [2].

The biosynthesis of PGs involves multiple enzymatically regulated reactions. The process is initiated through the release of arachidonic acid (AA) from the cell membrane by phospholipases. Subsequently, AA is converted to an intermediate substrate PGH_2 by the actions of cyclooxygenase (COX). Two distinct isoforms have been identified: COX-1 is constitutively expressed, whereas COX2 is induced by various stimuli such as proinflammatory cytokines and growth factors [5]. Once formed by COX-1 or -2, the unstable PGH_2 intermediate is metabolized by specific PG synthase enzymes, to generate the classical bioactive PGs, including PGE_2, PGD_2, $PGF_2\alpha$, PGI_2 and thromboxane [6].

There is a growing body of evidence suggesting that PGD_2 may have protective effects in OA and possibly other chronic articular diseases. For instance, treatment with PGD_2 enhances the expression of the cartilage-specific matrix components collagen type II and aggrecan [7] and prevents chondrocyte apoptosis [8]. In addition, we have recently shown that PGD_2 inhibits the induction of MMP-1 and MMP-13, which plays an important role in cartilage damage [9]. Thus, PGD_2 can mediate its chondroprotective effects not only through chondrogenesis enhancement, but also through inhibition of catabolic events. PGD_2 was also shown to exhibit anti-inflammatory properties. Indeed, increased levels of

PGD_2 are observed during the resolution phase of inflammation and the inflammation is exacerbated by COX inhibitors [10, 11]. The anti-inflammatory role of PGD_2 is supported by studies using PGD_2 synthase deficient and transgenic mice. The knockout animals show impaired resolution of inflammation, and transgenic animals have little detectable inflammation [12]. In addition, retroviral delivery of PGD_2 synthase suppresses inflammatory responses in a murine air-pouch model of monosodium urate monohydrate crystal-induced inflammation [13]. Some effects of PGD_2 can be mediated by its dehydration end product, 15-deoxy-delta12,14-PGJ_2 (15d-PGJ_2), which has been shown to exhibit potent anti-inflammatory and anti-catabolic properties [14]. PGD_2 exerts its effects principally by binding and activating two plasma membrane receptors, the D prostanoid receptor (DP) 1 [15] and chemoattractant-receptor-like molecule expressed on Th2 cells (CRTH2), also known as DP2 [16]. The effects of the PGD_2 metabolite 15d-PGJ_2 are mediated through mechanisms independent of and dependent on the nuclear peroxisome proliferator-activated receptor (PPAR) γ [14, 17, 18]. The biosynthesis of PGD_2 from its precursor PGH_2 is catalyzed by two PGD synthases (PGDSs); one is gluthatione independent, the lipocaline-type PGDS (L-PGDS); and the other is glutathione requiring, the haematopoietic PGDS (H-PGDS) [19]. L-PGDS (also called β-trace) is expressed abundantly in the central nervous system [20, 21], the heart [22], the retina [23], and the genital organs [24]. H-PGDS is expressed mainly in mast cells [25], megakaryocytes [26] and T helper (Th)2 lymphocytes [27]. So far, little is known about the expression and regulation of L-PGDS and H-PGDS in cartilage. To better understand the role of PGD_2 in the joint, we investigated the expression of H- and L-PGDS in normal and OA cartilage. Moreover, we explored the effect of IL-1, a key cytokine in the pathogenesis of OA, on L-PGDS expression in cultured chondrocytes.

MATERIALS AND METHODS

Reagents

Recombinant human (rh) IL-1 was obtained from Genzyme (Cambridge, MA). Cycloheximide was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). SB203580, SP600125, PD98059, SN-50 and N-[N-(3,5-diflurophenylacetate)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) were from Calbiochem (La Jolla, CA). PGD_2 was from Cayman Chemical Co. (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, foetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from either Bio-Rad (Mississauga, ON, Canada) or Sigma-Aldrich Canada (Oakville, ON, Canada).

Specimen selection and chondrocyte culture

Normal cartilage and synovial fluids were obtained at necropsy, within 12 hours of death, from donors with no history of arthritic diseases (n = 13, mean ± SD age: 64 ± 17 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. OA cartilage and synovial fluids were obtained from patients undergoing total knee replacement (n = 32, mean ± SD age: 67 ± 16 years). All OA patients were diagnosed on criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA [28]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors. Patients who had received intraarticular injections of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the informed consent form.

Chondrocytes were released from cartilage by sequential enzymatic digestion as previously described [29]. Briefly, this consisted of 2 mg/ml pronase for 1 hour followed by 1 mg/ml collagenase for 6 hours (type IV; Sigma-Aldrich) at 37°C in DMEM and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue

culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS. At confluence, the chondrocytes were detached, seeded at high density, and allowed to grow in DMEM, supplemented as above. The culture medium was changed every second day, and 24 hours before the experiment the cells were incubated in fresh medium containing 0.5% FCS. Only first passaged chondrocytes were used.

RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA from homogenized cartilage or stimulated chondrocytes was isolated using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR), dissolved in diethylpyrocarbonate (DEPC)-treated-H_2O and stored at -80°C until use. One μg of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as detailed in the manufacturer's guidelines. One fiftieth of the reverse transcriptase reaction was analyzed by real-time quantitative PCR as described below. The following primers were used: L-PGDS [GeneBank: NM000954] , sense 5'-AACCAGTGTGAGACCCGAAC-3', antisense 5'-AGGCGGTGAATTTCTCCTTT-3'; H-PGDS [GeneBank: NM014485], sense 5'-CCCCATTTTGGAAGTTGATG-3', antisense 5'-TGAGGCGCATTATACGTGAG-3; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [GeneBank: NM002046], sense 5'-CAGAACATCATCCCTGCCTCT-3', antisense 5'-GCTTGACAAAGTGGTCGTTGAG -3'.

Quantitative PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 nM of sense and antisense primers, 25 μl of SYBR® Green master mix (Qiagen, Mississauga, ON, Canada) and uracil-N-glycosylase (UNG, 0.5 Unit, Epicentre Technologies, Madison, WI). After incubation at 50°C for 2 min (UNG reaction), and at 95°C for 10 min (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 sec at 95°C for denaturation and 1

min for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative amounts of mRNA in normal and OA cartilage were determined using the standard curve method. Serial dilutions of internal standards (plasmids containing cDNA of target genes) were included in each PCR run, and standard curves for the target gene and for GAPDH were generated by linear regression using log (C_T) versus log (cDNA relative dilution). The C_T were then converted to number of molecules. Relative mRNA expression in cultured chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising 2 to the $-\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

Immunohistochemistry

Cartilage specimens were processed for immunohistochemistry as previously described [29]. The specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) of paraffin-embedded specimens were deparaffinized in toluene, and dehydrated in a graded series of ethanol. The specimens were then preincubated with chondroitinase ABC (0.25 U/ml in PBS pH 8.0) for 60 min at 37˚C, followed by a 30 min

incubation with Triton X-100 (0.3%) at room temperature. Slides were then washed in PBS followed by 2% hydrogen peroxide/methanol for 15 min. They were further incubated for 60 min with 2% normal serum (Vector Laboratories, Burlingame, CA) and overlaid with primary antibody for 18 hours at 4 °C in a humidified chamber. The antibody was a rabbit polyclonal anti-human L-PGDS (USBiological, Swampscott, MA), used at 10 μg/ml. Each slide was washed 3 times in PBS pH 7.4 and stained using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories). The color was developed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories) containing hydrogen peroxide. The slides were counterstained with eosin. The specificity of staining was evaluated by using antibody that had been preadsorbed (1 hour, 37°C) with a 20-fold molar excess of recombinant human L-PGDS (Cayman Chemical), and by substituting the primary antibody with non-immune rabbit IgG (Chemicon, Temecula, CA), used at the same concentration as the primary antibody. The evaluation of positive-staining chondrocytes was performed using our previously published method [29]. For each specimen, 6 microscopic fields were examined under 40X magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated and results were expressed as the percentage of chondrocytes staining positive (cell score).

Western blot analysis

Chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL). Twenty μg of total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a tris buffer (Tris-buffered

saline (TBS) pH 7.5, with 0.1% Tween 20). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and, finally, exposed to Kodak X-Omat film (Eastman Kodak Ltd, Rochester, NY). Bands on the films were scanned using the imaging system Chemilmager 4000 (Alpha Innotech, San Leonardo, CA) and the intensity of the L-PGDS bands were normalized by dividing them by the intensity of the β-actin band of the corresponding sample.

11β-$PGF_2\alpha$ and PGD_2assays.

The levels of 11β-PGF2α in hyaluronidase-treated synovial fluids and of PGD_2 in chondrocyte supernatants were determined using competitive enzyme immunoassays from Cayman Chemical. Assays were performed according to the manufacturer's recommendation.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was assessed by the 2-tailed Student's t-test. p values less than 0.05 were considered significant.

RESULTS

Expression of L-PGDS and H-PGDS in normal and OA cartilage

We first analyzed the levels of L-PGDS and H-PGDS messenger RNAs (mRNAs) in normal and OA cartilage using real-time quantitative RT-PCR. As shown in Fig. 1, cartilage predominantly express L-PGDS mRNA, and its levels of expression were ~3-fold higher in OA cartilage compared with normal cartilage. In contrast to L-PGDS, there was no statistically significant difference in the levels of H-PGDS mRNA between OA and normal cartilage (Fig. 1). In preliminary experiments, we showed that the amplification efficiency of tested genes and GAPDH were similar. The efficiencies for the amplification of each gene and the reference were approximately equal, ranging between 1.95 and 2.

Next, we used immunohistohemistry to analyze the localization and the expression level of L-PGDS and H-PGDS proteins in normal and OA cartilage. As shown in Fig. 2A and B, the immunostaining for L-PGDS was located in the superficial and upper intermediate layers of cartilage. Statistical evaluation for the cell score revealed a clear and significant increase in the number of chondrocytes staining positive for L-PGDS in OA cartilage (43 ± 6%, mean ± SEM) compared to normal cartilage (20 ± 4%, mean ± SEM). The specificity of the staining was confirmed using antibody that had been preadsorbed (1 hour, 37°C) with a 20-fold molar excess of the recombinant protein (Fig. 2C) or non-immune control IgG (data not shown). We were unable to detect H-PGDS protein expression in OA or normal cartilage using several commercially available antibodies directed against human H-PGDS. Together, these data indicate that the expression level of L-PGDS is increased in OA cartilage.

To assess the level of PGD_2 in synovial fluids from OA and normal donors, we quantified its major stable metabolite, 11β-$PGF_2\alpha$. We measured this metabolite because PGD_2 is unstable *in vivo* [30] and quantification of PGD_2 in synovial fluid can be unreliable. We

found higher level of 11β-$PGF_2\alpha$ in OA synovial fluid when compared with normal synovial fluid (Fig. 3), indicating that the production of PGD_2 is higher in OA synovial fluids. Together, these data indicate increased expression and activity of L-PGDS in OA tissues.

IL-1 induces L-PGDS expression in chondrocytes

IL-1 plays a major role in the cartilage physiology and in the pathogenesis of OA [2]; therefore, we examined its effects on the expression of L-PGDS in cultured OA chondrocytes. Cells were treated with IL-1 (100 pg/ml) for different time periods, and the levels of L-PGDS mRNA were quantified using real-time RT-PCR. IL-1-induced changes in gene expression were evaluated as -fold over control (untreated cells) after normalization to the internal control gene, GAPDH. As shown in Fig. 4A, treatment with IL-1 (100 pg/ml) enhanced L-PGDS mRNA expression in a time-dependent manner. L-PGDS mRNA expression started to gradually increase 24 h post-stimulation with IL-1, and remained elevated until 72 h. The induction of L-PGDS mRNA by IL-1 was also dose-dependent. A significant increase was observed at concentrations as low as 10 pg/ml and the maximal effect was reached at 100 pg/ml (Fig. 4B). To determine whether changes in mRNA levels were paralleled by changes in L-PGDS protein levels, we performed Western blot analysis. Consistent with its effects on L-PGDS mRNA, treatment with IL-1 led to a dose- and time-dependent increase in the L-PGDS protein expression (Fig. 4C and D). To establish whether the IL-1-induced increase in L-PGDS expression corresponded with an increase in PGDS activity, we measured PGD_2 levels in conditioned media. As shown in Fig. 4E and F, the increased expression of L-PGDS protein was accompanied by a time- and dose-dependent increase in PGD_2 production.

The up-regulation of L-PGDS mRNA expression in chondrocytes requires de novo protein synthesis

The lag period required for IL-1 to induce L-PGDS mRNA in chondrocytes contrasts with those required for other IL-1-inducible genes, the expression of which starts as early as 2-6 hours and reaches a maximum at 8-18 hours. This suggests that de novo protein synthesis is required for IL-1-induced L-PGDS expression. To evaluate this possibility, we examined the impact of the protein synthesis inhibitor cycloheximide (CHX). Chondrocytes were stimulated with IL-1 in the absence or presence of cycloheximide and the levels of L-PGDS mRNA were analyzed by real-time PCR. As shown in Fig. 5, treatment with CHX prevented IL-1-mediated up-regulation of L-PGDS mRNA expression. This suggests that to up-regulate L-PGDS expression in chondrocytes, IL-1 must induce the synthesis of one or more proteins.

JNK and p38 MAPKs and NF-κB pathways contribute to IL-1-induced up-regulation of L-PGDS

IL-1 exerts its effects acting through activation of the MAPK (Erk, JNK and p38) and NF-κB signalling cascades [31-35]. To evaluate the potential contribution of these pathways in IL-1-induced L-PGDS expression, we used specific pharmacological inhibitors. Chondrocytes were pretreated for 30 min with selective inhibitors for the above pathways, and then stimulated or not with IL-1 for 48 h. As shown in Fig. 6A, pretreatment with the p38 MAPK inhibitor SB203580 (1 μM), the JNK MAPK inhibitor SP600125 (10 μM), or the NF-κB inhibitor SN-50 (1 μM) suppressed IL-1-induced up-regulation of L-PGDS expression. In contrast, pretreatment with the p42/44 MAPK inhibitor PD98059 (10 μM), had no effect on IL-1-induced upregulation of L-PGDS. The concentration of the MAPK and NF-κB inhibitors used for these experiments had no significant effect on cell viability as indicated by the results of the MTT assay (data not shown). These results suggest that the activation of JNK and p38 MAPK as well as NF-κB is essential to the induction of L-PGDS by IL-1 in chondrocytes.

The Notch signalling pathway regulates diverse cellular processes including proliferation, differentiation and apoptosis [36] and was reported to contribute to the regulation of L-PGDS expression [37]. To determine whether this pathway participates in IL-1-induced L-PGDS expression in human chondrocytes, we assessed the effect of N-[N-(3,5-diflurophenylacetate)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT). DAPT is a γ-secretase inhibitor, which blocks cleavage of the intracellular domain of all Notch proteins, and is widely used to evaluate the effect of Notch inhibition [36]. As shown in Fig. 6B, pretreatment with DAPT dose-dependently prevented IL-1-induced L-PGDS protein expression, indicating the involvement of Notch signalling in this process. Notch inhibition was confirmed by transcriptional inhibition of its direct target gene Hes1 (data not shown).

PGD_2 down-regulated L-PGDS expression

To further characterize the regulation of L-PGDS expression in cartilage, we examined the effect of PGD_2, the end product of L-PGDS. Chondrocytes were stimulated with IL-1 in the absence or presence of increasing concentrations of PGD_2 for 48 h and the expression of L-PGDS was evaluated by Western blotting. As shown in Fig. 7, treatment with PGD_2 dose-dependently reduced IL-1-induced L-PGDS expression.

DISCUSSION

This is the first report demonstrating the presence of L-PGDS in human cartilage and that its levels are elevated in OA cartilage compared to normal cartilage. The proinflammatory cytokine IL-1 up-regulated, whereas PGD_2 down-regulated, the expression of L-PGDS in cultured chondrocytes. These findings suggest that L-PGDS may be implicated in the pathogenesis of OA.

In normal cartilage, L-PGDS immunostaining was located, only in a few cells in the superficial and middle zones. By contrast, in OA cartilage, the cell score was significantly higher, particularly in cartilage areas showing significant damage (fibrillation). Given the anti-inflammatory and anti-catabolic roles of PGD_2, it is reasonable to speculate that up-regulation of L-PGDS may act as a sort of chondroprotective mechanism. Increased expression of L-PGDS was described in other diseases such as atherosclerosis [22], multiple sclerosis [38], diabetes [39] essential hypertension [40], and Tay-Sachs and Sandhoff diseases [41]. Thus, L-PGDS expression is up-regulated in many pathologies.

The enhanced expression of L-PGDS in the superficial and middle zones of cartilage could potentially be due to the increased level of the proinflammatory cytokine IL-1 in these zones. Indeed, IL-1 which plays pivotal roles in the initiation and progression of OA has been shown to accumulate in these zones [42-46]. To prove this hypothesis, we performed cell culture experiments. Our results revealed that exposure to IL-1 led to a time and concentration-dependent up-regulation of L-PGDS expression and PGD_2 production. The up-regulation of L-PGDS expression by IL-1 was blocked by cycloheximide suggesting that this effect of IL-1 requires de novo protein synthesis and would be consistent with an indirect stimulatory mechanism.

The delayed induction of L-PGDS by IL-1 in chondrocytes is consistent with the recently reported anti-inflammatory and anti-catabolic properties of PGD_2. Indeed, the production of PGD_2 is markedly elevated during the resolution of inflammation in carrageenan-induced pleurisy in rats, and exogenous PGD_2 significantly reduces neutrophil levels in the inflammatory exudates [10, 11]. Enhanced production of PGD_2 was also described during

the resolution phase of the wound-healing process [47]. Cipollone et al [48] examined the expression of L-PGDS in atherosclerotic arteries and found lower expression of L-PGDS and higher expression of mPGES-1 in symptomatic plaques; and higher expression of L-PGDS and lower expression of mPGES-1 in asymptomatic ones. This suggests that the balance between PGD_2 and PGE_2 contributes to the pathology of atherosclerosis and that a shift toward PGD2 synthesis may have an anti-inflammatory role. This is supported by the observation that increased biosynthesis of PGD_2 is associated with reduced production of PGE_2 in several in vitro studies [49, 50]. Recently, two separate studies demonstrated anti-inflammatory properties of PGD_2 in an air pouch model of inflammation induced by monosodium urate monohydrate crystals [13, 51] Moreover, hPGDS knock-out mice fail to resolve a delayed-type hypersensitivity reaction [12]. In addition to its anti-inflammatory effects, PGD_2 was shown to induce the expression of collagen type II and aggrecan [7], to prevent apoptosis [8], and to inhibit the induction of MMP-1 and MMP-13 [52] in chondrocytes. Together, these data and those from the present study favour the hypothesis that the up-regulation of L-PGDS expression in chondrocytes may be part of a negative feedback control of inflammatory and catabolic responses activated by IL-1 in the joint.

The production of PGD_2 by chondrocytes is of particular interest since PGD_2 is readily converted to 15d-PGJ_2, a potent anti-arthritic agent [14]. 15dPGJ_2 down-regulates the expression of a number of inflammatory and catabolic mediators involved in the pathogenesis of OA, including, IL-1, TNF-α, iNOS and MMPs [14]. Moreover, many in vivo studies support a protective effect of 15d-PGJ_2 and other PPARγ ligands in experimental animal models of OA [53, 54]. Thus, the increased expression of L-PGDS can lead to the production of a PPARγ ligand in the joint. In contrast to classical PGs which induce their effects through binding to cell surface G protein-coupled receptors, 15d-PGJ_2 induces most of its effects through the nuclear receptor PPARγ. We have previously shown that PPARγ expression is reduced in OA cartilage and that IL-1 down-regulates its expression in chondrocytes [29], which may interfere with the protective effect of the PGD2 metabolite 15d-PGJ2. Therefore, the increased expression of L-PGDS observed in

our study may represent a compensatory mechanism to counter the reduced expression of PPARγ in OA and to limit local inflammatory and catabolic responses. Also, it should be noted that 15d-PGJ2 can induce many of its effects independently of PPARγ [14, 17, 18]. In addition, PGD2 can directly exert protective effects in OA before being metabolized into 15d-PGJ2. Indeed, we have recently demonstrated that human chondrocytes express functional DP1 and CRTH-2 and that PGD2 down-regulates MMP-1 and -13 expression through activation of the DP1 pathway [9].

To elucidate the mechanisms by which IL-1 up-regulates L-PGDS expression, we evaluated the roles played by downstream signalling cascades using specific pharmacological inhibitors. We found that JNK and p38 MAPK inhibitors blocked IL-1-induced L-PGDS up-regulation, whereas an inhibitor of the Erk MAPK was without effect. We also found that NF-κB blockade caused a significant decrease in IL-1-induced up-regulation of L-PGDS protein expression. These findings support the hypothesis that the JNK and p38 MAPKs, as well as the NF-κB pathways are involved in the up-regulation of L-PGDS expression by IL-1. Our results are concordant with previous reports that implicate activation of MAPKs (JNK and p38) and NF-κB in the up-regulation of L-PGDS in leptomeningel cells [55], endothelial cells [56] and macrophages [57]. The activation of JNK and p38 MAPK, and NF-κB pathways in chondrocytes has been shown to cause activation of their downstream transcription factors, including AP-1 and NF-κB [31-35]. Interestingly, the promoter region of the human L-PGDS contains binding sites for NF-κB and AP-1 [55, 56]. Therefore, one could speculate that up-regulation of L-PGDS expression by IL-1 could be mediated by AP-1 and NF-κB. Our results also demonstrate that the Notch signalling pathway positively contributes to IL-1-induced L-PGDS expression in chondrocytes because DAPT, a Notch signalling inhibitor, blocked this process. These findings contrast with previous data showing that the Notch pathway down-regulates L-PGDS expression in the brain-derived TE671 cells [37]. The reasons for these

discrepancies are presently unclear but are most likely due to cell-type differences or to differences in experimental conditions.

We also found that PGD_2 inhibits IL-1-induced L-PGDS expression. These results suggest that PGD_2 may exert a negative feedback mechanism to down-regulate L-PGDS expression and activity. Given that the levels of L-PGDS are elevated in OA cartilage and that IL-1 up-regulated its expression in chondrocytes, it is possible that the IL-1 effect prevails over that of PGD_2 in vivo during advanced stages of the disease. Indeed, the OA cartilage specimens used in this study were from donors with long-established OA. Further studies are clearly warranted to determine the expression profile of L-PGDS over the course of OA in animal models of the disease.

The concentrations of PGD_2 used to suppress IL-1-induced L-PGDS expression are likely much higher that those produced in synovial fluids. However, it should be noted that like other eicosanoids, PGD_2 functions as an autocrine and paracrine molecule and can readily reach pharmacological levels in the microenvironment of cells that produce it.

CONCLUSION

In conclusion our study has demonstrated for the first time that L-PGDS is up-regulated in OA cartilage. The pro-inflammatory cytokine IL-1 may be responsible for this up-regulation via a mechanism that seems to involve activation of the JNK and p38 MAPK and NF-κB signalling pathways. These results suggest that the increased expression of L-PGDS may play a protective role against articular inflammation and cartilage damage.

Abbreviations

CHX, cycloheximide; IL, interleukin; L-PGDS, lipocalin-type prostaglandin D synthase; PG, prostaglandin; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; OA, osteoarthritis; PCR, polymerase chain reaction;.

Competing interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. L-PGDS and H-PGDS mRNA levels in normal and OA human cartilage. RNA was extracted from normal (n=9) and OA (n=9) cartilage, reverse transcribed into cDNA, and processed for real-time PCR. The threshold cycle values were converted to the number of molecules, as described under Materials and Methods. Data were expressed as copies of gene's mRNA detected per 10000 GAPDH copies. *$p<0.05$ versus normal samples.

Figure 2. Expression of L-PGDS protein in normal and OA cartilage. Representative immunostaining of human normal (**A**) and OA cartilage (**B**) for L-PGDS protein. **C,** OA specimens treated with anti-L-PGDS antibody that was preadsorbed with a 20-fold molar excess of recombinant human L-PGDS (control for staining specificity). **D**, Percentage of chondrocytes expressing L-PGDS in normal and OA cartilage. The results are the mean ± SEM of 9 normal and 9 OA specimens. * $p<0.05$ versus normal cartilage.

Figure 3. Synovial levels of the PGD_2 metabolite 11β-$PGF_2\alpha$. 11β-$PGF_2\alpha$ levels were measured in synovial fluids from normal subjects and patients with OA. The results are expressed as pg/mg proteins and are the mean ± SEM of 7 normal subjects and 11 OA patients. * $p<0.05$ versus normal subjects.

Figure 4. Effect of IL-1 on L-PGDS expression in OA chondrocytes. Chondrocytes were treated with 100 pg/ml IL-1 for the indicated time periods, or with increasing concentrations of IL-1 for 48 h. **A, B,** Total RNA was isolated; reverse transcribed into cDNA; and L-PGDS, and GAPDH mRNAs were quantified using real-time PCR. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. Results are expressed as –fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of 4 independent experiments.

*p<0.05 compared with unstimulated cells. **C, D**, Cell lysates were prepared and analyzed for L-PGDS and β-actin proteins by Western blotting. Representative Western blots (upper panels). In the lower panels, the bands were scanned, and the L-PGDS band intensity values were normalized to the corresponding β-actin band intensity value. Data are expressed as -fold induction, considering 1 as the value of unstimulated cells, and represent the mean ± SEM of 4 independent experiments. *p<0.05 compared with unstimulated cells. **E, F**, Conditioned media was collected and analyzed for PGD_2 content. Results are expressed as the mean ± SEM of 4 independent experiments. *p<0.05 compared with unstimulated cells.

Figure 5. The IL-1-induced up-regulation of L-PGDS mRNA expression requires de novo protein synthesis. Chondrocytes were incubated with cycloheximide (10 μg/ml) for 30 minutes prior to stimulation with 100 pg/ml IL-1 for 48 hours. Total RNA was isolated, reverse transcribed into cDNA, and L-PGDS mRNA was quantified using real-time PCR. Results are expressed as -fold changes, considering 1 as the value of untreated cells, and represent the mean ± SEM of 4 independent experiments. *p<0.05 compared with cells treated with IL-1 alone.

Figure 6. Effect of MAPK, NF-κB, and Notch inhibitors on IL-1-induced up-regulation of L-PGDS expression. **(A, B)** OA chondrocytes were pretreated with SB203580 (1 μM), SP600125 (10 μM), PD98059 (10 μM), or SN-50 (1 μM) for 30 min **(A)**; or with increasing concentrations (1, 5 and 10 mM) of DAPT for 48 h **(B)** prior to stimulation with IL-1 (100 pg/ml). After 48 h, cell lysates were prepared and analyzed for L-PGDS and β-actin protein expression by Western blotting. Representative Western blot (upper panels). In the lower panel, the bands were scanned, and the L-PGDS band intensity values were normalized to the corresponding β-actin band intensity value. Data are expressed as -fold induction, considering 1 as the value of unstimulated cells, and represent

the mean ± SEM of four independent experiments. *p<0.05 compared with cells treated with IL-1 alone.

Figure 7. Effect of PGD_2 on IL-1-induced up-regulation of L-PGDS expression. OA chondrocytes were pretreated with increasing concentrations of PGD_2 for 30 min prior to stimulation with IL-1 (100 pg/ml). After 48 h, cell lysates were prepared and analyzed for L-PGDS and β-actin protein expression by Western blotting. Representative Western blot (upper panel). In the lower panel, the bands were scanned, and the L-PGDS band intensity values were normalized to the corresponding β-actin band intensity value. Data are expressed as -fold induction, considering 1 as the value of unstimulated cells, and represent the mean ± SEM of four independent experiments. *p<0.05 compared with cells stimulated with IL-1 alone.

