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# Regulation of cyclooxygenase-2 (COX-2 ) gene expression in normal human chondrocytes

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## SUMMARY

Cellular activation by proinflammatory stimuli results in, among other responses, increased prostaglandin synthesis which may be important in the etiopathogenesis of many immune and inflammatory diseases. Acting locally in a paracrine or autocrine fashion, prostaglandin E2 ($PGE_2$) can initiate and modulate cell and tissue responses involved in many physiological processes affecting essentially all organ systems. The rate-limiting step in the formation of prostanoids is the conversion of phospholipid derived arachidonic acid to $PGH_2$ (which is rapidly converted to $PGE_2$) by cyclooxygenase (COX). Two forms of COX have been identified: a constitutive cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2). They are integral, monotopic, endoplasmic-reticulum associated, homodimeric enzymes that possess heme-dependent perroxidase and cyclooxygenase activity.

While studying the role of protein kinase C-α (PKC-α) (major conventional isoform in chondrocytes) in the control of COX-2 gene expression in human chondrocytes (Miller et al., 1998), we observed that calphostin C (CalC), an inhibitor of PKC mimicked the stimulatory effects of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). When co-incubated, CalC and PMA produced additive effects in terms of COX-2 expression in human, phenotypically stable chondrocytes.

Calphostin C induces Activating protein 1 (AP-1), as judged by gel-shift analysis using a consensus of AP-1 oligonucleotide. Maximum induction of AP-1 was reached at a concentration of 250nmol/L of CalC. The major up-regulation phase was at 24 hours. In the presence of specific antibodies to c-Jun, JunB, JunD, c-Fos, and CREB/ATF, it was revealed that AP-1 complexes were probably c-Fos/c-Jun, c-

Fos/JunB, or c-Jun/JunB dimers. We performed transient transfection of c-jun promoter-chloramphenicol acetyltransferase (CAT) constructs harboring either wild-type (WT) AP-1 or mutant AP-1 sites, for testing the role of the AP-1 site present in the c-jun promoter in the induction of c-jun by CalC. It is shown that CalC induced a marked increase in CAT activity with WT AP-1 c-jun promoter CAT plasmid, but the response of mutant AP-1 was completely abrogated.

We demonstrated for the first time that COX-2 mRNA expression and COX-2 protein synthesis by human chondrocytes treated with CalC were stimulated in a complex kinetic pattern with an initial phase reaching a zenith at 8 hours, followed by a rapid decline with a nadir at 16 hours, and finally another rapid inductive phase with a maximum at 24 hours. The increase in COX-2 mRNA expression was accompanied by the induction of proto-oncogenes c-jun, junB, and c-fos (not junD or Fra-1). CalC suppressed the activity of the MAP kinase, ERK1/2 in a time-dependent fashion, suggesting that the Raf-1/MEKK1/MEK1/ERK1,2 cascade was compromised by CalC treatment. By contrast, CalC caused a dramatic increase in SAPK/JNK expression and activity, indicative of an activation of MEKK1/JNKK/SAPK/JNK pathway.

We conclude that in primary phenotypically stable human chondrocytes, COX-2 gene expression may be up-regulated by CalC, the inhibitor of PKC, through the stimulation of JNK/SAPK kinase activity. With the demonstration of CalC-induced inhibition of MAPK pathway, our results added to a growing list of signaling cascades affected by CalC that are PKC-independent. Calphostin C, can indeed be a useful chemical tool in certain situations and it has allowed us to better understand how COX-2, the target of virtually all nosteroital antiinflammatory drugs (NSAIDs), is regulated in primary nontransformed cell type.

**Résumé**

L' activation cellulaire par des stimuli pro-inflammatoires se traduit, entre autre, par une augmentation de la synthèse des prostaglandines. Celles-ci sont importantes dans l' étiopathogénèse de plusieurs maladies immunes ou inflammatoires. Agissant localement de façon autocrine ou paracrine, la $PGE_2$ peut initier et moduler des réponses cellulaires ou tissulaires impliquées dans plusieurs processus affectant pratiquement tous les systèmes organiques. La formation des prostanoïdes dépend essentiellement de la conversion de l' acide arachidonique en $PGH_2$ (rapidement métabolisé en $PGE_2$) par la COX. Deux formes de COX ont été identifiées: une première (COX-1), constitutive et une seconde (COX-2), inductible. On les retrouve de façon intégrale, monotopique sous forme d' homodimères, associées au réticulum endoplasmique et qui possèdent une activité peroxydase hème-dépendante ainsi qu'une activité COX.

Alors que nous étudions le rôle de PKC-α (principal isoforme de PKC chez les chondrocytes) sur le contrôle de l' expression génétique de la COX-2 (Miller et al., 1998), nous avons observé que la CalC imitait les effets stimulateurs PMA. Lorsque co-incubés, la CalC et le PMA avaient un effet additif sur l' expression de COX-2 chez les chondrocytes humain phénotypiquement stables.

D' après nos études de migration à retardement sur gel (' gel-shift' ), la CalC induirait l' expression du gène AP-1. Cette induction serait maximale après 24 heures et à une concentration de 250 nmol/L de CalC. L' utilisation d' anticorps spécifiques nous a permis de révéler que les complexes protéiques se liant au site AP-1 seraient probablement les dimères c-Fos/c-Jun, c-Fos/JunB ou c-Jun/JunB. Afin de tester le rôle du site AP-1, présent dans le promoteur du gène c-jun, ainsi que l' induction de c-jun par la CalC, nous avons effectué des transfections transitoires avec des constructions du promoteur de c-jun arborant un site AP-1 muté ou non, lié au gène reporteur CAT. Les résultats ont démontré que la CalC induisait une augmentation marquée de l' activité de CAT avec le plasmide contenant le site AP-1 de type sauvage, alors que, dans le cas du plasmide muté, la réponse était complètement inhibée.

Nous démontrons pour la première fois que lorsque les chondrocytes humains sont traités à la CalC, l' expression en ARNm de COX-2 ainsi que sa synthèse en protéines suivent un même patron cinétique. D' abord, on remarque une phase initiale atteignant son zénith à 8 heures, suivie par un déclin rapide avec le nadir à 16 heures, puis une autre phase rapide d' induction avec un maximum à 24 heures. L' augmentation de l' expression de l' ARNm de COX-2 était accompagnée de l' induction des proto-oncogènes c-jun, junB et c-fos (mais pas junD ni Fra-1). La Calphostine C supprime l' activité de la MAP kinase ERK1/2 de façon temporelle,

suggérant que la cascade Raf-1/MEKK1/MEKK1/MEK1/ERK1,2 serait compromise par le traitement de la CalC. Par opposition, la CalC provoque une augmentation drastique de l' expression et de l' activité de SAP/JNK, indiquant une activation de la voie MEKK1/JNKK/SAPK/JNK.

En conclusion, l' expression du gène COX-2 chez les chondrocytes humains phénotypiquement stables serait régulée par la CalC (un inhibiteur de PKC). Cette régulation se ferait via la stimulation de l' acvtivité kinasique de JNK/SAPK. En démontrant que la CalC inhibe la voie de MAPK, nos résultats viennent s' ajouter à une liste croissante de cascades de signalisation PKC-indépendantes. En fait, la CalC peut s' avérer un outil chimique trés utile dans certaines situations. En ce qui nous concerne, la CalC nous a permis de mieux comprendre comment la COX-2, cible de presque tous les anti-inflammatoires non-stéroïdiens, était régulée chez les chondrocytes humains en culture primaire.

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Article One

Article Two

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AP-1 | Activating protein 1 |
| AP-2 | Activating protein 2 |
| ATF | Activating transcription factor |
| bZIP | Basic region leucine zipper |
| CalC | Calphostin C |
| CAT | Chloramphenicol acetyltransferase |
| CBP | CREB binding protein |
| c/EBP | CCAAT enhancer binding protein |
| COX | Cyclooxygenase |
| COX-1 | Cyclooxygenase of type 1 |
| COX-2 | Cyclooxygenase of type 2 |
| cPLA$_2$ | Cytoplasmic phospholipase A$_2$ |
| CRE | CAMP-responsive element |
| CREB | CRE-binding protein |
| CS | Chondroitin sulfate |
| ECM | Extracellular matrix |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal regulated kinase |
| FN | Fibronectin |
| FRK | Fos-regulated kinase |

| | |
|---|---|
| GAG | Glycosaminoglycan |
| HA | Hyaluronic acid |
| IFN | Interferon |
| IGF | Insulin-like growth factor |
| IGFBP6 | Insulin-like growth factor binding protein 6 |
| IKK | IκB kinase |
| IL-1 | Interleukin-1 |
| JAK | Janus kinase |
| JNK | Jun N-terminal kinase |
| JUK-SAPK | NH-Terminal c-jun kinase-Stress activated protein kinase |
| kb | Kilobase |
| kDa | Kilodalton |
| KS | Keratan sulfate |
| LP | Link glycoprotein |
| MAPK | Mitogen-activated protein kinase |
| MAPKK | MAP kinase kinase |
| MAPKKK | MAP kinase kinase kinase |
| MEK | MAPK/ERK kinase |
| MEKK | MEK kinase |
| MMPs | Matrix metalloproteinases |
| MMP-2 | 72kDa gelatinase A |
| MMP-3 | Stromelysin 1 |
| MMP-9 | 92 kDa gelatinase B |
| MMP-10 | Stromelysin 2 |
| NF-κB | Nuclear factor kappa B |

| | |
|---|---|
| NIF | NF-κB-inducing kinase |
| NF-IL6 | Nuclear factor for interleukin-6 expression |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| OA | Osteoarthritis |
| OKA | Okadaic acid |
| PA | Plasminogen activator |
| PAI | Plasminogen activator inhibitor |
| PGHs | Prostaglandin H synthase |
| $PGE_2$ | Prostaglandin $E_2$ |
| PG | Proteoglycan |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| $PLA_2$ | Phospholipase $A_2$ |
| PMA | Phorbol 12-myristate 13-acetate |
| PMN | Polymorphonuclear leukocyte |
| PP1 | Phosphatase-1 |
| PP-2A | Phosphatase-2A |
| PSTK | Serine/threonine directed kinase |
| PSTP | Serine/threonine directed phosphatase |
| PTK | Protein tyrosine kinase |
| RA | Rheumatoid arthritis |
| SAPK | Stress-activated protein kinase |
| SP1 | Signaling protein-1 |
| SRE | Serum response element |
| SRF | Serum response factor |

| | |
|---|---|
| STAT | Signal transducers and activators of transcription |
| TCF | Ternary complex factor |
| TGF-β | Transforming growth factor-β |
| TIMPs | Tissue inhibitor of metalloproteinases |
| TMJ | Temporomandibular joint |
| TNF-α | Tumor necrosis factor-α |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| 3'-UTR | 3'-untranslated region |
| UV | Ultraviolet |

*TO MY MOTHER AND MY FATHER*

WAN BI AND GANSHENG ZHANG

*TO MY HUSBAND AND MY SON*

KELI SONG AND KEQING SONG

# CHAPTER ONE : INTRODUCTION

## 1.1 NORMAL CARTILAGE STRUCTURE, BIOCHEMISTRY, AND METABOLISM:

Articular cartilage is a very distinctive tissue containing chondrocytes embedded in an abundant extracellular matrix (ECM). The chondrocytes produce collagen, proteoglycans, and other components of the ECM. Cartilage is aneural, avascular, and alymphatic. Because cartilage is aneural, pain perception and proprioception in synovial joints are dependent on nerve endings in the synovium, capsule, muscles, and subchondral bone (Mankin and Brandt, 1989). Because cartilage is avascular and alymphatic, nutrition take-up and elimination of waste products are dependent on diffusion through the cartilage matrix to and from the synovial fluid (Fassbenger, 1987).The avascularity of cartilage is possibly maintained by endothelial cell growth inhibitors and protease inhibitors (Hamerman, 1989).

### 1.1.1 Extracellular matrix

The biochemical composition of the ECM varies considerably from individual to individual, from site to site, with depth, and with time, because the extracellular matrix is a dynamic system that is continuously exposed to anabolic and catabolic factors (Mankin and Radin, 1989). Moreover, the biochemical composition of the ECM varies per joint. The TMJ articular cartilage consists of fibrocartilage, whereas the articular cartilage in most other synovial joints consists of hyaline cartilage. The ECM of both hyaline and fibrocartilage consists of water, collagen, proteoglycans, structural glycoproteins, and small amounts of lipid and inorganic components. Water constitutes 60% to 80% of the total weight of hyaline cartilage (Mankin and Radin, 1989).

1.1.1.1.Collagens

Collagen accounts for the major proportion of the dry weight of articular cartilage (50%-90%) (Muir, 1995) and also constitutes over 60% of the dry weight of TMJ fibrocartilage (de Bont, 1985). The collagen fibrils are organized in sheets and bundles, creating a network (de Bont *et al.*, 1984). This collagen network is in general kept together by its basketweave, cross-links, and anchoring proteins, such as chondronectin and fibronectins (Howell *et al.*, 1992). The collagen network provides cartilage with its tensile strength and shape, and counteracts the swelling pressure of the highly hydrophilic proteoglycan (Maroudas, 1979). Collagen in articular cartilage is normally very long-lived (Maroudas *et al.*, 1992) and as a consequence undergoes gradual changes and decline in tensile strength with age (Kempson, 1991).

Type II collagens are extended extracellular proteins composed of three polypeptide chains (α-chains), each possessing a characteristic tripeptide sequence (gly-x-y) that forms a left-handed helix (Muir, 1995). Three α-chains in each molecule are twisted tightly into a right-handed helix to form a rope-like structure that is stabilized by hydrogen bonds, while peptide bonds are buried inside the helix. Glycine, placed at every third residue of the tripeptide sequence, is small enough to occupy the crowded interior of the helix, while frequently other amino acids are proline and hydroxyproline. Collagen precusors or procollagens are synthesised with large C- and N-terminal extensions which, among other functions, are involved in chain assembly necessary for triple helix formation. These extension pro-peptides are cleaved by specific procollagen peptidase after secretion but prior to fibril formation. Collagen fibrils are futher stabilized by cross links that involve lysine residues. Fibrillar collagen, which is the biologically functional form, results from a series of post translational

modifications, both intra-and extracellularly, that require a number of specific and non-specific enzymes (Muir, 1995).

Fibrocartilage predominantly contains type I collagen, or a combination of type I and type II collagen (Seyer and Kang, 1989). Type I and Type II collagen molecules are rather similar in structure, and pyridinoline cross links between fibrils are formed by both types of collagen. Their fibril morphology is somewhat different. In cartilage the fibrils formed from type II collagen are thinner than those from type I collagen. The free energy change is lower and fibril formation is slower than for type I collagen. An important difference is that type II collagen forms cross links with type IX collagen, a minor collagen also specific to cartilage (Muir, 1995). Type II collagen is the predominant collagen in articular cartilage and comprises up to 90% of the total collagen present. It is characterized by its ability to form a network of fibrils that entrap proteoglycan aggregates and appear as thin firils in the electron microscope. It is believed to be responsible for the high tensile strength characteristic of cartilage (Dean, 1991). Type IX collagen, is present at 10% of the total pool of collagens in cartilage. The molecule contains three collagenous and four noncollagenous domains derived from three genetically distinct chains (Dean, 1991). It is believed that type IX collagen function is diffusely distributed throughout the ECM, and also involved in the cross-linking of especially type II collagen fibrils to each other and to other ECM components (Dean, 1991). Type XI collagen, is present in very low amounts in cartilage and is structurally closely related to typeV collagen. Whereas type XI is found associated with type II collagen, type V is found with type I. Type XI collagen is distributed around the chondrocytes, and is involved in the organization of the fibrous components of the ECM and the exocytoskeleton of chondrocytes (Seyer and Kang, 1989; Dean, 1991) .

1.1.1.2 .Proteoglycans (PGs)

Proteoglycans constitute 20% to 40% of the dry weight of hyaline cartilage (Mankin and Brandt, 1989). PGs are complex macromolecules, consisting of a core protein with many glycosaminoglycan (GAG) side-chains of varying composition and chain length, linked with hyaluronic acid by link protein (Leonore *et al.*, 1995). In hyaline cartilage the GAG chains consist of 90% chondroitin 6-sulphate and keratan sulphate, and less than 5% of chondroitin 4-sulphate.

PGs are in general intertwined throughout the collagen network and are not only mechanically but also chemically entangled within this network (de Bont. 1985). In this way the proteoglycans "mask" the collagen fibrils. PGs are highly hydrophilic macromolecules with a high water-binding capacity. They are only constrained from full expansion by the tension of the collagen network (Maroudas, 1979). Loading of cartilage results in an increase of the internal hydrostatic pressure. When the hydrostatic pressure exceeds the osmotic pressure of the cartilage, water is squeezed out of the ECM, contributing to the lubrication of the joint surfaces. This so-called "weeping" lubrication is especially functional under high loads (Mankin and Brandt, 1989). The PGs in conjunction with the collagen network provide the cartilage with its resilience, elasticity, shear strength, and self-lubrication. However, PGs can also function as integral membrane receptors (Leonore *et al.*, 1995).

1.1.1.3 .Noncollagenous proteins

The structural glycoproteins, the noncollagen nonproteoglycan matrix glycoproteins, constitute 5% to 15% of the dry weight of hyaline cartilage (Mankin and Brandt, 1989). Structural glycoproteins in general interact with cellular receptors,

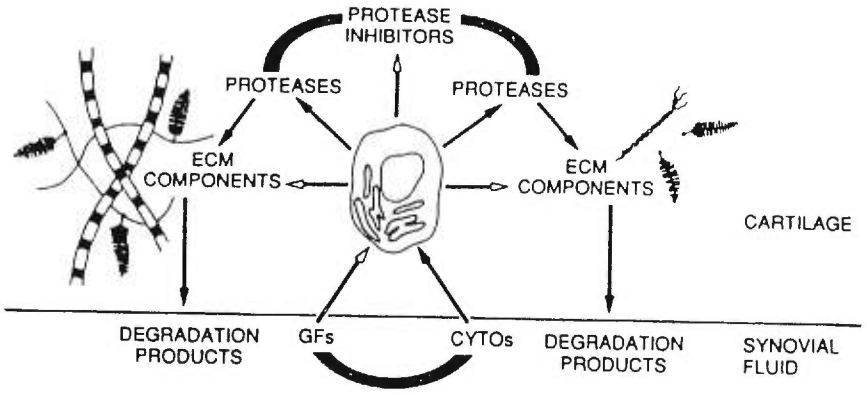
mainly of the integrin family, and regulate adhesion, migration, proliferation, and differentiation of the chondrocytes (Trelstas, 1989). The two main structural glycoproteins are fibronectin (FN) and laminin. FN is a large, adhesive glycoprotein that aggregates near the chondrocyte cell membrane and in the ECM. FN is also found in serum, where it functions as an opsonin and chemoattractant. FN is a polyvalent molecule with particular binding affinities for fibrin, collagen, heparin, components in bacterial cell coat, and cells (Trelstad, 1989). The binding of FN to cells is mediated by the integrins. Laminin is a polyvalent molecule prominent in basement membranes and as receptor-bound component of the cell surface.

Lipids and inorganic components constitute only a very small part of the dry weight of both hyaline and fibrocartilage.

### 1.1.2. Chondrocytes

The chondrocytes occupy about 5% of the cartilage volume, lack physical cell-to-cell contact (Dean, 1991). Each chondrocyte is surrounded by a territorial matrix containing abundant ground substance but only a few collagen fibrils (Fassbenger, 1987). Chondrocytes in general have relatively low metabolic activity. Therefore they are able to function under almost anaerobic conditions, but they are sensitive to toxic influences and unable to regenerate after major injury. However, chondrocytes do have some recuperative abilities (Fassbenger, 1987). Joint loading stimulates diffusion of chondrocyte nutrients and waste products through the cartilage matrix and is therefore essential for chondrocyte nutrition. Joint immobilization impairs matrix diffusion to an extent that chondrocyte nutrition may stop (Howell, 1989; Leonore *et al.*, 1995).

**Figure 1.1** Internal remodeling system of normal articular cartilage by the chondrocytes. Chondrocytes synthesize all ECM components, as well as degrade these components through production of precisely regulated amounts of proteases and protease inhibitors. Proteases not only degrade resident ECM components (*left*), but also newly synthesized ECM components (*right*). In normal cartilage homeostasis, anabolic (open arrows) and catabolic (arrows) processes are equated by a balance (beam) between proteases and protease inhibitors. Chondrocytes probably maintain this balance by equal amounts (beam) of growth factors and cytokines (Leonore *et al.*, 1995).


PROTEASE INHIBITORS
PROTEASES
PROTEASES
ECM COMPONENTS
ECM COMPONENTS
CARTILAGE
DEGRADATION PRODUCTS
GFs
CYTOs
DEGRADATION PRODUCTS
SYNOVIAL FLUID

Chondrocytes have the capacity to synthesize as well as to degrade all components of the ECM. Although once thought of as an inert tissue, articular cartilage is now recognized to be a dynamic system that is capable of remodeling under functional demands, and turnover of ECM components. The half-life of PGs, for example, varies from 1 week to 200 days (Fassbenger, 1987). This points to the existence of a presumably enzymatically mediated "internal remodeling system" by the chondrocytes. Stimulated by balanced levels of cytokines and growth factors, chondrocytes produce precisely regulated amounts of protease and protease inhibitors to induce normal turnover of ECM components.

Besides the synthesis and degradation of all ECM components, chondrocytes are capable of control over the location of ECM components adjacent to the chondrocytes through specific receptors in their cell membrane (Trelstad, 1989). These receptors effectively bind ECM components such as collagen and FN. Subsequently, these bound polyvalent macromolecules bind other ligands, thereby extending the linkage between the cell membrane and the pericellular matrix. The intracellular portions of the receptors interact with cytoplasmic structures, thereby providing a direct linkage, physically and functionally, with intracellular events (Trelstad, 1989). The cell membrane receptors binding ECM components are of the integrin family, or are integral membrane proteins. The integrins are a family of ubiquitous cell-surface ECM adhesion receptors that interact with a specific sequence of three amino acids, arginine-glycine-asparagine, with proteins (Woods *et al.*, 1994). Integrins bind many ECM components, including FN (Gardner, 1992; Anderson, 1992). Moreover, integrins mediate many cellular processes, including tissue morphogenesis, homeostasis, and repair (Woods *et al.*, 1994). Integral membrane proteins are receptors, consisting of ECM components that are intercalated with the cell membrane, such as syndecan and

heparan sulfate proteoglycan. (Trelstad, 1989). The extracellular domain of these PGs contains glycosaminoglycan chains capable of binding other ligands including ECM components and growth factors (Leonore *et al.*, 1995).

In conclusion, chondrocytes in general are multi functional cells, capable of synthesis and degradation of all ECM components and, to a certain extent, control over the location of these components in the ECM.

## 1.2 OSTEOARTHRITIS:

Osteoarthritis (OA) is a common disease of joints with complex etiology. This disease affects millions of people in North America and around the world. OA results from an imbalance between predominantly chondrocyte-controlled anabolic and catabolic processes, and is characterized by progressive degradation of the components of the ECM of the articular cartilage, associated with secondary inflammatory factors (Dijkgraaf *et al.*, 1995; Mankin and Brandt, 1992). The clinical features are the gradual development of joint pain, stiffness, and limitation of motion. The disease is classified as primary or idiopathic when it occurs in the absence of any known underlying predisposing factor. In contrast, secondary OA is that form of disease which follows an identifiable underlying local or systemic pathogenic factor (Moskowitz, 1993). OA results in loss of articular cartilage structure and function accompanied by attempted cartilage repair and bone remodeling. Currently, there is no well tolerated or successful treatment for OA; patients use analgesics, NSAIDs, physiotherapy and for some, eventually surgery for joint replacement. Because NSAIDs are the most commonly used drugs, they frequently cause side effects such as gastrointestinal bleeding and renal dysfunction. These complications are thought to be mediated by inhibition of

tissue-protective cyclooxygenase of type 1 (COX-1). Most commonly used NSAIDs are more active against COX-1 activity than against cyclooxygenase of type 2 (COX-2) (Meade *et al.*, 1993). One focus of recent research has been to develop a new generation of NSAIDs that are specific COX-2 inhibitors. Those inhibitors will help further delineate the role of COX-2 products in inflammatory diseases.

### 1.2.1 Etiopathogenesis of OA

Three main concepts concerning the etiopathogenesis of OA have been postulated, based on cartilaginous or extracartilaginous factors (Dean, 1991). The first concept is a mechanical approach that implies overloading (whether absolute or relative) or under loading of the cartilage. Absolute and relative overloading, as well as under loading, i.e. loading outside the individual physiologic range, may result in biomaterial failure of the ECM and/or chondrocyte injury (Bullough, 1992; Howell, 1989). Biomaterial failure, especially of the collagen network, due to failure of collagen fibrils per se or failure of anchoring proteins, will lead to increased water binding by proteoglycans and subsequent loss of proteoglycans. Chondrocyte injury will lead to an increase of degradative responses, with release of proteases, an increase of protease activators, and a decrease of protease inhibitors (Howell, 1989). The second concept implies failure of the chondrocyte-controlled internal remodeling system. According to this concept a primary insult, whether (bio)mechanical, biochemical, inflammatory, or immunologic in character, will disturb the balance between synthesis and degradation of ECM components by the chondrocyte. The chondrocyte is instigated to release increased amounts of proteolytic enzymes and probably decreased amounts of protease inhibitors, resulting in accelerated ECM breakdown (Howell, 1989; Moskowitz, 1992). The first two concepts are based on cartilaginous factors. The

third concept is based on extracartilaginous factors, and actually is a compilation of factors that alone or together may lead to cartilage degradation. These factors include reduction in the quality and/or quantity of synovial fluid, changes in the synovial membrane, subchondral bone microfractures, and vascular changes. Several other factors also have been proposed to play a role in the initiation, perpetuation, or pathogenesis of OA (Mankin and Brandt, 1989). These include age, alterations in the structure of the ECM, in the chondrocytic metabolic activity, or in its regulators; and possibly hypermobility of the affected joint (Mankin and Brandt, 1989; Hough and Sokoloff, 1989).

### 1.2.2 Cartilage metabolism of OA

Proteases or proteolytic enzymes play an important role both in maintaining normal tissue turnover and in degradation of ECM components of articular cartilage in the osteoarthritic process. They are capable of cleaving internal peptide bonds of proteins (Werb, 1989). Proteases may be found intracellularly, in lysosomes, or extracellularly. Four classes of proteases have been identified: 1) aspartic proteases ( including cathepsin D), 2) cysteine proteases (including cathepsin B and cathepsin L), 3) serine proteases (including plasminogen activator (PA), polymorphonuclear leukocyte (PMN) elastase, and cathepsin G), and 4) metalloproteases (including collagenase or matrix metalloprotease-1 [MMP-1], gelatinase A (72kDa type IV collagenase, or MMP-2, collagenase-3 [MMP-13] and stromelysin-1 or MMP-3). The aspartic and cysteine proteases are lysosomal enzymes and are most active at acid pH, whereas the serine and metalloproteases are most active at neutral pH (Werb, 1989). Proteases can be synthesized by chondrocytes, synovial cells, and inflammatory cells. The synthesis of proteases is mediated by a variety of cytokines, growth factors, and

hormones. All proteases are synthesized in inactive preforms that later require activation (Tyler and Radzio-Andzelm, 1992). The activated proteases can be inhibited by specific protease inhibitors, presumably synthesized by the chondrocytes (Howell *et al.*, 1992). Normal articular cartilage contains large amounts of protease inhibitors, including tissue inhibitor of metalloproteases (TIMPs), ex., TIMP-1, TIMP-2 and TIMP-3. They bind with high affinity and specificity to active and, in some cases, unactivated, zymogenic MMPs (ex. TIMP-2/proMMP-2 complex or TIMP-1/proMMP-9complex). An imbalance between protease and protease inhibitor levels has been postulated as a possible pathogenic pathway of OA (Dean *et al.*, 1989).

### 1.2.3 Cytokines and growth factors in OA

Cytokines and growth factors are soluble polypeptides capable of regulating growth, differentiation, and metabolic activity of cells (Howell *et al.*, 1992; Trowbridge and Emling, 1989). Generally, in articular cartilage cytokines (e.g. IL-1, tumor necrosis factor [TNF], and interferon [IFN]) exert a catabolic effect, whereas growth factors (including insulin-like growth factor [IGF], transforming growth factor [TGF], and fibroblast growth factor [FGF]) exert an anabolic effect. Cytokines induce synthesis of proteases, resulting in an increased rate of cartilage ECM degradation and consequently proteoglycan depletion, while reducing the rate of synthesis of proteoglycans and other ECM components. Cytokines act primarily through cell surface receptors, whose signal is subsequently mediated by nuclear oncoproteins to activate transcription of the gene (Pelletier *et al.*, 1993). Growth factors can antagonize the cytokine effect by increasing the rate of synthesis of ECM components (Tyler *et al.*, 1992) and TIMPs (Gunther *et al.*, 1994). The net effect depends on the relative concentration of each mediator present in the cartilage. Cytokines can be synthesized by chondrocytes,

synovial cells, and inflammatory cells. Cytokines can be antagonized by receptor antagonists (e.g., IL-1 ra) and by so-called soluble binding proteins (eg, TNF-BP) (Pelletier *et al.*, 1991). An imbalance between cytokine and cytokine inhibitor levels has been postulated as one of the possible pathogenic pathways of OA.