Figure 1:

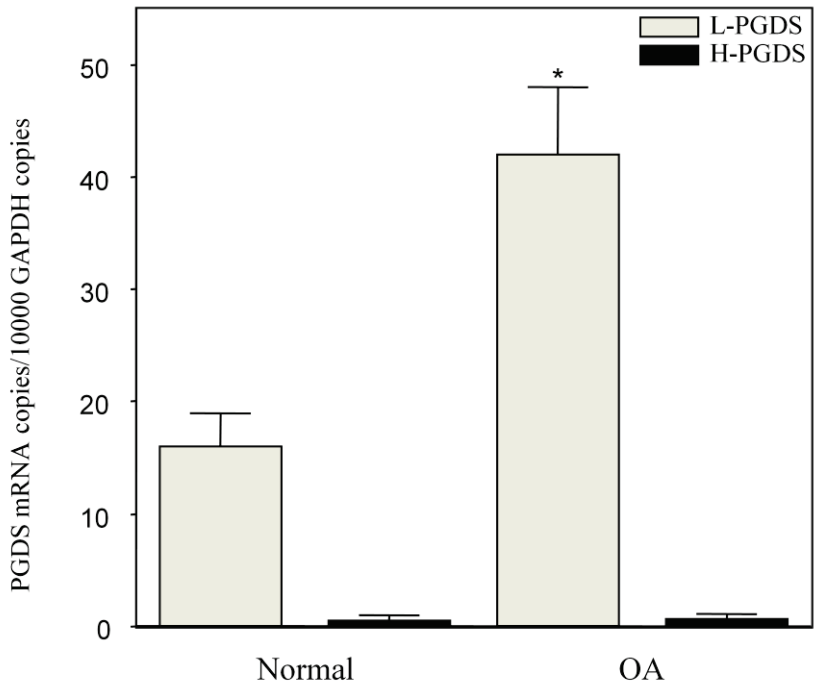

L-PGDS
H-PGDS
PGDS mRNA copies/10000 GAPDH copies
50
40
30
20
10
0
*
Normal
OA

Figure 2:

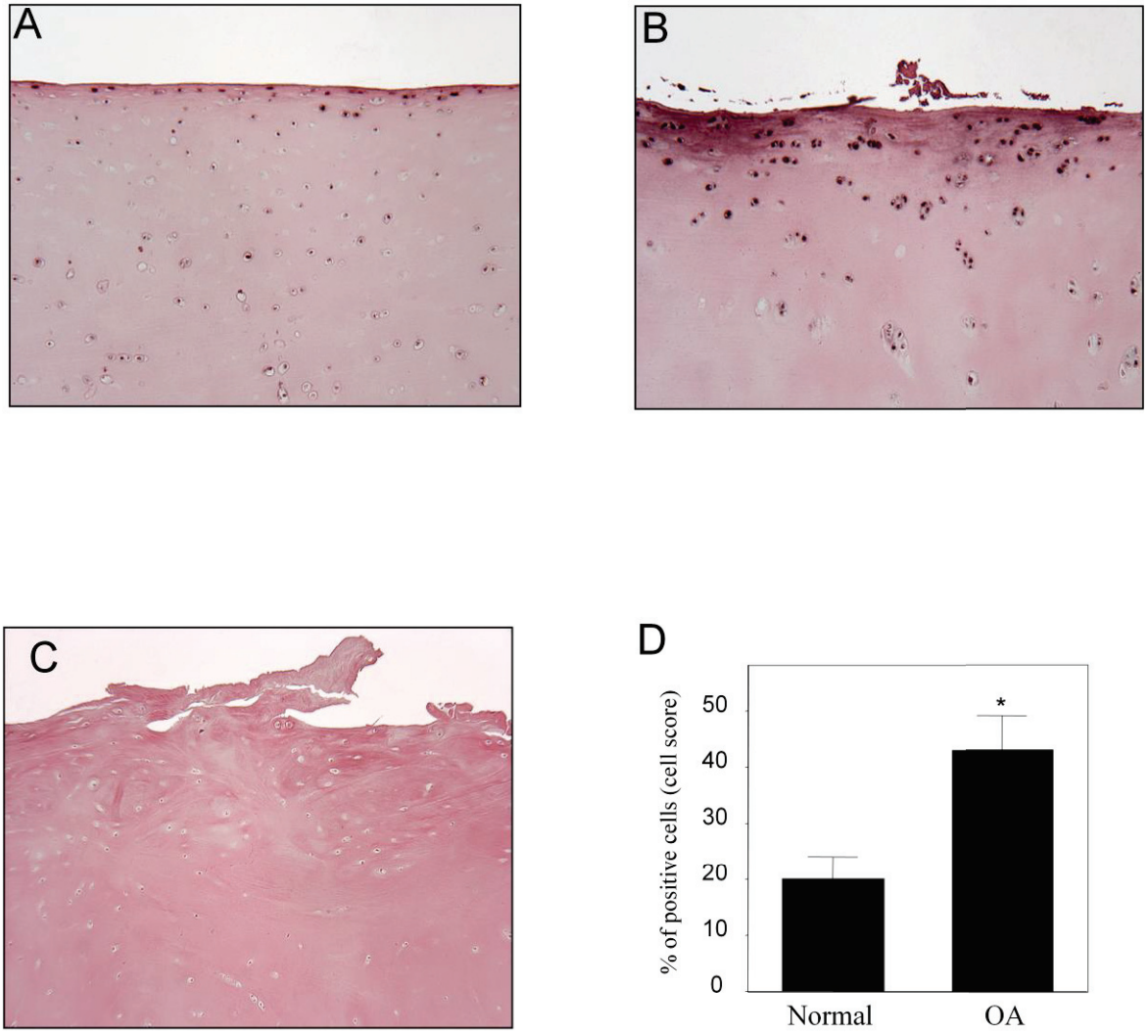


Figure 3 :

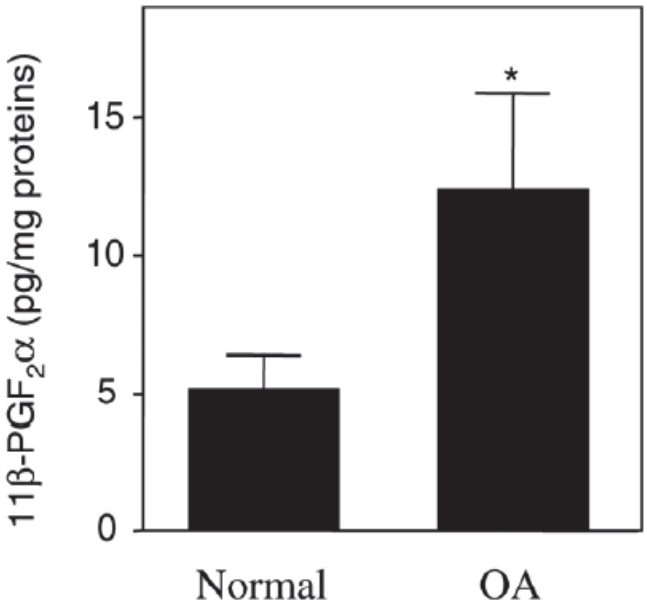


Figure 4:

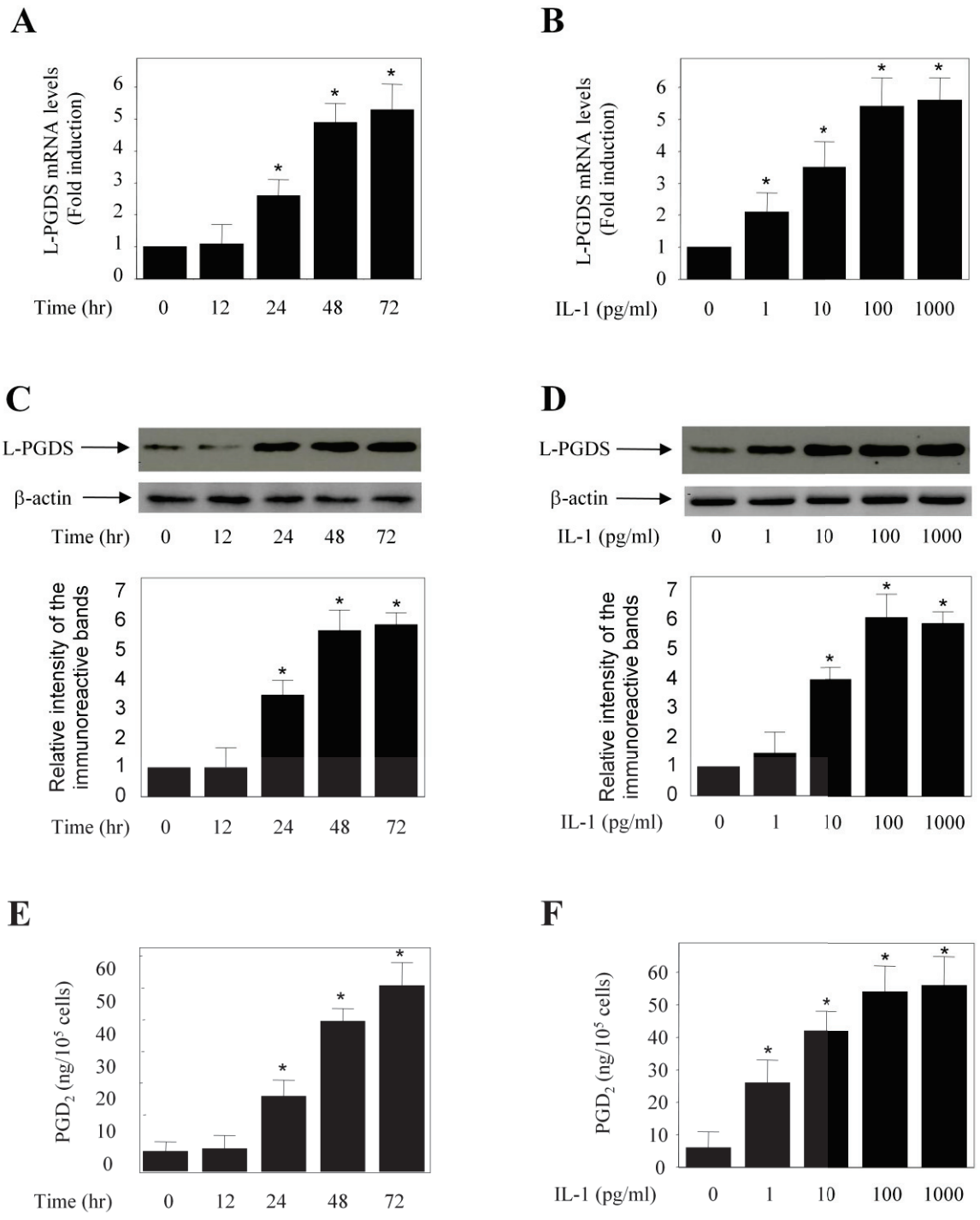

A
L-PGDS mRNA levels (Fold induction)
Time (hr) 0 12 24 48 72
B
L-PGDS mRNA levels (Fold induction)
IL-1 (pg/ml) 0 1 10 100 1000
C
L-PGDS
β-actin
Time (hr) 0 12 24 48 72
Relative intensity of the immunoreactive bands
Time (hr) 0 12 24 48 72
D
L-PGDS
β-actin
IL-1 (pg/ml) 0 1 10 100 1000
Relative intensity of the immunoreactive bands
IL-1 (pg/ml) 0 1 10 100 1000
E
PGD2 (ng/10^5 cells)
Time (hr) 0 12 24 48 72
F
PGD2 (ng/10^5 cells)
IL-1 (pg/ml) 0 1 10 100 1000

Figure 5:

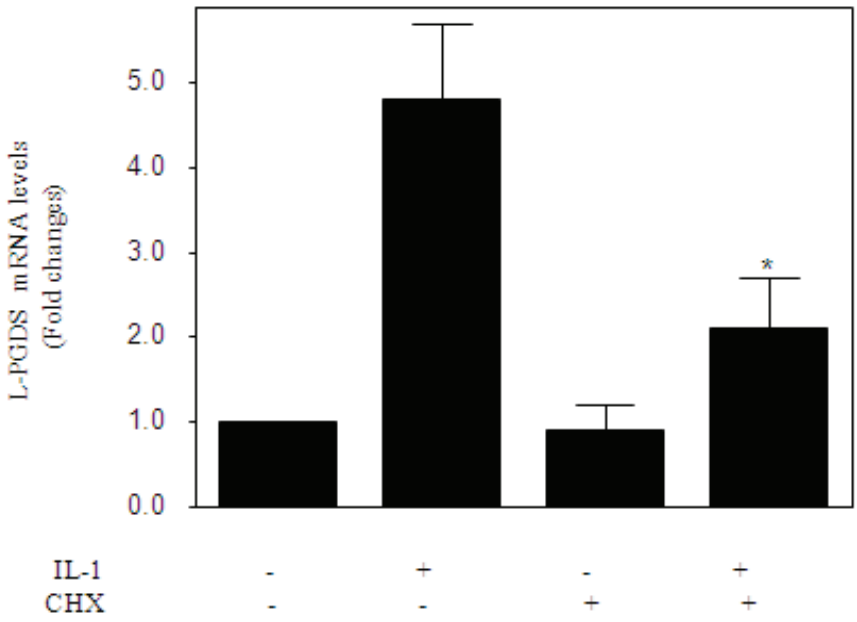
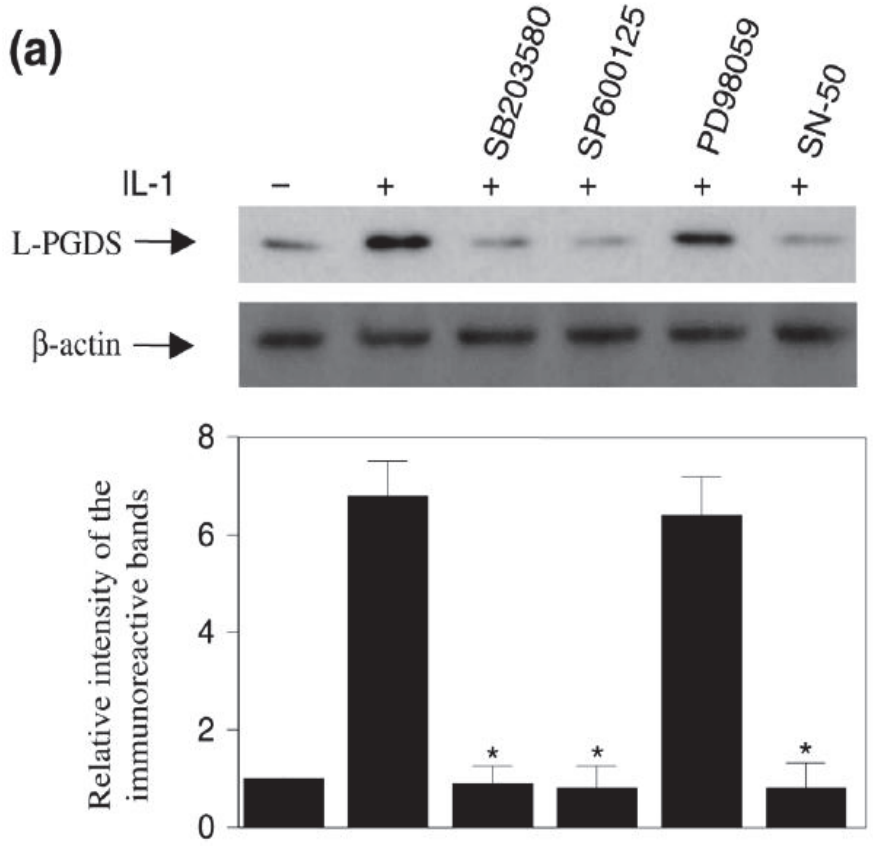


Figure 6:


(a)
SB203580
SP600125
PD98059
SN-50
IL-1
– + + + + +
L-PGDS
β-actin
Relative intensity of the immunoreactive bands
8
6
4
2
0
*
*
*

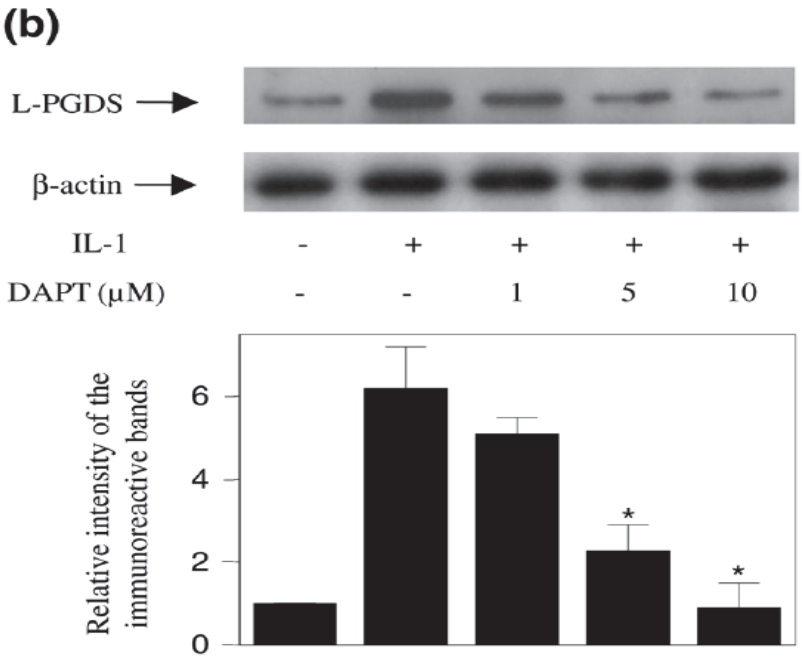
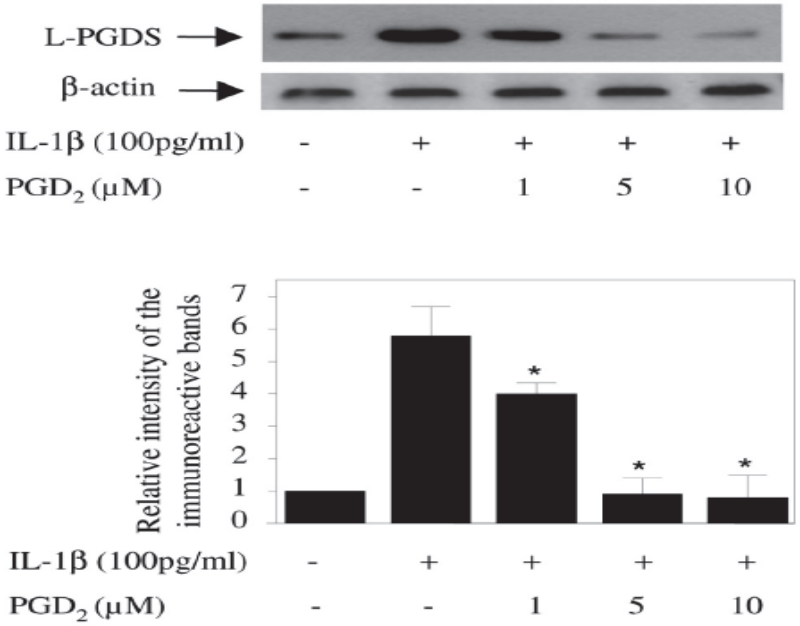

(b)
L-PGDS
β-actin
IL-1 - + + + +
DAPT (μM) - - 1 5 10
Relative intensity of the immunoreactive bands
0
2
4
6
*
*

Figure 7:


L-PGDS
β-actin
IL-1β (100pg/ml) - + + + +
PGD2 (μM) - - 1 5 10
Relative intensity of the immunoreactive bands
0
1
2
3
4
5
6
7
*
*
*
IL-1β (100pg/ml) - + + + +
PGD2 (μM) - - 1 5 10

CHAPITRE III : DISCUSSION

Le déséquilibre du métabolisme chondrocytaire caractéristique de l'OA en faveur du catabolisme conduit à la perte progressive des propriétés biomécaniques du cartilage articulaire, élément déclenchant d'une série de réactions au niveau de l'articulation. Cette perte cartilagineuse affecte les structures avoisinantes de différentes manières. Elle peut soit les affecter de manière directe, où sa dégradation met à nu l'os sous chondral et par conséquent lors du mouvement des frottements sont générés au niveau de cette structure. Comme elle peut les affecter indirectement où les fragments libérés de la dégradation entraînent une inflammation de la synoviale suite à la production de médiateurs inflammatoires qui peuvent agir de manière autocrine ou paracrine. Ces médiateurs inflammatoires sont représentés principalement par les cytokines inflammatoires, les PGs et les MMPs. Toutefois, des efforts ont été consentis afin d'étudier le rôle des MMPs au cours de l'OA. Les principales MMPs imliquées dans la maladie sont les MMP-1 et MMP-13.

Dans nos travaux de recherche nous avons porté un intérêt particulier à l'étude des MMP-1 et MMP-13. Comme nous nous intéresserons à leur régulation par le médiateur inflammatoire IL-1β produit au niveau du cartilage articulaire au cours de l'OA. Pour ce faire, les chondrocytes en culture sont notre matériel de base. Cependant, des travaux réalisés par *Bonaventure et al*, ont montré des inconvénients suite à l'utilisation de chondrocytes, en culture, ayant subi plusieurs passages. Selon ces travaux, les chondrocytes subissent un phénomène de dédifférenciation au cours de ces nombreux passages [297]. Suite à cela, nous n'utiliserons que des cultures primaires de chondrocyte dans nos expériences, afin de pallier à ce problème tout en bénificiant des avantages de l'utilisation des cultures.

Pour commencer, nous avons évalué l'effet de l'IL-1β sur la production de MMP-1 et MMP-13. Tel que présumé, le traitement des chondrocytes humains par l'IL-1β induit une augmentation de la production de MMP-1 et MMP-13 au niveau des chondrocytes en culture. Ces résultats concordent avec les données de la littérature dans la mesure où l'IL-1β est la cytokine majeure fortement produite au niveau de l'articulation OA, tel qu'il a été démontré dans de nombreux travaux de recherche [298], contribuant ainsi de manière importante au dévelopement de l'aspect inflammatoire de la maladie. De plus, l'IL-1β induit l'augmentaion de la production de nombreux médiateurs cataboliques, et anti-anaboliques. L'IL-1β exerce ses différents effets anti-anaboliques se traduisant par l'inhibition de la synthèse des protéoglycanes, ainsi que par la diminution de l'expression de l'aggrécane d'un ordre de 2 à 3 fois noté au niveau des explants de cartilage et des chondrocytes humains [113]. Dans ce même contexte, une étude réalisée par *Pfander et al*, a montré une diminution de plus de 40% de l'ARNm de l'aggrécane suite au traitement des chondrocytes OA avec l'IL-1β [105]. En outre son action sur l'aggrécane, l'IL1-β inhibe la synthèse de composants majeurs de la MEC représentés par le ColII [299]. Ce résultat a été confirmé par une étude réalisée par *Nawrat et al* qui, en utilisant des synoviocytes ont montré que le traitement par l'IL-1β conduit à une diminution de moitié de la synthèse du collagène [300]. De cette manière, et par l'inhibition de la synthèse de l'aggrécane et du ColII, l'IL-1β contribue de manière significative à l'inhibition de l'activité anabolique du chondrocyte.

À son action anti-anabolique, s'ajoute ses effets cataboliques par l'induction de la production des protéinases connues pour leur implication dans la dégradation du cartilage articulaire au cours de l'OA, entre autres la MMP-1, MMP-3 et MMP-13 [113]. Cette stimulation est conservée chez différentes espèces aussi bien chez les chondrocytes humains normaux que chez les chondrocytes OA [105]. La combinaison de l'ensemble des effets induits par l'IL-1β conduit à une sévère dégradation des tissus articulaires d'où la création de conditions caractéristiques et favorables pour le développement de l'OA [7]. De plus, des travaux réalisés par *Noh et al*, ont montré que le traitement de synoviocytes

(RA) avec l'IL-1β induit une augmentation de la production de MMPs [301]. Cette augmentation est également notée dans les travaux de *Hyeon G et al*, qui ont montré une augmentation de la production des MMPs sous l'effet de l'IL-1β chez les macrophages murins RAW264.7 [302].

En plus de ces effets, l'IL-1β agit également sur la production des PGs, dont la PGE_2 générée par la voie classique à partir de l'AA telle que décrite dans la première partie de cette thèse. La PGE_2 a fait l'objet de nombreux travaux de recherche l'incriminant dans la physiopathologie de l'OA.

Contrairement à la PGE_2 qui a fait l'objet de nombreux travaux de recherche dans l'OA, le rôle de la PGD_2 n'est presque pas connu. La PGD_2 est une molécule secrétée, synthétisée dans une variété d'organes comme le cerveau, la rate, le thymus, moelle osseuse, utérus, ovaire, oviducte, testicule, prostate, et épididyme [303-311]. Il s'agit du prostanoïde majeur produit au niveau du système nerveux central du rat, des autres mammifères ainsi que chez l'homme [312, 313], assurant de nombreuses fonctions physiologiques comme la régulation de la température corporelle [313], les fonctions olfactives, la libération d'hormones, la nociception [314], l'inhibition de l'aggrégation plaquettaire, le contrôle du mécansime de vasodilatation et de bronchochonstriction, la reproduction, la formation de la mémoire, et la régulation du sommeil [315, 316]. Le cycle veille-sommeil est accompagné de variations de la concentration de la PGD_2 au niveau du liquide céphalo-rachidien et ainsi des enzymes responsables de sa biosynthèse au niveau du cerveau [317].

Outre ces fonctions physiologiques, la PGD_2 est impliquée également dans plusieurs pathologies, notamment dans le paludisme cérébral [318] et la maladie du sommeil [319]. Il s'agit du prostanoïde produit par les mastocytes en réponse aux IgE et par conséquent impliqué dans plusieurs maladies inflammatoires telles que l'asthme [319], la conjonctivite [320], la rhinite [321], et les dermatites [322]. Il est probable que la PGD_2 agisse comme

une hormone où sa libération aux sites inflammatoires induit la mobilisation des éosinophiles de la moelle osseuse vers la circulation sanguine [323].

De plus, la PGD_2 joue également un rôle important dans la circulation par l'inhibition de l'agrégation plaquettaire au cours de maladies vasculaires ou par l'induction des éosinophiles lors de réponses allergiques [324]. La PGD_2 contribue au maintien de la progéniture en contrôlant la balance Th1/Th2 et la présentation de l'antigène (Ag) par les cellules dendritiques via ses récepteurs *D Prostanoid Receptor* 1 (DP1) et *Chemoattractant Receptor expressed on Th2 cells* (CRTH2) [325], elle réduit la sécrétion de l'IL-12 par les Th1, et affecte la synthèse des chemokines impliquées dans le chimiotactisme des Th1, plus particulièrement CXCL 10 [326, 327].

Tout comme les autres prostanoïdes, la biosynthèse de la PGD_2 résulte de deux réactions enzymatiques communes aux autres prostaglandines faisant intervenir la PLA2 et la COX2. La conversion de la PGH_2 en PGD_2 se fait sous l'action de PGDS qui catalysent l'isomérisation du groupe 9,11 endoperoxide de la PGH_2, précurseur commun de nombreux prosatnoïdes pour la production de la PGD_2 avec des groupements 9-hydroxy et 11-Keto [305, 328]. Il s'agit d'un métabolite avec une courte durée de vie pouvant être convertie par diverses réaction d'hydratation et d'isomérisation *in vitro*, ou encore par action enzymatique *in vivo,* aboutissant à des dérivés comportant des structures cycliques ou annulaires de type F,D ou J [329, 330].

Afin d'évaluer l'effet de la PGD_2 au niveau du cartilage articulaire, cette dernière a été utilisée en co-traitement avec l'IL-1β pour determiner la nature de l'effet exercé en présence de l'IL-1β: synergie, addition ou antagonisme.

Le cotraitement des cultures primaires de chondrocytes avec des concentrations croissantes de la PGD_2 en présence d'IL-1β entraîne une diminution de la production de MMP-1 et MMP-13 et donc la PGD_2 antagonise l'effet de l'IL-1β.

Afin de créer des conditions expérimentales se rapprochant le plus du contexte physiologique tout en gardant le contexte tissulaire, des explants de cartilage ont subi le même traitement à la PGD_2 et ont réagi de manière similaire aux chondrocytes cultivés en monocouche: par suppression de l'effet inducteur exercé par l'IL-1β sur la production de MMP-1 et MMP-13 au niveau des explants du cartilage articulaire de manière dose-dépendante. Cette diminution de production de la protéine n'est autre qu'une diminution de l'expression au niveau de l'ARNm tel que démontré par la RT-PCR qui est la résultante de la diminution de l'activité du promoteur sous l'effet de la PGD_2. L'ensemble de ces résultats pourrait nous amène à penser d'emblée que la PGD_2 pourrait avoir des effets anti-cataboliques de part l'antagonisme exercé en présence de l'IL-1β par l'inhibition de la production de MMP-1 et MMP-13 de manière dose-dépendante.

Dans ce même ordre d'idées, des travaux réalisés par *Jacob et al,* et *Tasaki et al*, ont appuyé le rôle pro-anabolique de la PGD_2 par l'induction de l'expression du ColII et de l'aggrécane [331, 332]. D'autres travaux réalisés par *Relic et al*, et *Pietila et al,* ont aussi évoqué son rôle dans la prévention de l'apoptose [333, 334]. En plus de son action augmentatrice de la différenciation chondrogénique et inductrice de la formation de la matrice au niveau du cartilage hyalin [331].

Outre ces effets, la PGD_2 exerce un effet anti-inflammatoire en s'opposant aux effets inducteurs exercés par l'IL-1β, notamment au cours de l'inflammation où une diminution de la PGD_2 en début d'inflammation et augmentation de cette dernière est notée lors de la phase de résolution [335-337]. La PGD_2 est parmi les PGs les plus abondantes au niveau du liquide synovial [281]. Elle est sécrétée par les cellules articulaires telles qu'elles soient, mastocytes synoviaux [338], synoviocytes [334], chondrocytes [339] ou encore les ostéoblastes [326]. Ce qui suggère que la PGD_2 possède un rôle dans le maintien de l'homéostasie du cartilage articulaire.

La biosynthèse de la PGD_2 est catalysée par des prostaglandines D-synthases, existant sous deux isoformes la H-PGDs et la L-PGDS ayant différentes localisations au niveau du corps humain. La H-PGDS est une protéine cytosolique de 26 kDa, active par des ions divalents dont le Ca^{2+} et Mg^{2+}, qui permettent la fixation du glutathion. La H-PGDS appartient à la classe Sigma gluthation S-transferase (GSTs); la seule protéine existant chez les vertébrés et est responsable de la production de la PGD_2 par les cellules immunitaires telles que les mastocytes, les cellules présentatrices d'antigènes et les cellules Th2 d'où son implication lors d'une inflammation [340]. L'ARNm de l'H-PGDS est également retrouvé au niveau des tissus périphériques, notamment le placenta, le poumon, le foie fœtal, rate, thymus, moelle osseuse et oviducte des vertébrés tel que le rat. Le gène humain est d'une taille de 41 kb situé sur le chromosome 4q21-22. Cette isoforme présente de grandes similitudes avec l'enzyme gluthation sigma S-transferase (GST) exprimée chez les invertebrés comme les insectes et nématodes, son expression est moindre au niveau du cœur, de ganglions lymphatiques, et la moelle osseuse. Tandis que cette dernière est augmentée au niveau du foie fœtal, ceci indique son rôle important dans l'hématopoïèse [340-343].

La deuxième isoforme, la L-PGDS (EC5.3.99.2) est une glycoprotéine sécrétoire de 27 kDa présente au niveau des membranes cellulaires d'où son nom ectoprotéine intervenant dans l'étape finale de la biosynthèse de la PGD_2 [344] en présence de groupement sulfudryl. Structuralement, elle est apparentée à la superfamille des lipocalines, possèdant trois résidus cystéine Cys65, Cys89, et Cys186 conservées chez toutes les espèces. La L-PGDS a pour caractéristique le transport des molécules lipophiles. Les cystéines aux positions 89 et 186 forment un pont disulfure conservé dans la plupart des lipocalines et dont la déstabilisation n'entraîne aucune perte d'activité. La Cys65 est unique à la L-PGDS et n'existe chez aucun autre membre des lipocalines [328]. La différence entre cette synthase et les autres membres du groupe réside au niveau du groupe SH du résidu cystéine appartenant au site actif qui peut être activé ou encore inhibé lors de certaines réactions physiologiques. Cette inhibition est réversible et peut se faire par des

ions de sélenium inoganiques tétravalents, comme elle peut être levée par des composés sulfudryls [345, 346]. La L-PGDS est la seule enzyme dans le groupe des lipocalines transportant les lipides comme, le rétinol et la β-lactoglobuline [313]. Elle est présente dans le plasma siminal, l'ascite, le sérum, l'urine, et le liquide amniotique [325], produite également au niveau du cerveau [347, 348] et par certains types cellulaires, notamment les adipocytes, ce qui laisse supposer son implication dans la tolérance du glucose, dans le diabète de type 2. Les souris présentant une déficience au niveau du gène L-PGDS montrent des anomalies dans ces fonctions [316, 328].

Afin de mieux caractériser la fonction de la PGD_2 au niveau du cartilage articulaire et en raison de l'absence de données sur l'expression des PGDS responsables de sa biosynthèse, nous avons cherché à évaluer dans un premier temps l'expression des PGDS aussi bien au niveau du cartilage normal qu'au niveau du cartilage OA. Après avoir déterminé la forme impliquée, nous avons été plus en profondeur afin de mieux définir les mécanismes moléculaires incriminés dans la régulation**.**

L'analyse de l'expression de l'ARNm de la H-PGDS et la L-PGDS, a montré que le cartilage articulaire, qu'il soit normal ou OA, exprime préférentiellement et de façon dominante la L-PGDS. La H-PGDS, quant à elle est très faiblement exprimée voire inexistante. Cette expression de L-PGDS connaît une augmentation au niveau du cartilage OA comparativement au cartilage normal tel que démontré par l'immunohistochimie avec une localisation préférentielle au niveau de la zone superficielle et la phase supérieure de la zone intermédiaire. En raison des effets anticataboliques et anti-inflammatoires de la PGD_2 discutés auparavant, il était logique qu'une augmentation de l'expression de L-PGDS soit observée lors de l'OA comme un méchanisme chondroprotecteur. Ces résultats observés au cours de l'OA concordent avec ceux obtenus dans le cas d'autres maladies inflammatoires, notamment l'athérosclerose [349], *multiple sclerosis* [350], le diabète [351], l'hypertension [352], ainsi que dans les maladies de Taysachs et Sandhoff [353], qui connaissent une augmentation de l'expression de L-PGDS.

L'expression préférentielle de la L-PGDS au niveau de la zone superficielle et la partie supérieure de la couche intermédiaire peut être liée à l'augmentation de l'expression de l'IL-β au niveau de ces zones, à l'origine des effets délétères [159, 354-357]. Dans le but de confirmer cette hypothèse, nous avons évalué l'effet de l'IL-1β sur l'expression de la L-PGDS.

Le traitement des cultures de chondrocytes avec l'IL-1β à différents intervalles de temps a montré une augmentation de l'expression de L-PGDS sous l'effet de l'IL-1β de manière temps dépendant à partir de 12h post induction et avec un pic à 72h. De même, le traitement avec des concentrations croissantes d'IL-1β montre une augmentation de l'expression de L-PGDS de manière dose-dépendante. Ces résultats indiquent que la L-PGDS est régulée positivement par l'IL-1β de manière dose et temps dépendants. Ce qui pourrait expliquer leur co-localisation au niveau du cartilage articulaire, et ce qui mène à penser que l'IL-1β exerce une boucle de rétroaction positive. Cette induction par l'IL-1β est bloquée sous l'action de la cycloheximide (CHX), ce qui indique que son induction nécessite de *novo* une synthèse de protéines et donc que l'IL-1β agit de manière indirecte sur la L-PGDS.