### 1.2.4 Prostaglandins and OA

Prostaglandin $E_2$ ($PGE_2$) is an inflammatory mediator. Activated chondrocytes and synoviocytes are capable of producing large quantities of $PGE_2$ which, when released into surrounding tissues apparently exacerbate joint inflammation, and induce, among other processes, bone resorption and modulation of the immune response (Campbell *et al.*, 1990). Recent studies suggest that the eicosanoid may also be involved in many cartilage anti-catabolic processes as well. For example $PGE_2$ is able to reverse proteoglycan degradation induced by IL-1 in bovine and human articular cartilage explant cultures (Dingle, 1993). In addition, $PGE_2$ inhibits the expression and release of IL-1β and TNFα by inflammatory synoviocytes and macrophages (Knudsen *et al.*, 1986). Perhaps most importantly, $PGE_2$ also potently suppresses the expression and synthesis of collagenase and stromelysin by human synoviocytes ( DiBattista *et al.*,1994; DiBattista *et al.*, 1995).

In the nanomolar range, $PGE_2$ favors the synthesis of collagen type II, and proteoglycans (Raisz *et al.*, 1993; O' Keefe *et al.*, 1992; DiBattista *et al.*, 1996; Dingle, 1991). Synthetic prostaglandins and $PGE_2$ can reverse the negative effects of NSAIDS (e.g. indomethacin) on proteoglycan and collagen metabolism (Dingle, 1991). The effect of $PGE_2$ on chondrocyte collagen type II expression and synthesis is likely to be indirect since the promoter region of the gene has no canonical $PGE_2$-sensitive

(read cAMP) response elements. Indeed, $PGE_2$ stimulates the synthesis of collagenase digestible proteins in chondrocytes via an autocrine feed-back loop involving $PGE_2$ up-regulated insulin-like growth factor-1(IGF-1) (DiBattista *et al.*, 1996). It is known that the latter growth factor can potently stimulate collagen type II expression and synthesis. In this connection, the bioactivity of IGF-1 is governed at several levels including the presence of extracellular, high-affinity IGF-binding proteins (BPs, IGFBP-1 through to IGFBP-6) which modify the interaction of IGF-1 with its receptor (Jones *et al.*, 1995). The circulating or local levels of IGFBPs are regulated ontogenetically, by various endocrine factors, and by specific proteases that compromise the functionality of the IGFBPs (Cohen *et al.*, 1991; Lamson *et al.*, 1993).

Thus, collectively, $PGE_2$ may favor the maintenance of chondrocytes in a terminally differentiated phenotype under conditions where elements or factors conspire to destabilize homeostasis. Prostaglandin $E_2$ acts locally, in an autocrine/paracrine fashion, and may serve as a bridge linking catabolic and anabolic regulatory processes.

## 1.3 CYCLO-OXYGENASE

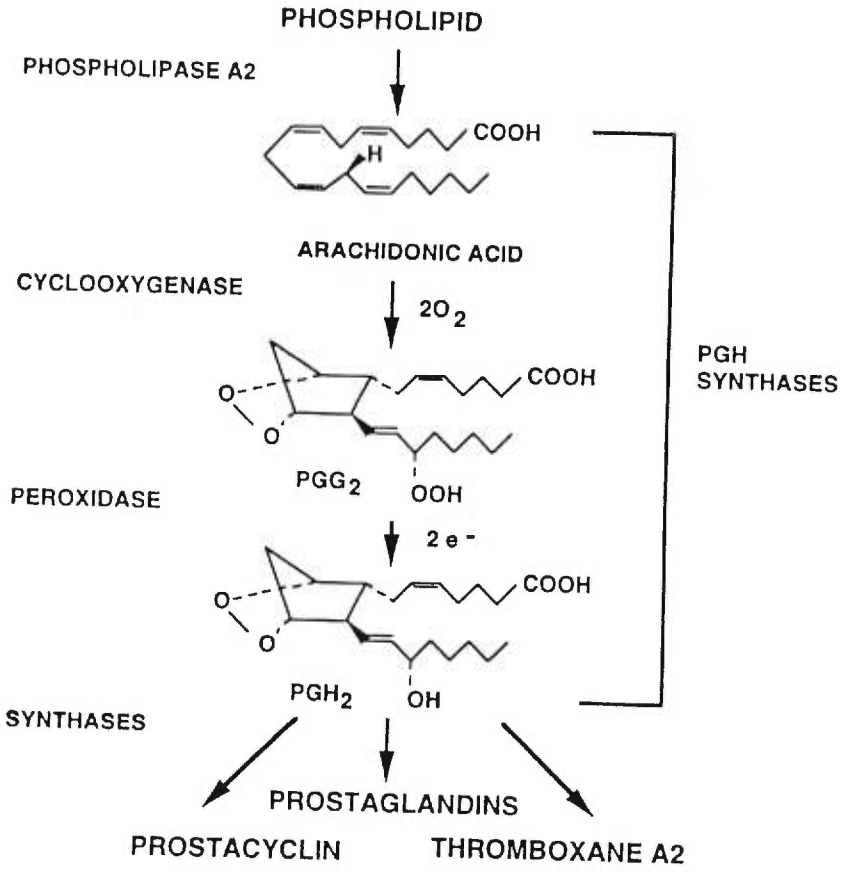
Cyclo-oxygenases (COXs) are key prostaglandin biosynthetic enzymes. Prostaglandins are important mediators of inflammation whose synthesis is initiated by release of arachidonic acid from cell membranes catalyzed principally by non-pancreatic phospholipase $A_2$. Arachidonic acid is then converted to $PGH_2$ by cyclooxygenase (COX; also known as PGH sythase), the central enzyme in the prostaglandin synthesis pathway. $PGH_2$ is rapidly converted into one of several prostanoids, such as $PGE_2$, $PGD_2$ and $PGF_2$ (DeWitt, 1991). Prostaglandin biosynthesis

has been implicated in the pathophysiology of cardiovascular disease, cancer and inflammatory disease (Marnett, 1990; Makheja, 1992; Abramson, 1991). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and indomethacin directly target COX (Vane *et al.*, 1987; Smith *et al.*, 1991). The COX enzyme is a integral membrane protein located primarily in the endoplasmic reticulum (Smith and Marnett, 1991). There are two isoenzymes encoded by two related genes, COX-1 and COX-2. The COX-1 gene is constitutively and ubiquitously expressed, while the COX-2 gene is only expressed at high levels upon induction by growth factors, cytokines, and extracellular stimuli associated with cell activation (Smith, 1992).

### 1.3.1 Arachidonic acid and prostaglandin

Prostaglandins are derived primarily from arachidonate hydrolyzed from the sn-2 position of membrane phospholipids by the action of phospholipase $A_2$ ($PLA_2$). These enzymes are generally calcium-dependent and have been found both intra- and extracellularly. By hydrolyzing the sn-2 bond in glycerophospholipids, $PLA_2$s release fatty acids. One such fatty acid, the arachidonic acid, generates the substrates for the initiation of the arachidonic acid cascade that produce various eicosanoids(i.e., Prostaglandins, leukotrienes and thrombxanes) many of which are potent mediators of inflammation. Phospholipases $A_2$ include both the relatively low molecular weight (=14kDa) type I and type II enzymes and the more recently described form known as cytoplasmic $PLA_2$ ($cPLA_2$). When arachidonic acid is released from the membrane lipids of most cell types by the action of non-pancreatic phospholipase $A_2$ (typeII)

**Figure 1.2.** Prostanoid biosynthetic pathway (Smith *et al.*,1996)


PHOSPHOLIPID
PHOSPHOLIPASE A2
COOH
H
ARACHIDONIC ACID
CYCLOOXYGENASE
2O2
COOH
O
O
PGG2
OOH
PEROXIDASE
2 e -
COOH
O
O
PGH2
OH
PGH
SYNTHASES
SYNTHASES
PROSTAGLANDINS
PROSTACYCLIN
THROMBOXANE A2

(Smith *et al.*, 1991; Smith, 1989), the enzyme prostaglandin $H_2$ synthase (PGHS) catalyzes the first committed, rate-limiting step by converting arachidonic acid to prostaglandin $H_2$ ($PGH_2$) which is then rapidly metabolized by tissue-specific isomerases to one of several prostanoids. $PGE_2$ is the predominant prostaglandin in synovial fluids and tissues, and is formed by a simple non-oxidative isomerization by PGE synthase (Smith, 1991).

### 1.3.2 Molecular structure of COX-1 and COX-2

Cyclooxygenase is a homodimer comprised of three independent folding units; an amino-terminal epidermal growth factor domain, a membrane binding motif and an enzymatic domain (Picot *et al.*, 1994). There are actually two isoenzymes, COX-1 and COX-2, which have about 61% homology at the amino acid sequence level but differ markedly in their regulation and tissue distribution (DeWitt, 1991; Holtzmaan *et al.*, 1992; Meade *et al.*, 1993; Otto *et al.*, 1993). COX-1 and COX-2 are polypeptides of 599 and 604 amino acids (70kDa), respectively, including the putative n-terminal signal peptide sequence. COX-1 mRNA (2.8kb) is not inducible and thus COX-1 (chromosome 9, 11 exons and10 introns) has the characteristics of a constitutive gene. COX-2 gene (mRNA 4.1kb) consists of 10 exons and 9 introns; the largest exon in the COX-2 gene encodes the entire 3'-UTR, containing 22 copies of the "AUUUA" RNA instability element. Sequence analysis of 5'-flanking region has shown several potential transcription regulatory sequences, including a TATA box, a C/EBP motif, two AP-2 sites, three SP1 sites, two NF-κB sites, a CRE motif and Ets-1 site. These studies serve as a basis for future studies on transcriptional and post-transcriptional mechanisms of COX-2 gene regulation (Appleby *et al.*, 1994).

### 1.3.3 Characteristics of COX-1 and COX-2

Cycloxygenase is a membrane-bound, bifunctional enzyme that catalyzes the conversion of arachidonic acid to prostaglandin $G_2$ by its cyclooxygenase activity and prostaglandin $G_2$ to prostaglandin $H_2$ by peroxidase activity.

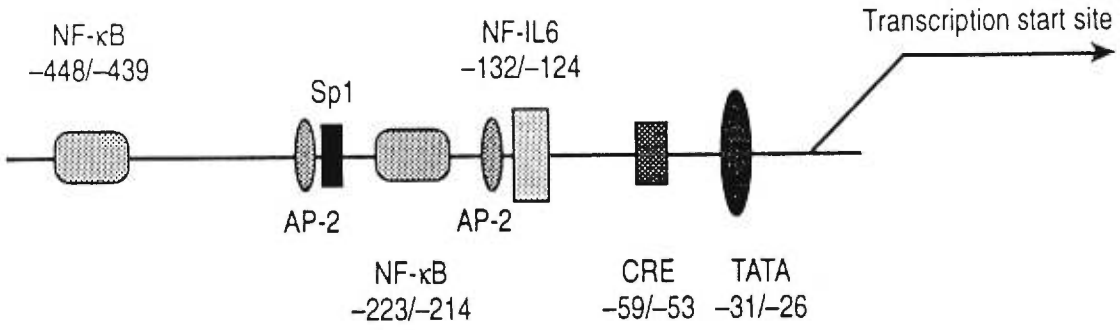
COX-1 and COX-2 subserve different physiologic functions largely because of the striking differences in their tissue expression and regulation.

COX-1 displays the characteristics of a "housekeeping" gene, such as a TATA-less promoter. The COX-1 promoter has not shown any significant transcription (Smith and De Witt, 1995). COX-1 is constitutively expressed in almost all tissues. It occurs as part of an endoplasmic reticulum (ER) prostanoid biosynthetic system, which forms prostanoids that act extracellularly as "local" hormones functioning through cell surface G protein-linked receptors to mediate acute "housekeeping" responses to circulating hormones (Smith *et al.*, 1996). This isoform appears to be responsible for the production of prostaglandins important for homeostatic functions, such as maintaining the integrity of the gastric mucosa and mediating normal platelet function.

In sharp contrast, COX-2 is an "immediate early" gene that is rapidly inducible and tightly regulated. Under basal conditions, COX-2 expression is highly restricted with the highest levels of expression in the brain and kidney. COX-2 expression is dramatically increased during inflammation and dysregulated proliferation. In 1989 Simmons et al (Simmons *et al.*, 1989) identified an inducible form of COX-2. This cyclooxygenase isoform was identified by differential screening of a phorbol ester-

stimulated Swiss-3T3 fibroblast cDNA library (Kujubu *et al.*, 1991) and subsequently by many other groups. COX-2 expression is inducible by a wide range of extracellular and intracellular stimuli, including lipopolysaccharide (Fu *et al.*, 1990; Lee *et al.*, 1992; O' Sullivan *et al.*, 1992), forskolin (Kujubu and Herschman, 1992), interleukin-1 (IL-1), tumor necrosis factor (TNF) (Coyne *et al.*, 1992; Geng *et al.*, 1995; Jones *et al.*, 1993), serum (DeWitt and Meade, 1993; Ryseck *et al.*, 1992), epidermal growth factor (EGF) (Hamasaki *et al.*, 1993); synaptic activity (Yamagata *et al.*, 1993), transforming growth factor-α (TGF-α) (DuBois *et al.*, 1994), human chorionic gonadotropin (Sirois, 1989), interferon-γ (Riese *et al.*, 1994) , platelet activating factor, retinoic acid (Bazan *et al.*, 1994) and endothelia (Kester *et al.*, 1994). Additionally, the formation of COX-2 protein parallels the increase in prostanoid production that is commonly the result of mitogenic stimulation in a wide variety of cell types. Prostaglandin production increases rapidly in rat intestinal epithelial cells after stimulation with mitogens such as TGF-α or EGF (DuBois *et al.*, 1994). COX-2 probably has two roles. One role, involving a subpopulation of COX-2 localized with COX-1 on the lumenal surface of the ER, is to augment the function of COX-1 (or to substitute for COX-1 in cells lacking this isoform). The other role, involving the subpopulation of COX-2 present on the lumenal surface of the inner membrane of the nuclear envelope, may operate as part of a unique nuclear prostanoid biosynthetic system to form products that act through nucleoplasmic or nuclear membrane targets in association with cell differentiation and replication (Smith *et al.*, 1996).