Dans ce même contexte, des travaux réalisés par *Mathurin et al,* ont montré également une co-localisation de la L-PGDS avec l'arrestine-3 qui intervient dans sa régulation chez les lignées cellulaires HEK293 et MG-63 [358]. Les arrestines sont impliquées dans de nombreux processus physiologiques, notamment dans l'inhibition de l'ostéoclastogénèse [281, 358] la régulation de l'IL-1β [339, 358].

Quant à l'induction retardée de la L-PGDS sous l'effet de l'IL-1β, ceci pourraît être expliqué par les travaux réalisés par *Gilroy et al*, et *Ianaro et al*, qui ont montré une augmentation de sa production durant la phase de résolution de l'inflammation lors de *carrageenan-induced pleurisy* chez le rat. De plus, un apport exogène en PGD_2 réduit le niveau des neutrophiles dans l'exsudat inflammatoire [335, 336]. Ceci pourrait expliquer que l'apparition tardive de la PGD_2 est liée à la résolution de l'inflammation.

S'ajoute à cela, les nombreux travaux mettant en valeur le rôle anti-inflammatoire des PGDS, notamment des expériences utilisant des souris PGD_2 synthase déficientes et un autre modèle de souris transégiques pour la PGDS générées afin d'analyser la durée et la sévérité de la réaction d'hypersensibilité retardée. Ces expériences ont appuyé le rôle anti-inflammatoire de la PGD_2 dans la mesure où les souris PGDS déficientes présentent une phase inflammatoire plus sévère, contrairement aux souris transgéniques présentant une faible inflammation [359]. Par ailleurs, une expression ectopique de la PGDS a permis de réduire l'inflammation dans un modèle murin [360]. Ces effets anti-inflammatoires de la PGD_2 ont été également appuyés par des travaux réalisés sur ce même modèle de cristaux d'urate de sodium monohydratés induisant l'inflammation [360, 361] avec d'autres travaux réalisés sur des souris H-PGDS déficientes qui ne parviennent pas à résoudre l'inflammation résultante de la réaction d'hypersensibilité retardée [359]. L'ensemble de ces données avec celles obtenues dans notre étude pourrait nous indiquer que les PGDS, de façon plus précise la L-PGDS au niveau des chondrocytes, exerce une boucle de contrôle négative sur les effets inflammatoires et cataboliques induits par l'IL-1β.

La combinaison des données de notre étude avec celles de la littérature appuyent l'idée que les PGs peuvent être divisés en PGs pro-inflammatoires, comme la PGE_2 synthétisée par la PGES, ou encore anti-inflammatoire à titre d'exemple la PGD_2 synthétisée par les PGDS, comme il vient d'être abordé plus haut. Cependant, une balance pourrait exister entre les différentes formes où l'apparition de l'une pourrait compromettre la synthèse de l'autre, en raison qu'elles partagent une voie de synthèse commune qui semble être profondément orientée vers la génération d'une forme ou de l'autre selon les conditions environnantes. Cependant, il a été remarqué qu'il existerait une relation étroite entre la PGE_2 et la PGD_2.

Des travaux réalisés par *Cipollone et al,* ont établi le type de lien existant entre la PGE_2 et la PGD_2 dans le cas d'artères athérosclérotiques où une faible expression de L-PGDS et des taux élevés de mPGES-1 sont produits lors de plaques symptomatiques, contrairement aux plaques asymptomatiques où une forte expression de L-PGDS et une faible de mPGES-1 sont notées. La balance entre la PGE_2 et la PGD_2 pourrait contribuer à ce type de pathologie [362]. Cette balance a été démontrée dans plusieurs travaux de recherche notamment ceux de *Fournier et al*, et *Matsumoto et al*, [363, 364]. La génération de la PGE_2 précédant la PGD_2 est liée à l'établissement de l'inflammation, tandis que l'apparition de la PGD_2 retardée est plutôt liée à la résolution de l'inflammation comme rapporté dans les travaux réalisés par *Kapoor et al*, lors de la cicatrisation [337]. Cette augmentation de la PGD_2 au cours de la résolution de l'inflammation apparaît être dû à une augmentation de la production de PPARγ et donc une synergie entre la PGD_2 et PPARγ pourrait exister afin d'induire la cicatrisation. La chronologie de la production entre la PGE_2 et la PGD_2, indique un rôle différé de la COX2 entre pro-inflammatoire via la génération de la PGE_2 et anti-inflammatoire via la génération de la PGD_2 [337]. Les résultats obtenus dans cette étude appuyent le rôle anti-inflammatoire de la PGD_2 [335, 360, 365-367]. Paradoxalement, l'utilisation d'antagonistes des récepteurs de la PGD_2 sont préconisés pour le traitement de l'asthme et des rhinites [368], indiquant le rôle pro-inflammatoire que pourrait jouer la PGD_2 lors de maladies allergiques [369].

Toutefois, et à l'opposée des résultats de notre étude qui avaient montré une expression tardive de la L-PGDS, une étude réalisée par *Joos et al*, [370] a montré que la soumission d'explants de cartilage à un stress mécanique entraîne une augmentation de la production de la PGD_2, suite à l'application de la contrainte. Cette élévation de production de la PGD_2 n'est pas accompagnée de l'augmentation de l'expression de PGDS dans les 24h suivant la stimulation. Ceci suggère une expression précoce de PGDS qui finit par disparaître dans les 24h suivant la stimulation et ce malgré la présence de l'IL-1β. Donc le stress mécanique pourrait exercer une boucle de rétrocation négative sur l'IL-1β induisant

l'expression de PGDS. Cette augmentation de la production de PGD_2 suite à l'application d'une contrainte mécanique sur les explants de cartilage est corrélée avec l'augmentation de cette dernière dans les travaux réalisés par *Gallant et al*, [371] qui ont montrée une augmentation de sa production au niveau de l'urine ainsi qu'au niveau du sérum de patients ayant eu des fractures. L'augmentation de la production de PGD_2 est accompagnée de l'augmentation de la L-PGDS. L'ensemble de ces résultats nous amène à penser que la production de la PGD_2 au niveau des explants du cartilage a pour objectif le rétablissmement de l'homéostasie cellulaire cartilagineuse. Tandis que son augmentation suite à la fracture peut suggérer que la PGD_2 soit impliquée dans la réparation osseuse, et par conséquent dans le contrôle de l'anabolisme osseux.

Pour voir si cette forme de PGDS est effectivement active au niveau du cartilage articulaire, nous avons procédé au dosage de la forme la plus stable de la PGD_2 representée par le $9\alpha\text{-}11\beta PGF_{2\alpha}$ au niveau du liquide synovial et qui est inactive sur les récepteurs de la PGD_2 [372]. Les résultats montrent que la production de ce métabolite concorde parfaitement avec l'augmentation de l'expression de la L-PGDS. Ce qui confirme que cette dernière est la seule isoforme responsable de la synthèse de la PGD_2 au niveau des tissus articulaires.

Comme rapporté dans plusieurs travaux de recherche [121, 373-376], l'IL-1β est la cytokine responsable d'induire ses effets via différentes voies de signalisation, entre autres p38MAPK, ERK, *c-jun N-terminal kinase* (JNK), et enfin *Nuclear Factor-kappa-B* (NF-κB). L'implication de ces différentes voies de signalisation a été élucidée par l'utilisation d'inhibiteurs pharmacologiques spécifiques. Les inhibiteurs des voies JNK et p38MAPK ont bloqué l'augmentation de l'expression de L-PGDS induite par l'IL-1β. De même que pour l'inhibiteur de la voie NF-κB qui diminue l'expression de L-PGDS sous l'effet de l'IL-1β, contrairement à l'inhibiteur de la voie ERK/MAPK qui n'a montré aucun effet notable. Ces résultats révèlent l'implication des voies JNK, et p38MAPK dans l'augmentation de l'expression de L-PGDS sous l'effet de l'IL-1β ainsi que la voie NF-κB.

L'activation de ces voies chez les chondrocytes induit l'activation des facteurs de transcription dont la protéine -1 (AP-1) et NF-κB [326, 331, 333, 334, 338]. Comme le promoteur de L-PGDS humain comporte des sites pour AP-1 et NF-κB [377, 378], on présume que l'effet de l'IL-1β sur L-PGDS, pourraît être médié par les facteurs AP-1 et NF-κB.

Or, les résultats obtenus de nos expériences montrent clairement que la PGD_2 s'oppose aux effets de l'IL-1β. Au moment où l'IL-1β induit l'expression de L-PGDS via les voies p38 MAPK, JNK MAPK et NF-κB, la PGD_2 à son tour diminue la production induite de la L-PGDS par l'IL-1β. Ce qui nous permet de supposer que la PGD_2 inhibe les voies de signalisation (p38 MAPK, JNK MAPK et NF-κB) via lesquel l'IL-1β exerce une induction de l'expression de la L-PGDS, ceci d'une part.

D'autre part, l'activation transcriptionnelle des MMPs, à l'exception de MMP-2 et MMP-11, est aussi dépendante du facteur de transcription AP-1 [172]. L'inhibition des voies (p38 MAPK, JNK MAPK et NF-κB) par la PGD_2 peut être à l'origine de la diminution de l'expression des MMPs, via lesquelles l'IL-1β induit l'expression des MMPs, tel que rapporté dans les travaux de *Pelletier et al*, qui supportent le fait que l'IL-1β induit l'expression des MMPs, notamment la MMP-1, par les voies p38 MAPK et JNK MAPK [172]. Ceci explique que leur inhibition est à l'origine de l'antagonisme exercé par la PGD2 via l'IL-1β sur l'expression de la L-PGDS, mais aussi que l'inhibition de ces voies peut probablement être la cause de la diminution de la production de MMP-1 et MMP-13, dont l'activation transcriptionnelle est AP-1 dépendante.

Ces résulats obtenus chez les chondrocytes sont confirmés chez d'autres types cellulaires dont les cellules leptoméningées [377], les cellules endothéliales [378], et les macrophages [379]. L'IL-1β exerce ses effets en implicant les voies MAPKs (JNK and p38) et NF-κB dans l'augmentation de l'expression de L-PGDS. En plus de ces voies, la

voie Notch est impliquée dans la régulation de nombreux processus physiologiques [380] et est impliquée également dans la régulation positive de la L-PGDS par l'IL-1β tel que démontré par l'utilisation de l'inhibiteur spécifique DAPT qui montre une inhibition de l'induction de la L-PGDS par l'IL-1β. Paradoxalement, cette voie régule négativement l'expression de L-PGDS au niveau d'autres types cellulaires, notamment les cellules TE671 qui dérivent du cerveau [381], ceci peut être expliqué par les différences existantes entre les différents types cellulaires ou encore à des différences dans les conditions expérimentales.

Toujours dans l'objectif de mieux caractériser la régulation de la L-PGDS, nous avons évalué l'effet de son métabolite final la PGD_2, utilisé à des concentrations croissantes sur les chondrocytes en culture suivant le traitement par l'IL-1β. Les résultats obtenus ont montré une diminution de la production de L-PGDS sous l'effet de la PGD_2. Les concentrations de la PGD_2 conduisant à l'effet observé sont plus élevées que celles produites au niveau du liquide synovial. Par cet effet, on déduit que la PGD_2 inhibe l'induction de la L-PGDS sous l'effet de l'IL-1β et ainsi son activité, ce qui nous mène à suggérer que la PGD_2 exerce une boucle de rétroaction négative, pouvant agir de manière autocrine ou paracrine dans le contexte cellulaire physiologique atteignant des niveaux pharmacologiques. La diminution de l'expression de L-PGDS sous l'effet de la PGD_2, et son induction par l'IL-1β, de même que son augmentation notée au niveau du cartilage arthrosique, nous mène à déduire que l'effet de l'IL-1β emporte sur celui de la PGD_2 *in vivo,* pendant les phases tardives de la maladie car les échantillons humains ne sont récupérés que lors d'un stade très avancé suite au remplacement de l'articulation du genou. Il serait sans doute pertinent d'étudier le profil d'expression de la L-PGDS au fil du temps.

Bien que la PGD_2 puisse exercer ses effets via différents récepteurs, notamment récepteur du tromboxane A2(TP) ainsi qu'au récepteur de la PGE_2, EP3 [382], ses effets sont exercés principalement via deux récepteurs. Le premier récepteur est couplé aux protéines G, appelé DP1 et membre de la famille des récepteurs prostanoïdes cloné en 1995

par *Boie et al* [383]. Il s'agit d'un récepteur via lequel la PGD_2 exerce de nombreux effets. Ce récepteur intervient dans la réponse immunitaire en affectant le processus de maturation et de migration des cellules dendritiques, le retardement de l'apoptose des éosinophiles ainsi que l'inhibition fonctionnelle des cellules dendritiques dont l'activation empêche la reconstitution osseuse et diminue la production de l'ostéoprotégérine par les ostéoblastes humains. L'action via DP1 peut conduire à une série de réactions lors de l'asthme par l'induction de la vasodilatation, de la bronchochonstriction et l'inhibition de l'aggrégation plaquettaire [384] et enfin la diminution de la production d'INF-γ et d'IL-2 produits par les lymphocytes CD4+ et CD8+ au cours des allergies [326].

La PGD_2 joue un rôle immuno-régulateur lors de la réponse innée via DP1 exprimée par les cellules dendritiques iNKT en réponse à un superagoniste (α-GalCer) [385]. Il possède également un rôle dans la protection du cerveau du nouveau-né de l'hypoxie-ischémique en prévenant la dégénération des cellules endothéliales [240].

En plus de l'action de la PGD_2 via DP1, cette dernière peut également agir via un second récepteur CRTH2 décrit pour la première fois en 2001 [386]. Différemment du DP, le CRTH2 est un membre de la famille des « *Chemoattractant receptors* » présentant une plus grande homologie avec les récepteurs fMLP et C5a qu'avec la famille des récepteurs DP, son activation conduit à une mobilisation du Ca^{2+} intracellulaire. Le CRTH2 humain est composé de 395 acides aminés avec un poids moléculaire de 43 kDa comprenant sept domaines transmembranaires caractéristiques des récepteurs couplés à la protéines G alpha *i/*0 avec deux sites de glycosylations à l'extrémité NH2-terminale et une longue partie cytoplasmique comportant plusieurs sites de phosphorylation par les protéines kinases C. Ces sites semblent jouer un rôle dans l'activation du récepteur [386] retrouvé dans les organes périphériques notamment le cerveau, le cœur, l'estomac, le foie, le placenta, et thymus fœtal. Chez l'homme, ce récepteur est exprimé préférentiellement sur les lymphocytes de type 2, les éosinophiles et les basophiles possédant un rôle également dans le chimiotactisme *in vitro* [372].

L'expression de ces récepteurs au niveau des chondrocytes humain n'a fait guère l'objet de recherche. Pour cela nous avons examiné leur expression au niveau des chondrocytes obtenus de donneurs différents. Les résultats obtenus démontrent clairement l'expression de DP1 et CRTH2 par les chondrocytes humains comparativement au contrôle positif. En plus des chondrocytes, d'autres cellules de l'articulation, les ostéoclastes expriment ces deux types de récepteurs et le DP1 semble jouer un rôle dans la résorption osseuse. Cependant, l'activation de ces récepteurs par des agonistes spécifiques DP1 et CRTH2 inhibe l'ostéoclastogénèse. Cet effet est renversé par l'utilisation d'antagonistes spécifiques à DP1 ou CRTH2 en présence de la PGD_2 endogène. Ces deux récepteurs sont bien exprimés et sont fonctionnels au niveau de l'os.

Pour confirmer la fonctionnalité de ces récepteurs au niveau du cartilage articulaire, les chondrocytes ont été traités avec des concentrations croissantes de la PGD_2. Tel que précisé dans la littérature la liaison de la PGD_2 à son récepteur DP1, tout comme les autres récepteurs couplés aux protéines G, conduit à une élévation de l'AMPc intracellulaire [387], ce qui a pour conséquence l'activation de la PKA. Tandis que l'action de la PGD_2 via le CRTH2 entraîne la mobilisation du calcium intracellulaire suite à l'ouverture de canaux calciques [240]. Les effets respectifs résultant de l'activation de l'un comme l'autre des récepteurs ont été observés chez les chondrocytes humains suite à leur traitement par la PGD_2, indiquant ainsi la fonctionnalité des deux recepteurs.

L'objectif de ces expériences ne se limite pas à démontrer l'expression et la fonctionnalité des récepteurs au niveau des chondrocytes humains, mais aussi de confirmer que la PGD_2 outre ses effets anti-inflammatoires connus au cours de l'OA [388], exercés via ses métabolites de la série J, dont le 15d-PGJ2, est capable par elle-même d'induire ces effets recherchés sous sa forme brute par son action directe via ses récepteurs.

En vu de déterminer de manière précise lequel des deux récepteurs est impliqué dans les effets de la PGD_2 conduisant à la diminution de la production et de l'expression de MMP-1 et MMP-13, nous avons eu recours à l'utilisation d'agonistes spécifiques dont le BW245C exerçant ses effets via DP1 [389, 390] et le DK-PGD_2 présentant une plus grande

sélectivité pour CRTH2 que pour DP1 et est légèrement moins puissant que la PGD_2 dans l'activation de ce récepteur [372, 391], en absence de la PGD_2. De manière intéressante, nous avons eu une reproduction des effets obtenus sous l'action de la PGD_2 sur MMP-1 et MMP-13, malgré l'absence de la PGD_2. Cet effet est mimé par le BW245C, qui diminue la production de MMP-1 et MMP-13 de manière dose-dépendante, ce qui suggère que la PGD_2 exerce ses effets via DP1 et non via CRTH2. Ces résultats sont appuyés par d'autres travaux indiquant le rôle anti-inflammatoire de la PGD_2 via ce récepteur, notamment ceux réalisés par *Matsuoka et al* [392].

Afin de confirmer l'implication de DP1 dans l'effet de la PGD_2, les chondrocytes sont traités par des concentrations croissantes d'anticorps polyclonal anti-DP1 et les cellules sont stimulées par l'IL-1β en absence ou en présence de la PGD_2. Les niveaux de MMP-1 et MMP-13 sont évalués dans le milieu. Il semble que l'anticorps polyclonal bloque l'effet inhibiteur induit par la PGD_2 de manière dose-dépendante, au moment où le traitement avec l'anticorps anti-CRTH2 n'exerce aucun effet sur l'expression de MMP-1 et MMP-13. Bien que l'anticorps anti-CRTH2 ne soit pas capable de s'opposer aux effets exercés par la PGD_2 sur les MMPs, ce dernier est capable d'arrêter la mobilisation du calcium intracellulaire suite à la stimulation de la PGD_2, démontrant ainsi sa spécificité pour le récepteur. Malgré que l'effet de la PGD_2 sur la production et l'expression de MMP-1 et MMP-13 est clairement reproduit par l'agoniste de DP1, le BW245C de manière très comparable à celle résultante du traitement à la PGD_2, on ne peut toutefois exclure la possibilité que la PGD_2 pourrait induire cette diminution par un méchanisme DP1 indépendant.

Tel que décrit dans la littérature, la PGD_2 peut être convertie de manière non enzymatique en 15-deoxy-Δ12,14-PGJ2 [330], qui est à son tour responsable de l'inhibition de l'induction de MMP-1 et MMP-13 dans différents types cellulaires y compris au niveau des chondrocytes, entre autres chez la lignée chondrocytaire SW1353 [190]. Cet effet inhibiteur est observé également au niveau des chondrocytes de rat où le 15d-PGJ2 inhibe

l'induction de la MMP-3 et la MMP-9 sous l'effet de l'IL-1β et du TNF-α, de même que pour la dégradation des proéoglycanes [393]. De ce fait, il serait souhaitable que d'autres travaux soient réalisés dans un objectif de déterminer si l'effet observé est exercé vraisemblablement par une méchanisme DP1 dépendant ou indépendant.

Dans cette optique, l'utilisation d'agents ayant pour effets d'induire l'AMPc intracellulaire, effet observé suite à l'activation du récepteur DP1, conduit à une diminution de la production de MMP-1 et MMP-13. Cet effet est par contre supprimé par l'utilisation d'inhibiteurs de la PKA (H89, KT520). Ces résultats indiquent que la voie DP1/cAMP/PKA est la voie par laquelle la PGD_2 exerce ses effets.

L'ensemble de ces résultats indique que la PGD2 représente une cible intéressante au cours de l'OA, non seulement via ses dérivées, mais aussi via sa forme brute tel que démontré: par l'inhibition de la production de MMP-1 et MMP-13 fortement impliquées dans le catabolisme observé lors de la maladie et principaux agents dégradant le cartilage articulaire. Les effets de la PGD_2 sont médiés via le récepteur DP1, mettant en œuvre la voie cAMP /PKA.

En raison des effets chondroprotecteurs, proanaboliques, anti-inflammatoires et anticataboliques tel qu'on vient de démontrer, la PGD_2 pourrait représenter une cible thérapeutique intéressante pour le traitement des maladies arthritiques, non seulement via son action directe sous sa forme brute PGD_2 sur le récepteur DP1 dans le cas de notre étude, mais aussi grâce à sa conversion au $15dPGJ_2$, considéré comme agent antiarthritique [244].

Cependant, afin de confirmer nos résultats obtenus *in-vitro*, une étude complémantaire *in-vivo* s'avère nécessaire. Malgré les recherches intensives sur l'OA, il n'existe actuellement aucun consensus, ni sur le mode ni sur les espèces les plus pertinents pour l'homme. L'absence d'un tel modèle standard de l'OA, provient entre autres d'une mauvaise compréhension de l'étiologie de la maladie, mais aussi de l'absence de traitement

efficace chez les humains qui pourrait être utilisé pour évaluer la pertinence des modèles animaux existants. Donc, en absence de modèle standard, je propose d'utiliser, le modèle animal, Cochon d'Inde Hartley. Cette espèce est le modèle de choix pour la validation de nos résultats *in-vitro*. La raison du choix de cette espèce est le fait qu'elle développe l'OA de manière spontannée [394, 395]. Le compartiment affecté est le compartiment médial du genou, ce qui est semblable à l'homme. De plus, différemment aux autres espèces d'OA induite ou spontannée, le cochon d'Inde exprime les deux MMPs majeures impliquées au cours de l'OA, la MMP-1 et MMP-13 [396]. Un suivi du profil d'expression de la H- et L-PGDS, 15-LOX1, 15-LOX2 et aussi PPARγ, avec un dosage de la PGD2 doit être réalisé chez cette espèce à différents intervalles de temps.

Contrairement aux autres PGs exerçant leurs effets via des récepteurs couplés aux protéines G, le 15d-PGJ_2 connu pour ses effets anti-inflammatoires [335, 397], pourrait représenter une cible interessante dans le traitement des maladies inflammatoires. Cette molécule exerce ses effets via un méchanisme PPARγ dépendant ou indépendant [244, 398, 399], par l'inhibition de l'activation de NF-κB [400].

Cependant, le 15d-PGJ_2 à des concentrations de l'ordre de la micromole promeut la résolution de l'inflammation par sa capacité à diminuer l'expression de médiateurs inflammatoires comme l'IL-1β, TNF-α, iNOS et de médiateurs cataboliques dont les MMPs [244] , ainsi que dans l'induction de l'apoptose [400], l'inhibition de la COX2 [372]. De plus, plusieurs travaux réalisés *in vivo* ont démontré l'effet protecteur du 15d-PGJ_2 ainsi que des autres ligands de PPARγ [401, 402]. Outre ces effets, le 15d-PGJ_2 reverse l'effet inhibiteur de l'IL-1β sur la production de COX2 et de iNOS et par conséquent sur leur produits la PGE_2 et NO au niveau des chondrocytes humains arthrosiques, tel que démontré dans des travaux ultérieurs de notre laboratoire, ainsi que dans d'autres [403-405]. Ce même effet est exercé par le 15d-PGJ_2 au niveau des chondrocytes de rat [398, 403-407]. L'inhibition de la production de la PGE_2 ne se fait pas uniquement via l'inhibition de la production de la COX2, mais aussi via l'inhibition de la mPGES1 qui catalyse sa biosynthèse. Cet effet est observé au niveau des chondrocytes

humains arthrosiques [408]. De plus, la PGD_2 et son métabolite le $15dPGJ_2$ entraînent l'apoptose des neutrophiles et des macrophages lors d'une atteinte pulmonaire (pleurésie) chez le rat [335].

D'autres études ont mis le point sur l'implication de la PGD_2 et de son métabolite, le $15dPGJ_2$ dans l'apoptose, leur rôle dans ce processus n'est par contre pas toujours clair, dans la mesure où ils préviennent l'apoptose au niveau des chondrocytes humains normaux via l'inhibition de NF-κB, ce qui semble se produire via la voie MAPK ERK1/2. Mais aussi, de façon plus spécifique le 15d-PGJ_2 induit l'apoptose via la voie p53 au niveau des chondrocytes OA, RA mais aussi normaux. Ceci est en contradiction avec leur effet inhibiteur observé [409, 410]. Bien que le 15d-PGJ_2 puisse exercer ses effets via un méchanisme PPARγ indépendant, nombreux sont les travaux qui supportent son effet anti-inflammatoire via un méchanisme PPARγ dépendant.

CONCLUSION ET PERSPECTIVES

Les résultats obtenus de nos recherches apportent un éclairage nouveau sur l'une des nombreuses cibles moléculaire impliquées dans la physiopathologie de l'OA et dont le rôle restait encore méconnu. Nos travaux ont montré pour la première fois l'expression des deux récepteurs à la PGD_2 et leur fonctionnement au niveau des chondrocytes humains. Cependant, la PGD_2 possède des effets anti-cataboliques au cours de l'OA par l'inhibition de la production des MMP-1 et MMP-13, les principales MMPs responsables de l'altération de l'architecture matricielle et à l'origine de la perte des propriétées biomécaniques du cartilage articulaire. Cette inhibition se fait via la voie DP1/AMPc/PKA. En sus de cet effet, on pourrait spéculer que la PGD_2 pourrait être une cible intéressante dans l'OA par l'antagonisme qu'elle peut exercer sur l'IL-1β en s'opposant aux différentes cascades cataboliques et inflammatoires entrainées par cette dernière.

Afin de completer ces travaux, il serait opportun de déterminer la nature des effets exercés par DP1 *in vivo* au cours de l'OA. Le traitement d'un modèle animal avec une OA induite par un agoniste DP1 permettrait d'évaluer l'effet exercé par DP1, d'abord par une évaluation histologique, en utilisant les critères de l'OARSI, qui permettraient de voir la progression globale de l'OA, mais aussi de voir l'évolution des différents médiateurs cataboliques dont les MMP-1 et MMP-13, inflammatoires, iNOS, mPGES et COX2 et anaboliques comme le ColII. Les résultats récupérés de cette étude seront comparés aux résultats obtenus à partir d'un modèle animal DP1 déficient, chez qui une évaluation histologique doit être préalablement réalisée. L'expression des différents médiateurs inflammatoires, cataboliques et aussi anaboliques, doit aussi être effectuée afin de pouvoir comparer avec le modèle OA, ayant subi un traitement avec l'agoniste DP1.

Dans la deuxième partie de nos travaux nous avons également démontré pour la première fois que les prostaglandines D-synthases responsables de la biosynthèse de la PGD_2 sont

exprimées au niveau du cartilage articulaire normal et OA et que la L-PGDS est l'isoforme responsable de la biosynthèse de la PGD_2 au niveau du cartilage articulaire du fait que son expression soit plus importante au niveau du cartilage OA comparativement au cartilage normal. De plus, la L-PGDS semble être régulée positivement par l'IL-1β. Cet effet est exercé de manière indirecte et plusieurs voies de signalisation semblent être impliquées.

Collectivement, ces résultats nous mènent à penser qu'il serait d'un grand profit de continuer à explorer cette voie par la génération de souris L-PGDS transgéniques OA. Une évaluation histologique sur un intervalle de temps bien déterminé ne dépassant pas les trois mois, tenant compte de la vitesse de progression considérable de l'OA, chez les modèles induits, est recommandée chez ces souris et sera comparée à des souris OA présentant une expression normale de la L-PGDS. De plus, un suivi du profil d'expression des médiateurs inflammatoires COX-2, mPGES, iNOS; cataboliques, MMP-1 et MMP-13 et anaboliques, tel que ColII à des intervalles de temps bien déterminés post-induction de l'OA doivent être réalisés et comparés à des souris OA normales (L-PGDS exprimée normalement). La comparaison des scores histologiques permet d'abord de déterminer, si la L-PGDS *in-vivo* semble améliorer ou du moins stabiliser la progression de l'OA. Tandis que la comparaison du profil d'expression des différents médiateurs, pourrait déterminer via quelle voie la L-PGDS exerse ses effets, à l'origine des résultats obtenus (histologie).

En résumé, les résultats obtenus de notre étude indiquent l'importance que pourrait avoir la PGD_2 dans la physiopathologie de l'OA et l'intérêt porté pour une étude plus complète sur cette prostaglandine.

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ANNEXES

ANNEXE 1 :

Histone deacetylase inhibitors suppress interleukin-1beta-induced nitric oxide and prostaglandin E2 production in human chondrocytes

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Histone deacetylase inhibitors suppress interleukin-1β-induced nitric oxide and prostaglandin E_2 production in human chondrocytes

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Summary

Objective: Overproduction of nitric oxide (NO) and prostaglandin E_2 (PGE_2) plays an important role in the pathogenesis of osteoarthritis (OA). In the present study, we determined the effect of trichostatin A (TSA) and butyric acid (BA), two histone deacetylase (HDAC) inhibitors, on NO and PGE_2 synthesis, inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 expression, and nuclear factor (NF)-κB DNA-binding activity, in interleukin-1β (IL-1)-stimulated human OA chondrocytes, and on IL-1-induced proteoglycan degradation in cartilage explants.

Methods: Chondrocytes were stimulated with IL-1 in the absence or presence of increasing concentrations of TSA or BA. The production of NO and PGE_2 was evaluated using Griess reagent and an enzyme immunoassay, respectively. The expression of iNOS and COX-2 proteins and mRNAs was evaluated using Western blotting and real-time reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. Proteoglycan degradation was measured with dimethymethylene blue assay. Electrophoretic mobility shift assay (EMSA) was utilized to analyze the DNA-binding activity of NF-κB.

Results: HDAC inhibition with TSA or BA resulted in a dose-dependent inhibition of IL-1-induced NO and PGE_2 production. IL-17- and tumor necrosis factor-α (TNF-α)-induced NO and PGE_2 production was also inhibited by TSA and BA. This inhibition correlated with the suppression of iNOS and COX-2 protein and mRNA expression. TSA and BA also prevented IL-1-induced proteoglycan release from cartilage explants. Finally, we demonstrate that the DNA-binding activity of NF-κB, was induced by IL-1, but was not affected by treatment with HDAC inhibitors.

Conclusions: These data indicate that HDAC inhibitors suppressed IL-1-induced NO and PGE_2 synthesis, iNOS and COX-2 expression, as well as proteoglycan degradation. The suppressive effect of HDAC inhibitors is not due to impaired DNA-binding activity of NF-κB. These findings also suggest that HDAC inhibitors may be of potential therapeutic value in the treatment of OA.

Key words: Chondrocytes, Nitric oxide, Inducible nitric oxide synthase, Prostaglandin E_2, Cyclooxygenase-2, Histone deacetylases, Nuclear factor-κB.

Abbreviations: BA butyric acid, COX-2 cyclooxygenase-2, HDAC histone deacetylase, IL interleukin, iNOS inducible nitric oxide synthase, NF-κB nuclear factor-κB, NO nitric oxide, OA osteoarthritis, PGE_2 prostaglandin E2, TNF-α tumor necrosis factor-α, TSA trichostatin A.