**Figure 1.3.** Schematic representation of the COX-2 promoter/enhancer region (Bazan *et al.*, 1996)


NF-κB
–448/–439
Sp1
NF-IL6
–132/–124
Transcription start site
AP-2
AP-2
NF-κB
–223/–214
CRE
–59/–53
TATA
–31/–26

### 1.3.4 Regulation of COX-2 expression

Regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels. The COX-2 message has an extensive 3'UTR having at least two distinct polyadenylation sites and 22 Shaw-Kamen 5'-AUUUn-A-3' motif (Appleby *et al.*, 1994). The latter sequences are believed to be associated with message instability and rapid turnover (Beelman and Parker, 1995). Sequence analysis of the 5'-flanking region has shown several potential transcription regulatory sequences, including a TATA box, a c/EBP motif, two AP-2 sites, 3 SP-1 sites, two NF-κB sites, a CRE motif and Ets-1 site (Appleby *et al.*, 1994). Despite this wealth of structural information, it is still unclear how the COX-2 gene is regulated by external stimuli in terms of signaling pathway. Recently, Inoue et al (Inoue *et al.*, 1995) showed that induction of COX-2 expression in vascular endothelial cells by phorbol ester or LPS involved the COX-2 CRE and NF-IL6 sites, and that c/EBP δ functioned as a transacting factor, perhaps in association with CREB. Yamamoto et al (Yamamoto *et al.*, 1995) suggested that NF-κB and NF-IL6, through binding to their cognate response elements, mediate COX-2 induction by tumor necrosis factor-α (TNF-α ) in MC3T3E1 cells. Xie and Herschman' s results indicated that the signal pathway leading to src-induced COX-2 transcription involves both Ras/MEKK-1/JNK and Ras/Raf-1/ERK pathway (Xie and Herschman, 1995) . COX-2 gene transcription induced by each class of agonists is likely mediated by multiple, complex signaling pathways leading to the binding of nuclear transcription activators to specific binding motifs (Kenneth, 1996). In our laboratory, recently we identified some potential signaling pathways involved in the regulation of COX-2 gene expression in phenotypically stable human chondrocytes (Miller *et al*, 1998). This was achieved with the use of two specific serine/threonine phosphatase (type 1 and type 2A) inhibitors,

namely okadaic acid (OKA) and calyculin A, which provided a cellular environment favouring an increased level of protein phosphorylation. One major MAP kinase cascade, the Raf/MEKK/MEK/ERK pathway, was inhibited by treatment with phosphatase inhibitors while another, the MEKK1/JNKK/JNK/SAPK pathway, was fully activated. In addition, PKA activity was stimulated, which was probably responsible for the Raf/MEKK/MEK/ERK pathway shutdown because PKA inhibits Raf kinase activity (Cook and McCormik. 1993).

## 1.4 SIGNAL TRANSMISSION FROM CELL SURFACE TO THE NUCLEUS

To activate or repress transcription, transcription factors must be located in the nucleus, bind to DNA, and interact with the basal transcription apparatus. Accordingly, extracellular signals that regulate transcription factor activity may affect one or more of these processes. Most commonly, regulation is achieved by reversible phosphorylation. Post-translational modification of protein by phosphorylation plays an important role in the regulation of many cellular processes including gene expression (Hunter and Karin. 1992; Hunter, 1995). Considerable evidence accrued over the years has implicated phosphorylation reactions as the molecular basis for a large number of intracellular signaling cascades. Biological processes that depend on reversible phosphorylation require not only protein kinases but also protein phosphatases, and the cellular concentration of serine/threonine directed kinases (PSTK) (but not tyrosine specific kinase) is approximately equal to that of serine/PSTP (Hunter, 1995). Therefore targeted substrate proteins are specifically phosphorylated at cognate sites by protein kinases and dephosphorylated by substrate-specific phosphatases. Phosphorylation of a transcription factor by several different kinases (or by a kinase linked to more than one pathway) is a simple mechanism that allows different signals to converge at the same

factor (Hill and Hunter 1995) .Two general mechanisms have evolved for the rapid and accurate transmission of signals from cell-surface receptors to the nucleus, both involving protein phosphorylation. One mechanism depends on the regulated translocation of activated protein kinases from the cytoplasm to the nucleus, where they phosphorylate target transcription factors, like protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). In the second mechanism, transcription factors are kept in a latent state in the cytoplasm and are translocated into the nucleus upon activation, as nuclear factor kappa B ( NF-κB), and Janus kinase (JAKs) and signal transducers and activators of transcription ( STAT) (Karin and Hunter 1995).

### 1.4.1 Protein kinase A

PKA is a serine-threonine protein kinase that catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected proteins. The amino acids phosphorylated by PKA are marked by the presence of two or more basic amino acids on their amino-terminal side. Covalent phosphorylation of the appropriate amino acids in turn regulates the activity of the target protein (Bruce *et al.*, 1994). PKA is found in all animal cells and is thought to account for all of the effects of cyclic AMP in most of these cells. The substrates for PKA differ in different cell types (Bruce *et al.*, 1994).

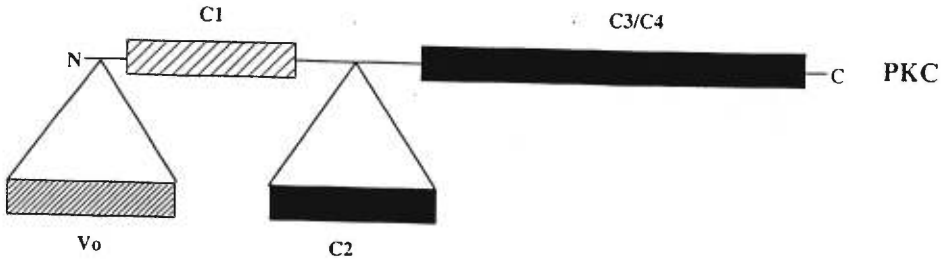
One of the simpler modes of signal transmission from surface receptors to the nucleus is illustrated by proteins that bind to a DNA sequence known as cAMP-response elements (CREs); these are known as CRE-binding protein (CREBs). The activation of certain G-protein-coupled receptors causes a build-up of intracellular

cAMP. The binding of to the regulatory subunit of the tetrameric PKA liberates its catalytic subunit, which then translocates to the nucleus (Nigg *et al.*, 1985). Once in the nucleus, the PKA catalytic subunit phosphorylates CREB which is thought to bind constitutively to functional CREs upstream of certain cAMP-regulated genes on residue Ser 133, thereby stimulates its ability to activate transcription (Gonzalez and Montminy, 1989). PKA also stimulates the transactivating potential of the related protein CREM (CRE modulator) by phosphorylation at a similar, functionally equivalent site (Karin and Hunter 1995).

### 1.4.2 Protein kinase C

Protein kinase C (PKC), also a serine/threonine kinase, mediates the effects of a large number of hormones, growth factors and cytokines, and is thus considered a key factor in the regulation of cellular proliferation and differentiation (Clemens *et al.*, 1992; Kratzmeier *et al.*, 1996). PKC is generally thought to be activated by signal transduction system that produce diacylglycerol (DAG), such as certain tyrosine kinase and G protein-coupled receptors (Clemens *et al.*, 1992; Bell, 1986; Hug and Sarre, 1993; Dekker and Parker 1994). PKC is also the major target for the well-known tumor-promoting agent 12-O-tetradecanoyl-phorbol acetate (TPA), a phorbol ester, which acts in a fashion very similar to DAG (Blumberg *et al.*, 1984; Nishzuka, 1984; Bell and Burns, 1991; Gschwendt *et al.*, 1991). The downstream targets of activated PKC are only partially identified and include other protein kinases (Dekker and Parker 1994; Yamagushi *et al.*, 1995) and the transcription factor AP-1(Boyle *et al.*, 1991; Angel and Karin, 1996; Karin, 1995).

**Figure 1.4** Domain structure of PKC isotype (Dekker and Parker 1995)


C1
C3/C4
N
C
PKC
Vo
C2

1.4.2.1 Protein kinase C structure and classification

Members of the PKC family are a single polypeptide chain, comprised of an N-terminal regulatory region (approximately 20-40 kDa) and a C-terminal catalytic region (approximately 45 kDa). They have four conserved domains: C1-C4 (Coussens *et al.*, 1986). Each is a functional module, and many unrelated proteins have one or the other (Newton, 1995). The C1 domain contains a Cys-rich motif, duplicated in most isozymes, that forms the diacylglycerol/phorbol ester binding site (Bell and Burns, 1991); this domain is immediately preceded by an autoinhibitory pseudosubstrate sequence (House and Kemp, 1987); the C2 domain contains the recognition site for acidic lipids and, in some isozymes, the $Ca^{2+}$-binding site (Newton, 1995). The C3 and C4 domains form the ATP-and substrate-binding lobes of the kinase core (Tayor and Radzio-Andzelm,1994).

Multiple discrete subspecies of PKC have been defined. These subspecies show subtly different enzymological properties, differential tissue expression, and specific intracellular localization (Nishizuka, 1988). The PKC family now comprises at least 12 members, which can be divided into two groups (Hug and Sarre, 1993): group A, the $Ca^{2+}$-dependent or conventional PKCs (cPKCs) $\alpha$, $\beta$I, $\beta$II and $\gamma$; and group B, the $Ca^{2+}$-independent or novel PKCs (nPKCs) $\delta$, $\varepsilon$, $\zeta$, $\eta$, $\theta$, $\tau$, $\lambda$, $\mu$. The cPKC enzymes of group A have four conserved (C1 to C4) and five variable (V1 to V5) regions. The C1 region is a putative membrane-binding domain (Ono *et al.*, 1989). The $Ca^{2+}$ region appears to be related to the $Ca^{2+}$ sensitivity of the enzyme. The C3 region contains the catalytic site. The C4 region seems to be necessary for recognition of the substrate to be phosphorylated. The cPKC enzymes are activated by $Ca^{2+}$, phophatidylserine, and diacylglycerol or phorbol esters, and this activation is enhanced further by cis unsaturated fatty acids and lysophosphophatidylcholine. The nPKC enzymes of group

B, which lack the C2 region, do not require $Ca^{2+}$. The enzymes are activated by micelles composed of phosphatidylserine and diacylglycerol or phorbol esters (Nishizuka, 1988; Schaap and Parker, 1990; Saido *et al.*, 1990). The ε subspecies is activated by cis unsaturated fatty acids but the δ subspecies is not. On the other hand, the subspecies ζ and λ have only one cysteine-rich zinc finger-like motif (Nishzuka, 1988; Liyanage *et al.*, 1992; Ono *et al.*, 1989; Ways *et al.*, 1992) are dependent on phosphatidylserine but not affected by diacylglycerol, phorbol esters, or $Ca^{2+}$ (Ono *et al.*, 1989; Ways et al., 1992). The α, βI, βII, δ, ε and ζ isoforms seem to be widely distributed (Hug and Sarre, 1993; Wetsel *et al.*, 1992; Nishzuka, 1988; Wada *et al.*, 1989). However, most cell types express only a certain subset of these isotypes (Hug and Sarre, 1993; Wetsel *et al.*, 1992). PKC γ has so far only been found in neural tissues (Hug and Sarre, 1993; Wetsel *et al.*, 1992; Nishzuka, 1988; Wada *et al.*, 1989). Such differences in distribution suggest a divergence in function between isotypes (Dekker *et al.*, 1994).

1.4.2.2 Protein kinase C function

PKC typically phosphorylates serine or threonine residues in basic sequences. In the nucleus, the signal pathway through PKC appears to be indispensable for the control of gene expression and the cell cycle. Some members of the PKC family may function in the regulation of specific points of the cell cycle (Watanabe *et al.*, 1995). In the membrane, crucial roles have been assigned to PKC in down-regulation of receptors, modulation of ion channels, release of hormones and neurotransmitters, and exocytosis (Nishzuka, 1986). Biochemical and electrophysiological evidences for a role of PKC in modulating $Na^{+}$ channels have been presented (West *et al.*, 1991). The distinct modes of activation, together with the apparent patterns of tissue expression

and intracellular localization of the various PKC subspecies, imply specialized functions in cell signaling.

PKC participates in inflammatory processes and immune responses. The signaling pathway through PKC is essential for the activation of platelets, neutrophils, macrophages, lymphocytes, and fibroblasts, and for the function of vasculoendothelial system (Nishzuka, 1986).

1.4.2.3 Protein kinase C regulation

The function of PKC is regulated by two equally important mechanisms. First, the binding of ligands or, in some cases, the substrate activates the enzyme by removing the pseudosubstrate from the substrate-binding site. Second, the enzyme is rendered catalytically competent by phosphorylations, that align residues for catalysis and localize PKC to the cytosol (Newton, 1995).

1.4.2.4 Protein kinase C inhibitor

PKC activation and translocation can be blocked by a group of highly selective PKC inhibitors such as CalC. CalC is a polycyclic hydrocarbon (perylenequinoes) isolated from *Cladosporium cladosporioides* which binds specifically and with high affinity (IC50=50 nmol/L) to the DAG site in the regulatory (unique) domain of PKC (Kobayashi *et al.*,1989;Bruns *et al.*, 1991). The inhibitor has been used extensively and has proven to be a useful probe in the study of PKC-dependent signaling pathways and gene expression (Das and White, 1997; Xu and Clark, 1997). Furthermore, it has been cited for its potential as a useful drug in cancer treatment and various forms of arthritis because it is pro-apoptotic in many cell types (Lee *et al.*, 1995; Yang *et al.*, 1995; Kobayashi *et al.*, 1989). A biochemical rationale for the action of CalC in this regard

remains to be clarified although proto-oncogene expression (e.g. c-jun) has been implicated (Gamou *et al.*, 1995; Freemerman *et al.*, 1996).

### 1.4.3 Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine specific, proline directed, protein kinases which are activated by a wide spectrum of extracellular stimuli. MAPK activation is achieved through kinase cascades, which include a MAPK kinase (MAPKK or MEK) and a MAPKK/MEK kinase (MAPKKK/MEKK). These cascades serve as information relays, connecting cell-surface receptors to specific transcription factors and other regulatory proteins, thus allowing extracellular signals to regulate the expression of specific genes. Genetic and biochemical analyses have revealed many tiers in the regulation of the activities of MAPKs, as well as different routes that lead to the activation of an individual MAPK (Su and Karin, 1996). Currently, three distinct MAPK cascades are known in vertebrates, the target MAPKs are the extracellular signal-regulated kinase (ERKs), the Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and the p38/Mpk2, Fos-regulating kinase (FRK) (Karin and Hunter 1995).

#### 1.4.3.1The ERK1/2 MAPK cascade

ERK1/2 activity is most strongly stimulated by growth factors that activate tyrosine kinase receptors (Cobb and Goldsmith, 1995; Segar and Krebs, 1995). ERK1/2 activation is mediated by the specific protein kinases MAPK/ERK kinase (MEK)1/2, which are members of the MAPK kinase (MAPKK) supergene family (Crews *et al.*, 1992; Zheng and Guan, 1993). MEK1/2 themselves are activated through phosphorylation by three distinct MAPKK kinases (MAPKKKs): Raf (Moodie *et al.*,

1993; Kyriakis *et al.*, 1992), c-Mos (Pham *et al.*, 1995) and MEK kinase (MEKK) 1 (Lange-Carter *et al.*, 1993). The major pathway by which tyrosine-kinase-mediated signals are directly relayed to the ERK1/2 cascade is through Ras-mediated recruitment of Raf to the plasma membrane (Egan and Weinberg, 1993). Ras activation is dependent on the tyrosine-kinase-mediated membrane translocation of the guanine nucleotide exchange factor SOS by the Grb2 adaptor protein, which connects SOS to the tyrosine-phosphorylated receptor (Egan and Weinberg, 1993). The recruitment of upstream signaling components to the vicinity of the activated cell-surface receptor appears to be a generally important mechanism for activation of a MAPK cascade by different upstream activators. Once ERKs is in the nucleus, the ERKs phosphorylate several substrates including ternary complex factor (TCF), the transcription factor that mediates *c-fos* induction (Gille *et al.*, 1992, Marais *et al.*, 1993).

Although tyrosine kinases play a major role in up-regulating the ERK1/2 cascade, a cAMP-mediated signaling pathway has been reported to down-regulate the ERK1/2 cascade in Rat 1 cell (Cook and McCormik, 1993). This down-regulation by cAMP was attributed to the inhibition of Raf-1 activation by Ras.

1.4.3.2 The JNK MAPK cascade

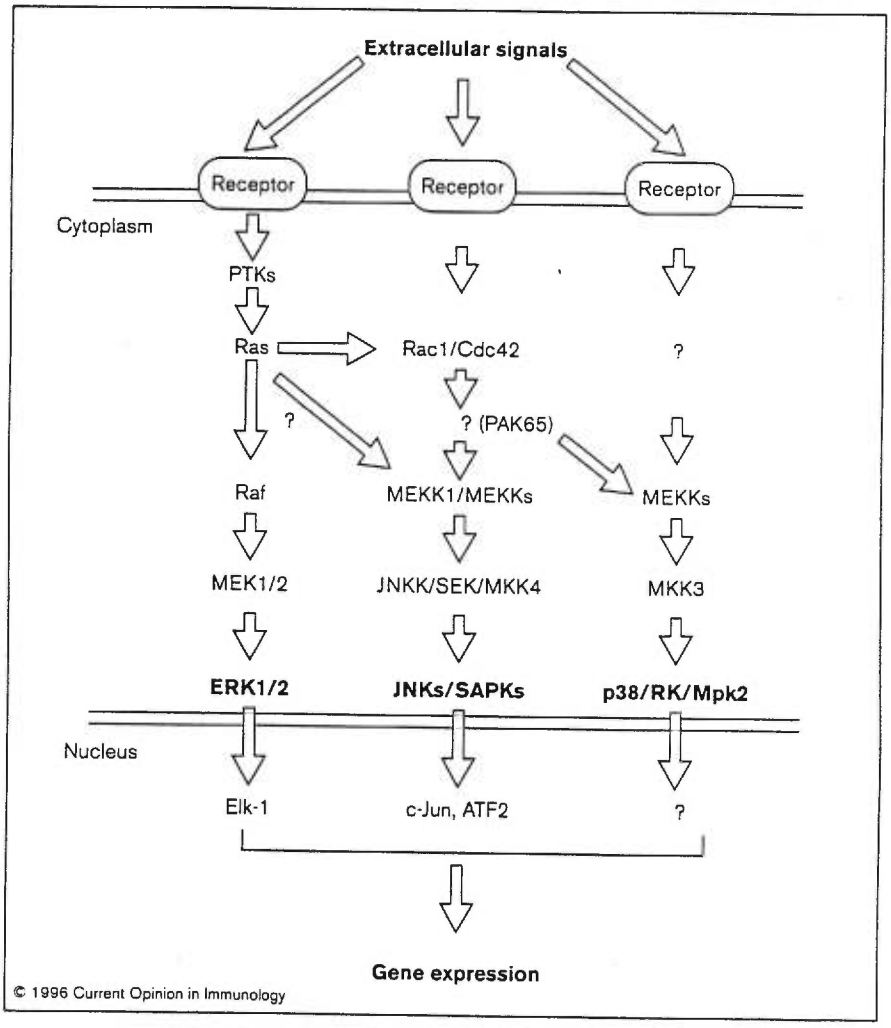
The JNK/SAPKs appear to be responsible for stimulation of c-Jun transcriptional activity, by phosphorylation of residues Ser 63 and Ser 73 in *c-jun*' s activation domain (Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Hibi *et al.*, 1993; Adler *et al.*, 1995). Consistent with the different sequences surrounding their activating phosphorylation sites, the JNKs are not phosphorylated by MEK1/2, but by another MAPKK, named SEK1 (Sanchez *et al.*, 1994), MKK4 (Derijard *et al.*, 1995) or JNKK1 (Lin *et al.*, 1995). JNKK1 activity is stimulated by some, but not all, of the

JNK-activating agonists, suggesting the existence of additional JNKKs (Lin *et al.*, 1995). Like other MAPKKs, JNKK1 is phosphorylated and activated by a MAPKK kinase (MAPKKK), MEKK1 (Sanchez *et al.*, 1994; Lin *et al.*, 1995). Low-level expression of truncated and even nearly full-length MEKK1 results in efficient JNK activation without a significant increase in ERK1/2 activity (Minden *et al.*, 1994). Very strong JNK activation is observed after irradiation of cells with either UV light, treatment with certain translational inhibitors, such as anisomycin (Cano *et al.*, 1994), exposure of interleukin (IL)-1 (Raingeaud *et al.*, 1995), Co-stimulatory activation of T cells (Su *et al.*, 1994), ischemia reperfusion (Pombo *et al.*, 1994), and exposure to alkylating agents (Kharnanda *et al.*, 1995). Less potent activation is observed after treatment of cells with tumor necrosis factor (TNF)-α and exposure to heat shock (Kyriakis *et al.*, 1994).

1.4.3.3 p38/RK/Mpk2 MAPK cascade

A 38kDa protein was found to be the major tyrosine-phosphorylated protein following LPS treatment (Han *et al.*, 1993). Sequence of the cDNA indicated that the 38kDa polypeptide is a new member of the MAPK group (Han *et al.*, 1994). In addition to LPS and IL-1, p38 is activated in response to osmotic shock and by and large responds to the same agonists that activate the JNKs (Lin *et al.*, 1995). The similarity between the regulation of p38 and that of JNK is not surprising, because JNKK1 (MKK4) is also a direct activator of p38. In addition, another MAPKK, MKK3, which activates only p38 and not JNK or ERK, has been identified (Derijard *et al.*, 1995).

**Figure 1.5** Summary of signal transduction by the following subgroups of MAPKS: ERK1/2, JNKs/SAPKs and p38/RK/Mpk2 (Su and Karin 1996)


Extracellular signals
Receptor
Receptor
Receptor
Cytoplasm
PTKs
Ras
Rac1/Cdc42
?
?
? (PAK65)
Raf
MEKK1/MEKKs
MEKKs
MEK1/2
JNKK/SEK/MKK4
MKK3
ERK1/2
JNKs/SAPKs
p38/RK/Mpk2
Nucleus
Elk-1
c-Jun, ATF2
?
Gene expression
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### 1.4.4 NF-κB activation, JAKs and STAT

An alternative strategy for transmitting signals from the cell surface to the nucleus is regulated by nucleus translocation of transcription factors that are stored as inactive cytoplasmic complex, first demonstrated for NF-κB (Baeuerle and Baltimore, 1988). In non-stimulated cells, the different NF-κB complexes are held in the cytoplasm by interaction with the IκB inhibitors. The latter may function by masking the nucleus translocation sequence within the Rel-homology domain of NF-κB proteins (Beg and Baldwin, 1993). Following cell stimulation by inflammatory mediators, the NF-κB-IκB complex dissociates and NF-κB dimers are rapidly translocated to the nucleus. It has been proven that the first step in NF-κB activation is IκB phosphorylation, IκB protein has two isoforms, IκBα and IκBβ. Both of isoforms share common properties but also exhibit significant differences. For example, IκBα and IκBβ interact with the same spectrum of Rel proteins, inhibit their DNA binding, and restrict their distribution to the cytoplasm. Degradation of IκBα occurs with all of the NF-κB inducers tested (Baldwin, 1996), while IκBβ responds to only a subset (Good and Sun, 1996). IκBα is a stronger inhibitor of NF-κB than is IκBβ. IκB-α is rapidly phosphorylated, then ubiquitinated, and finally degraded by proteasomes (Chen *et al.*, 1996; DiDonato *et al.*, 1996). Activation of the NF-κB requires the successive action of NF-κB-inducing kinase (NIK) and IκB kinase (IKK). The IκB kinase-β (IKK-β) phosphorylates the α and β members of the IκB family on serines residues 32 and 36 of IκB-α and serines 19 and 23 of IκB-β, whereas IKK-α phosphorylates serine 19 of IκB-β quite poorly. IKK-α and IKK-β may normally exist as a heterodimer that can interact directly with the upstream kinase NIK. All three kinase are components of the

large 700-900-kD IκB kinase complex, and each is essential for activity of the complex (Woronicz *et al.*, 1997).

A second family of transcription factors whose activity is regulated by their compartmentalization are the signal transducers and activators of transcription (STATs). The STATs are activated in response to a large variety of cytokines and growth factors (Darnell *et al.*, 1994). Currently, six STAT genes are known. The mechanism of STAT activation has been most extensively studied in the case of IFN signaling. The binding of IFN-α or IFN-β to their respective receptors results in recruitment and activation of Janus kinase 1 (JAK1) and TYK2 or JAK1 and JAK2, respectively. For example, activation of JAK1 and TYK2 results in phosphorylation of the IFN-α receptor followed by association of STAT1α / STAT1β and STAT2 with specific phosphotyrosines on the receptor via their SH-2 domains. The receptor-bound STATs are then phosphorylated, presumably by the receptor-associated JAKs, and this results in dimerization and nuclear translocation of the STAT1α/β-STAT2 dimer. In the nucleus, these activated STAT dimers associate with p48 to form trimeric interferon-stimulated gene factor 3 complexes which bind to interferon-stimulated gene response elements and activate transcription of IFN-α inducible genes. IFN-γ, on the other hand, induces only STAT1α phosphorylation and does not affect STAT2 (Shuai *et al.*, 1992; Shuai *et al.*, 1993). In both cases, tyrosine phosphorylation precedes nuclear translocation.

## 1.5 TRANSCRIPTION FACTOR: Activating protein 1 (AP-1) and CRE binding protein (CREB)

Extracellular signals modulate the activity of many different types of transcription factors. One important group of signal-regulated transcription factors are the bZIP proteins, so named because of their conserved basic (B) and leucine zipper (Zip) domains that are required for DNA binding and dimerization, respectively (Vinson *et al.*, 1989). These sequence-specific factors have a modular structure consisting of distinct and separable DNA binding, dimerization, and transcriptional activation domains (Lamb and Mcknight, 1991; Angel and Karin,1991). The most studied members of this superfamily are AP-1 (Jun/Fos) and CREB/ATF proteins that control gene expression by binding to the TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element (TRE; this element has the base sequence TGACTCA) and cyclic AMP (cAMP) response element (CRE; this element has the base sequence TGACGTCA), respectively (Angel and Karin,1991; Montminy *et al.*, 1990; Karin, 1992). Regulation by bZIP proteins can involve a variety of complex mechanisms-transcriptional, combinatorial, temporal and post-translational -that affect the level and the repertoire of the factors expressed in a given cell as well as their DNA binding and transcriptional activation function (Karin and Smeal 1992).

### 1.5.1 The composition and function of AP-1 and CREB

AP-1 was originally defined as a DNA-binding activity recognizing the TRE and responsible for transcriptional induction of a number of genes in response to an activation of PKC ( Su and Karin, 1996). AP-1 consists of a collection of structurally related transcription factors, which belong to the Jun and Fos families; Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) bZIP protein ( Vogt and Bos, 1990; Angel and Karin, 1991; Karin *et al.*, 1997), these associate to form a variety of homo- and

heterodimers, all of which recognize the TRE (Angel and Karin, 1991). Like all members of the bZIP family, the AP-1 components must dimerize prior to DNA binding. The Jun proteins bind DNA as either homodimers or Jun-Jun heterodimers, whereas the Fos proteins must heterodimerize with one of the Jun proteins, since they cannot form stable Fos-Fos homo- or heterodimers (Angel and Karin, 1991). Owing to their increased stability, the Jun-Fos dimers exhibit more DNA-binding activity and *trans*-activation capability than the corresponding Jun-Jun dimers (Angel and Karin, 1991; Smeal *et al.*, 1989). Among the Jun proteins, c-Jun is the most potent transcriptional activator, either as a homodimer or in combination with c-Fos (Chiu *et al.*, 1989; Nakabeppu and Nathans, 1991).The Fos proteins also vary in their ability to activate transcription in combination with c-Jun, c-Fos and FosB being much more potent than Fra1 or Fra2 (Nakabeppu and Nathans, 1991; Suzuki, 1991).

All of these dimers are thought to contribute to AP-1 activity and participate to varying extents in its regulation by extracellular stimuli (Angel and Karin, 1991). The different AP-1 factors may regulate different target genes and thus execute distinct biological functions. For example, both *c-fos* and *c-jun* are "immediate-early" genes, whose transcription is rapidly induced independently of *de novo* protein synthesis following cell stimulation (Su and Karin 1996). AP-1 should be regarded as a nuclear messenger that mediates the actions of signal transduction pathways stimulated by growth factors, hormones, cytokines and neurotransmitters, most of which are initiated with the activation of either tyrosine kinases or phospholipid turnover.

The cAMP-regulated transcription factor, CREB, was originally defined as a sequence-specific DNA activity that binds to CREs within the promoters of cAMP-inducible genes and mediates their induction in response to activation of the PKA

pathway (Karin, 1992). The family of CRE-binding proteins consists of at least eight members, of which only CREB has been established as a mediator of cAMP action (Karin,1992). A new member of CREB family , CREM, acts as dominant-negative inhibitors of cAMP response elements, while others act positively. Also, AP-1 complexes can bind the CRE. In general, the CREBs do not bind TREs. However, several of them can form heterodimers with the Jun/Fos proteins that can bind both type of elements. For example, Jun-ATF dimers or ATF homodimers prefer to bind to the CRE (Hai and Curran, 1991).