Introduction

Osteoarthritis (OA) is the most common joint disorder and a leading cause of disability among the elderly population. It is characterized by progressive degenerative structural changes in articular cartilage, leading to loss of joint function. It is also characterized by excessive production of several inflammatory mediators[1–3]. Among these mediators, the pro-inflammatory cytokine interleukin-1β (IL-1) plays a pivotal role in the pathophysiology of OA. It induces a cascade of inflammatory and catabolic events in chondrocytes including the synthesis of prostaglandin E_2 (PGE_2) and nitric oxide (NO). IL-1 also alters chondrocyte anabolism by suppressing the synthesis of proteoglycan and collagen and by enhancing the production of matrix metalloproteinases (MMPs)[1–3].

NO is synthesized from L-arginine by a family of NO synthases (NOSs) of which three isoforms have been identified. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while the inducible NOS (iNOS) is expressed following stimulation with a variety of inflammatory agents such as endotoxins or cytokines[4]. NO promotes inflammation by enhancing the production of inflammatory cytokines[5] and PGE_2[6] and by reducing

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the synthesis of endogenous IL-1 receptor antagonist (IL-1 Ra)[7]. NO is also considered a potent catabolic agent in OA since it inhibits collagen and proteoglycan synthesis[8,9], stimulates the production and activation of MMPs[10] and induces chondrocyte apoptosis[11]. Accordingly, the *in vivo* selective inhibition of iNOS in an experimental model of OA reduces the joint structural changes and the expression of several inflammatory and catabolic factors, including IL-1 and MMP-1[12].

The biosynthesis of PGE_2 from arachidonic acid (AA) involves multiple enzymes including, cyclooxygenases (COXs). Two isoforms of COX have been identified: COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by various stimuli such as endotoxins, growth factors and pro-inflammatory cytokines[13]. PGE_2 is the most abundant prostanoid in arthritic joint and one of the major catabolic mediators involved in cartilage resorption. PGE_2 elicits cartilage resorption by enhancing the activation and production of MMPs and the degradation of cartilage matrix components[14,15] and by promoting chondrocyte apoptosis[16]. In addition PGE_2 mediates pain responses and potentiates the effects of other inflammatory mediators[13].

Acetylation and deacetylation of nucleosomal histones play an important role in the regulation of gene expression[17,18]. The histone acetylation status is controlled by the opposing actions of two classes of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs). Acetylation of histones loosens nucleosomal structures, thereby promoting gene transcription. In contrast, deacetylation of histones stabilizes nucleosomal structures and represses gene transcription[17,18]. However, emerging evidence indicates that gene regulation by acetylation/deacetylation is more dynamic and complex, and that HATs can act as repressors and HDAC as activators of transcription. Indeed, global analysis of gene expression has shown that inhibition of HDAC activity results both in induction and repression of gene expression[19–24].

In recent years, significant interest has emerged in the inhibition of HDAC activity as a possible anti-cancer treatment. HDAC inhibitors induce growth arrest, differentiation and apoptosis of cancer cells *in vitro* and reduce the growth of experimental tumors *in vivo*[25,26]. Presently, several HDAC inhibitors are in clinical trials for the treatment of solid and hematological tumors[27,28]. In addition to their anti-cancer effects, recent studies have demonstrated that HDAC inhibitors modulate inflammatory responses. For instance, HDAC inhibitors reduce the production of IL-1, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells[29,30]. Likewise, HDAC inhibitors prevent LPS-induced production of TNF-α, IL-6 and reactive oxygen species in neuroglia cultures, and primary microglia[31–33]. HDAC inhibitors have also been reported to suppress IL-12 production in dendritic cells and macrophages[34]. However, it is currently unknown whether HDAC inhibitors regulate inflammatory responses in articular chondrocytes.

Since excessive production of the inflammatory mediators NO and PGE_2 plays an important role in the pathogenesis of OA, we assessed the effect of two HDAC inhibitors, trichostatin A (TSA) and butyric acid (BA), on the production of NO and PGE_2 in primary cultured human chondrocytes stimulated with IL-1. We additionally analyzed the expression of iNOS and COX-2 as well as the binding activity of transcription factor nuclear factor-κB (NF-κB).

Materials and methods

REAGENTS

Recombinant human (rh) IL-1β was obtained from Genzyme (Cambridge, MA, USA), rhTNF-α and rhIL-17 were from R&D Systems (Minneapolis, MN, USA). TSA and BA were from Sigma–Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from either Sigma–Aldrich Canada or Bio-Rad (Mississauga, ON, Canada).

SPECIMEN SELECTION AND CHONDROCYTE CULTURE

Human normal cartilage (from femoral condyles) was obtained at necropsy, within 12 h of death, from donors with no history of arthritic disease ($n = 7$, mean ± SD age: 54 ± 16 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. Only those with no alterations were further processed. Human OA cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement ($n = 47$, mean ± SD age: 66 ± 12 years). All OA patients were diagnosed according to the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA[35]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors. Patients who had received intra-articular injections of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as previously described[36]. In brief, this consisted of 2 mg/ml pronase for 1 h followed by 1 mg/ml collagenase (type IV; Sigma–Aldrich) for 6 h at 37°C in DMEM and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS. At confluence, the chondrocytes were detached, seeded at high density, and allowed to grow in DMEM, supplemented as above. The culture medium was changed every second day, and 24 h before the experiment the cells were incubated in fresh medium containing 0.5% FCS. Only first passaged chondrocytes were used.

NO AND PGE_2 DETERMINATIONS

The nitrite levels, used as an indicator of NO production, were determined using the Griess assay as previously described[36]. The levels of PGE_2 were determined using a PGE_2 enzyme immunoassay from Cayman Chemical (Ann Arbor, MI, USA). The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

WESTERN BLOT ANALYSIS

Chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethansulfonylfluorid (PMSF), 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Twenty microgram of total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a Tris buffer [Tris-buffered saline (TBS) pH 7.5, with 0.1% Tween 20]. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak Ltd, Rochester, NY, USA).

RNA EXTRACTION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from stimulated chondrocytes was isolated using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate (DEPC)-treated-H_2O and stored at −80°C until use. One microgram of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as

detailed in the manufacturer's guidelines. One fiftieth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: iNOS, sense 5′-ACATTGATGAGAAGC TGTCCCAC-3′ and antisense 5′-CAAAGGCTGTGAGTCCTGCAC-3′; COX-2, sense 5′-TGTGTTGACATCCAGATCAC-3′ and antisense 5′-ACAT CATGTTTGAGCCCTGG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5′-CAGAACATCATCCCTGCCTCT-3′ and antisense 5′-GCTTGACAAAGTGGTCGTTGAG-3′.

REAL-TIME PCR

Real-time PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 nM of sense and antisense primers, 25 μl of SYBR® Green master mix (QIAGEN, Mississauga, ON, Canada) and uracil-*N*-glycosylase (UNG, 0.5 Unit, Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 min (UNG reaction), and at 95°C for 10 min (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real-time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative mRNA expression in chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising two to the $-\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

PROTEOGLYCAN RELEASE

Cartilage proteoglycan degradation was assessed by measuring sulfated glycosaminoglycan (GAG) released into culture media using dimethyl methylene blue (DMMB) with chondroitin sulfate as a standard[37]. Results are expressed as μg of GAG released/mg cartilage.

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared as previously described[38]. Briefly, chondrocytes were washed in ice-cold phosphate buffered saline (PBS) and gently scraped in ice-cold hypotonic buffer containing 10 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES)–KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiotheritol (DTT), 1 mM PMSF, 1 mM Na_3VO_4 and 10 μg/ml of aprotinin, leupeptin, and pepstatin. The cells were allowed to swell on ice and the nuclei were recovered by brief centrifugation. The pellets were resuspended in high salt buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol, 0.5 mM PMSF, 1 mM Na_3VO_4 and 10 μg/ml of aprotinin, leupeptin, and pepstatin, followed by incubation on ice for 20 min. The nuclear extracts were recovered by centrifugation and protein concentration was determined using the Bradford method (Bio-Rad). A synthetic double-stranded oligonucleotide containing the κB consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ was end-labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P] adenosine triphosphate (ATP). The mutant competitor oligonucleotide had the following sequence with a 1 bp substitution (underlined): 5′-AGTTGAGGC-GACTTTCCCAGGC-3′. The binding buffer consisted of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol and 2.5 μg poly (dI–dC). Binding reactions were conducted with 5 μg nuclear extract and 100,000 cpm ^{32}[P]-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 μl. In supershift assays, the antibody top65 (1 μg/reaction) was incubated with the reaction mixture for 1 h at 4°C before the addition of ^{32}[P]-labeled oligonucleotide. In cold competition assays, 50-fold molar excess of cold wild-type or mutant oligonucleotide was used. Binding complexes were resolved on non-denaturating 6% polyacrylamide gel electrophoresis in Tris–borate buffer system, after which the gels were fixed, dried, and subjected to autoradiography.

STATISTICAL ANALYSIS

Data are expressed as the mean ± S.E.M. Statistical significance was assessed by the two-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

TSA AND BA ATTENUATE IL-1-INDUCED NO AND PGE_2 PRODUCTION IN HUMAN CHONDROCYTES

Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of two HDAC inhibitors, TSA and BA, and the production of NO was evaluated using Griess reagent. As shown in Fig. 1(A), treatment with either TSA or BA suppressed IL-1-induced NO production in a dose-dependent manner. Similarly, the production of PGE_2 was dose-dependently suppressed in the presence of each HDAC inhibitor [Fig. 1(B)]. In another set of experiments, we found that TSA and BA also dose-dependently inhibited IL-1-induced NO and PGE_2 production in normal chondrocytes, ($n = 3$, data not shown). The observed inhibition was not a result of reduced cell viability as confirmed by the methyl thiazolyl tetrazolium (MTT) assay (data not shown).

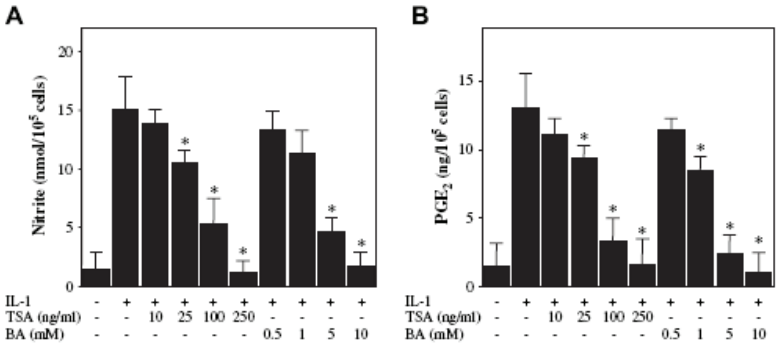

Fig. 1. HDAC inhibitors TSA and BA prevent IL-1-induced NO and PGE_2 release from chondrocytes. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 24 h. Conditioned media were collected and analyzed for NO (A) and PGE_2 (B) release. Results are expressed as percentage of control (i.e., cells treated with IL-1 alone) and are the mean ± S.E.M. from four independent experiments. *$P < 0.05$ compared with cells treated with IL-1 alone.

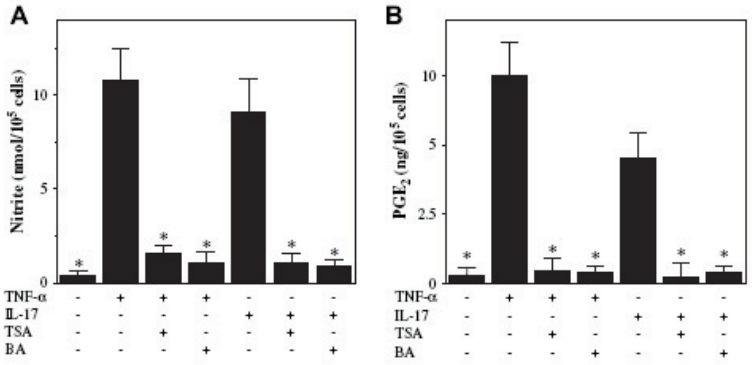

Fig. 2. TSA and BA suppress TNF-α and IL-17-induced iNOS and COX-2 protein expression. Chondrocytes were treated with TNF-α (1 ng/ml) or IL-17 (100 ng/ml) in the absence or presence of TSA (250 ng/ml) or BA (10 mM) for 24 h. Culture media were collected and analyzed for the production of NO (A) and PGE_2 (B). Results are expressed as mean ± S.E.M. of three independent experiments. *$P < 0.05$ compared with cells treated with TNF-α or IL-17 alone.

TSA AND BA INHIBIT TNF-α AND IL-17-INDUCED NO AND PGE_2 PRODUCTION

The pro-inflammatory cytokines TNF-α and IL-17 also contribute to the pathogenesis of OA and are potent inducers of NO and PGE_2 production[1–3]. Therefore, we examined whether HDAC inhibition could also attenuate TNF-α and IL-17-induced NO and PGE_2 production. As shown in Fig. 2, stimulation of chondrocytes with TNF-α or IL-17 dramatically increased the production of NO and PGE_2. Interestingly, the induction of NO and PGE_2 production by TNF-α or IL-17 was almost completely abolished after treatment with TSA or BA. These data suggest that the suppressive effect of HDAC inhibitors was not specific to IL-1, and that HDAC inhibitors might target common pathways implicated in NO and PGE_2 production.

TSA AND BA DECREASE IL-1-INDUCED iNOS AND COX-2 EXPRESSION IN CHONDROCYTES

To determine whether the inhibition of IL-1-induced NO and PGE_2 production is due to reduced iNOS, and COX-2 protein expression, the effects of HDAC inhibitors on the expression of both proteins were analyzed by Western blotting. Under basal conditions, iNOS and COX-2 proteins were undetectable and treatment with IL-1 resulted in a strong induction of both protein expressions (Fig. 3). Consistent with their effects on NO and PGE_2 production, HDAC inhibitors prevented the induction of iNOS and COX-2 protein expression by IL-1, in a concentration-dependent manner (Fig. 3). The levels of β-actin and COX-1 were not influenced by IL-1 alone or in combination with each HDAC inhibitors (Fig. 3). IL-1-induced iNOS and COX-2 protein expression was also inhibited by TSA and BA in normal chondrocytes ($n = 3$, data not shown). As expected, the induction of iNOS and COX-2 proteins by TNF-α or IL-17 was also suppressed by each HDAC inhibitor (Fig. 4).

Next, we used real-time PCR to determine whether HDAC inhibitors modulate iNOS and COX-2 mRNAs' induction. The relative expression level of each gene mRNA was plotted as fold changes over untreated control cells. GAPDH gene expression was used for normalization. As expected, IL-1 induced a marked increase of both iNOS and COX-2 mRNA levels (Fig. 5). Treatment with either TSA or BA dose-dependently suppressed the induction of iNOS and COX-2 mRNA expression (Fig. 5), suggesting that HDAC inhibitors exert their effects at the transcriptional

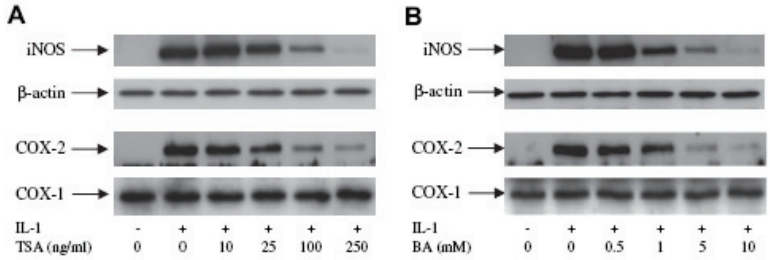

Fig. 3. TSA and BA decrease IL-1-induced iNOS and COX-2 protein expression. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA (A) or BA (B) for 24 h. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. In the lower panels the blots were stripped and re-probed with specific anti-β-actin or anti-COX-1 antibodies. The blots are representative of similar results obtained from four independent experiments.

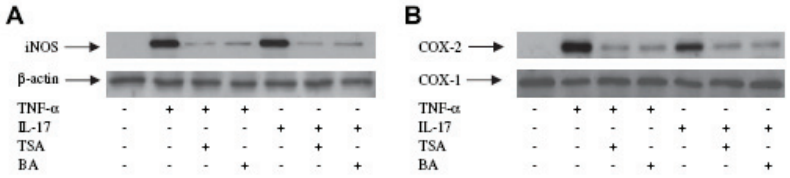

Fig. 4. TSA and BA suppress TNF-α and IL-17-induced iNOS and COX-2 protein expression. Chondrocytes were treated with TNF-α (1 ng/ml) or IL-17 (100 ng/ml) in the absence or presence of TSA (250 ng/ml) or BA (10 mM) for 24 h. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. In the lower panels, the blots were stripped and re-probed with specific anti-β-actin or anti-COX-1 antibodies. The blots are representative of similar results obtained from three independent experiments.

level. Similar results were observed with normal chondrocytes ($n = 3$, data not shown).

TSA AND BA PREVENT IL-1-INDUCED PROTEOGLYCAN DEGRADATION IN CARTILAGE EXPLANTS

To investigate the effect of HDAC inhibitors on IL-1-induced proteoglycan degradation, cartilage explants were incubated in DMEM with 10% FBS for 48 h and then transferred to medium containing 0.5% FBS and re-incubated for an additional 48 h. Thereafter, the explants were stimulated with IL-1 in the absence or presence of increasing concentrations of TSA or BA for 72 h, and GAG release into the supernatants was determined[37]. As shown in Fig. 6, IL-1-induced GAG release was inhibited in a dose-dependent manner by either TSA or BA.

TSA AND BA DO NOT IMPAIR NF-κB-BINDING TO THE iNOS AND COX-2 PROMOTERS

The transcription factor NF-κB, mainly composed of p50 and p65 dimers, plays a pivotal role in mediating the effects of IL-1 in chondrocytes[39,40]. Therefore, we performed EMSA to determine whether HDAC inhibitors modulate IL-1 effects by interfering with the DNA-binding activity of NF-κB. Nuclear extracts from chondrocytes treated with IL-1 alone, or in combination with increasing concentrations of HDAC inhibitors for 1 h, were used for these assays. As shown in Fig. 7, IL-1 treatment induced a prominent increase in the DNA-binding activity of NF-κB. Interestingly, treatment with each HDAC inhibitor did not decrease the DNA-binding activity of NF-κB at any concentration point. The specificity of DNA-binding was confirmed using unlabeled wild-type and mutant oligonucleotides. The specificity was further evidenced using supershift assays and a specific anti-p65 antibody. Together these data suggest that HDAC inhibitors modulate IL-1 effects in chondrocytes without interfering with the DNA-binding activity of NF-κB.

Discussion

In the present study we demonstrated that inhibition of HDACs by two structurally unrelated HDAC inhibitors, TSA and BA, results in a dose-dependent suppression of IL-1-induced NO and PGE_2 production. The inhibition of NO and PGE_2 production was concomitant with the suppression of iNOS and COX-2 expression at both the protein and mRNA levels. We also showed that this inhibition is not associated with changes in NF-κB DNA-binding activity.

The effects of HDAC inhibition on the production of NO and the expression of iNOS have been examined in a few recent studies. Yu *et al.* demonstrated that TSA inhibited

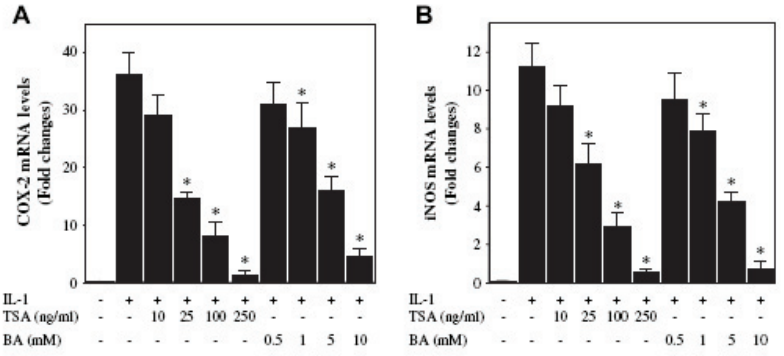

Fig. 5. TSA and BA decrease IL-1-induced iNOS and COX-2 mRNA expression. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 6 h. Total RNA was isolated, cDNA was synthesized; and iNOS and COX-2 mRNAs were quantified using real-time PCR. GAPDH gene expression was used for normalization. The results are expressed as − fold changes considering one as the value of untreated cells. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. The results are expressed as mean ± S.E.M. of four independent experiments. *$P < 0.05$ compared with cells treated with IL-1 alone (control).

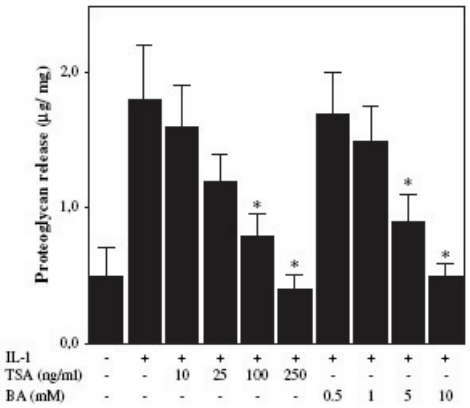

Fig. 6. TSA and BA suppress IL-1-induced cartilage proteoglycan degradation. Cartilage explants were stimulated with 1 ng/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 72 h. Proteoglycan degradation was assessed by assaying aliquots of culture media for GAG release. Results are expressed as μg GAG/mg cartilage and are the mean ± S.E.M. from four independent experiments. *$P < 0.05$ compared with cells treated with IL-1 alone.

IL-1- or LPS + IFN-γ-induced NO production in mesangial cells and RAW 264.7 cells[41]. Leoni *et al.*[29] showed that another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), dose-dependently attenuated NO release from mouse peritoneal macrophages stimulated by the combination of TNF-α and IFN-γ. Similarly, Larsen *et al.*[42] found that TSA and SAHA prevented the production of NO and the expression of iNOS in the β-cell line INS-1 and in intact rat islets treated with IL-1 + IFN-γ[42] On the other hand, TSA and SAHA have been shown to enhance LPS-induced production of NO in microglial cells[43], and butyrate was reported to increase the expression of iNOS and the production of NO in response to treatment with LPS + IFN-γ in intestinal epithelial cells[44]. The reasons for these discrepancies are unclear but may be attributable to several factors including cell type and stimulation conditions. We also demonstrated that TSA and BA prevented IL-1-induced PGE_2 release in chondrocytes. Furthermore, TSA and BA suppressed IL-1-induced COX-2 expression at the mRNA and protein levels. These findings are in agreement with previous studies showing that HDAC inhibitors prevented the induction of PGE_2 production and COX-2 expression in several cell types[45,46].

Pro-inflammatory cytokines TNF-α and IL-17 are also believed to contribute to the pathogenesis of OA and are strong inducers of NO and PGE_2 synthesis in chondrocytes[1–3]. Interestingly, HDAC inhibitors blocked the production of NO and PGE_2 in chondrocytes treated with either TNF-α or IL-17. Thus, the suppression of NO and PGE_2 synthesis by HDAC inhibitors is not specific to IL-1 and is independent of the nature of the stimulus that triggers NO and PGE_2 production.

Several studies have demonstrated that HDAC inhibitors suppress the production of a number of pro-inflammatory cytokines *in vitro* and *in vivo*. For example, treatment with SAHA or ITF2357 decreased the release of IL-1, IL-12, TNF-α and IFN-γ from LPS-stimulated human peripheral blood mononuclear cells[29,30]. TSA treatment was also reported to prevent the expression of IL-8 in Caco-2 cells[47] and of IL-12 in lung epithelial cells stimulated with LPS[48]. *In vivo*, SAHA dose-dependently reduced the circulating levels of the pro-inflammatory cytokines TNF-α, IL-1, and IL-6 in an endotoxemia model[29]. In addition to their anti-inflammatory effects, HDAC inhibitors display

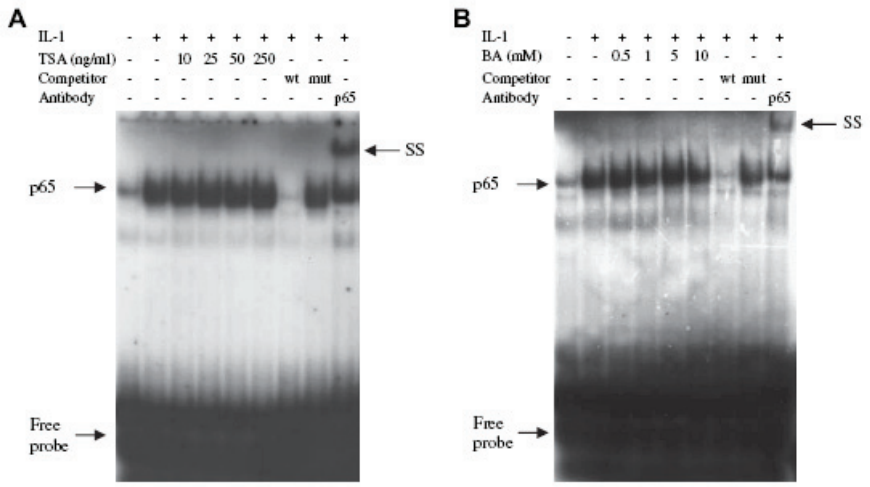

Fig. 7. Effect of TSA and BA on DNA-binding activity of NF-κB. Confluent chondrocytes were treated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of TSA (A) or BA (B) for 1 h. Nuclear extracts were prepared and incubated with a ^{32}[P]-labeled oligonucleotide containing the NF-κB sequence. Specificity of binding was confirmed using 50-molar excess of wild-type and mutated unlabeled oligonucleotides. The supershifted (SS) band is indicated. The autoradiograph shown is representative of similar results obtained from four independent experiments.

chondroprotective properties. Indeed, we demonstrated here that treatment with TSA or BA prevents IL-1-induced proteoglycan degradation in cartilage explants. Moreover, Young *et al.*[49] showed that HDAC inhibitors blocked the induction of several enzymes responsible for cartilage degradation, including MMP-1, MMP-13, a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS) -4, -5 and -9 and prevented cartilage degradation in an explant assay[49]. Together these data suggest that HDAC inhibitors may prevent cartilage destruction in arthritis. Indeed, HDAC inhibitors prevent cartilage damage in models of adjuvant-induced arthritis[50] and autoantibody-mediated arthritis[51]. Protective effects of HDAC inhibitors on cartilage were also observed in collagen-induced arthritis models[52].

The transcription factor NF-κB is important in the induction of iNOS and COX-2 by pro-inflammatory cytokines and stimuli in chondrocytes, and the 5′-flanking regions of both *iNOS* and *COX-2* genes contain binding sites for NF-κB[39,40]. In the present study, we demonstrated that IL-1 enhances the binding activity of NF-κB p65. Interestingly, treatment with TSA or butyrate did not affect the binding activity of NF-κB, suggesting that HDAC inhibitors influence NF-κB-dependent gene expression down-stream of DNA-binding in chondrocytes. These results are in accordance with previous reports showing that HDAC inhibitors did not affect the DNA-binding activity of NF-κB in IL-1-stimulated mesangial cells[41], and Caco-2 cells[47], as well as in LPS-stimulated N9 microglia cells[43]. In contrast, other groups have reported that HDAC inhibitors reduced the DNA-binding activity of NF-κB in A549 cells[53] and human colon cell lines[54] treated with pro-inflammatory cytokines. Several reasons may explain this dichotomy including the differences in time exposure to HDAC inhibitors and the model used.

There are a number of potential mechanisms by which HDAC inhibitors could inhibit IL-1-induced iNOS and COX-2 expression. First, HDAC inhibitors may down-regulate gene expression by altering local chromatin structure secondary to increased histone acetylation. Secondly, the suppressive effect of HDAC inhibitors could be mediated by hyperacetylation of transcription factors or signaling molecules that participate in IL-1-induced iNOS and COX-2 expression. Finally, gene products induced by HDAC inhibitors may also interfere with the signaling pathways involved in iNOS and COX-2 expression. Regardless of the exact mechanism by which HDAC inhibitors down-regulate IL-1-induced NO and PGE_2 production, these results are very interesting from a pharmacological point of view since inhibitors of PGE_2 and NO production are a promising class of compounds with therapeutic potential for OA.

In conclusion, we have shown that HDAC inhibitors suppress IL-1-induced NO and PGE_2 production, iNOS and COX-2 expression as well as proteoglycan degradation. The mechanism by which HDAC inhibitors attenuate IL-1-effects is independent of the DNA-binding activity of the transcription factor NF-κB. These data also suggest that HDAC inhibitors represent a promising new class of compounds in the treatment of OA.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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ANNEXE 2

Human articular chondrocytes express 15-lipoxygenase-1 and -2: potential role in osteoarthritis

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Research article

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Human articular chondrocytes express 15-lipoxygenase-1 and -2: potential role in osteoarthritis

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Abstract

Introduction 15-Lipoxygenases and their metabolites have been shown to exhibit anti-inflammatory and immunomodulatory properties, but little is known regarding their expression and function in chondrocytes. The objective of this study was to evaluate the expression of 15-lipoxygenase-1 and -2 in human articular chondrocytes, and to investigate the effects of their metabolites 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids on IL-1β-induced matrix metalloproteinase (MMP)-1 and MMP-13 expression.

Methods The expression levels of 15-lipoxygenase-1 and -2 were analyzed by reverse transcription PCR and Western blotting in chondrocytes, and by immunohistochemistry in cartilage. Chondrocytes or cartilage explants were stimulated with IL-1β in the absence or presence of 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids, and the levels of MMP-1 and MMP-13 protein production and type II collagen cleavage were evaluated using immunoassays. The role of peroxisome proliferator-activated receptor (PPAR)γ was evaluated using transient transfection experiments and the PPARγ antagonist GW9662.

Results Articular chondrocytes express 15-lipoxygenase-1 and -2 at the mRNA and protein levels. 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids dose dependently decreased IL-1β-induced MMP-1 and MMP-13 protein and mRNA expression as well as type II collagen cleavage. The effect on MMP-1 and MMP-13 expression does not require *de novo* protein synthesis. 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids activated endogenous PPARγ, and GW9662 prevented their suppressive effect on MMP-1 and MMP-13 production, suggesting the involvement of PPARγ in these effects.

Conclusions This study is the first to demonstrate the expression of 15-lipoxygenase-1 and -2 in articular chondrocytes. Their respective metabolites, namely 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids, suppressed IL-1β-induced MMP-1 and MMP-13 expression in a PPARγ-dependent pathway. These data suggest that 15-lipoxygenases may have chondroprotective properties by reducing MMP-1 and MMP-13 expression.

Introduction

Osteoarthritis (OA) is the most common form of arthritis, accounting for a large proportion of disability in adults. The destruction of articular cartilage is a typical pathological characteristic of the disease [1,2]. and is believed to be largely mediated by proteases belonging to the matrix metalloproteinase (MMP) family of enzymes [3]. The MMPs can be classified into at least five main groups, including the collagenases

AP: activator protein; C_T: threshold cycle; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HETE: hydroxyeicosatetraenoic acid; HODE: hydroxy octadecadienoic acid; IL: interleukin; LOX: lipoxygenase; MMP: matrix metalloproteinase; NF-κB: nuclear factor-κB; OA: osteoarthritis; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PPAR: peroxisome proliferator-activated receptor; PPRE: peroxisome proliferator-activated receptor-responsive element; SD: standard deviation; TNF: tumor necrosis factor; UNG: uracil-N-glycosylase.

(MMP-1, -8, and -13), the gelatinases (MMP-2 and -9), the stromelysins (MMP-3, -10, and -11), the matrilysins (MMP-7 and -26), and the membrane-bound-type MMPs (MMP-14, -15, -16, -17, -24, and -25). Among the MMPs, two collagenases, namely MMP-1 and MMP-13, are considered key players in the pathogenesis of OA because they have the unique ability to cleave most components of cartilage matrix, including collagen and aggrecan [3-5]. The expression levels of MMP-1 and MMP-13 are upregulated in arthritic tissues [6,7], and the pro-inflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α, and IL-17, which are also upregulated in OA tissues, are known to induce strongly the production of both MMPs in articular chondrocytes [6-8]. Inhibition of MMP has been considered a therapeutic strategy in arthritis, but most clinical trials have yielded disappointing results [9-11]. Thus, identification of factors and pathways that modulate MMP-1 and MMP-13 expression in chondrocytes is critical to our understanding the pathogenesis of OA and may lead to the development of new therapeutic targets for the treatment of the disease.