### 1.5.2 Regulation of AP-1 and CREB

In addition to TPA, AP-1 activity is induced by a variety of polypeptide hormones, growth factors, cytokines and neurotransmitters (Angel and Karin, 1991). These agents activate signaling pathways that are initiated with either stimulation of membrane-associated tyrosine kinases or phospholipid turnover, the latter giving rise to increased PKC activity (Cantley *et al.*, 1991). In addition, AP-1 activity is elevated in cells that express a variety of transforming oncogenes, whose products act as constitutively activated intermediates in the signal transduction pathway that transmits information from cell-surface tyrosine kinases to the nucleus. These signaling pathways mainly affect AP-1 activity at two levels: transcriptional and post-transcriptional. First, transcription of the *fos* genes, which is very low in most non-stimulated cells, is induced in response to a variety of extracellular stimuli. The most rapid induction is exhibited by c-*fos*, the expression of which is also highly transient while induction of other fos genes, such as *fra*-1, is somewhat slower and longer lasting (Angel and Karin, 1991; Hai and Curran, 1991). Most of the signals stimulate *c-fos* transcription through the serum response element (SRE). Induction of fos

transcription results in increased synthesis of Fos proteins, which combine with pre-existing Jun proteins to form more stable heterodimers and thereby increase the level of AP-1 binding activity (Angel and Karin, 1991). Most of the signals that stimulate AP-1 activity induce c-*jun* transcription, which usually is longer lasting than c-*fos* induction (Angel and Karin, 1991). The persistent induction of c-*jun* is presumably due to the ability of c-Jun to autoregulate its expression by binding to a TRE in the c-*jun* promoter (Angel *et al.*, 1988). Expression of *jun*B is also stimulated by extracellular stimuli but appears to respond to different signals than those that affect c-*jun*. In most cells, expression of *jun*D is constitutive (Angel and Karin, 1991). The differential responsiveness and induction kinetics of the various jun and fos genes result in the formation of different AP-1 complexes at different times after cell stimulation.

In comparison to the AP-1 proteins, expression of the CREB proteins so far appears to be more or less constitutive and relatively unresponsive to extracellular stimuli. Therefore the major level of control affecting these proteins is post-translational (Karin, 1992). All of the signals known to stimulate CREB activity *in vivo* are those which activate the PKA pathway (Montminy *et al.*, 1990). The regulation of this pathway is much less complicated than that of signaling pathways centered around the Ras proteins. PKA activity is directly stimulated by elevation of cAMP levels; cAMP binds to the regulatory subunit of this tetrameric enzyme, leading to its dissociation and liberation of the catalytic subunit. This results in the activation of the catalytic subunit and its translocation to the nucleus where it can phosphorylate CREB. The activation of CREB activity is rapid, generally peaking within 30 minutes and declining gradually over 24 hours (Karin, 1992). Also, ATF-1, which has a regulatory domain similar to the one of CREB, but lacks the glutamine activation domain present in CREB, is activated by the catalytic subunit of PKA (Rehfuss *et al.*, 1996). The ATF-

2 protein, which has a different regulatory domain, responds to a different type of signal.

## 1.6 RESEARCH HYPOTHESIS:

AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos proto-oncogene families (Angel *et al.*, 1991). These proteins, which belong to the bZIP group of DNA binding proteins (Johnson *et al.*, 1989), associate to form a variety of homo- and heterodimers that bind to a common site. AP-1 was found as a transcription factor that mediates gene induction by the phorbol ester tumor promoter 12-*O*-tetradecanoylphobol-13-acetate (TPA) and hence the name TRE (TPA response element) for its recognition site. Also AP-1 activity was found to be induced by many other stimuli, including growth factors, cytokines, T cell activators, neurotransmitters, and UV irradiation. Several mechanisms are involved in induction of AP-1 activity and may be classified as those that increase the abundance of AP-1 components and those that stimulate their activity (Karin, 1995).

Increased prostaglandin synthesis may be an important component in the pathogenesis of arthritic diseases and COX-2 in the first induced enzyme in the pathway that leads to prostanoid synthesis. Also this inducible COX-2, linked to inflammatory cell types and tissues, is believed to be the target enzyme for the anti-inflammatory activity of NSAIDs (Masferrer *et al.*,1995; Gierse *et al.*, 1996). COX-2 is rapidly induced by tumor promoters, growth factors, cytokines and mitogens in many cell model systems (Crofford *et al.*, 1994; Kubuju *et al.*, 1991; O'Banion *et al.*, 1992). Regulation of COX-2 expression has been shown to occur at both

transcriptional and post-transcriptional levels. AP1 is known to mediate gene induction of COX-2 in many cell types (Xie and Herschman, 1995; Miller *et al.*, 1998).

We recently identified some potential signaling pathways involved in the regulation of COX-2 gene expression in phenotypically stable human chondrocytes (Miller *et al.*, 1998). Okadaic acid is a specific serine/threonine phosphatase (type I and 2A) inhibitor. The inhibition of phosphatase-1 (PP-1)/phosphatase-2A (PP-2A) results in a shutdown of MEKK1/MEK1/ERK cascade probably as a result of an increase in PKA activity, which is known to inhibit Raf-1, a MEKK1 kinase. By contrast, there is a concomitant activation of the other proline-directed MAP kinase pathway, MEKK1/JNKK/SAPK/JNK, in addition to increased levels of PKA (Miller *et al.*, 1998). While studying the role of PKC-α (major conventional isoform in chondrocytes) in the control of COX-2 gene expression in human chondrocytes, we observed that CalC mimicked the stimulatory effects of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). So we decided to use a highly selective PKC inhibitor like CalC, that is a polycyclic hydrocarbon (perylenequinones) isolated from *Cladosporium cladosporioides* which binds specifically and with high affinity to the DAG site in the regulatory (unique) domain of PKC (Bruns *et al.*, 1991) as a probe to evaluate the expression of COX-2 in human chondrocytes. When co-incubated, CalC and PMA produced additive effects in terms of COX-2 expression in human, phenotypically stable chondrocytes. The inhibitor has been used extensively and has proven to be a useful probe in the study of PKC-dependent signaling pathway and gene expression (Das and White, 1997; Xu and Clark, 1997). Nothing is known about the signal transduction pathways controlling COX-2 expression in human chondrocytes treated with CalC and this project represents novel and original observations. We hypothesize, that CalC up-regulates COX-2 gene expression by inducing AP-1 synthesis which in

turn transactivates genes either containing AP-1 sites in their promoters or containing other enhancer elements that bind AP-1 (e.g. COX-2 and the CRE).

The specific aims of my research were 1) to determine whether CalC could indeed up-regulate AP-1 expression and synthesis, TRE binding, and AP-1 transactivation of c-jun gene through the major TRE site to explain some of the biochemical effects of CalC in human chondrocytes. 2) to elucidate the signal transduction pathway controlling COX-2 gene expression, and, in particular, the role of PKC with the aid of a specific pharmacological inhibitor CalC and activator, PMA.

# CHAPTER TWO: ARTICLES

# CALPHOSTIN C INDUCES AP1 SYNTHESIS AND AP1-DEPENDENT *C-JUN* TRANSACTIVATION IN NORMAL HUMAN CHONDROCYTES INDEPENDENT OF PROTEIN KINASE C-a INHIBITION: ROLE OF C-JUN N-TERMINAL KINASE.

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**Running title:** Activator protein 1 (AP1) induction by Calphostin C.

**ABSTRACT**:

The specific PKC inhibitor, calphostin C (CalC), has anti-cancer and anti-arthritic properties attributable to the arrest of the cell cycle and induction of apoptosis. We explored a biological rationale for these properties of CalC by examining its effects on proto-oncogene expression, specifically AP1 complexes, in normal human chondrocytes in comparison with the tumour promoter, phorbol ester (PMA). Exploratory studies confirmed the anti-PKC effects of CalC since equal molar concentrations of CalC blocked the PMA-induced translocation of PKC-a from the cytosolic to the membrane fraction. CalC induction of AP1, as judged by gel-shift analysis using a consensus AP1 oligonucleotide, was bi-phasic with an initial, rapid increase (maximum 4h), followed by a decline reaching its nadir after 16h, and finally a major up-regulation phase which plateaued at 24 h. Maximum induction of AP-1 was reached at a concentration of 250 nmol/L of CalC. CalC did not block PMA-induced AP1 synthesis. Gel shift analysis in the presence of specific antibodies to c-Jun, JunB, JunD, c-Fos, and CREB/ATF revealed that the AP1 complexes were probably c-Fos/c-Jun, c-Fos/JunB, or c-Jun/JunB dimers. Northern analysis confirmed that *c-jun* , *jun* B and *c-Fos* were the principal proto-oncogenes induced by CalC. To confirm that *c-jun* induction occurs at the transcriptional level and to examine the role of the AP1 site present in the *c-jun* promoter in the induction of *c-jun* by CalC, we performed transient transfections of *c-jun* promoter-CAT constructs harbouring either wild-type (WT) AP1 regulatory element sites or mutant AP1 sites. CalC (250 nmol/L) induced a marked increase in CAT activity (i.e. promoter activation) with WT AP1 *c-jun* promoter-CAT plasmids, but the response was completely abrogated when using constructs where the AP1 site was mutated. PMA produced similar results but the induction of the WT AP1 *c-jun* promoter-CAT plasmid was smaller. CalC (250 nmol/L) inhibited MAPK (p42/44) activity while stimulating c-Jun N-terminal kinase activity in a time-frame co-

incident with the activation of AP1. We conclude that CalC induces signalling pathways that activate AP1 and transactivate genes harbouring AP1 enhancer sites independent of PKC. We submit that the reported anti-tumourogenic and anti-proliferative effects of CalC may not be related to PKC inhibition.

**Key words**: Chondrocytes, Calphostin C, c-Jun N terminal kinase, AP1, protein kinase C.

**INTRODUCTION:**

Activator protein-1 (AP1) is a DNA sequence-specific transcriptional activator and an important mediator of cellular responses to growth factors, serum, and tumour-promoting phorbol esters (Angel et al, 1987; Curran and Franza, 1988). AP1 is a dimeric protein consisting of c-Jun (c-Jun, JunB, JunD) and c-Fos (Fra-1, FosB, c-Fos) family members that enhances gene transcription in promoters harbouring the so-called phorbol ester response element (TRE) having the consensus sequence TGA(C/G)TCA (Curran and Franza, 1988; Angel et al, 1988, Nakabeppu et al, 1988). Whereas c-Jun proteins can form stable homodimers, c-Fos proteins cannot and must complex with c-Jun family members. Furthermore, c-Jun proteins can heterodimerize with CREB/ATF family members as well and the latter heterodimers can transactivate target genes at TRE sites (Benbrook and Jones, 1994; Gupta et al, 1995).

Following induction by phorbol ester or UV light, AP1 binds rapidly (30 min to 2 h) to the TRE and increases promoter activity in target genes (e.g. *c-jun*, collagenase) without any increase in the synthesis of c-Jun or c-Fos (Angel et al, 1987). This is the result of post-translational modifications involving the specific phosphorylation of amino acid residues (e.g. N-terminal ser-63 and ser-73) of c-Jun and c-Fos (C-terminal thr 232) which endow the proteins with increased transcriptional activity (Curran and Franza, 1988). The *c-jun* promoter has a rather uncomplicated structure with most of its inducers operating through the *c-jun* TRE. Activation of AP1 leads to auto up-regulation of the *c-jun* gene through the TRE site and amplification of the original signal (Angel et al, 1988).

AP1 phosphorylation may be accomplished by a group of proline-directed mitogen-activated protein kinases (MAP) (Karin, 1995; Hunter and Karin, 1992), called Jun N-terminal, stress-activated kinase (JNK/SAPK). c-Fos, in fact is not a

good substrate for JNK but instead is phosphorylated specifically and productively by the Fos regulating kinase (FRK) (Deng and Karin, 1994). Depending on the cell type, the activity of JNK may be controlled by $Ca^{++}$ and protein kinase C (PKC)-dependent pathways (Su et al, 1994). Protein kinase C represents a family of isoenzymes with at least 11 members (categorized as conventional (4) and novel (8) isoforms) and are single polypeptides comprised of an N-terminal regulatory region and a C-terminal catalytic domain (Dekker et al, 1995). Maximizing the activity of conventional isoforms of PKC is dependent on the presence of $Ca^{++}$, phospholipids (principally phosphatidylserine, PS) and diacylglycerol (DAG), which in turn are generated in the cell by a diversity of transduction mechanisms promoting lipid hydrolysis (Nishizuka, 1992; Kiley and Jaken, 1994; Newton, 1995). Phorbol esters are potent inducers of PKC by virtue of their affinity for the DAG site in the regulatory domain of the enzyme (Newton, 1995), and these observations have linked directly and indirectly PKC activation to AP1 phosphorylation and promoter transactivation (Su et al, 1994).

Though the regulatory mechanisms are quite complex, involving a number of steps including multiple auto and trans phosphorylations, a hallmark of conventional PKC activation is the translocation of the mature form from the cytoplasmic compartment to the membrane (Newton, 1995). Membrane translocation is mediated by DAG ( and phorbol esters) and PS binding to the cognate domains in the enzyme (Kiley and Jaken, 1994; Newton, 1995). Protein kinase C activation and translocation can be blocked by a group of highly selective PKC inhibitors like Calphostin C (CalC). Calphostin C is a polycyclic hydrocarbon (perylenequinones) isolated from *Cladosporium cladosporioides* which binds specifically and with high affinity ($IC_{50}$=50 nmol/L) to the DAG site in the regulatory (unique) domain of PKC (Kobayashi et al, 1989; Bruns et al, 1991). The inhibitor has been used extensively

and has proven to be a useful probe in the study of PKC-dependent signaling pathways and gene expression (Das and White, 1997; Xu and Clark, 1997). Furthermore, it has been cited for its potential as a useful drug in cancer treatment and various forms of arthritis because it is pro-apoptotic in many cell types (Lee et al, 1995; Yang et al, 1995; Kobayashi et al, 1989). A biochemical rationale for the action of CalC in this regard remains to be clarified although proto-oncogene expression (e.g.*c-jun*) has been implicated (Gamou and Shimizu, 1994; Freemerman et al, 1996).

While studying the role of PKC-a (major conventional isoform in chondrocytes) in the control of cyclooxygenase -2 (COX-2) gene expression in human chondrocytes (Miller et al, 1998 in press, and unpublished observations), we observed that CalC mimicked the stimulatory effects of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). When co-incubated, CalC and PMA produced additive effects in terms of COX-2 expression in human, phenotypically stable chondrocytes.

Since AP1 is known to mediate gene induction of COX-2 (Xie and Herschman, 1995; Miller et al, 1998 in press) in many cell types, we sought to determine whether CalC could indeed up-regulate AP1 expression and synthesis, TRE binding, and AP1 transactivation of *c-jun* gene through the major TRE site as a rationale to explain some of the biochemical effects of CalC.