Lipoxygenases (LOXs) are a family of enzymes that incorporate molecular oxygen at specific positions into unsaturated fatty acids. In human tissues, three major LOXs have been characterized and named according to the carbon position of arachidonic acid oxygenation [12,13]: 5-LOX, 12-LOX, and 15-LOX. Two different human 15-LOXs have been identified that differ in tissue distribution and substrate preferences. 15-LOX-1 is expressed in reticulocytes, eosinophils, skin, and macrophages [14,15]. 15-LOX-2 has been detected in prostate, lung, skin, and cornea [16]. 15-LOX-1 preferentially converts linoeic acid to 13(S)-hydroxy octadecadienoic acid (HODE), whereas 15-LOX-2 essentially converts arachidonic acid to 15(S)-hydroxyeicosatetraenoic acid (HETE) [16].

Several studies have documented that 15-LOXs and their metabolites exhibit anti-inflammatory and immunomodulatory properties. For instance, 15-HETE and 13-HODE were shown to inhibit the production of leukotriene-B_4 and reactive oxygen species by stimulated neutrophils [17], and the production of IL-8 by colonic cells [18]. In addition, 15-LOX metabolites suppress the production of TNF-α, a key cytokine in the pathogenesis of arthritis [19,20], and mediate the effects of the T-helper-2 cytokine IL-4 [21,22]. The 15-LOX metabolites 15-HETE and 13-HODE are also ligands for the peroxisome proliferator-activated receptor (PPAR)γ [23,24]. PPARγ is a unique member of the ligand-dependent nuclear receptor family that has been implicated in the modulation of critical aspects of development and homeostasis. We and others have shown that PPARγ activation inhibits the expression of a number of genes involved in the pathogenesis of OA, including IL-1β, TNF-α, MMP-1, MMP-13, inducible nitric oxide synthase, and microsomal prostaglandin E synthase-1 [25-28], and is protective in animal models of OA [29].

The expression of 15-LOXs and the roles played by their metabolites have been characterized in various tissues and cell types [12-16]. However, little is known regarding the expression and function of 15-LOXs in human cartilage. This study was undertaken to investigate the expression of 15-LOXs in human articular OA chondrocytes and to define the effect of their metabolites 15-HETE and 13-HODE on IL-1β-induced MMP-1 and MMP-13 production. We provide evidence that both 15-LOX-1 and 15-LOX-2 are expressed in human OA chondrocytes. We also demonstrate that 13-HODE and 15-HETE suppressed IL-1β-induced MMP-1 and MMP-13 expression and type II collagen cleavage. These data suggest that 15-LOXs may play a role in preventing the cartilage destruction observed in OA.

Materials and methods

Reagents

Recombinant human IL-1β was obtained from Genzyme (Cambridge, MA, USA), and recombinant human TNF-α and recombinant human IL-17 from R&D Systems (Minneapolis, MN, USA). GW9662, 13(S)-HODE, 15(S)-HETE, anti-15-LOX-1 and 15-LOX-2 antibodies were from Cayman Chemical Co. (Ann Arbor, MI, USA). Cycloheximide was from Sigma-Aldrich Canada (Oakville, Ontario, Canada), and Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, Ontario, Canada). All other chemicals were purchased from either Sigma-Aldrich Canada or Bio-Rad (Mississauga, Ontario, Canada).

Specimen selection and chondrocyte culture

Human OA cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement (n = 23; mean ± standard deviation [SD] age 68 ± 13 years). All OA patients were diagnosed in accordance with the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA [30]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of nonsteroidal anti-inflammatory drugs or selective cyclo-oxygenase-2 inhibitors. Patients who had received intra-articular injections of steroids were excluded. The Clinical Research Ethics Committee of the Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion, as previously described [26]. In brief, this consisted of 2 mg/ml pronase for 1 hour followed by 1 mg/ml collagenase (type IV; Sigma-Aldrich) for 6 hours at 37°C in DMEM and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS.

Confluent chondrocytes were detached by trypsinization, seeded at 3.5×10^5 cells per well in 12-well culture plates (Costar, Corning, NY, USA) or at 7×10^5 cells per well in six-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 hours. Cells were washed and incubated for an additional 24 hours in DMEM containing 0.5% FCS, before stimulation with either IL-1β alone or in combination with 13-HODE or 15-HETE. 13-HODE and 15-HETE, supplied in ethanol at 1 mg/ml, were air-dried and dissolved in dimethyl sulfoxide at 10 mg/ml. Control cells were treated with the highest concentration of dimethyl sulfoxide (0.14%) as vehicle control. In another set of experiments, chondrocytes were pretreated for 30 minutes with vehicle, cycloheximide, or GW9662 before stimulation. The levels of MMP proteins released in supernatants were determined 24 hours after stimulation, whereas MMP mRNA levels were determined at 8 hours. Only first passaged chondrocytes were used.

RNA extraction and PCR analyses

Total RNA was isolated using the TRIzol® reagent (Invitrogen), in accordance with the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate-treated water and stored at -80°C until use. One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, Ontario, Canada), as detailed in the manufacturer's guidelines. One-fifth of the reverse transcriptase reaction was analyzed by traditional PCR or real-time quantitative PCR. The following primers were used: 15-LOX-1, sense 5'-TTGGTTATTTCAGCCCCCATC-3' and antisense 5'-TGTGTTCACTGGGTGCAGAGA-3'; 15-LOX-2, sense 5'-GCATCCACTGATTGGACCTT-3' and antisense 5'-GCTGGCCTTGAACTTCTGAC-3'; MMP-1, sense 5'-CTGAAAGTGACTGGGAAACC-3' and antisense 5'-AGAGTTGTCCCGATGATCTC-3'; MMP-13, sense 5'-CTT AGA GGT GAC TGG CAA AC-3' and antisense 5'-GCC CAT CAA ATG GGT AGA AG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-CAGAACATCATCCCTGCCTCT-3' and antisense 5'-GCTTGACAAAGTGGTCGTTGAG-3'.

Quantitative PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 nmol/l of sense and antisense primers, 25 μl of SYBR® Green master mix (QIAGEN, Mississauga, Ontario, Canada), and uracil-N-glycosylase (UNG; 0.5 units; Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 minutes (UNG reaction) and at 95°C for 10 minutes (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 seconds at 95°C for denaturation and 1 minute for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA), allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative mRNA expression in chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising 2 to the power of $-\Delta\Delta C_T$. Each PCR reaction generated only the expected specific amplicon, as shown by the melting temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions for each independent experiment. In conventional PCR, the mixtures were incubated at 95°C for 1 minute followed by 35 cycles each at 94°C/30 seconds and 60°C/1 minute, with a final elongation step at 60°C/8 minutes. Controls for reverse transcription and PCR amplifications were included. PCR product (10 μl/50 μl) reactions were separated on a 1.8% agarose gel and stained with ethidium bromide.

Western blot analysis

Chondrocytes were lysed in ice-cold lysis buffer (50 mmol/l Tris-HCl [pH 7.4], 150 mmol/l NaCl, 2 mmol/l EDTA, 1 mmol/l PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mmol/l Na_3VO_4, and 1 mmol/l NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 minutes. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Twenty micrograms of total cell lysate was subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mmol/l Tris-HCl (pH 7.5) containing 150 mmol/l NaCl, 0.1% Tween 20, and 5% (weight/volume) nonfat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a Tris buffer (Tris-buffered saline [pH 7.5], with 0.1% Tween 20). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak Ltd, Rochester, NY, USA).

Immunohistochemistry

Cartilage specimens were processed for immunohistochemistry, as described previously [26]. The specimens were fixed in

4% paraformaldehyde and embedded in paraffin. Sections (5 μm) of paraffin-embedded specimens were deparaffinized in toluene, and dehydrated in a graded series of ethanol. The specimens were then pre-incubated with chondroitinase ABC (0.25 U/ml in phosphate-buffered saline [PBS; pH 8.0]) for 60 minutes at 37°C, followed by a 30-minute incubation with Triton X-100 (0.3%) at room temperature. Slides were then washed in PBS followed by 2% hydrogen peroxide/methanol for 15 minutes. They were further incubated for 60 minutes with 2% normal serum (Vector Laboratories, Burlingame, CA, USA) and overlaid with primary antibody for 18 hours at 4°C in a humidified chamber. Each slide was washed three times in PBS (pH 7.4) and stained using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories). The color was developed with 3,3'-diaminobenzidine (Vector Laboratories) containing hydrogen peroxide. The slides were counterstained with eosin. The specificity of staining was evaluated by substituting the primary antibody with nonimmune IgG (Chemicon, Temecula, CA, USA) at the same concentration as the primary antibody. The evaluation of positive-staining chondrocytes was performed using our previously published method [26]. For each specimen, six microscopic fields were examined under 40× magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated, and the results were expressed as the percentage of chondrocytes staining positive (cell score).

Plasmids and transient transfection

The PPRE-luciferase construct containing three PPAR-responsive elements (PPREs) cloned upstream of the thymidine kinase promoter (PPRE-Tk-luciferase) was generously provided by Dr CK Glass (University of California, San Diego, CA, USA). β-Galactosidase reporter vector under the control of SV40 promoter (pSV40-β-galactosidase) was from Promega (Madison, WI, USA). Transient transfection experiments were performed using FuGene-6 (1 μg DNA: 4 μl FuGene 6; Roche Applied Science, Laval, Quebec, Canada), in accordance with the manufacturer's recommended protocol. Briefly, chondrocytes were seeded 24 hours before transfection at a density of 6×10^5 cells/well in six-well plates and transiently transfected with 1 μg of the reporter construct and 0.5 μg of the internal control pSV40-β-galactosidase. Six hours later, the cells were rinsed in PBS and changed to medium containing 0.5% FCS for an additional 18 hours. The cells were then treated with increasing concentrations of 13-HODE or 15-HETE for 18 hours. In these conditions, transfection efficiency typically ranges between 40% and 50%. After harvesting, luciferase activity was determined and normalized to β-galactosidase activity. All of the transfection experiments were repeated at least three times in duplicate.

Matrix metalloproteinase-1 and -13 determination

The levels of MMP-1 and MMP-13 in conditioned media were determined by specific ELISAs (R&D Systems Inc, Minneapolis, MN, USA). All measurements were performed in duplicate.

Extraction and assay for cleavage of type II collagen

Cartilage explants were digested to extract cleaved type II collagen, as previously described [31]. Briefly, after treatment the harvested cartilage was incubated overnight at 37°C with 1.0 mg/50 mg cartilage of α-chymotrypsin in 50 mmol/l Tris-HCl (pH 7.6; with the following proteinase inhibitors: 1 mmol/l EDTA, 1 mmol/l iodoacetamide, and 10 μg/ml pepstatin A). After the α-chymotrypsin activity was inhibited with N-tosyl-L-phenylalanine-chloromethyl ketone (Sigma) for 20 minutes, the samples were centrifuged and the supernatants assayed for type II collagen degradation using a C2C ELISA kit (IBEX, Montreal, Quebec, Canada).

Statistical analysis

Data are expressed as the mean ± SD. Statistical significance was assessed using the two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

Human OA articular chondrocytes express both 15-LOX-1 and -2

To investigate whether human articular chondrocytes express 15-LOX-1 and -2, total RNA from cultured chondrocytes, derived from four different OA patients, was subjected to reverse transcription PCR analysis using specific primers for 15-LOX-1 and -2. As shown in Figure 1a, the expression of 15-LOX-1 and -2 mRNAs was detected in the four chondrocyte preparations. No PCR products were obtained with control reactions performed in the absence of the cDNA or reverse transcriptase (Figure 1a). To further confirm the expression of 15-LOX-1 and -2 in chondrocytes, we analyzed their expression at the protein level. Western blot analysis with total protein extracts revealed the presence of both isoforms in all examined chondrocyte preparations (Figure 1b, c).

To examine whether chondrocytes express 15-LOX-1 and -2 *in vivo*, we performed immunohistochemical analysis using OA cartilage. The positive immunostaining for 15-LOX-1 (Figure 2a) and 15-LOX-2 (Figure 2d) was located mainly in the superficial and intermediate zones of the cartilage. Statistical evaluation of the cell score revealed lower immunostaining for 15-LOX-1 (mean ± SD: 36.2% ± 17.6%) than for 15-LOX-2 (mean ± SD: 43.7% ± 19.2%), but these differences were not significant. The specificity of staining was confirmed using nonimmune control IgG (Figure 2c, f). These observations demonstrate the *in vivo* expression of 15-LOX-1 and -2 proteins in OA cartilage.

13-HODE and 15-HETE inhibited IL-1β-induced MMP-1 and MMP-13 expression in chondrocytes

To examine the effects of 15-LOX-1 and -2 metabolites on MMP-1 and MMP-13 release, chondrocytes were stimulated with IL-1β in the absence or presence of increasing concentrations of 13-HODE or 15-HETE, and the levels of MMP-1 and MMP-13 proteins in conditioned media were determined

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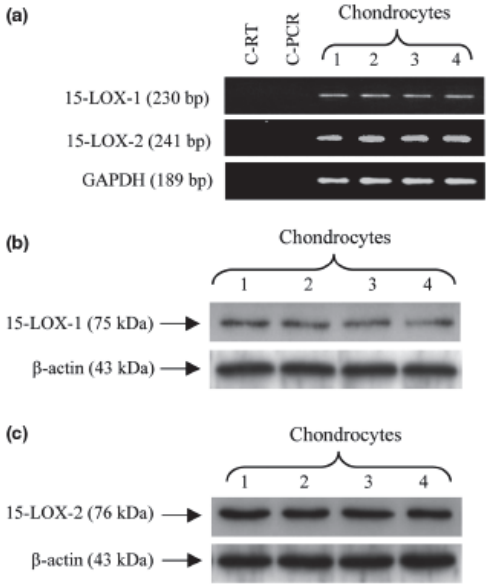
Figure 1



Human articular chondrocytes express both 15-LOX-1 and 15-LOX-2. **(a)** Chondrocytes were isolated from OA knee cartilage and maintained as monolayer culture for 7 to 10 days. Total RNA was prepared, reverse transcribed into cDNA, and processed for PCR using specific primers for 15-LOX-1, 15-LOX-2, and GAPDH. PCR products were resolved on a 1.8% agarose gel and stained with ethidium bromide. C-RT and C-PCR are negative controls for the reverse transcription and PCR reaction, respectively. **(b, c)** Chondrocytes were isolated from OA knee cartilage and lysates were prepared after 7 to 10 days in culture. Samples with equal amounts of total proteins (20 μg per lane) were immunoblotted with specific anti-15-LOX-1 (panel b) and anti-15-LOX-2 (panel c) antibodies (upper sections). The blots were stripped and reprobed with a specific anti-β-actin antibody (lower sections). bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOX, lipoxygenase; OA, osteoarthritis.

by ELISA. As shown in Figure 3a, b, the production of MMP-1 and MMP-13 was dose dependently reduced in the presence of 13-HODE or 15-HETE. The concentrations of 13-HODE and 15-HETE utilized did not affect chondrocyte viability, as judged using the MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (data not shown). Taken together, these findings suggest that 15-LOX metabolites may constitute novel endogenous negative regulators of MMP-1 annd MMP-13 expression in chondrocytes.

In addition to IL-1, the pro-inflammatory cytokines TNF-α and IL-17 also contribute to the pathogenesis of OA and are potent inducers of MMP-1 and MMP-13. Therefore, we examined whether 13-HODE and 15-HETE could also attenuate TNF-α and IL-17-induced MMP-1 and MMP-13 production in chondrocytes. As shown in Figure 3c–e, the induction of MMP-1 and MMP-13 production by TNF-α or IL-17 was dose dependently diminished in the presence of 13-HODE or 15-HETE. These data suggest that the suppressive effect of 13-HODE and 15-HETE is not specific to IL-1, and is independent of the nature of the stimulus that triggers MMP-1 and MMP-13 production.

13-HODE and 15-HETE suppress IL-1-induced type II collagen cleavage

Next, we assessed the effects of 13-HODE and 15-HETE on IL-1-induced type II collagen cleavage. Cartilage explants were treated with IL-1β in the absence or presence of increasing concentrations of 13-HODE or 15-HETE for 5 days, and type II collagen degradation was determined using a specific commercial kit that measures C2C epitopes of type II collagen. As shown in Figure 4, treatment with 13-HODE or 15-HETE dose-dependently prevented IL-1-induced type II collagen cleavage.

Suppression of IL-1β-induced MMP-1 and MMP-13 expression by 13-HODE and 15-HETE does not require *de novo* protein synthesis

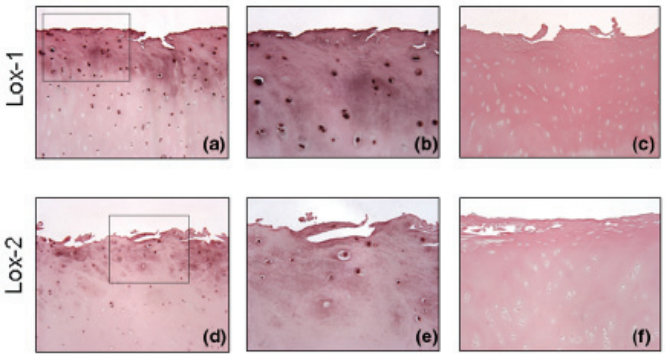
To investigate the effects of 13-HODE and 15-HETE on IL-1β-induced MMP-1 and MMP-13 mRNA expression, we used real-time PCR. Consistent with their effects on MMP-1 and MMP-13 protein production, 13-HODE and 15-HETE dose-dependently suppressed IL-1β-induced MMP-1 and MMP-13 mRNA expression (Figure 5a, b), suggesting that these effects occur at the transcriptional level.

To evaluate whether the effect of 13-HODE and 15-HETE on IL-1β-induced MMP-1 and MMP-13 expression is direct or indirect, we tested the impact of the protein synthesis inhibitor cycloheximide. Chondrocytes were pretreated with cycloheximide for 30 minutes and stimulated with IL-1β alone or in combination with either 13-HODE or 15-HETE for 8 hours. The levels of MMP-1 and MMP-13 mRNAs were analyzed by real-time PCR. As shown in Figure 5c, pretreatment with cycloheximide did not affect 13-HODE and 15-HETE-mediated inhibition of IL-1β-induced MMP-1 and MMP-13 expression, suggesting that their effect was a direct primary effect through pre-existing factors and was not dependent on *de novo* protein synthesis.

13-HODE and 15-HETE suppressed IL-1β-induced MMP-1 and MMP-13 production in a PPARγ dependent manner

The 15-LOX metabolites 13-HODE and 15-HETE are ligands for PPARγ, and PPARγ activation was reported to suppress IL-1β-induced MMP-1 and MMP-13 production [26,27]. To test the possibility that PPARγ is involved in the suppressive effect of 13-HODE and 15-HETE on MMP-1 and MMP-13 production, we first examined their effects on the transcriptional activity of endogenous PPARγ in chondrocytes. Chondrocytes were transiently transfected with a luciferase reporter con-

Figure 2



Expression of 15-LOX-1 and 15-LOX-2 in human OA cartilage. Representative immunostaining of human osteoarthritis (OA) cartilage for **(a)** 15-LOX-1 and **(d)** 15-LOX-2. **(b, e)** Higher magnification views of the area indicated within the broken line rectangle in panels a and d, respectively. **(c, f)** Cartilage treated with nonimmune control IgG at the same concentration as the primary antibody (control for staining specificity). (Magnification: ×100 for panels a, c, d and f; ×250 for panels b and e). The results are representative of four separate experiments performed with cartilage samples from four different donors. LOX, lipoxygenase.

struct containing three copies of a consensus PPRE, and treated with increasing concentrations of 13-HODE and 15-HETE. As illustrated in Figure 6a, treatment with 13-HODE and 15-HETE dose dependently increased the activity of the synthetic promoter. These data confirm the presence of inducible PPARγ-dependent transcriptional responses in chondrocytes. Next, we examined the effect of GW9662, a selective and irreversible PPARγ antagonist. Chondrocytes were pre-incubated with increasing concentrations of GW9662 before addition of 13-HODE or 15-HETE and were subsequently stimulated with IL-1β. As shown in Figure 6b, GW9662 dose-dependently relieved the suppressive effect of 13-HODE and 15-HETE on IL-1β-induced MMP-1 and MMP-13 protein production. Taken together, these results strongly suggest that 13-HODE and 15-HETE inhibit IL-1β-induced MMP-1 and MMP-13 production through a PPARγ-dependent mechanism.

Discussion

In the present study, we report for the first time that articular OA chondrocytes express 15-LOX-1 and -2. Treatment with 13-HODE and 15-HETE, the major products of 15-LOX-1 and -2, respectively, suppressed IL-1β-induced MMP-1 and MMP-13 expression and type II collagen degradation. Taken together, these findings strongly suggest a chondroprotective role for 15-LOXs by negatively regulating the expression of MMP-1 and MMP-13.

In addition to their chondroprotective properties observed in this study, 15-LOX metabolites were shown to exhibit potent anti-inflammatory effects. For instance, 15-HETE inhibits polymorphonuclear neutrophil degranulation and superoxide production elicited by N-formylmethionylleucylphenylalaline, platelet-activating factor and leukotriene B_4 [17]. In addition, 15-HETE prevents polymorphonuclear neutrophil migration across IL-1β or TNF-α-activated endothelium [32] and TNF-α-induced expression of several adhesion molecules, including intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin [33]. On the other hand, 13-HODE attenuates the production of reactive oxygen species in macrophages [34], the production of IL-8 in colonic epithelial cells [18], and the ability of dendritic cells to activate interferon-γ secretion by T lymphocytes [35]. Moreover, 13-HODE and 15-HETE were shown to mediate the suppressive effect of the anti-inflammatory cytokine IL-4 on inducible nitric oxide synthase expression in macrophages [21] and IL-2 production in T lymphocytes [22]. In addition to 13-HODE and 15-HETE formation, 15-LOXs are involved in the generation of the potent anti-inflammatory molecules lipoxins, resolvings, and protectins [36]. Thus, 15-LOXs can dampen inflammation through production of distinct classes of anti-inflammatory and pro-resolution lipid mediators.

The protective effect of 15-LOXs is further supported by results from studies using transgenic animals. Over-expression of 15-LOX in rabbits reduced inflammation and tissue damage in atherosclerosis [37] and peritonitis [38]. In rats, over-expression of 15-LOX suppressed renal inflammation and preserved organ function in experimental glomerulonephritis [39]. These data, together with our findings that 15-LOX metabolites block MMP production, suggest that these lipids may have protective effects in OA *in vivo*. Further studies using cartilage-specific 15-LOX-null mice will be required to elucidate the role of 15-LOXs in cartilage integrity and the pathogenesis of OA.

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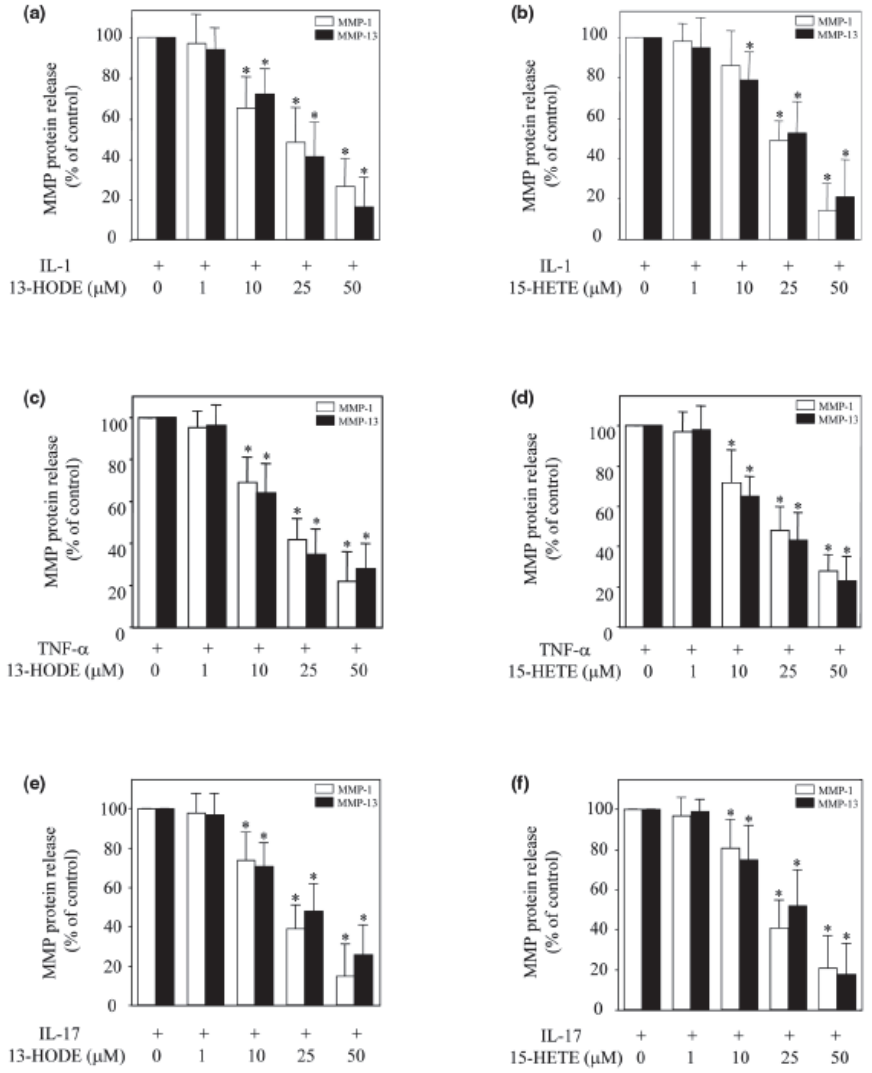
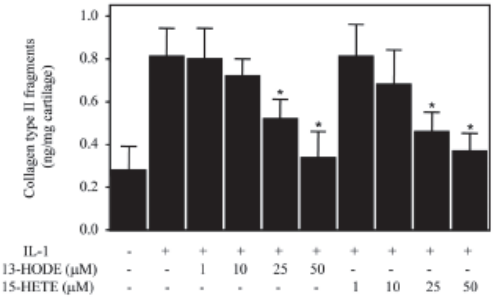
Figure 3



13-HODE and 15-HETE downregulate induction of MMP-1/MMP-13 protein synthesis by IL-1β, TNF-α and IL-17. **(a, b)** Chondrocytes were stimulated with IL-1β (100 pg/ml), **(c, d)** TNF-α (0.1 ng/ml), or **(e, f)** IL-17 (10 ng/ml) in the presence of vehicle (dimethyl sulfoxide at a maximum concentration of 0.14%) or increasing concentrations of 13-HODE (panels a, c, and e) or 15-HETE (panels b, d, and f) for 24 hours. The levels of MMP-1 and MMP-13 proteins in conditioned media were measured using ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells treated with IL-1β, TNF-α or IL-17 alone, and are the mean ± standard deviation of at least three independent experiments. *$P < 0.05$ versus cells treated with IL-1β, TNF-α, or IL-17 alone. HETE, hydroxyeicosatetraenoic acid; HODE, hydroxy octadecadienoic acid; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.

Figure 4

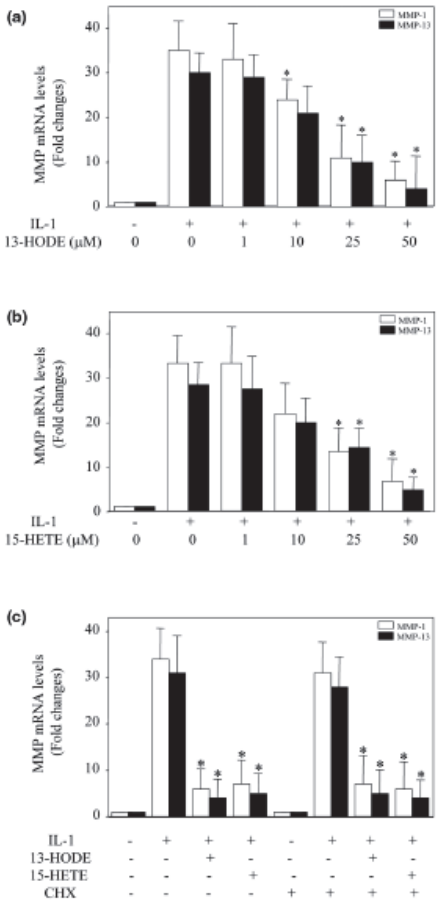


13-HODE and 15-HETE downregulate IL-1β-induced type II collagen degradation cleavage. Cartilage explants were stimulated with 1 ng/ml IL-1β in the presence of the control vehicle dimethyl sulfoxide or increasing concentrations of 13-HODE or 15-HETE for 5 days. Type II collagen degradation was assessed by quantification of C2C epitopes of type II collagen in cartilage explants. Data are the mean ± standard deviation of three independent experiments. *$P < 0.05$ versus cartilage explants treated with IL-1β alone. HETE, hydroxyeicosatetraenoic acid; HODE, hydroxy octadecadienoic acid.

Several factors are known to modulate 15-LOX expression. For instance, IL-4 and IL-13, increase the expression of 15-LOX-1 and -2 in a number of cell types, including monocytes/macrophages, T lymphocytes and several cancer cell lines [40-45]. Moreover, chromatin modifications that play pivotal roles in the regulation of gene expression were reported to modulate 15-LOX expression. Histone acetylation appears to upregulate 15-LOX expression [46] whereas DNA methylation downregulates 15-LOX expression [47]. Whether these factors and conditions contribute to the modulation of 15-LOX expression in chondrocytes is among our ongoing research projects.

13-HODE and 15-HETE are potent endogenous activators and ligands for PPARγ [23,24]. Using a PPRE reporter plasmid in transient transfection experiments, we confirmed the capability of the above 15-LOX products to activate PPARγ in human chondrocytes. We also showed that pretreatment with an irreversible pharmacological PPARγ antagonist GW9662 overcame the inhibitory effect of 13-HODE and 15-HETE on IL-1β-induced MMP release. These results are consistent with previous findings showing that PPARγ activation suppresses MMP production in several cell types, including chondrocytes [26] and synovial fibroblasts [27]. Altogether, these data strongly suggest that 13-HODE and 15-HETE suppress IL-1β-induced MMP-1 and MMP-13 by chondrocytes through activation of PPARγ. The expression of MMP-1 and MMP-13 are essentially regulated by the transcription factors activator protein (AP)-1 and nuclear factor-κB (NF-κB), and analysis of the 5'-flanking regions of these genes has demonstrated the presence of numerous putative binding sites for AP-1 and NF-κB [3]. On the other hand, previous studies showed that acti-

Figure 5



Downregulation of IL-1β-induced MMP-1/MMP-13 expression by 13-HODE and 15-HETE does not require *de novo* protein synthesis. **(a, b)** Chondrocytes were treated with 100 pg/ml IL-1β in the presence of the control vehicle dimethyl sulfoxide or increasing concentrations of 13-HODE (panel a) or 15-HETE (panel b) for 8 hours. **(c)** Chondrocytes were pretreated with control vehicle dimethyl sulfoxide or cycloheximide (10 μg/ml) for 30 minutes before stimulation with 100 pg/ml IL-1β in the absence or presence of 50 μmol/l 13-HODE or 15-HETE for 8 hours. Total RNA was isolated, reverse transcribed into cDNA, and MMP-1 and MMP-13 mRNAs were quantified using real-time PCR. The housekeeping gene GAPDH was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. Results are expressed as fold changes, considering 1 as the value of untreated cells, and are the mean ± standard deviation of three independent experiments. *$P <$ 0.05 versus cells treated with IL-1β alone. CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxy octadecadienoic acid; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.