## RESULTS

### Calphostin C (CalC) inhibits PMA-induced PKC-α translocation in human chondrocytes

We previously reported that CalC inhibits PMA-induced PKC activity in human chondrocytes in culture (Di Battista et al, 1996). With the use of specific antibodies (conventional PKC-α, ßI, ßII, g), we determined that PKC-α is the major conventional identifiable isoform in chondrocytes on a quantitative basis, although there were trace levels of PKC-ßI (data not shown). In order to test the response of chondrocytic PKC-α to phorbol ester treatment, primary human chondrocytes were incubated with 300 nmol/L of PMA for 0 to 60 min. PMA (300 nmol/L) induced a time-dependent depletion of the PKC-α protein from the cytosolic fraction which began after 2-3 min and was nearly complete after 60 min. There was a concomitant, reciprocal accumulation in isolated membrane fractions of PKC-α (Fig. 1A). When CalC (250 nmol/L) was pre-incubated with chondrocytes in culture for 1h prior to the addition of PMA, PKC-α translocation was prevented (Fig. 1B).

### Calphostin C (CalC) induces AP1 activity in nuclear extracts

CalC induction of AP1, as judged by gel-shift analysis using a consensus AP1 oligonucleotide, was bi-phasic with an initial, rapid cycloheximide insensitive increase (maximum 4h), followed by a decline reaching its nadir after 16h, and finally a major up-regulation phase which plateaued at 24 h (Fig.2A). Maximum induction of AP1 was reached at a CalC concentration of 250 nmol/L of CalC (Fig. 2B). PMA (300 nmol/L) increased AP1 binding activity, but this latter response was not inhibited by preincubation for 1h with CalC (250 nmol/L) (Fig. 2C). Radioinert AP1/TRE consensus oligonucleotide (B, lane 7) and *c-jun* AP1/TRE oligonucleotide

(C, lane 5) displaced the shifted bands completely. Gel shift analysis with specific antibodies to c-Jun, JunB, JunD, c-Fos, and CREB/ATF revealed that the AP1 complexes were probably c-Fos/c-Jun, c-Fos/JunB, or c-Jun/JunB dimers as the AP-1 shift patterns were displaced by these respective antibodies and not by JunD or CREB/ATF antibodies (Fig.2D). There were no "supershift" patterns to slower moving complexes as such suggesting that these antibodies compete or titrate their cognate proteins and reduce homo/heterodimer formation and binding to the AP1 oligomer. Treatment of chondrocytes with CalC did not significantly increase binding of nuclear extracts to NF-κB, c/EBP, SP1, OCT-1, or AP2 consensus oligonucleotides (data not shown).

**Calphostin C (CalC) stimulates *c-jun* and *jun*B mRNA expresson**

Although AP1 complexes are often composed of the gene products of *c-jun* and *c-fos*, other members of the latter protooncogene families may also be present. We wished to confirm and extend our studies on the identity of the AP1 complexes by mRNA analysis of CalC-treated chondrocytes. Calphostin C upregulated the expression of *c-jun* and *c-fos* protooncogene mRNA expression (Fig.3) in a time-dependent fashion beginning at about 4h, with the zenith being reached at 8h and declining thereafter (Fig.3). *Jun* B increased gradually with a peak being observed at 24h but was followed by a precipitous decline so that after 48h there were no detectable *jun*B transcripts (Fig. 3). There was no similar upregulation of *Jun*D or *fra*-1 mRNA, and cycloheximide treatment did not inhibit *c-jun* mRNA expression but, in fact, induced a small super-induction (data not shown).

**Induction of *c-jun* promoter constructs by Calphostin C (CalC)**

To confirm that *c-jun* induction occurs at the transcriptional level and to examine the role of the AP1 site present in the *c-jun* promoter in the induction of *c-*

*jun* by CalC, we performed transient transfections of *c-jun* promoter-CAT constructs harbouring either wild-type (wt) AP1 regulatory element sites or mutant AP1 sites. CalC (250 nmol/L) induced a marked increase in CAT activity (i.e. promoter activation) with wt AP1 *c-jun* promoter-CAT plasmids, but the response was completely abrogated when constructs where the AP1 site was mutated were used (Fig. 4). PMA (300 nmol/L) caused a somewhat smaller increase in the level of CAT activity, and the increase was not abrogated by co-incubation with CalC (Fig. 4).

**Calphostin C inhibits MAP kinase (p42/44) activity but stimulates JNK/SAPK**

In order to explore the possibility that CalC may stimulate non-PKC signaling pathways associated with AP1 activation (directly or indirectly), we examined the effects of the inhibitor on two distinct proline-directed MAPK cascades, namely the MAP kinase pathway and JNK/SAPK pathway. To do so, we measured the activity of last kinase in each of the cascades, MAP kinase p42/44 also known as ERK1 and ERK2, respectively, and JNK/SAPK. As shown in Fig. 5, CalC (250 nmol/L) inhibited in a time-dependent fashion the phosphorylation of Elk-1, a specific substrate of MAP kinase. Under the same experimental conditions, however, CalC increased the specific phosphorylation of c-Jun by JNK/SAPK (Fig.5).

## DISCUSSION

We recently identified some potential signaling pathways involved in the regulation of COX-2 gene expression in phenotypically stable human chondrocytes (Miller et al, 1998 in press). This was achieved with the use of two specific serine/threonine phosphatase (type 1 and 2A) inhibitors, namely okadaic acid (OKA) and calyculin A, which provided a cellular environment favouring an increased level of protein phosphorylation. One major MAP kinase cascade, the Raf/MEKK/MEK/ERK pathway, was inhibited by treatment with the phosphatase inhibitors while another, the MEKK1/JNKK/JNK/SAPK pathway, was fully activated. In addition, PKA activity was stimulated, which was probably responsible for the Raf/MEKK/MEK/ERK pathway shutdown because PKA inhibits Raf kinase activity (Cook and McCormick, 1993). Two major transcription factor families were activated as a result of the activation of MEKK1/JNKK/JNK/SAPK and PKA pathways, namely AP1 and CREB/ATF, respectively. These latter transacting factors play a pivotal role in the transactivation of the COX-2 gene in human chondrocytes by binding to the CRE in the promoter region (Xie and Herschman, 1995; Inoue et al, 1994). Interestingly, PKC was not activated by OKA treatment, which was at first puzzling since PMA, a potent activator of PKC, strongly increases AP1 and COX-2 synthesis. In order to resolve this conundrum, we designed the present set of experiments to elucidate the role of PKC with the use of CalC, a specific inhibitor of the enzyme which binds not to the catalytic region, as most inhibitors do, but to the regulatory domain thereby increasing specificity immeasureably (Kobayashi et al, 1989; Bruns et al, 1991). To our surprise, CalC mimicked the action of PMA in terms of AP1 synthesis while inhibiting the translocation of the principal chondrocyte PKC isoenzyme, PKC-α. Furthermore,

CalC could induce transactivation through an AP1 site in the *c-jun* promoter. Although CalC has been shown to induce *c-jun* and *c-fos* mRNA in other cell types (Gamou and Shimizu,

1994; Freemerman et al, 1996), to our knowldge this is the first demonstration that the inhibitor can induce AP1 activation.

We interpreted our results to mean that CalC acts on signaling pathways independent of PKC to induce AP1. Recent evidence suggests that CalC can activate glycogen synthase kinase-3 (GSK-3) A431 at concentrations that inhibit PKC, but is inhibitory at higher concentrations (Lee and Yang, 1996). The latter data provided us with interesting possibilities since GSK-3 can indeed phosphorylate c-Jun (Boyle et al, 1991). However, the kinase phosphorylates a threonine residue (239) in a domain of c-Jun that inhibits its ability to transactivate target promoters (Boyle et al, 1991). Furthermore, GSK-3 is cytoplasmic making it an unlikely candidate for a physiological nuclear c-Jun protein kinase (Hunter and Karin, 1992). On the other hand, co-expression of GSK-3α or ß with c-Jun decreases its ability to transactivate an AP1-dependent reporter gene (de Groot et al, 1992), suggesting that GSK-3 may play a role in regulating c-Jun in vivo, although whether this occurs via direct c-Jun phosphorylation is unclear.

Some of the most important kinases regulating AP1 DNA binding and transactivating ability are the MAP kinases, specifically JNK/SAPK, FRK, and possibly ERK1,2 (Karin , 1995). They are capable of phosphorylating c-Jun and family members at the correct Ser residue (s) in the transactivation domain of the protein (Kallunki et al, 1996). The activity of these latter kinases are controlled by upstream kinases such as MEKK and JNNK/SEK (Cobb and Goldsmith, 1995). It

would seem that CalC increases AP1 DNA binding and transactivating ability by stimulating JNK/SAPK activity; ERK1and 2 were inhibited by CalC under our experimental conditions and thus can be ruled out as mediators of AP1 activation. C-*fos* mRNA was induced by CalC, and may be the major protooncogene stimulated, although c-Fos is a poor substrate for JNK/SAPK (Deng and Karin, 1994). Since the inhibitor has the ability to activate proline-directed kinases, it is possible that FRK would also be stimulated in tandem with JNK/SAPK, although we have previously shown that the cellular levels of FRK in human chondrocytes are quite low (unpublished observations).

Our results suggest that CalC increases *c-jun* promoter activity though a TRE site since a mutation in the sequence completely abrogated the response (in addition to evidence above). The TRE site in the *c-jun* promoter differs by one base pair from the consensus TRE and, though it has a high affinity for conventional AP-1 dimers, c-Jun/ATF2 dimers are more efficiently bound (Angel et al, 1987; Gupta et al, 1995). We have no evidence that CalC can stimulate the synthesis and phosphorylation of CREB/ATF family members and, in fact, preliminary data in our laboratory seem to suggest an inhibitory pattern with respect to CREB and ATF, at least on a short- term basis (unpublished observations). However, conventional AP1 complexes can still potently transactivate through the TRE site in a manner quite similar to c-Jun/ATF2, and this fact may explain our results with CalC on *c-jun* promoter activation.

Calphostin C was classified as a specific PKC inhibitor based on *in vitro* studies in which the inhibitor was reacted with purified preparations of various conventional isoforms of PKC (Kobayashi et al, 1989). It was subsequently shown that photoactivation was essential for maximum inhibitory activity (Bruns et al, 1991). There were however, reports that CalC was not active with intact cells in

culture (Gamou and shimizu, 1994), and many of its effects were due to cytotoxicity. We have shown that CalC inhibits PKC activity *in vitro* and *in vivo* (Di Battista et al, 1996) and, in this present study, we show that the inhibitor blocks cytoplasmic to membrane translocation of PKC-α in human chondrocytes in culture. Though we have identified another PKCß I isoform expressed in chondrocytes, we have no evidence of its contribution to cellular PKC activity or of its sensitivity to CalC under our experimental condition.

The use of CalC with cells in culture have led to observations of cell toxicity, which may be not surprising since, in the presence of light, the molecule reacts with molecular oxygen to form free radicals. This can lead to membrane destruction and cell death. Other studies have demonstrated an effect of CalC akin to apoptosis, particularly in human glioma cells (Ikemoto et al, 1995; Freemerman et al, 1996). Ikemoto et al showed that the inhibitor (100 nmol/L) blocked PKC activity within 2-8h and then stimulated $Ca^{++}/Mg^{++}$-dependent endonuclease activity after 16 to 24h , a harbinger of DNA fragmentation and apoptosis. Furthermore, there was a down-regulation of the protooncogene Bcl-2 (anti-apoptotic) expression and synthesis prior to cell death. Our cultures of human chondrocytes are more resistant to CalC than glioma cells to the extent that, even at concentrations up to 600 nmol/L, we observed no cellular toxicity. A preliminary check of cell extracts of human chondrocytes revealed firstly, that the cells produce relatively large amounts of Bcl-2 and secondly, the levels are unaffected by CalC treatment (unpublished observations).

From the pathophysiological point of view, the net effect of activation of the PKC-dependent signaling cascade on cartilage tissue homeostasis is still unclear. However, recent evidence obtained from animal models of osteoarthritis suggests that PKC activation is chondroprotective (Hamanishi et al, 1996; Kimura et al,

1994). For example, the synthesis of proteoglycans (PG), which are basic building blocks of cartilage matrix, is stimulated by phorbol esters. Transfection of chondrocytes with a PKC-a expression vector resulted in markedly elevated PG synthesis (Kimura et al, 1994). The stimulation of keratan sulfate production by a histamine H1 agonist was inhibited by a PKC inhibitor and activated by phorbol ester (Fukuda et al, 1991). It was recently observed that the PKC-a isoenzyme appears in larger quantities in osteoarthritic chondrocytes relative to normal chondrocytes (Hamanishi et al, 1996). Interestingly, activation of PKC abrogated the IL-1-induced stimulation of PG breakdown and inhibition of PG synthesis by cartilage in organ culture (Arner et al, 1991).

In summary, CalC can induce AP1 synthesis and transactivation of AP1 sensitive target genes in human chondrocytes probably through the stimulation of JNK/SAPK kinase activity. With the demonstration of CalC-induced inhibition of the MAPK pathway, our results add to a growing list of signalling cascades affected by CalC that are PKC-independent.

# MATERIALS AND METHODS

## Chemicals

Diethylpyrocarbonate (DEPC), polyvinylpyrrolidone (PVP), Ficoll, salmon testes DNA, ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), sodium acetate, ethidium bromide (EtBr), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Calphostin-C and PMA were supplied by Calbiochem (San Diego, CA). Bio-Rad (Mississauga, Ont) supplied acrylamide, bis-acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), bromophenol blue, coommassie blue G-250, and glycerol. Dulbecco's modified Eagles' medium (DMEM), HEPES, heat-inactivated fetal calf serum (FCS), stock antibiotic/antimycotic mixture (10,000 units/ml of penicillin base, 10,000 μg/ml of streptomycin base, and 50 μg/ml of amphotericin-B), agarose and phenol came from GIBCO BRL (Gaithersburg, MD). Fisher-Scientific (Montréal, Québec, Canada) provided the TRIS (tris-(hydroxymethyl)-aminomethane), NaCl, $MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, isopropanol, acetic acid, methanol and ethanol.

## Cell culture

Normal cartilage from tibial plateaus and femoral condyles were obtained at necropsy from the knee joint of human cadavers within 12 hours of death. To ensure that only normal tissue was used, cartilage specimens were examined thoroughly, both macroscopically and microscopically. Only those with neither lesions nor alterations were processed further. Human chondrocytes were released from articular cartilage by sequential enzymatic digestion as previously described (Di Battista et al, 1991). Tissue specimens were incubated at 37°C with filter-sterilized solutions of pronase or trypsin (1 mg/ml, 1.5 h) and collagenase (1 mg/ml, 6 h) in

DMEM containing 10% heat-inactivated FCS and an antibiotic mixture (100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/mL amphotericin ß). Cell viability was determined by the trypan blue exclusion test. In order to maintain a terminally differentiated chondrocyte phenotype, primary cultures were seeded at high density, and were used at a confluent and stationary phase (1-2 days).