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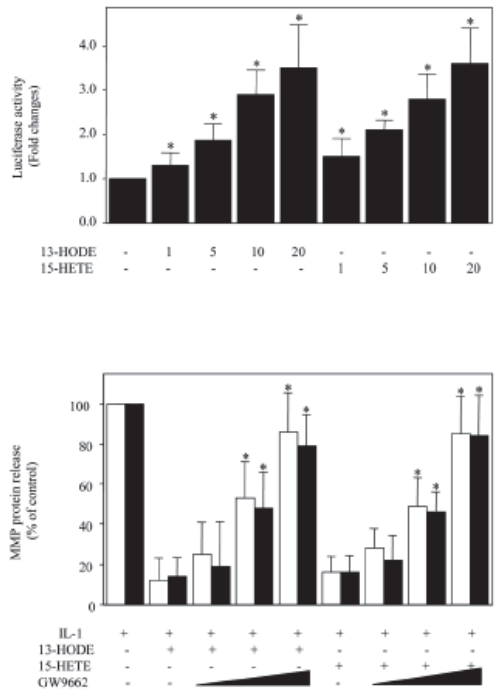
Figure 6



13-HODE and 15-HETE suppressed IL-1β-induced MMP-1/MMP-13 production in a PPARγ dependent manner. **(a)** 13-HODE and 15-HETE activate endogenous PPARγ in human chondrocytes. Chondrocytes were transiently transfected with a reporter construct containing three copies of a consensus PPRE placed upstream from the Tk-luciferase reporter ($PPRE_3$-Tk-Luc) along with the internal control pSV40-β-gal using FuGene 6 transfection reagent. Six hours later, the cells were washed and changed to medium containing 0.5% fetal calf serum for an additional 18 hours. Transfected cells were then treated with the control vehicle dimethyl sulfoxide or increasing concentrations of 13-HODE or 15-HETE for 18 hours. Luciferase activity values were determined and normalized to β-galactosidase activity. Results are expressed as fold changes, considering 1 as the value of unstimulated cells, and are the mean ± standard deviation of three independent experiments. *$P < 0.05$ versus unstimulated cells. **(b)** PPARγ antagonist (GW9662) prevented the suppressive effect of 13-HODE and 15-HETE on IL-1β-induced MMP-1 and MMP-13 release. Chondrocytes were pretreated with increasing concentrations (1, 5, and 10 μmol/l) of GW9662 for 30 minutes. Then, the cells were treated with or without IL-1β (100 pg/ml) for 24 hours in the absence or the presence of 50 μmol/l 13-HODE (panel a) or 50 μmol/l 15-HETE (panel b). The levels of MMP-1 and MMP-13 proteins in conditioned media were measured using ELISA. Results are expressed as the percentage of control, considering 100% as the value of cells treated with IL-1β alone, and are the mean ± standard deviation of four independent experiments. *$P < 0.05$ versus cells treated with IL-1β and 13-HODE or 15-HETE. HETE, hydroxyeicosatetraenoic acid; HODE, hydroxy octadecadienoic acid; MMP, matrix metalloproteinase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor-responsive element.

vation of PPARγ suppresses the transcriptional activity of AP-1 and NF-κB [48]. Therefore, it is possible that activation of PPARγ by 13-HODE and 15-HETE reduces transcriptional activity of AP-1 and NF-κB, leading to diminished production of MMP-1 and MMP-13 (Figure 7). Another possible mechanism through which 13-HODE and 15-HETE may downregulate MMP expression could involve the promotion of mRNA decay. Indeed, 15-LOX metabolites were reported to downmodulate lipopolysaccharide-induced TNF-α expression by enhancing mRNA decay [19]. Alternatively, 15-LOX products could prevent IL-1β-induced MMP-1 and MMP-13 expression by interfering with key signalling pathways. In this context, 15-LOX metabolites were shown to inhibit protein kinase C activity and translocation [20,49], and protein kinase C was shown to contribute to MMP-1 and MMP-13 expression [50,51].

15-HETE and 13-HODE are synthesized by a number of cell types such as macrophages, neutrophils and chondrocytes [52]. They have also been detected *in vivo* in several pathophysiological fluids, including sputum from chronic bronchitis patients [53], cerebrospinal fluid from patients with Alzheimer's disease [54], bronchoalveolar lavage fluids from patients with asthma [55] and scleroderma lung disease [56]. Apart from a report by Walenga and coworkers [57], who found that the levels of 15-HETE increase to about 1 μmol/l in blood stimulated with various agents, the concentrations of 15-HETE and 13-HODE detected in most pathophysiological fluids (1 to 100 nmol/l) were lower than those used in the present study (1 to 50 μmol/l). However, it should be noted that, like other eicosanoids, 13-HODE and 15-HETE function as autocrine and paracrine molecules and can readily reach pharmacological levels in the microenvironment of cells that produce them. Moreover, synovial fibroblasts [58] and osteoblasts [59] express 15-LOX and may represent additional sources for the production of 15-LOX metabolites within the joint. Also, we cannot exclude the possibility that low concentrations of 13-HODE and 15-HETE can synergize with each other or with other 15-LOX derivatives to suppress inflammatory and catabolic responses in the joint.

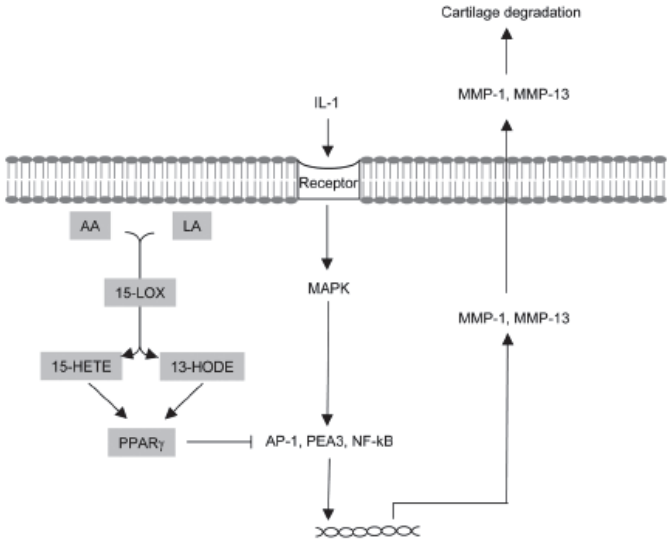
Conclusions

We demonstrated that 15-LOX-1 and -2 are expressed in OA articular chondrocytes. Treatment with 13-HODE and 15-HETE, the respective metabolites of 15-LOX-1 and -2, suppressed IL-1β-induced MMP-1 and MMP-13 production. These effects do not require protein synthesis and are mediate by PPARγ. These data suggest that 15-LOXs and their metabolites may have therapeutic promise in OA by preventing the production of cartilage-degrading enzymes.

Competing interests

The authors declare that they have no competing interests.

Figure 7



Schematic representation of the suppressive effect of 15-LOX metabolites on MMP-1/MMP-13 expression. Pro-inflammatory cytokines such as IL-1 interact with their respective receptors that activate MAPK signalling and downstream transcription factors, resulting in the transcription of MMP-1 and MMP-13 genes. 15-LOX convert AA and LA to 15-HETE and 13-HODE, which then activate PPARγ. Activated PPARγ antagonizes the transcriptional activity of AP-1, NF-κB and PEA3, which results in the inhibition of the expression of their target genes (for instance, MMP-1 and MMP-13). AA, arachidonic acid; AP, activator protein; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxy octadecadienoic acid; LA, linoeic acid; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PEA3, Polyoma Enhancer Activator 3; PPAR, peroxisome proliferator-activated receptor.

Authors' contributions

NC conceived the study, designed and carried out cell and real-time reverse transcription PCR experiments and some immunohistochemistry experiments. NZ contributed to the study design, carried out immunoassays and some cell experiments. MB participated in the study design and data analysis. JM-P, J-PP and ND helped to obtain tissues, and participated in the study design and in some immunohistochemistry experiments. HF conceived, designed and coordinated the study, carried out some cell experiments, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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ANNEXE 3:

Contribution of H3K4 methylation by SET-1A to interleukin-1-induced cyclooxygenase 2 and inducible nitric oxide synthase expression in human osteoarthritis chondrocytes

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Contribution of H3K4 Methylation by SET-1A to Interleukin-1–Induced Cyclooxygenase 2 and Inducible Nitric Oxide Synthase Expression in Human Osteoarthritis Chondrocytes

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Objective. **To investigate the role of histone H3 lysine 4 (H3K4) methylation in interleukin-1β (IL-1β)–induced cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in human osteoarthritic (OA) chondrocytes.**

Methods. **Chondrocytes were stimulated with IL-1, and the expression of iNOS and COX-2 messenger RNA and proteins was evaluated by real-time reverse transcriptase–polymerase chain reaction analysis and Western blotting, respectively. H3K4 methylation and the recruitment of the histone methyltransferases SET-1A and MLL-1 to the iNOS and COX-2 promoters were evaluated using chromatin immunoprecipitation assays. The role of SET-1A was further evaluated using the methyltransferase inhibitor 5′-deoxy-5′-(methylthio)adenosine (MTA) and gene silencing experiments. SET-1A level in cartilage was determined using immunohistochemistry.**

Results. **The induction of iNOS and COX-2 expression by IL-1 was associated with H3K4 di- and trimethylation at the iNOS and COX-2 promoters. These changes were temporally correlated with the recruitment of the histone methyltransferase SET-1A, suggesting an implication of SET-1A in these modifications. Treatment with MTA inhibited IL-1–induced H3K4 methylation as well as IL-1–induced iNOS and COX-2 expression. Similarly, SET-1A gene silencing with small interfering RNA prevented IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 expression. Finally, we showed that the level of SET-1A expression was elevated in OA cartilage as compared with normal cartilage.**

Conclusion. **These results indicate that H3K4 methylation by SET-1A contributes to IL-1–induced iNOS and COX-2 expression and suggest that this pathway could be a potential target for pharmacologic intervention in the treatment of OA and possibly other arthritic diseases.**

Osteoarthritis (OA) is the most common form of arthritis and is a leading cause of disability in the elderly (1). Clinical manifestations of OA may include pain, stiffness, and reduced joint motion. Pathologically, OA is characterized by progressive degeneration of articular cartilage, synovial inflammation, and subchondral bone remodeling. These processes are thought to be largely mediated through excess production of proinflammatory and catabolic mediators. Among these mediators, interleukin-1β (IL-1β) has been demonstrated to be predominantly involved in the initiation and progression of the disease (2–4). One mechanism through which IL-1 exerts its effects is by up-regulating the expression

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of genes encoding for inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) and the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) (2–4).

The production of NO is an important component in the pathogenesis of OA, and increased levels of nitrite/nitrate have been observed in the synovial fluid and serum of arthritis patients (5). The biosynthesis of NO is catalyzed by a group of enzymes known as NO synthases (NOS). There are 3 distinct NOS. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while the iNOS is expressed following stimulation with a variety of inflammatory agents, such as endotoxins or cytokines (6). NO participates in the pathogenesis of arthritis by inducing chondrocyte apoptosis (7) and matrix metalloprotease (MMP) production (8) and by suppressing the synthesis of collagen and proteoglycans (9). In addition, NO enhances the production of inflammatory cytokines (5) and PGE_2 (10) and reduces the synthesis of endogenous IL-1 receptor antagonist (IL-1Ra) (11). The important role of NO in the pathogenesis of OA is further supported by the finding that selective inhibition of iNOS in an experimental model of OA reduces the structural changes and the expression of several inflammatory and catabolic factors (12).

Like NO, PGE_2 contributes to the pathogenesis of arthritis through several mechanisms, including upregulation of MMP (13) and IL-1 (14) production, enhancement of the degradation of cartilage matrix components (15), and promotion of chondrocyte apoptosis (16). In addition, PGE_2 mediates pain responses and potentiates the effects of other mediators of inflammation (17). COX is the key enzyme in the biosynthesis of PGE_2, and 2 isoforms have been identified. COX-1 is constitutively expressed in a wide variety of tissues and is responsible for housekeeping functions. In contrast, COX-2 is undetectable in most normal tissues, but is rapidly induced by growth factors and proinflammatory cytokines, such as IL-1 and tumor necrosis factor α (TNFα) (17). COX-2 expression and activity are increased in cartilage from OA patients, and this is thought to play a primary role in the pain and inflammation associated with the disease (18). Moreover, COX-2 inhibitors have been extensively used in the treatment of OA.

Posttranslational modifications of nucleosomal histones, including acetylation, methylation, phosphorylation, and sumoylation, play important roles in the regulation of gene transcription through remodeling of chromatin structure (19,20). To date, histone acetylation and methylation are among the most studied and best characterized modifications. Unlike acetylation, which is generally associated with transcriptional activation, histone-lysine methylation is associated with either gene activation or repression, depending on the specific residue modified (21–24). For instance, methylation of the histone H3 lysine-4 (H3K4) is commonly associated with transcriptional activation, whereas methylation of H3K9 correlates with transcriptional repression (21–24). In addition, H3K4 can be mono-, di-, or trimethylated, with the di- and trimethylated forms being the most positively correlated with transcriptional activation (21–24).

H3K4 methylation is catalyzed by the action of a family of histone methyltransferases (HMTs) that share a conserved SET domain, which was named for its presence in diverse *Drosophila* chromatin regulators: Su(var)3–9, Enhancer of Zeste (E[z]) and Trithorax (Trx). Several specific H3K4 methyltransferases have been identified and characterized, including SET-1A, SET-1B, and 4 mixed-lineage leukemia (MLL) family HMTs (MLL-1, MLL-2, MLL-3, and MLL-4). Among them, only SET-1A and MLL-1 are able to di- and trimethylate H3K4 (25–28).

Although the induction of iNOS and COX-2 expression by IL-1 in chondrocytes is well documented (2–4), the role of histone methylation in their regulation remains undefined. In this study, we examined the role of H3K4 methylation in IL-1–induced iNOS and COX-2 expression in chondrocytes.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant human IL-1 was obtained from Genzyme. Aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, and 5′-deoxy-5′-(methylthio)adenosine (MTA) were from Sigma-Aldrich Canada. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal calf serum (FCS), and TRIzol reagent were from Invitrogen. Antibodies against iNOS and COX-2 were purchased from Cayman Chemical. Antibody against β-actin was from Santa Cruz Biotechnology. Antibodies against histone H3 and against mono-, di-, and trimethylated H3K4 were from Upstate/Millipore. Anti–SET-1A and anti–MLL-1 antibodies were from Bethyl Laboratories. Polyclonal rabbit anti-mouse IgG coupled with horseradish peroxidase (HRP) and polyclonal goat anti-rabbit IgG coupled with HRP were from Pierce.

Specimen selection and chondrocyte culture. Normal human cartilage (from femoral condyles) was obtained at necropsy, within 12 hours of death, from donors who had no history of arthritic diseases (n = 14; mean ± SD age 59 ± 13 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. Only those found to be free of alterations by both methods were further processed. OA cartilage was

obtained from patients undergoing total knee replacement surgery (n = 48; mean ± SD age 63 ± 19 years). All OA patients were diagnosed with knee OA according to the criteria developed by the American College of Rheumatology (29). At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors. Patients who had received intraarticular injection of steroids were excluded.

The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues. Informed consent was obtained from each donor or from an authorized third party.

Chondrocytes were released from cartilage by sequential enzymatic digestion, as previously described (30). Cells were seeded at 3.5×10^5/well in 12-well culture plates (Costar) or at $6–7 \times 10^5$/well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 hours. Cells were washed and incubated for an additional 24 hours in DMEM containing 0.5% FCS before stimulation with IL-1.

Protein extraction and Western blot analysis. Histones were extracted from the cells as previously described (31). Briefly, cells were washed with phosphate buffered saline (PBS) and lysed with ice-cold lysis buffer containing 10 m*M* HEPES-KOH, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 1.5 m*M* PMSF, 1 m*M* Na_3VO_4, and 10 μg/ml of aprotinin, leupeptin, and pepstatin. Sulfuric acid was added to a concentration of 0.2*N*, and the resultant supernatant was collected and dialyzed twice against 0.1*M* acetic acid and 3 times against sterile water.

Whole-cell lysates were prepared and analyzed as previously described (30).

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) analysis. Total RNA from stimulated chondrocytes was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, the isolated RNA was treated with RNase-free DNase I (Ambion). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes), dissolved in diethylpyrocarbonate-treated-H_2O and stored at –80°C until used. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Fermentas) as detailed in the manufacturer's guidelines. One-fiftieth of the RT reaction was analyzed by real-time PCR as described below. The following primers were used: for iNOS, 5′-ACATTGATGAGAAGCTGTCCCAC-3′ (sense) and 5′-CAAAGGCTGTGAGTCCTGCAC-3′ (antisense); for COX-2, 5′-TGTGTTGACATCCAGATCAC-3′ (sense) and 5′-ACATCATGTTTGAGCCCTGG-3′ (antisense); and for GAPDH, 5′-CAGAACATCATCCCTGCCTCT-3′ (sense) and 5′-GCTTGACAAAGTGGTCGTTGAG-3′ (antisense).

Real-time PCR analysis. Real-time PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 n*M* sense and antisense primers, 25 μl of SYBR Green Master Mix (Qiagen), and 0.5 units of uracil *N*-glycosylase (UNG; Epicentre Technologies). After incubation at 50°C for 2 minutes (UNG reaction), and at 95°C for 10 minutes (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 seconds at 95°C for denaturation and 1 minute for annealing and extension at 60°C). Incorporation of SYBR Green dye into the PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems), allowing determination of the threshold cycle (C_t), at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with 1 peak, indicating the specificity of the amplification. A C_t value was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative messenger RNA (mRNA) expression in chondrocytes was determined using the $\Delta\Delta C_t$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_t value was first calculated by subtracting the C_t value for the housekeeping gene GAPDH from the C_t value for each sample. A $\Delta\Delta C_t$ value was then calculated by subtracting the ΔC_t value of the control (unstimulated cells) from the ΔC_t value of each treatment. Fold changes compared with the control were then determined by raising 2 to the $-\Delta\Delta C_t$ power. Each PCR reaction generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on 2 separate occasions for each independent experiment.

Chromatin immunoprecipitation (ChIP) assay. The ChIP experiments were performed according to the ChIP protocol provided by Upstate/Millipore and previously published protocols (32,33). The primer sequences used were as follows: for the iNOS promoter, 5′-ATGAACTGCCACCTTGGACT-3′ (sense) and 5′-GTTTTCGACTCGCTACAAAGTT-3′ (antisense); for the COX-2 promoter, 5′-AAGACATCTGGCGGAAACC-3′ (sense) and 5′-ACAATTGGTCGCTAACCGAG-3′ (antisense); and for the MMP-13 promoter, 5′-ATTTTGCCAGATGGGTTTTG-3′ (sense) and 5′-CTGGGGACTGTTGTCTTTCC-3′ (antisense).

RNA interference. Specific small interfering RNA (siRNA) for SET-1A, MLL-1, or scrambled control was obtained from Dharmacon. Chondrocytes were seeded in 6-well plates at 6×10^5 cells/well and incubated for 24 hours. Cells were transfected with 100 n*M* siRNA using HiPerFect Transfection Reagent (Qiagen) following the manufacturer's recommendations. The medium was changed 24 hours later, and the cells were incubated for an additional 24 hours before stimulation with 100 pg/ml of IL-1 for 2 hours or 20 hours.

Immunohistochemistry. Cartilage specimens were processed for immunohistochemistry as previously described (30). The specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) of paraffin-embedded specimens were deparaffinized in toluene and dehydrated in a graded series of ethanol. The specimens were then preincubated with chondroitinase ABC (0.25 units/ml in PBS, pH 8.0) for 60 minutes at 37°C, followed by a 30-minute incubation with 0.3% Triton X-100 at room temperature. Slides were then washed in PBS followed by 2% hydrogen peroxide/methanol for 15 minutes. They were further incubated for 60 minutes with 2% normal serum (Vector) and overlaid with primary antibody for 18 hours at 4°C in a humidified chamber. The antibody was a rabbit polyclonal anti-human SET-1A (Bethyl Laboratories), which was used at 10 μg/ml.

Each slide was washed 3 times in PBS, pH 7.4, and

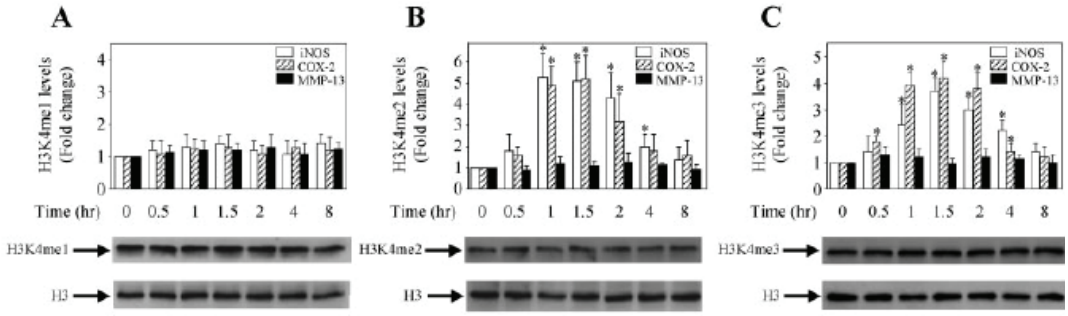

Figure 1. Effect of interleukin-1 (IL-1) on histone H3K4 methylation at the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2 promoters. Confluent chondrocytes were treated with 100 pg/ml of IL-1 for the indicated time periods. Chromatin immunoprecipitation (ChIP assays coupled with real-time polymerase chain reaction were performed using antibodies specific for **A**, monomethylated H3K4 (H3K4me1), **B** dimethylated H3K4 (H3K4me2), and **C**, trimethylated H3K4 (H3K4me3). The results (shown at the top) are expressed as the fold change in H3K4 mono-, di-, and trimethylation at the iNOS, the COX-2, or the matrix metalloprotease 13 (MMP-13) promoter relative to untreated cells. Values are the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P < 0.05$ versus unstimulated cells, by Student's 2-tailed *t*-test. In addition, confluent chondrocytes were treated as indicated, and histones were extracted and immunoblotted for mono-, di-, and trimethylated H3K4 as well as unmodified H3. Shown at the bottom are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results.

stained using the avidin–biotin complex method (Vectastain ABC kit; Vector). The color was developed with 3,3′-diaminobenzidine (Vector) containing hydrogen peroxide. The slides were counterstained with eosin. The specificity of staining was evaluated by using antibody that had been preadsorbed (1 hour at 37°C) with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200–1250 of human SET-1A (Bethyl), and by substituting nonimmune rabbit IgG (Chemicon) for the primary antibody at the same concentration. The evaluation of positive-staining chondrocytes was performed using our previously published method (30). For each specimen, 6 microscopic fields were examined under 40× magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated, and

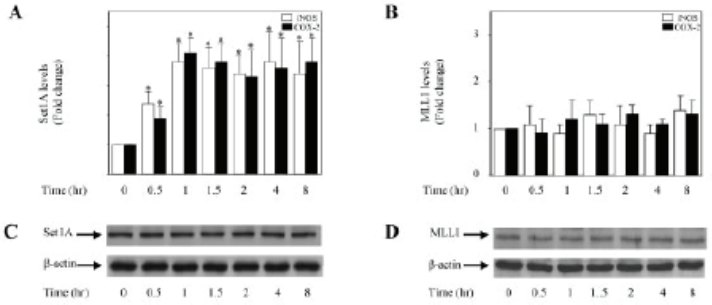

Figure 2. Effect of IL-1 on the recruitment of SET-1A and mixed-lineage leukemia 1 (MLL-1) to the iNOS and COX-2 promoters. **A** and **B**, Confluent chondrocytes were treated with 100 pg/ml of IL-1 for the indicated time periods, and ChIP assays were performed using specific anti–SET-1A (**A**) and anti–MLL-1 (**B**) antibodies. Results are expressed as the fold change in SET-1A and MLL-1 binding to the iNOS and COX-2 promoters relative to untreated cells. Values are the mean ± SD of 4 independent experiments. * = $P < 0.05$ versus unstimulated cells, by Student's 2-tailed *t*-test. **C** and **D**, Confluent chondrocytes were treated as indicated, and cell lysates were prepared and analyzed for SET-1A (**C**) and MLL-1 (**D**) protein expression by Western blotting. Blots were then stripped and reprobed with a specific anti–β-actin antibody. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

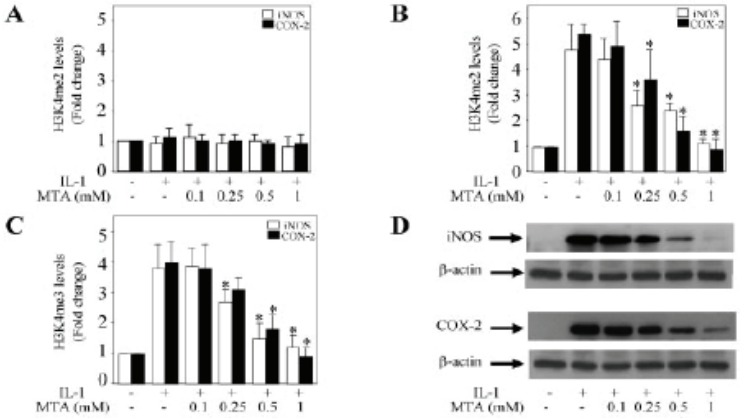

Figure 3. Effect of 5′-deoxy-5′-(methylthio)adenosine (MTA) on IL-1–induced H3K4 methylation and COX-2 and iNOS protein expression. Chondrocytes were pretreated for 1 hour with control vehicle (*N,N*-dimethylformamide; maximum concentration 0.05%) or with increasing concentrations of MTA prior to stimulation with 100 pg/ml of IL-1 for 1.5 hours (**A–C**) or 20 hours (**D**). **A–C,** ChIP assays, coupled with real-time polymerase chain reaction analyses, were performed using antibodies specific for mono-, di-, and trimethylated H3K4. Results are expressed as the fold change in H3K4 mono-, di-, and trimethylation at the iNOS and COX-2 promoters relative to untreated cells. Values are the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P < 0.05$ versus IL-1–treated cells, by Student's 2-tailed *t*-test. **D,** Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. Blots were then stripped and reprobed with a specific anti–β-actin antibody. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

the results were expressed as the percentage of chondrocytes staining positive (cell score).

Statistical analysis. Results of the real-time PCR and ChIP analyses are expressed as the mean ± SD, and statistical significance was assessed by Student's 2-tailed *t*-test. Results of the immunohistochemical analyses are expressed as the median (range), and statistical analysis was performed using the nonparametric Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Induction of iNOS and COX-2 expression by IL-1 in cultured human chondrocytes. We first examined the effect of IL-1 on iNOS and COX-2 mRNA expression in human OA chondrocytes. Cells were stimulated with IL-1 for various time periods, and the levels of iNOS and COX-2 mRNA were determined by real-time RT-PCR. IL-1–induced changes in gene expression were expressed as the fold change over control (untreated cells) after normalization to the internal control GAPDH. Treatment with IL-1 (100 pg/ml) induced iNOS mRNA expression in a time-dependent manner. Levels of mRNA for iNOS started to gradually increase at 2 hours after stimulation to reach a peak at 6 hours. With the longer incubation times, we observed a gradual decline in the mRNA levels starting at 8 hours. Similarly, treatment with IL-1 led to a time-dependent increase in COX-2 mRNA (data available upon request from the author). COX-2 mRNA was rapidly and significantly induced at 1 hour following stimulation with IL-1, reached the maximum at 6 hours and started to decrease at 8 hours (data available upon request from the author).

Next, we performed Western blot analysis to determine whether changes in mRNA levels were paralleled by changes in iNOS and COX-2 protein levels. Consistent with its effects on iNOS and COX-2 mRNA, IL-1 induced the expression of iNOS and COX-2 protein in a time-dependent manner (data available upon request from the author). By 4 hours poststimulation, iNOS protein levels were significantly increased. These levels were further increased up to 8 hours and remained

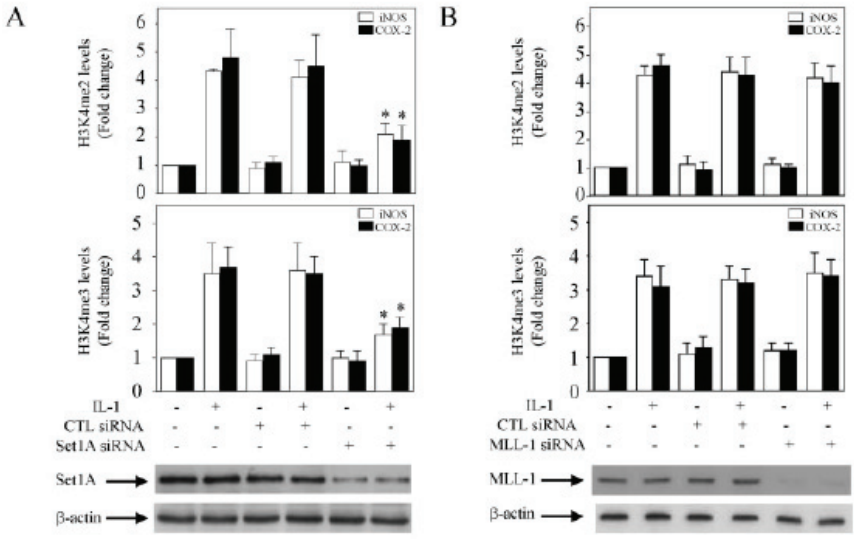

Figure 4. Effect of SET-1A silencing on IL-1–induced H3K4 methylation at the COX-2 and iNOS promoters. Chondrocytes were transfected with 100 n*M* SET-1A small interfering RNA (siRNA) (**A**), mixed-lineage leukemia 1 (MLL-1) siRNA (**B**), or control (CTL) scrambled siRNA. At 48 hours posttransfection, cells were left untreated or were treated for 1.5 hours with 100 pg/ml of IL-1. ChIP assays, coupled with real-time polymerase chain reaction analyses, were performed using antibodies specific for dimethylated (top) or trimethylated (middle) H3K4. Results are expressed as the fold change in H3K4 di- and trimethylation at the iNOS and COX-2 promoters relative to untreated cells. Values are the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P < 0.05$ versus nontransfected cells stimulated with IL-1, by Student's 2-tailed *t*-test. Knockdown of SET-1A and MLL-1 was confirmed by Western blotting using antibodies specific for SET-1A and MLL-1 (bottom). Blots were then stripped and reprobed with a specific anti–β-actin antibody. See Figure 1 for other definitions.

elevated until 24 hours. The induction of COX-2 protein expression occurred earlier (2 hours poststimulation) than iNOS protein expression, reached the maximum at 8 hours, and remained constant until 24 hours (data available upon request from the corresponding author). These results confirmed that IL-1 is a potent inducer of iNOS and COX-2 expression in chondrocytes (2–4).

IL-1 enhancement of H3K4 dimethylation and trimethylation, but not monomethylation, at the iNOS and COX-2 promoters. Recent studies have provided abundant evidence indicating that histone methylation plays an important role in the regulation of gene expression and that H3K4 di- or trimethylation is strongly correlated with transcriptional activation when found at promoter sites (21–24). To determine whether H3K4 methylation might be involved in IL-1–induced COX-2 and iNOS transcription, we performed ChIP assays. Chondrocytes were stimulated with IL-1 for various time periods, and formaldehyde cross-linked DNA–proteins were immunoprecipitated using antibodies specific for mono-, di-, or trimethylated H3K4. Control Ig and no antibodies were used as controls. DNA isolated from the immunoprecipitates was analyzed by real-time PCR using specific primers spanning the transcription start site (+1), the TATA box, and the binding sites of several transcription factors in the proximal regions of the iNOS (bp −256 to +24), COX-2 (bp −270 to +7), and MMP-13 (bp −220 to + 7) promoters.

As shown in Figures 1A–C, treatment with IL-1 enhanced the levels of di- and trimethylated H3K4 at the iNOS and COX-2 promoters in a time-dependent manner. In contrast, the levels of H3K4 methylation at the MMP-13 promoter remained unchanged, indicating that the observed modifications at the iNOS and COX-2 promoters are specific. The levels of di- and trimethylated H3K4 at the iNOS and COX-2 promoters were

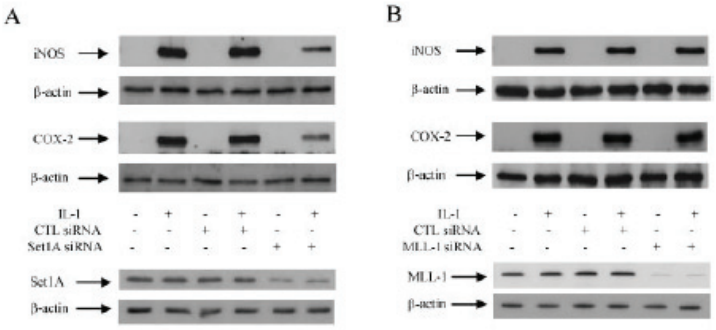

Figure 5. Effect of SET-1A silencing on IL-1–induced COX-2 and iNOS protein expression. Chondrocytes were transfected with 100 n*M* SET-1A small interfering RNA (siRNA) (**A**), mixed-lineage leukemia 1 (MLL-1) siRNA (**B**), or control (CTL) scrambled siRNA. At 48 hours posttransfection, cells were left untreated or were treated for 20 hours with 100 pg/ml of IL-1. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. Blots were then stripped and reprobed with specific anti–β-actin or anti–COX-2 antibodies. SET-1A and MLL-1 silencing was confirmed by Western blotting using antibodies specific for SET-1A and MLL-1. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

significantly increased at 0.5 hours after IL-1 stimulation, reached a maximum at 1–2 hours, and returned to a near basal level by 8 hours, whereas the level of monomethylated H3K4 did not appreciably change following IL-1 stimulation (Figure 1A). No immunoprecipitable COX-2 or iNOS promoter DNA was detected with the control Ig and with the no antibodies controls (data not shown). The induction of H3K4 di- and trimethylation by IL-1 at the iNOS and COX-2 promoter paralleled the increased transcription of iNOS and COX-2 (data available upon request from the author), suggesting that enhanced H3K4 di- and trimethylation may play a key role in IL-1–induced iNOS and COX-2 expression.

To determine whether the changes in H3K4 methylation seen at the iNOS and COX-2 promoters were not secondary to events causing global H3K4 methylation, we investigated the effect of IL-1 on global H3K4 methylation in chondrocytes. Cells were stimulated with IL-1 for various time periods, histones were extracted, and the levels of H3K4 methylation were measured by Western blot analysis using specific antibodies for mono-, di-, or trimethylated H3K4. As shown in Figure 1, the levels of mono-, di-, or trimethylated H3K4 were high in untreated chondrocytes, and treatment with IL-1 did not significantly change these levels. These results indicate that the alterations in H3K4 methylation seen in the ChIP assays were not due to nonspecific global histone modifications and are specific for the iNOS and COX-2 promoters.

IL-1–enhanced recruitment of the H3K4 methyltransferase SET-1A to the iNOS and COX-2 promoters. SET-1A and MLL-1 are H3K4-specific methyltransferases capable of di- and trimethylating H3K4 (25–28). Hence, we performed ChIP assays in IL-1–treated chondrocytes to examine the recruitment of SET-1A and MLL-1 to the iNOS and COX-2 promoters. As shown in Figure 2A, treatment with IL-1 resulted in sustained recruitment of SET-1A at the promoters of iNOS and COX-2. In contrast, IL-1 had no effect on the recruitment of MLL-1 to either promoter (Figure 2B), suggesting that the H3K4 methyltransferase that is involved in H3K4 methylation at the iNOS and COX-2 promoters is SET-1A. No immunoprecipitable COX-2 or iNOS promoter DNA was detected with the control Ig and no antibodies controls (data not shown). Strikingly, SET-1A was recruited to the promoters of iNOS and COX-2 when the levels of di- and trimethylated H3K4 increased (Figures 1B and C), and this recruitment correlated well with the increased transcription of iNOS and COX-2 (data available upon request from the author). Immunoblotting of cell lysates did not show any changes in the levels of SET-1A protein (Figures 2C and D), suggesting that the enhanced recruitment of SET-1A to the iNOS

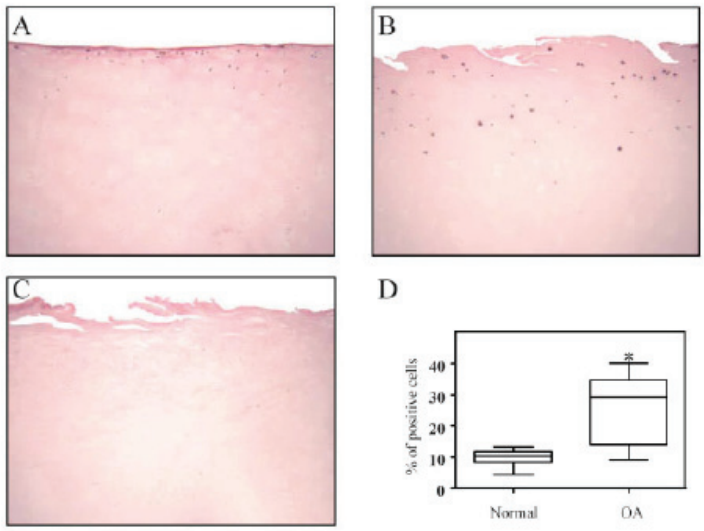

Figure 6. Expression of SET-1A protein in normal and osteoarthritic (OA) cartilage. **A** and **B**, Knee cartilage specimens from a normal donor (**A**) and a patient with OA (**B**) were immunostained for SET-1A protein. **C**, Knee cartilage specimen from a patient with OA was treated with anti–SET-1A antibody that had been preadsorbed with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200–1250 of human SET-1A (control for staining specificity). Representative sections are shown. Original magnification × 100. **D**, The percentage of chondrocytes expressing SET-1A in normal and OA cartilage samples was determined. Results are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. * = $P <$ 0.05 versus normal cartilage, by Mann-Whitney U test.

and COX-2 promoters seen with the ChIP assays was not due to increased expression of SET-1A protein. Together, these data suggest an implication of SET-1A in IL-1–induced H3K4 methylation and iNOS and COX-2 expression.

MTA reduction of IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 protein expression. The previous data suggest that SET-1A is involved in H3K4 di- and trimethylation and may contribute to the induction of iNOS and COX-2 expression. To test this, we first investigated the effect of MTA, a histone methyltransferase inhibitor (34), on IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters. Chondrocytes were pretreated with increasing concentrations of MTA for 1 hour, before stimulation with IL-1 for an additional 1.5 hours. The status of H3K4 methylation at the iNOS and COX-2 promoters was evaluated using ChIP assays with antibodies against mono-, di-, and trimethylated H3K4. We found that MTA treatment dose-dependently decreased IL-1–induced di- and tri-methylation of H3K4 (Figures 3B and C), which had increased during transcriptional activation. However, MTA treatment did not change the level of H3K4 monomethylation, which was not affected during transcriptional activation of iNOS and COX-2 (Figure 3A).

Next, we investigated the effect of MTA on IL-1–induced iNOS and COX-2 protein expression. Chondrocytes were pretreated with increasing concentrations of MTA for 1 hour, before stimulation with IL-1 for 20 hours. As shown in Figure 3D, treatment with MTA dose-dependently suppressed the IL-1–induced iNOS and COX-2 expression. This reduction was coincident with the decline in H3K4 methylation following treatment with MTA. The inhibition observed was not a result of reduced cell viability, as confirmed by MTT assay (data not shown). These findings strongly suggest that the SET-1A methyltransferase activity contributes

to IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 expression.

Prevention of IL-1–induced H3K4 methylation at the iNOS and COX-2 promoter as well as iNOS and COX-2 protein expression by siRNA-mediated depletion of SET-1A. To confirm the role of SET-1A, we examined the impact of its silencing by siRNA on IL-1–induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters. Chondrocytes were transfected with the scrambled control siRNA, siRNA for SET-1A, or siRNA for MLL-1, and after 48 hours of transfection, the cells were stimulated or were not stimulated with IL-1 for 1.5 hours. SET-1A knockdown reduced IL-1–induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters (Figure 4A). In contrast, MLL-1 silencing had no effect (Figure 4B). These results support the notion that SET-1A mediates IL-1–induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters.

Moreover, SET-1A silencing also markedly suppressed IL-1–induced iNOS and COX-2 expression (Figure 5A), whereas MLL-1 knockdown did not affect iNOS and COX-2 expression (Figure 5B). Taken together, these data strongly suggest that SET-1A contributes to IL-1–induced iNOS and COX-2 expression through up-regulation of H3K4 di- and trimethylation.

Elevated SET-1A protein levels in OA cartilage. To determine whether SET-1A levels were altered under conditions of OA, we performed immunohistochemical analysis on cartilage sections from OA patients and normal donors. As shown in Figures 6A and B, the immunostaining for SET-1A was located in the superficial and upper intermediate zones. Statistical evaluation of the cell score revealed a significant increase in the number of chondrocytes staining positive for SET-1A in OA cartilage (n = 14) as compared with normal cartilage (n = 14). The specificity of the staining was confirmed using an antibody that had been preadsorbed (1 hour at 37°C) with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200–1250 of human SET-1A (Figure 6C) or nonimmune control IgG (data not shown).

DISCUSSION

The present study is the first to show that the induction of iNOS and COX-2 expression by IL-1 is accompanied by increased H3K4 di- and trimethylation at the iNOS and COX-2 promoters. These modifications correlated with the recruitment of SET-1A to the iNOS and COX-2 promoters. Blocking methyltransferase activity or reducing the expression level of SET-1A abrogated IL-1–induced H3K4 methylation, as well as iNOS and COX-2 expression. Taken together, these results indicate that H3K4 methylation by SET-1A participates in IL-1–induced iNOS and COX-2 expression and suggest that this pathway may represent a therapeutic target in OA.

Our finding that IL-1–induced transcriptional activation of iNOS and COX-2 is associated with H3K4 di- and trimethylation is consistent with recent studies showing that transcriptional activation of a number of inducible inflammatory genes correlates with increased methylation of H3K4 at target promoters. For instance, the induction of monocyte chemotactic protein 1 (MCP-1) and TNFα by the proinflammatory astrocyte-derived protein S100B or TNFα in THP-1 cells is strongly associated with H3K4 methylation (35). Similarly, H3K4 methylation was reported to be increased at the promoters of TNFα and iNOS upon stimulation of the murine macrophage cell line RAW 264.7 and Kupffer cells with lipopolysaccharide (36). Increased methylation of H3K4 was also observed at promoters of MMP-1 in phorbol 12-myristate 13-acetate–treated T98G cells (31), IL-6 and MCP-1 in TNFα-treated vascular smooth cells (37), class II major histocompatibility complex in IFNγ-treated colon 26 cells (38), and IL-17 in CD4+ T helper cells treated with a combination of transforming growth factor β1 and IL-6 (39).

Several histone methyltransferases have been identified, among which SET-1A and MLL play dominant roles in the di- and trimethylation of H3K4 (25–28). Therefore, we examined the effect of IL-1 on the recruitment of SET-1A and MLL-1 to the iNOS and COX-2 promoters. ChIP results demonstrated that IL-1 enhanced the recruitment of SET-1A to the iNOS and COX-2 promoters, whereas the level of MLL-1 was not affected. Interestingly, the recruitment of SET-1A to the iNOS and COX-2 promoters was concomitant with the appearance of di- and trimethylated H3K4 at these sites, indicating that H3K4 methylation in response to IL-1 could be mediated by SET-1A. It is noteworthy that SET-1A appeared to be maintained at the iNOS and COX-2 promoters when the levels of di- and trimethylated H3K4 decreased. This suggests that specific H3K4 demethylases or inhibitors of SET-1A activity are recruited to the iNOS and COX-2 promoters and contribute to decreased H3K4 di- and tri-methylation.

The correlation between SET-1A recruitment and H3K4 di- and trimethylation suggests that SET-1A is implicated in these modifications and that H3K4 methylation by SET-1A contributes to IL-1–induced iNOS and COX-2 expression. Indeed, we found that

MTA, a protein methyltransferase inhibitor (34), prevented IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters and suppressed IL-1–induced iNOS and COX-2 protein expression. Moreover, the siRNA-mediated knockdown of SET-1A diminished the IL-1–induced di- and trimethylation of H3K4 and blocked the expression of iNOS and COX-2. Collectively, these results suggest that SET-1A contributes to IL-1–induced iNOS and COX-2 expression by enhancing H3K4 methylation.

In addition to H3K4, methylation of H3K9, H3K27, H3K36, and H3K79 is also known to modulate gene transcription. Like H3K4, methylation of H3K36 and H3K79 is associated with transcriptional activation, whereas methylation of H3K9 and H3K27 is associated with transcriptional repression (21–24). Although the role of these modifications in the effects of IL-1 is still unknown, we cannot exclude the possibility that they may also be involved in iNOS and COX-2 transcription.

We also demonstrated that the levels of SET-1A were increased in OA cartilage as compared with normal cartilage. Interestingly, OA chondrocytes in these zones were shown to express elevated levels of iNOS and COX-2 (15,40,41). These data, together with the implication of SET-1A in the transcriptional activation of iNOS and COX-2 in cultured chondrocytes, suggest that increased expression of SET-1 may be among the mechanisms that mediate the up-regulation of iNOS and COX-2 OA cartilage.

There are a number of mechanisms by which H3K4 methylation could mediate the transcriptional activation of iNOS and COX-2. One possibility is that H3K4 methylation promotes transcriptional activation by enhancing the acetylation of neighboring histones by histone acetyltransferases and by preventing the binding of the NuRD deacetylase complex (42,43). Alternatively, methylated H3K4 may serve as a docking site for the recruitment of chromatin-remodeling complexes such as the nucleosome remodeling factor (44), and the chromo–ATPase/helicase–DNA binding domain 1 (45). Finally, H3K4 methylation can activate transcription by facilitating the assembly of active transcription complexes. Indeed, the basal transcription complex TFIID can directly bind to the trimethylated H3K4 via the plant homeodomain finger of its subunit TAF-3 (46), and the methyltransferase SET-1A was reported to associate with RNA polymerase II (47).

In addition to histones, nonhistone proteins, especially transcription factors, have been identified as targets for methylation (48). In this context, Yang et al (49) reported that methylation of the RelA subunit of NF-κB, which is critically involved in the induction of iNOS and COX-2 in chondrocytes, by the lysine methyltransferase SET-7/9 inhibits NF-κB activity by inducing the degradation of RelA. On the other hand, Li et al (35) reported that SET-7/9 associates with the NF-κB p65 and up-regulates the expression of a subset of NF-κB target genes. Whether methylation of NF-κB contributes to the transcriptional activation of iNOS and COX-2 genes in chondrocytes remains to be determined.

In conclusion, the present study provides, to our knowledge, the first evidence that H3K4 methylation by SET-1A contributes to the induction of iNOS and COX-2 expression by IL-1. SET-1A may therefore be a novel therapeutic target for osteoarthritis and other human conditions associated with increased expression of iNOS and COX-2.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Fahmi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. El Mansouri, Fahmi.

Acquisition of data. El Mansouri, Chabane, Zayed, Fahmi.

Analysis and interpretation of data. El Mansouri, Chabane, Zayed, Kapoor, Benderdour, Martel-Pelletier, Pelletier, Duval, Fahmi.

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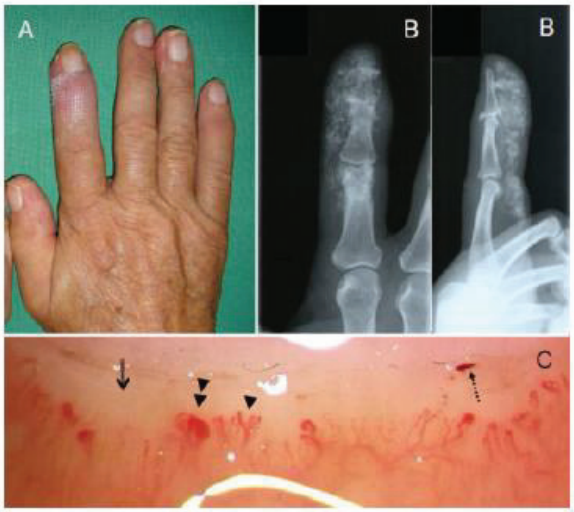
Clinical Images: Calcinosis and capillaries



The patient, a 77-year-old man, presented with a 6-month history of calcinosis cutis involving both hands and the right foot. Th alterations began in the index finger of the right hand, with the development of a digital ulcer. At presentation, the patient denie having symptoms compatible with Raynaud's phenomenon. The thumb and index finger of the right hand were swollen; there wa no sclerodactyly (A). The patient reported no sicca syndrome or gastrointestinal symptoms, and no swelling of the parotid an lacrimal glands. The C-reactive protein level was 18 mg/liter and the erythrocyte sedimentation rate was 30 mm/hou Immunofluorescence testing on HEp-2 cells revealed positive antinuclear antibodies (titer 1:1,280) with a fine speckled pattern. SS and SSB were positive by enzyme-linked immunosorbent assay, but scleroderma-specific antibodies (i.e., topoisomerase centromere, RNA polymerase III, PM/Scl, fibrillarin, Th/To, and ribonucleoprotein) were negative. Radiography demonstrate subcutaneous calcifications in several fingers of the right hand (B). Results of Schirmer's test, barium swallow, echocardiograph and computed tomography of the chest were normal; lung function tests showed no signs of restrictive or obstructive lung diseas Myositis, peripheral artery disease, renal disease, hyperparathyroidism, diabetes mellitus, myeloma, and malignancy were ruled ou Nailfold capillaroscopy revealed a severe microvasculopathy with capillary rarefaction and avascular fields (**arrow** with **double line** giant capillaries (**double arrowhead**), ramified, bushy capillaries (**arrowhead**), and capillary hemorrhages (**arrow** with **dotted lin** (C). In summary, this patient had evidence of an incipient undifferentiated connective tissue disease, with calcinosis cutis in th absence of Raynaud's phenomenon. The case highlights the importance of nailfold capillaroscopy in the detection of an underlyin microvasculopathy and the differential diagnosis of subcutaneous calcifications.

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ANNEXE 4:

Valproic acid suppresses interleukin-1ß-induced microsomal prostaglandin E2 synthase-1 expression in chondrocytes through upregulation of NAB1

Valproic Acid Suppresses Interleukin-1ß-induced Microsomal Prostaglandin E_2 Synthase-1 Expression i Chondrocytes Through Upregulation of NAB1

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ABSTRACT. ***Objective.*** Microsomal prostaglandin E_2 synthase-1 (mPGES-1) catalyzes the terminal step in the biosynthesis of PGE_2. Early growth response factor-1 (Egr-1) is a key transcription factor in the regulation of mPGES-1, and its activity is negatively regulated by the corepressor NGF1-A-binding protein-1 (NAB1). We examined the effects of valproic acid (VA), a histone deacetylase inhibitor, on interleukin 1ß (IL-1ß)-induced mPGES-1 expression in human chondrocytes, and evaluated the roles of Egr-1 and NAB1 in these effects.

Methods. Chondrocytes were stimulated with IL-1 in the absence or presence of VA, and the level of mPGES-1 protein and mRNA expression were evaluated using Western blotting and real-time reverse-transcription polymerase chain reaction (PCR), respectively. mPGES-1 promoter activity was analyzed in transient transfection experiments. Egr-1 and NAB1 recruitment to the mPGES-1 promoter was evaluated using chromatin immunoprecipitation assays. Small interfering RNA (siRNA) approaches were used to silence NAB1 expression.

Results. VA dose-dependently suppressed IL-1-induced mPGES-1 protein and mRNA expression as well as its promoter activation. Treatment with VA did not alter IL-1-induced Egr-1 expression, or its recruitment to the mPGES-1 promoter, but prevented its transcriptional activity. The suppressive effect of VA requires *de novo* protein synthesis. VA induced the expression of NAB1, and its recruitment to the mPGES-1 promoter, suggesting that NAB1 may mediate the suppressive effect of VA. Indeed, NAB1 silencing with siRNA blocked VA-mediated suppression of IL-1-induced mPGES-1 expression.

Conclusion. VA inhibited IL-1-induced mPGES-1 expression in chondrocytes. The suppressive effect of VA was not due to reduced expression or recruitment of Egr-1 to the mPGES-1 promoter and involved upregulation of NAB1. (First Release Jan 15 2011; J Rheumatol 2011;38:492–502; doi:10.3899/jrheum.100907)

Key Indexing Terms:
MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 CHONDROCYTES
VALPROIC ACID NGF1-A-BINDING PROTEIN-1

Prostaglandin E_2 (PGE_2) plays an important role in the pathophysiology of arthritis, and excessive levels have been reported in serum and synovial fluids from patients with osteoarthritis (OA) and rheumatoid arthritis (RA)[1]. PGE_2 contributes to the pathogenesis of arthritis by induci tilage proteoglycan degradation[2], enhancing the acti and production of matrix metalloproteases (MMP)[3], promoting chondrocyte apoptosis[4]. PGE_2 is also invol neoangiogenesis and mediates pain responses[5].

The biosynthesis of PGE_2 from arachidonic acid re 2 enzymatic activities. Cyclooxygenase (COX) en convert arachidonic acid into PGH_2, which is in tu merized to PGE_2 by PGE synthase (PGES) enzyme isoforms of the COX enzyme, COX-1 and COX-2 been identified. COX-1 is constitutively expressed i tissues, whereas COX-2 is inducible by various s including proinflammatory signals[6]. At least 3 c PGES isoforms have been cloned and characterized, i ing cytosolic PGES (cPGES), microsomal P (mPGES-1), and mPGES-2[7]. cPGES is constitutive ubiquitously expressed and is functionally couple COX-1, promoting immediate production of PGE_2[8]. I

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trast, mPGES-1 is markedly upregulated by inflammatory or mitogenic stimuli and is functionally coupled with COX-2, promoting delayed PGE_2 production[9]. mPGES-2 is constitutively expressed in various cells and tissues and can be coupled with both COX-1 and COX-2[10]. We and others have previously shown that the level of mPGES-1 is elevated in articular tissues from patients with OA and RA and in animal models of arthritis[11,12,13,14]. Moreover, mPGES-1 deficiency was protective in animal models of chronic inflammation, pain, and arthritis[15,16], which implicates mPGES-1 as a potential target for therapeutic intervention in arthritis.

The expression of mPGES-1 is upregulated in several cell types after treatment with proinflammatory stimuli such as interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) and is downregulated by antiinflammatory glucocorticoids[9,11,17]. Transcriptional induction of mPGES-1 is primarily controlled by the transcription factor early growth response factor-1 (Egr-1)[18,19]. Like most transcription factors, the activity of Egr-1 is negatively regulated by corepressor proteins. Two corepressors of Egr-1, NGF1-A-binding proteins (NAB)1 and NAB2, have been identified[20,21].

Acetylation and deacetylation of histone and nonhistone proteins play a critical role in the control of gene transcription[22,23]. The acetylation status is determined by interplay between histone acetyltransferases (HAT) and histone deacetylases (HDAC). In general, histone acetylation is associated with transcription activation through relaxed chromatin structure, whereas histone deacetylation is associated with transcription repression via chromatin condensation[22,23]. Recent studies, however, have revealed that transcription activation is not necessarily associated with histone acetylation and that HDAC activity can also activate transcription. For instance, global analysis of gene expression showed that inhibition of HDAC activity results in both induction and repression of gene expression[24,25,26]. In addition, genome-wide genetic studies with yeast demonstrated clearly that HDAC are required in both transcriptional activation and repression[27,28]. Finally, inhibition of HDAC activity was reported to repress transcription in several cell types including chondrocytes[29,30,31,32,33,34,35,36].

We examined the effect of valproic acid (VA), an HDAC inhibitor[37], on IL-1-induced mPGES-1 expression in human OA chondrocytes. We showed that VA suppressed IL-1-induced mPGES-1 expression without interfering with the expression or the recruitment of Egr-1 to the mPGES-1 promoter. Further, we provide evidence that the suppressive effect of VA on IL-1-induced mPGES-1 expression involves upregulation of NAB1.

MATERIALS AND METHODS

Reagents and antibodies. Human recombinant (rh) IL-1 was obtained from Genzyme (Cambridge, MA, USA). TNF-α and IL-17 were purchased from R&D Systems (Minneapolis, MN, USA). VA, cycloheximide (CHX) aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal calf serum (FCS) and Trizol reagent were supplied by Invitrogen (Burlington, ON, Canada). Plasmid DNA was prepared using a kit from Qiagen (Mississauga, ON, Canada). FuGene-6 transfection reagent was from Roche Applied Science (Laval, QC, Canada). The luciferase reporter assay system was from Promega (Madison, WI, USA). Anti-mPGES-1 and anti-cPGES antibodies were from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against Egr-1, NAB1, NAB2, and ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit anti-mouse immunoglobulin G (IgG) coupled with horseradish peroxidase (HRP) and polyclonal goat anti-rabbit IgG with HRP were from Pierce (Rockford, IL, USA). All other chemicals were purchased from Fisher Scientific or Bio-Rad (Mississauga, ON, Canada).

Chondrocyte isolation and treatment. Articular cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement (n = 49, mean age 67 ± SD 17 yrs). Informed consent had been obtained from patients with OA for the use of their tissues for research purposes. All OA patients were diagnosed according to the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA[38]. At the time of surgery, patients had symptomatic disease requiring medical treatment in the form of nonsteroidal antiinflammatory drugs or selective COX-2 inhibitors. Patients who had received intraarticular injection of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as described[33]. Briefly, small pieces of cartilage were incubated with 2 mg/ml pronase for 1 h followed by 1 mg/ml type IV collagenase (Sigma-Aldrich) for 6 h at 37°C in DMEM and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS.

Confluent chondrocytes were detached by trypsinization, seeded at 3.5×10^5 cells per well in 12-well culture plates (Costar, Corning, NY, USA), or at 7×10^5 cells per well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 h. Cells were washed and incubated for an additional 24 h in DMEM containing 0.5% FCS and pretreated with VA, trichostatin A (TSA), or BA for 30 min, before stimulation with IL-1, TNF-α, or IL-17. In another set of experiments chondrocytes were pretreated for 30 min with CHX, before stimulation with IL-1 or VA. The expression level of mPGES-1 protein was determined 24 h after stimulation, whereas the level of mPGES-1 messenger RNA (mRNA) was determined at 8 hours. Only first-passaged chondrocytes were used.

PGE_2 determination. Levels of PGE_2 were determined using a PGE_2 enzyme immunoassay (EIA; Cayman Chemical). The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

Protein extraction and Western blot analysis. For histone extraction, chondrocytes were washed with phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer containing 10 mM hydroxyethyl-piperazine ethanesulfonic acid potassium hydroxide (HEPES-KOH), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1.5 mM PMSF, 1 mM Na_3VO_4, and 10 μg/ml aprotinin, leupeptin, and pepstatin. Sulfuric acid was added to a concentration of 0.2 N and the resultant supernatant was collected and dialyzed twice against 0.1 M acetic acid and 3 times against sterile water. For whole-cell lysate preparation, chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce). Then 20 μg of total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose

membrane (Bio-Rad). After blocking in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20, and 5% (wt/vol) nonfat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with Tris-buffered saline (TBS), pH 7.5, with 0.1% Tween 20. The blots were then incubated with HRP-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA).

RNA extraction and cDNA synthesis. Total RNA was isolated from chondrocytes using the TRIzol reagent, and dissolved in 20 μl of diethylpyrocarbonate-treated H_2O. One microgram of total RNA was treated with RNase-free DNase and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase. One-fifth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: mPGES-1: sense 5'-GAA GAA GGC CTT TGC CAA C-3' and antisense 5'-GGA AGA CCA GGA AGT GCA TC-3'; cPGES: sense 5'-GCA AAG TGG TAC GAT CGA AGG-3' and antisense 5'-TGT CCG TTC TTT TAT GCT TGG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-CAG AAC ATC ATC CCT GCC TCT-3' and antisense 5'-GCT TGA CAA AGT GGT CGT TGA G-3'.

Real-time PCR. Real-time PCR analysis was performed in a total volume of 50 μl containing cDNA template, 200 nM of sense and antisense primers, and 25 μl of SYBR® Green master mix (Qiagen). Incorporation of SYBR Green dye into PCR products was monitored in real time using a GeneAmp 5700 sequence detector (Applied Biosystems, Foster City, CA, USA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After incubation at 95°C for 10 min to activate the AmpliTaq Gold enzyme, the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension). After PCR, dissociation curves were generated, with one peak indicating the specificity of the amplification. A C_T value was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems). Data were expressed as fold-changes relative to control conditions (unstimulated cells) using the $\Delta\Delta C_T$ method as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control from the ΔC_T value of each treatment. Fold-changes compared with the control (unstimulated cells) were then determined by raising 2 to the $\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on 2 separate occasions from at least 3 independent experiments.

Transient transfection. The mPGES-1 promoter construct (–538/–28) was provided by Dr. T. Smith (University of California, Los Angeles, CA, USA). Egr-1 expression vector was donated by Dr. Y. Chen (Morehouse School of Medicine, Atlanta, GA, USA). Expression plasmids for NAB1 and NAB2 were provided by Dr. J. Savren (University of Wisconsin, Madison, WI, USA). The ß-galactosidase reporter vector under the control of SV40 promoter (pSV40-ß-gal) was from Promega. Transient transfection experiments were performed using FuGene-6 transfection reagent according to the manufacturer's recommendation (Roche Applied Science). Briefly, chondrocytes were seeded 24 h prior to transfection at a density of 3×10^5 cells/well in 12-well plates and transiently transfected with 1 μg of the mPGES-1 promoter construct and 0.5 μg of the internal control pSV40-ß-gal. Six hours later, the medium was replaced with DMEM containing 1% FCS. At 1 day after transfection, the cells were treated with IL-1 in the absence or presence of VA for 18 h. In the overexpression experiments, the amount of transfected DNA was kept constant by using the corresponding empty vector. At the end of the indicated treatment, the cells were washed twice in ice-cold PBS and extracts were prepared for luciferase reporter assay. Luciferase activity was normalized for transfection efficiency using the corresponding ß-galactosidase activity.

RNA interference. Small interfering RNA (siRNA) specific for NAB1 and scrambled control were obtained from Dharmacon (Lafayette, CO, USA). Chondrocytes were seeded in 12-well plates at 3×10^5 cells/well and incubated 24 h. Cells were transfected with 100 nM siRNA using the HiPerFect Transfection Reagent (Qiagen) following the manufacturer's recommendations. The medium was changed 24 h later and the cells were incubated an additional 24 h before stimulation with IL-1 in the absence or presence of VA. Cell lysates were prepared and analyzed for mPGES-1 or NAB1 protein expression by Western blotting.

Chromatin immunoprecipitation (ChIP) assay. The ChIP experiments were performed according to the ChIP protocol provided by Upstate/Millipore Biotechnology and published protocols[39,40]. The primer sequences used were mPGES-1 promoter sense 5'-CCC GGA GAC TCT CTG CTT C-3' and antisense 5'-TCA ACT GTG GGT GTG ATC AGC-3'.

Statistical analysis. Data are expressed as the mean ± SD. Statistical significance was assessed by 2-tailed Student t test. P values < 0.05 were considered statistically significant.

RESULTS

VA suppressed IL-1-induced mPGES-1 protein expression. Blocking histone deacetylation with specific inhibitors modulated gene expression in several cell types[29,30,31,32,33,34,35,36]. To determine whether HDAC inhibitors can modulate PGE_2 production and mPGES-1 expression in chondrocytes, cells were stimulated with IL-1 in the absence or presence of increasing concentrations of VA, and the release of PGE_2 and the expression of mPGES-1 protein were evaluated by EIA and Western blotting, respectively. As shown in Figure 1A, stimulation with IL-1 dramatically increased PGE_2 production and mPGES-1 protein expression. Treatment with VA suppressed IL-1-induced PGE_2 release and mPGES-1 protein expression in a dose-dependent manner. In contrast, the expression of cPGES protein was not affected by these treatments. To determine whether VA inhibits HDAC activity in chondrocytes, we examined its effect on the acetylation of histone H3 protein. As shown in Figure 1B, treatment with VA increased histone H3 protein acetylation in a dose-dependent manner. Thus, VA suppressed IL-1-induced mPGES-1 expression and enhanced histone H3 acetylation in chondrocytes. Treatment of chondrocytes by 2 additional HDAC inhibitors, butyric acid (BA) and VA, also suppressed IL-1-induced mPGES-1 expression in a dose-dependent manner (Figure 1C).

To examine whether the inhibitory effect of VA on mPGES-1 expression was specific for IL-1, we assessed its effects on TNF-α- and IL-17-induced mPGES-1 protein expression. Interestingly, VA suppressed the induction of mPGES-1 expression by both TNF-α and IL-17 (Figure 1D), indicating that its effect was not restricted to IL-1. These data indicate that VA can downregulate the induction of mPGES-1 expression in human chondrocytes. The concentrations of VA utilized did not affect chondrocyte viability as judged by the trypan blue exclusion and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (data not shown).

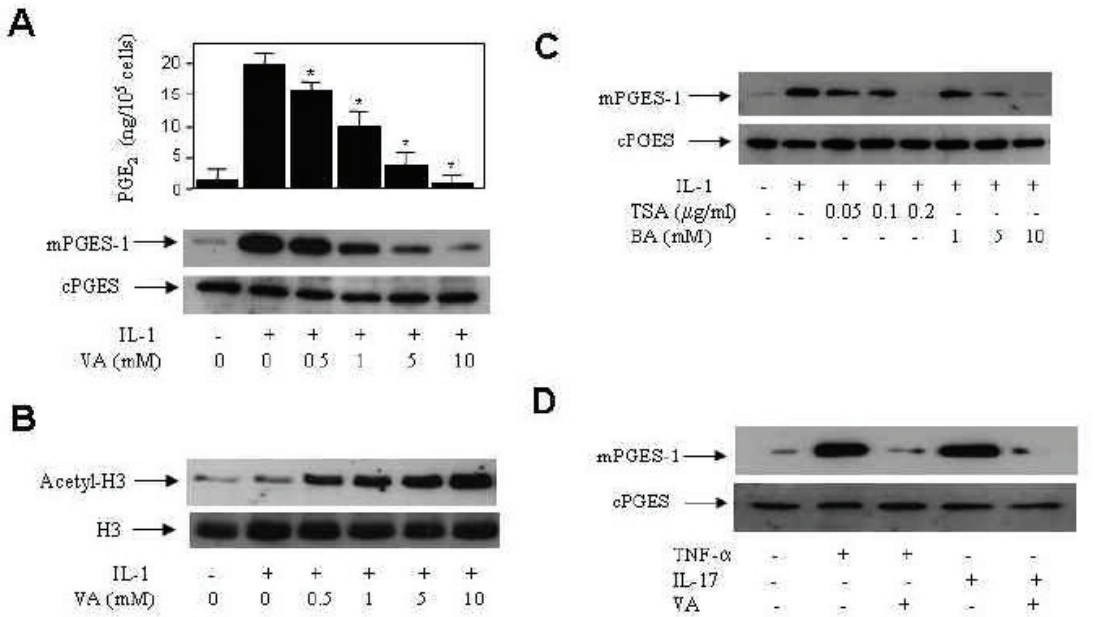

Figure 1. Valproic acid (VA) suppressed interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression in chondrocytes. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 20 hours. Conditioned media were collected and analyzed for PGE_2 release. Results are expressed as mean ± SEM from 4 independent experiments (*$p < 0.05$ compared with cells treated with IL-1 alone). Cell lysates were prepared and analyzed for mPGES-1 protein expression by Western blotting. B. Chondrocytes were treated as indicated, histones were extracted and immunoblotted for acetyl-H3. C, D. Chondrocytes were treated with IL-1 in the absence or presence of increasing concentrations of trichostatin A or butyric acid (C) or with 1 ng/ml TNF-α or 100 ng/ml IL-17 in the absence or presence of VA (10 mM) (D) for 20 hours. Cell lysates were prepared and analyzed for mPGES-1 protein expression by Western blotting. In the lower panels the blots were stripped and reprobed with specific anti-cPGES (A, C, D) or anti-histone H3 (B) antibodies. Blots are representative of similar results obtained from 4 independent experiments.

VA prevented IL-1-induced mPGES-1 expression at the transcriptional level. To determine whether the suppressive effect of VA was due to inhibition of mPGES-1 mRNA induction, chondrocytes were stimulated with IL-1 in the absence or presence of increasing concentrations of VA, and the level of mPGES-1 mRNA expression was determined using real-time PCR. The relative expression level of mPGES-1 mRNA was plotted as fold-change over control untreated cells. GAPDH gene expression was used for normalization. As expected, treatment with IL-1 resulted in a marked increase (~12-fold) of the mPGES-1 mRNA level, but this effect was dose-dependently attenuated in the presence of VA (Figure 2A), suggesting that VA exerts its effects at the transcriptional level. To confirm this, we carried out transient transfection experiments. Chondrocytes were transfected with the human mPGES-1 promoter-luciferase reporter gene and then stimulated with IL-1 in the absence or presence of VA. As shown in Figure 2B, IL-1 induced the luciferase activity of the mPGES-1 promoter and this activation was dose-dependently reduced by VA, consistent with its effect on mPGES-1 mRNA expression. Taken together, these data indicate that the suppressive effect of VA on IL-1-induced mPGES-1 takes place, at least in part, at the transcriptional level.

VA did not target Egr-1 expression and recruitment to the mPGES-1 promoter, but prevented its ability to transactivate the mPGES-1 promoter. The transcription factor Egr-1 plays a key role in the induction of mPGES-1 expression[18,19]; therefore, we considered whether the inhibition of IL-1-induced mPGES-1 expression by VA was due to prevention of Egr-1 expression. Chondrocytes were incubated with IL-1 in the absence or presence of VA, and cell extracts were prepared and analyzed by Western blotting. As shown in Figure 3A, IL-1 strongly induced the expression of Egr-1, and this effect was not altered in the presence of VA, indicating that the suppressive effect of VA is not due to reduced expression of Egr-1.

To determine whether VA affects the recruitment of Egr-1 to the endogenous mPGES-1 promoter, we performed ChIP assays. Chondrocytes were stimulated with IL-1 in the absence or presence of VA, and formaldehyde cross-linked DNA proteins were immunoprecipitated with an anti-Egr-1 antibody. No-antibody and non-immune serum were used as controls. DNA isolated from the immunoprecipitates was

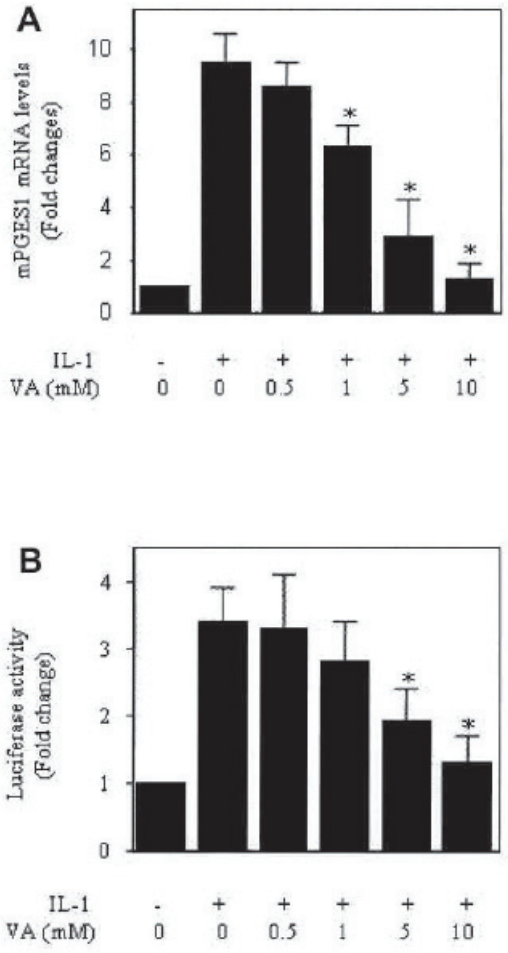

Figure 2. Valproic acid (VA) inhibited interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression at the transcriptional level. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 8 hours. Total RNA was isolated and reverse-transcribed into cDNA, and mPGES-1 levels were quantified using real-time PCR. GAPDH gene expression was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. B. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and the internal control pSV40-ß-gal (0.5 μg/well) using FuGene 6 transfection reagent. The next day, transfected cells were treated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of VA for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent the mean ± SD of 4 independent experiments [*p < 0.05 compared with cells treated with IL-1 alone (control)].

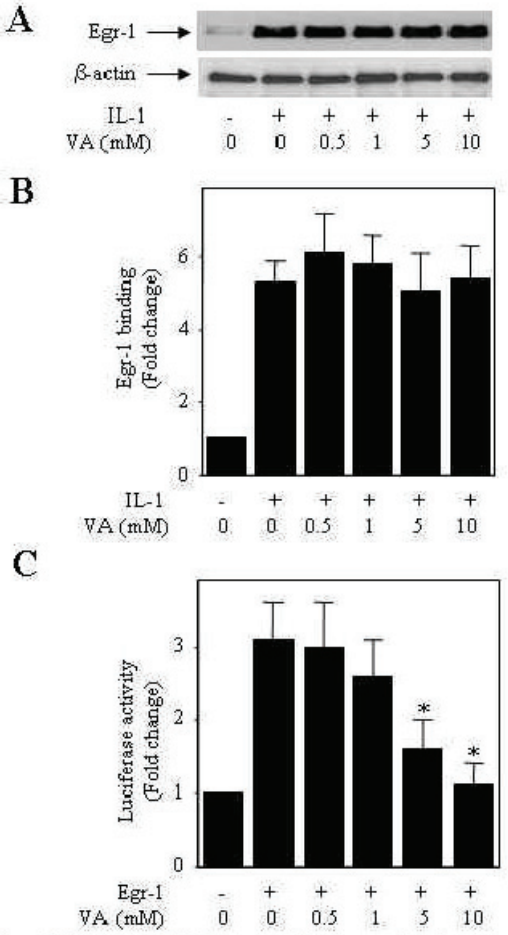

Figure 3. Valproic acid (VA) did not target Egr-1 expression and recruitment to the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter, but prevented its transcriptional activity. A. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 2 hours. Cell lysates were prepared and analyzed for Egr-1 protein by Western blotting. In the lower panel the blots were stripped and reprobed with specific anti-ß-actin antibody. This blot is representative of similar results obtained from 4 independent experiments. B. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of VA for 1 hour. ChIP assays, coupled with real-time PCR, were performed using a specific anti-Egr-1 antibody. Results are expressed as fold-changes of Egr-1 binding to the mPGES-1 promoter relative to untreated cells and represent the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions ($p < 0.05$ compared with untreated cells). C. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and an expression vector for Egr-1 (100 ng/ml) together with the internal control pSV40-ß-gal (0.5 μg/well) using FuGene 6 transfection reagent. The next day, transfected cells were treated with the indicated concentrations of VA for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of cells transfected with the reporter construct alone, and represent the mean ± SD of 4 independent experiments [*p < 0.05 compared with cells transfected with Egr-1 (control)].

analyzed by real-time PCR using primers amplifying the mPGES-1 promoter region (bp –142 to –37) that harbors Egr-1 binding sites. As shown in Figure 3B, treatment with IL-1 enhanced (3.2-fold) the binding of Egr-1 to the endogenous mPGES-1 promoter. However, IL-1-induced Egr-1 binding was not affected by VA. We failed to detect immunoprecipitable mPGES-1 promoter DNA with the no-antibody and non-immune serum controls (data not shown). Therefore, VA inhibited IL-1-induced mPGES-1 expression by mechanisms independent of, or in addition to, impaired expression or recruitment of Egr-1 to the mPGES-1 promoter.

Next, we investigated the effect of VA on the ability of Egr-1 to transactivate the mPGES-1 promoter. Chondrocytes were cotransfected with the mPGES-1 promoter and an expression vector for Egr-1 and then left untreated or treated with inceasing concentrations of VA. As shown in Figure 3C, overexpression of Egr-1 highly increased the mPGES-1 promoter activity. Interestingly, treatment with VA dose-dependently attenuated Egr-1-mediated activation of the mPGES-1 promoter. Together, these data suggest that VA inhibits mPGES-1 expression by interfering with the Egr-1 transcriptional activity.

VA-mediated inhibition of IL-1-induced mPGES-1 expression requires de novo protein synthesis. To determine whether the inhibitory effect of VA on IL-1-induced mPGES-1 expression requires *de novo* protein synthesis, we tested the effect of the protein synthesis inhibitor cycloheximide (CHX). Chondrocytes were pretreated with CHX for 30 minutes and stimulated with IL-1 in the absence or presence of VA for 8 hours. The levels of mPGES-1 mRNA were analyzed by real-time PCR. As shown in Figure 4, pretreatment with CHX blocked VA-mediated inhibition of IL-1-induced mPGES-1 expression, suggesting that the suppressive effect of VA was an indirect effect and was dependent on *de novo* protein synthesis.

NAB1 contributes to the suppression of IL-1-induced mPGES-1 by VA. The ability of CHX to block VA-mediated suppression of Egr-1-induced mPGES-1 expression suggests that VA induces the synthesis of one or more proteins that suppress Egr-1 activity. Possible candidates that may be involved in the suppressive effect of VA are NAB1 and NAB2. NAB1 and NAB2 negatively regulate the activity of Egr-1 and suppress the transcription of Egr-1-dependent target genes[20,21].

To determine whether NAB1 and/or NAB2 were involved in the suppressive effect of VA on IL-1-induced mPGES-1 expression, we first examined their ability to repress Egr-1-induced mPGES-1 promoter activation in chondrocytes. Cells were cotransfected with the mPGES-1 promoter and an expression vector for Egr-1 together with increasing concentrations of vectors encoding for NAB1 or NAB2. As shown in Figure 5A, overexpression of Egr-1 greatly increased the mPGES-1 promoter activity. Interestingly, cotransfection with NAB1 or NAB2

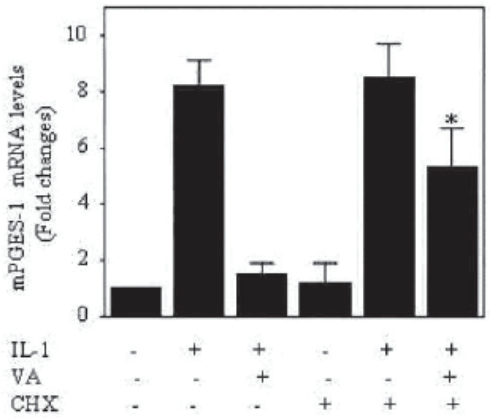

Figure 4. Downregulation of interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) expression by valproic acid (VA) requires *de novo* protein synthesis. Chondrocytes were pretreated with control vehicle dimethylsulfoxide or cycloheximide (10 μg/ml) for 30 minutes prior to stimulation with 100 pg/ml IL-1 in the absence or presence of 10 mM VA for 8 hours. Total RNA was isolated, reverse-transcribed into cDNA, and mPGES-1 mRNA was quantified using real-time PCR. The housekeeping gene GAPDH was used for normalization. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent the mean ± SD of 3 independent experiments (*$p < 0.05$ compared with cells treated with IL-1 and VA).

dose-dependently reduced Egr-1-mediated activation of the mPGES-1 promoter. Similarly, overexpression of NAB1 or NAB2 dose-dependently abrogated IL-1-mediated activation of the mPGES-1 promoter (Figure 5B). These experiments demonstrated that NAB1 and NAB2 proteins inhibit both Egr-1- and IL-1-mediated activation of the mPGES-1 promoter in chondrocytes.

Next we analyzed the effect of VA on NAB1 and NAB2 expression in chondrocytes. Cells were treated with VA for different time periods, and the expression of NAB1 and NAB2 proteins was evaluated by Western blotting. As illustrated in Figure 6A, VA enhanced NAB1 expression in a time-dependent manner. NAB1 protein expression started to increase 0.5 hours post-stimulation, reached the maximum at 1 hour, and remained elevated until 12 hours. In contrast, VA had no significant effect on the expression levels of NAB2 (Figure 6A).

Transcriptional repression by NAB proteins requires their recruitment to target promoters through interaction with Egr-1[41,42]. Therefore, we examined whether VA promotes NAB1 recruitment to the endogenous mPGES-1 promoter. As shown in Figure 6B, treatment with either VA or IL-1 alone had no effect on the binding of NAB1 to the mPGES-1 promoter. However, the combined treatment of IL-1 and VA induced the recruitment of NAB1 to the mPGES-1 promoter. We did not detect obvious recruitment

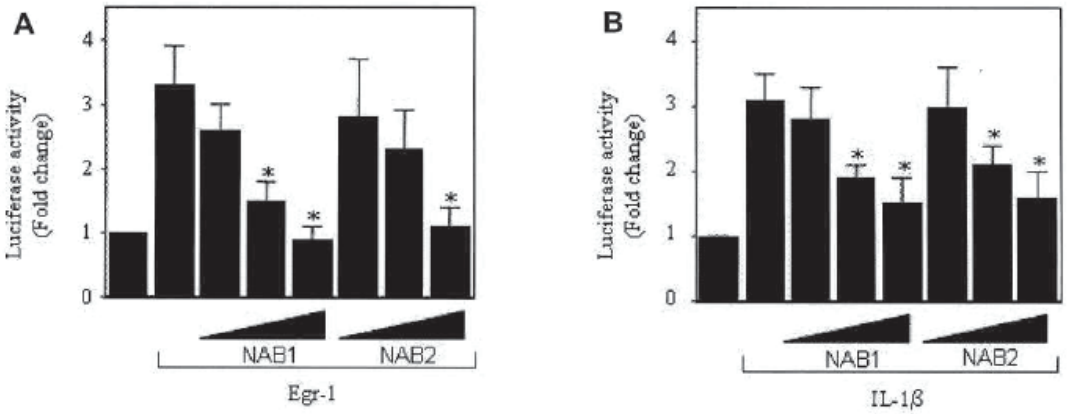

Figure 5. NAB1 and NAB2 suppress Egr-1 and interleukin 1 (IL-1)-mediated activation of the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter. A. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well), the internal control pSV40-ß-gal (0.5 μg/well), an expression vector for Egr-1 (100 ng/ml), and increasing concentrations (0.1, 0.5, and 1 μg/ml) of expression vectors for NAB1 (A) or NAB2 (B). The total amount of transfected DNA was kept constant by addition of the empty vector. Total cellular extracts were prepared 40 hours after transfection, and luciferase activity values were determined and normalized to ß-gal activity. Results are expressed as fold-changes, considering 1 as the value of cells transfected with the reporter construct alone, and represent mean ± SD of 4 independent experiments [*p < 0.05 compared with cells transfected with Egr-1 alone (control)]. B. Chondrocytes were cotransfected with the human mPGES-1 promoter (1 μg/well) and the internal control pSV40-ß-gal (0.5 μg/well) together with increasing concentrations of an expression vector for NAB1 or NAB2. The total amount of transfected DNA was kept constant by addition of the empty vector. The next day, transfected cells were treated with IL-1 (100 pg/ml) for 18 hours. Luciferase activity values were determined and normalized to ß-galactosidase activity. Results are expressed as fold-changes, considering 1 as the value of untreated cells, and represent mean ± SD of 4 independent experiments [*p < 0.05 compared with cells treated with IL-1 alone (control)].

of NAB2 to the mPGES-1 promoter. Thus, VA induced NAB1 recruitment to the mPGES-1 promoter in the presence of IL-1, suggesting that NAB1 may contribute to the suppressive effect of VA on mPGES-1 expression.

NAB1 silencing with small interfering RNA (siRNA) blocked VA-mediated suppression of IL-1-induced mPGES-1 expression. To confirm the involvement of NAB1 in the observed effect of VA, we evaluated the effect of NAB1 silencing by siRNA on VA-mediated suppression of IL-1-induced mPGES-1 expression. Chondrocytes were transfected with the scrambled control siRNA or siRNA for NAB1 and after 24 hours of transfection, the cells were stimulated with IL-1 in the absence or presence of VA. As shown in Figure 6C, transfection with NAB1 siRNA antagonized the suppressive effect of VA on IL-1-induced mPGES-1 expression, whereas transfection with scrambled control siRNA had no effect. NAB1 protein levels were almost completely suppressed in chondrocytes transfected with NAB1 siRNA compared to cells transfected with scrambled siRNA, confirming NAB1 gene silencing (Figure 6, lower panel).

These results support the notion that upregulation of NAB1 contributes to the suppression of IL-1-induced mPGES-1 expression by VA.

DISCUSSION

In this study, we demonstrate that treatment of human chondrocytes with VA, an HDAC inhibitor, suppressed IL-1-induced mPGES-1 expression at the transcriptional level. The inhibitory effect of VA was not associated with reduced expression of Egr-1 or its recruitment to the mPGES-1 promoter, and requires *de novo* protein synthesis. In addition, we identify NAB1 as an essential factor that mediates the suppressive effect of VA. To our knowledge, this is the first study to demonstrate that VA suppresses IL-1-induced mPGES-1 expression by upregulating the expression of NAB1.

Recently, a number of studies have demonstrated that HDAC inhibitors modulate inflammatory responses. For instance, HDAC inhibitors including VA reduced lipopolysaccharide-induced production of IL-1, TNF-α, and interferon-γ (IFN-γ) in human peripheral blood mononuclear cells[29], and production of TNF-α, IL-6, and reactive oxygen species in neuroglia cultures and primary microglia[30,31]. HDAC inhibitors have also been reported to suppress IL-12 production in dendritic cells and macrophages[32]. Further, we and others have reported that they downregulate inducible nitric oxide synthase and COX-2 expression in several cell types, including chondrocytes[33]. *In vivo*, HDAC inhibitors dose-dependently reduced the circulating levels of the proinflammatory cytokines TNF-α, IL-1, and IL-6 in an endotoxemia model[34]. In addition to their antiinflammatory properties, HDAC inhibitors exhibit chondroprotective effects. Indeed, Young, *et al*[35] showed that HDAC inhibitors blocked the induction of several enzymes responsible for cartilage degradation, includ-

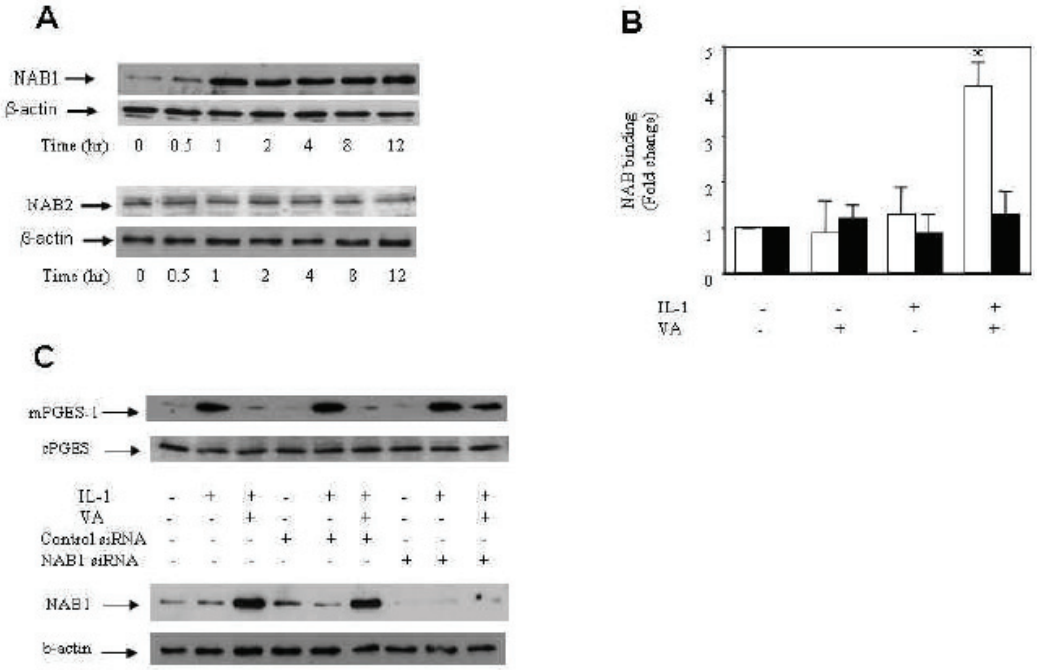

Figure 6. NAB1 contributes to the suppression of interleukin 1 (IL-1)-induced microsomal prostaglandin E_2 synthase-1 (mPGES-1) by valproic acid (VA) A. VA upregulates NAB1 expression in human chondrocytes. Chondrocytes were treated with 10 mM VA for the indicated time periods. Cell lysates were prepared and analyzed for NAB1, NAB2, and ß-actin proteins by Western blotting. The blots are representative of similar results obtained from 4 independent experiments. B. VA promotes NAB1 recruitment to the microsomal prostaglandin E_2 synthase-1 (mPGES-1) promoter. Chondrocytes were treated with 10 mM VA for the indicated time periods. ChIP assays, coupled with real-time PCR, were performed using specific antibodies against NAB1 and NAB2. The results are expressed as fold-changes of NAB protein binding to the mPGES-1 promoter relative to untreated cells and represent the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions (*$p < 0.05$ compared with untreated cells). C. NAB1 is required for suppression of IL-1-induced mPGES-1 expression by VA. Chondrocytes were transfected with 200 nM of scrambled control siRNA or siRNA for NAB1. At 24 hours post-transfection, cells were washed and left untreated or treated with IL-1 in the absence or presence of VA (10 mM) for 20 hours. Cell lysates were prepared and analyzed for the expression level of mPGES-1 and cPGES (upper panel). Specific knockdown of NAB1 was confirmed by Western blotting using antibodies specific for NAB1 (lower panel). Blots are representative of similar results obtained from 4 independent experiments.

ing MMP-1, MMP-13, and a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS)-4, -5 and -9, and prevented cartilage degradation in an explant assay[35]. Moreover, we recently showed that HDAC inhibitors downregulate IL-1-induced proteoglycan degradation in cartilage explants[33]. More recently, Grabiec, *et al*[36] reported that HDAC inhibitors suppressed the production of TNF-α and IL-6 by macrophages from patients with RA[36]. Together these data suggest that HDAC inhibitors may have protective effects in arthritis. Indeed, they inhibit joint swelling, synovial inflammation, and bone and cartilage destruction in autoantibody-mediated arthritis[43] and collagen-induced arthritis models[44,45]. We have extended these observations by showing that VA inhibits IL-1-induced mPGES-1 expression in human chondrocytes. Moreover, the induction of mPGES-1 expression by TNF and IL-17 was also inhibited by VA, suggesting that VA could repress mPGES-1 expression independently of the stimulus. The repressive effect of VA on IL-1-induced mPGES-1 expression occured at the transcriptional level, as determined by real-time RT-PCR analysis and transient transfection assays.

The transcriptional induction of mPGES-1 is primarily controlled by Egr-1 through 2 Egr-1 binding motifs located in the proximal region of the mPGES-1 promoter[18,19]. Therefore, we examined whether inhibition of Egr-1 expression and/or recruitment to the mPGES-1 promoter could be the mechanism by which VA prevents IL-1-induced mPGES-1 expression. Our results demonstrated that VA did not affect IL-1-induced Egr-1 expression and recruitment to the mPGES-1 promoter, indicating that VA acts at a step downstream of Egr-1 expression and recruitment to the mPGES-1 promoter. Indeed, VA inhibits both Egr-1 and IL-1-mediated activation of the mPGES-1 promoter.

We also demonstrated that inhibition of *de novo* protein synthesis blocked VA-mediated suppression of IL-1-induced mPGES-1 expression, suggesting that the sup

pressive effect of VA is not direct, but rather indirect, through transcription of a target gene that suppresses mPGES-1 expression. Potential candidates that may mediate the suppressive effect of VA are the corepressors of Egr-1 activity, namely NAB1 and NAB2, known to suppress the expression of Egr-1-dependent genes[20,21]. We found that treatment with VA caused a rapid upregulation of NAB1 expression, whereas the level of NAB2 was not affected, suggesting that the upregulation of NAB1 contributes to the suppressive effect of VA on IL-1-induced mPGES-1 expression. Interestingly, NAB1 expression was reported to be upregulated by 2 antiinflammatory agents: glucocorticoids and aspirin-triggered lipoxin analog[46,47].

Further, we found using ChIP assays that NAB1 is recruited to the −142/−37 region (which contains 2 Egr-1 binding sites) of the mPGES-1 promoter when the cells are stimulated with the combination of IL-1 and VA, but not with IL-1 or VA alone. This suggests that NAB1 is recruited to the mPGES-1 promoter by Egr-1. Indeed, it has been shown that Egr-1 and NAB1 can directly interact *in vitro* and *in vivo* and that the association of Egr-1 with NAB1 is involved in the repression of several Egr-1-dependent genes[41,42]. Altogether, these results strongly suggest that upregulation of NAB1 expression and its recruitment to the mPGES-1 promoter mediates the suppressive effect of VA on IL-1-induced mPGES-1 expression. This is further supported by the fact that silencing of NAB1 by specific siRNA blocked the downregulation of IL-1-induced mPGES-1 expression by trichostatin A. This study is the first to our knowledge to show that NAB1 expression is upregulated by VA, and that NAB1 is important for the suppressive effect of VA on IL-1-induced mPGES-1 expression.

It should be noted that siRNA-mediated silencing of NAB1 did not completely block the suppressive effect of VA on IL-1-induced mPGES-1 expression, suggesting that additional NAB1-independent mechanisms also contribute to the suppressive effect of VA. Indeed, we recently showed that HDAC4 contributes to mPGES-1 expression in synovial fibroblasts, and that trichostatin A suppresses IL-1-induced mPGES-1 expression by interfering with this enzyme's activity[40]. Modulation of Egr-1 activity or expression by HDAC inhibitors is not mutually exclusive; both may operate concomitantly. Further investigation will be needed to elucidate whether VA modulates mPGES-1 expression in chondrocytes by targeting the activity of HDAC4 or other isoforms.

There are a number of potential mechanisms by which VA could increase NAB1 expression. One possibility is that VA activates NAB1 transcription through inhibition of HDAC activity and subsequent histone hyperacetylation at the NAB1 promoter. Indeed, several studies reported that VA can activate transcription by enhancing histone acetylation at target gene promoters[48,49,50]. Alternatively, VA may induce NAB1 expression through hyperacetylation of transcription factor or signaling molecules involved in NAB1 expression. Of note, HDAC inhibitors including VA have been shown to modulate the expression of a number of genes by increasing the acetylation levels of key transcription factors[51,52]. Finally, VA can upregulate NAB1 expression through NAB1 mRNA stabilization. Additional molecular and biochemical studies are needed to delineate the mechanisms by which VA modulates NAB1 expression.

Our data show that suppression of IL-1-induced mPGES-1 expression by VA was not due to altered expression or recruitment of Egr-1 to the mPGES-1 promoter, but instead, was likely due to the repression of Egr-1 transcriptional activity through upregulation of NAB1 expression.

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