**Northern blot analysis of mRNA**

Total cellular RNA was isolated (1 x $10^6$ cells = 10-20 µg RNA) using the Trizol (Gibco) reagent. Generally, 10-15 µg of total RNA was resolved on 0.9% agarose-formaldehyde gel and transferred to Hybond-N™ nylon membranes (Amersham Canada Ltd. Oakville, ON.) in 20 X SSC buffer, pH 7 by vacuum blotting. After prehybridization for 24 h, hybridizations were carried out at 50-57°C (depending on the gene) for 24-36 hrs, followed by high-stringency washing as previously described (Di Battista et al, 1994). The following probes, labelled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization: Human *jun* B and D were 1.6 and 1.0 EcoRI and AccI/EcoRI cDNA fragments, respectively, from pBluescipt SK-, and were kindly provided by Dr. D. Skup (Louis-Charles Simard Research Center, Montreal, Quebec, Canada). The mouse *c-jun* 1.8 kb cDNA probe, provided by Dr. D. Edwards (University of Calgary, Alberta, Canada) was extracted from a pUC19 plasmid using EcoRI. The 1.3 kb cDNA mouse *c-fos* probe was originally cloned into pGEM II (Dr. J.P. Pujol, Université de Caen, France) and was excised for labelling using Pst I and BclI. The mouse *fos-B* 1.6 cDNA probe was kindly provided by Dr. D. Skup. All mouse probes were previously shown to hybridize specifically with human mRNA (Miller et al, 1998 in press). All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA) for semi-quantitative measurements, with

the relative amount of test mRNA normalized to the level of 28S/18S rRNA (negative image of EtBr staining pattern of membrane). Calphostin C modulates the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), actin and tubulin mRNA in a time dependent fashion and, as such, they are ill-suited as controls. The expression is stable for up to 2 h but increases to 125-140% of the control values after 24h (data not shown).

**Preparation of cell extracts and Western blotting:**

Isolation of cytosolic and particulate fractions of PKC was conducted according to previously described methods (Rzymliewicz et al, 1996). Fifty-100 μg of cellular extract (in RIPA 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 sodium orthovanadate, and 1 mM NaF) from control and treated cells were then subjected to SDS-PAGE through 10% gels (final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham). Following blocking (with 5% BLOTTO) and washing, the membranes were incubated for either 2 h at RT, or overnight at 4$^{o}$C, with primary antibodies (see below) in TTBS containing 0.25% BLOTTO. Second anti-rabbit or anti-mouse antibody-HRP conjugates (1:2000 dilutions) were subsequently incubated with membranes for 1 h at RT, and then washed extensively for 30-40 min with TTBS, and a final rinsing with TBS at RT. Following incubation with an ECL chemiluminescence reagent (Amersham), membranes were prepared for autoradiography and exposed to Kodak X-Omat film, then subjected to laser scanning densitometry for semi-quantitative analysis. PKC isoenzyme antibodies are products of Santa Cruz Biotechnology (Santa Cruz, CA) and were used at dilutions suggested by the manufacturer.

**Gel-retardation experiments**

Control and treated chondrocytes in 4 well cluster plates (3-5 x $10^6$ cells/well) were carefully scraped into 1.5 ml of ice-cold PBS and pelleted by brief centrifugation as previously described (Ney et al, 1990). The cellular pellet was gently resuspended in 200-400 µL of ice-cold hypotonic lysis buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Pefabloc$^{TM}$, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 1 mM NaF and 1% Nonidet P-40. Cells were allowed to swell on ice for 10 min, vortexed vigorously for 10 s, and nuclei recovered by brief centrifugation at 3,000 x g for 60 s. The nuclear pellets were resuspended in 25 µl of high salt extraction buffer containing 20 mM HEPES-KOH, pH 7.9, 0.42 M NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM EDTA, 25% glycerol, 0.5 mM Pefabloc$^{TM}$, and 10 µg/ml each of aprotinin, leupeptin and pepstatin, followed by incubation on ice for 45 min with intermittent vortexing. The nuclear extracts were recovered by centrifugation at 16,000 x g for 30 min at 4$^{o}$C and stored at -86$^{o}$C until used.

Double-stranded oligonucleotides containing consensus and promoter specific sequences (synthesized, annealed and purified by HPLC) were end-labeled with [g-$^{32}$P]ATP using T4 polynucleotide kinase (Pharmacia, Montreal, Quebec, Canada). The sense sequences of the oligos used were as follows: AP-1 consensus; 5'-CGC TTG ATG AGT CAG CCG GAA-3': AP-1 *c-jun*; 5'-CGC TTG ATG AGAT CAG CCG GAA-3': NF-kB consensus; 5'-AGT TGA GGG GAC TTT CCC AGG C-3': CRE; 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3': SP-1; 5'-ATT CGA TCG GGG CGG GGC GAG C-3': AP2; 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3': c/EBP; 5'-CAC CGG GCT TAC GCA ATT TTT TTA A-3': OCT-1; 5'-TGT CGA ATG CAA ATC ACT AGA A-3'. 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 15 µg of nuclear extract and 100,000 cpm of $^{32}$P-labeled oligonucleotide probe at 22$^{o}$C for

20 min in a final volume of 10 µl. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried and prepared for autoradiography.

**Analysis of protein kinase activity**

Chondrocytes were incubated with CalC (250 nM) for 0-48h and then rinsed with ice-cold PBS and scraped from the plates, pelleted and extracted into RIPA 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. Generally 20-40 µg of protein was used per assay (linear range 0-100 µg).

The mitogen-activated protein kinase (MAPK, p 44/42) and the stress activated protein kinase (SAPK/JNK) assays were also measured by nonradioisotopic procedures (New England Biolabs, Beverly, MA) by taking advantage of phospho specific antibodies. In the former case, a phospho-specific antibody to MAPKs (Tyr204) were used to selectively immunoprecipitate active MAPKs (p42/44, ERK-1 and ERK-2) from chondrocyte lysates and, the latter immunoprecipitates were then incubated with an Elk-1 (substrate) fusion protein in the presence of ATP and kinase buffer. Phosphorylation of Elk-1 at Ser383 was measured by Western blotting using a phospho-specific Elk-1(Ser383) antibody. Ser383 of Elk-1 is a major phosphorylation site for MAP kinase and is required for Elk-1-dependent transcriptional activity (Karin, 1995; Cobb and Goldsmith, 1995). For SAPK/JNK assays, an N-terminal c-JUN (1-89)-GST fusion protein, bound to glutathione sepharose beads, was used to selectively "pull-down" SAPK/JNK from chondrocytes lysates. The fusion protein is known to have a high-affinity site for SAPK/JNK binding (Kallunki et al, 1996). The beads were then mixed with ATP and kinase buffer, and c-JUN phosphorylation was selectively measured using phospho-specific c-JUN antibody. The latter antibody specifically detects SAPK-induced phosphorylation of c-JUN at Ser63, a site important for c-Jun-dependent transcriptional activity (Kallunki et al, 1996).

**Cell transfections and reporter assays**

Transient transfection experiments were conducted in 60 mm culture dishes with 5 x $10^5$ cells that were serum starved for 24h before use. Transfections were conducted by the calcium phosphate/DNA coprecipitation method followed by glycerol shock for 45 sec. Cells were re-exposed to a complete culture medium for 16h prior to the addition of increasing concentrations of CalC (250 nM) for an additional 20h. Transfection efficiencies were controlled by co-transfection with 0.5 µg of pRSV-ßgal, a ß-galactosidase reporter vector under the control of RSV-LTR promoter. The following promoter constructs were used: *c-jun* promoter: pBLCAT3 vectors with -79/+170 *jun*-CAT and -79/+170 ΔAP1 *jun*-CAT where the wild type (wt) AP1 consensus sequence is mutated (gift from Dr. M. Karin). CAT assays were measured by a specific CAT ELISA (Boehringer Mannheim, Québec, Canada). CAT values were normalized to the level of ß-galactosidase activity.

**Statistical analysis**

All results are expressed as mean ± standard deviation of between 2 to 5 separate experiments. Statistical significance was assessed using the Student's t-test and significant differences were confirmed only when the probability was less than or equal to 5%.

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## LEGENDS

**FIG. 1: Calphostin C (CalC) inhibits PMA-induced PKC-a translocation in human chondrocytes.**

Normal human chondrocytes were seeded at high density ($1.2 \times 10^6$ cells per culture plate well) and confluence (stationary phase) was reached in 1-3 days. (A) The cells were then serum starved for 24h and then incubated with 300 nmol/L of PMA for 0, 2.5, 5, 10, 15, 30, and 60 min. In (B) 250 nmol/L of CalC was pre-incubated with the cells 1h prior to the addition of PMA. Cellular proteins from the cytosolic and particulate fractions were extracted as described in the *Materials and Methods* section and analyzed by Western blotting using a specific anti-human PKC-a antiserum (1:1000 dilution).

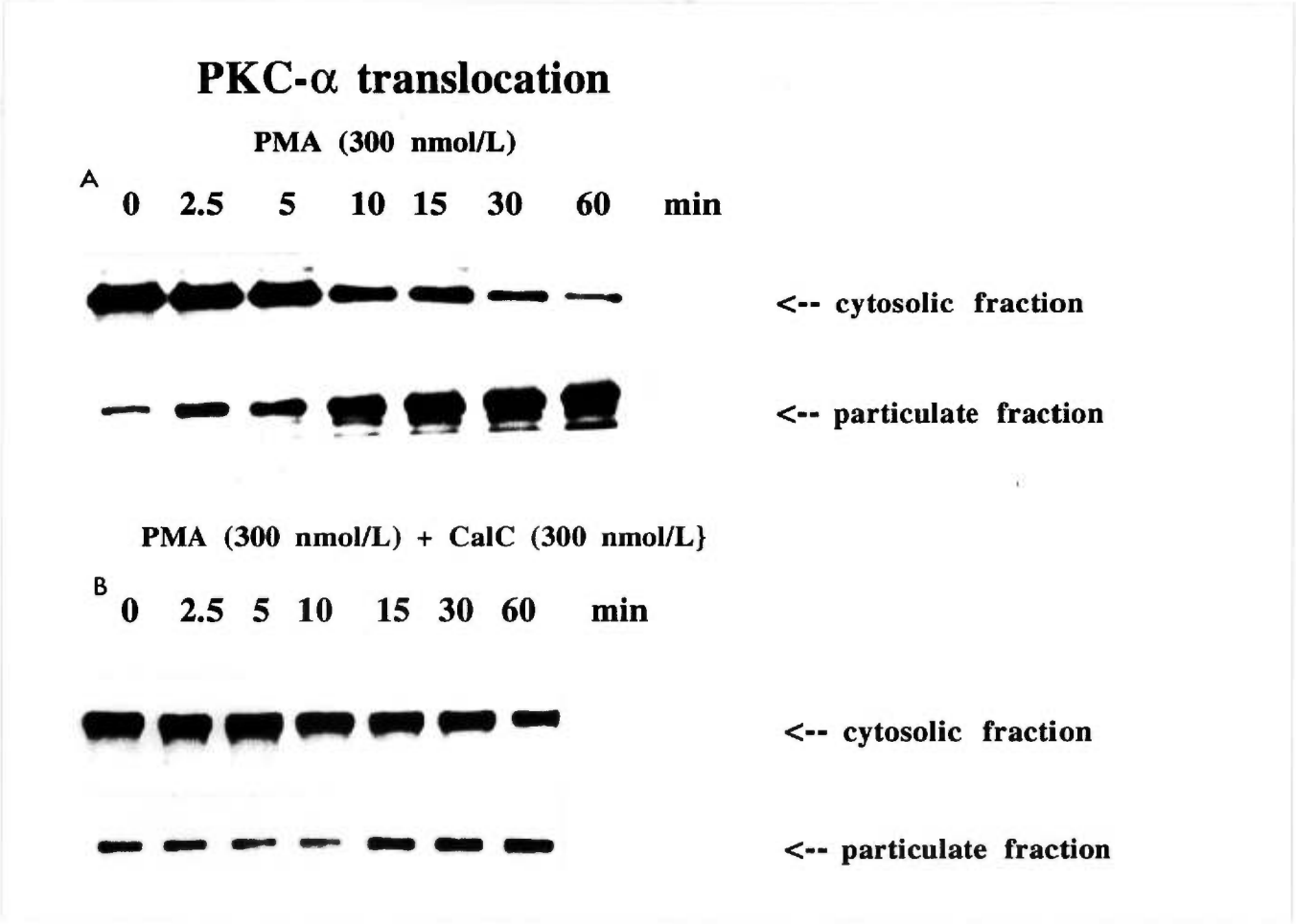

PKC-α translocation
PMA (300 nmol/L)
A
0 2.5 5 10 15 30 60 min
<-- cytosolic fraction
<-- particulate fraction
PMA (300 nmol/L) + CalC (300 nmol/L}
B
0 2.5 5 10 15 30 60 min
<-- cytosolic fraction
<-- particulate fraction

**FIG. 2: Time course and dose-response of CalC induction of AP-1 consensus sequence nuclear binding activity**.

Normal chondrocytes were seeded at high density and confluence was reached in 1-3 days. The cells were then serum starved for 24h prior to experimentation. In (A), cells were incubated with 250 nmol/L of CalC for 0, 1, 2, 4, 8, 16 and 24 h. In (B), dose-response experiments were conducted over a 24h period with 0, 50, 100, 200, 400, and 600 nmol/L. In (C), cells were incubated with vehicle, PMA (P) 300 nmol/L, PMA 300 nmol/L + CalC (C) 250 nmol/L, or CalC 250 nmol/L for 24h. In (D), cells were incubated in the presence or absence of 250 nmol/L of CalC for 24 h. Subsequently, in A, B, C, and D, nuclei were then isolated by hypotonic lysis of the chondrocytes, and proteins extracted into high salt buffer as described in Materials and Methods. Fifteen μgs of nuclear protein were incubated with $^{32}$P-labeled AP1 consensus sequence. In lane 7 of (B, from left), 1 fold molar excess of radioinert homologous AP1 (TRE) consensus oligonucleotide was added to the reaction mixture, while in lane 5 of (C, from left), 1 fold molar excess of radioinert *c-jun* AP1 (TRE) oligonucleotide was added. In lanes 3, 4, 5, 6, and 7 of (D, from left), nuclear extracts were preincubated for 20 min at RT with 2 μg of antibodies against human c-Jun, JunD, c-Fos, JunB, and CREB/ATF, respectively before the addition of radiolabeled probe as described in the *Materials and Methods* section. Mobility shifts were verified on 6% polyacrylamide gels run at 4$^{o}$C followed by gel drying and autoradiography.

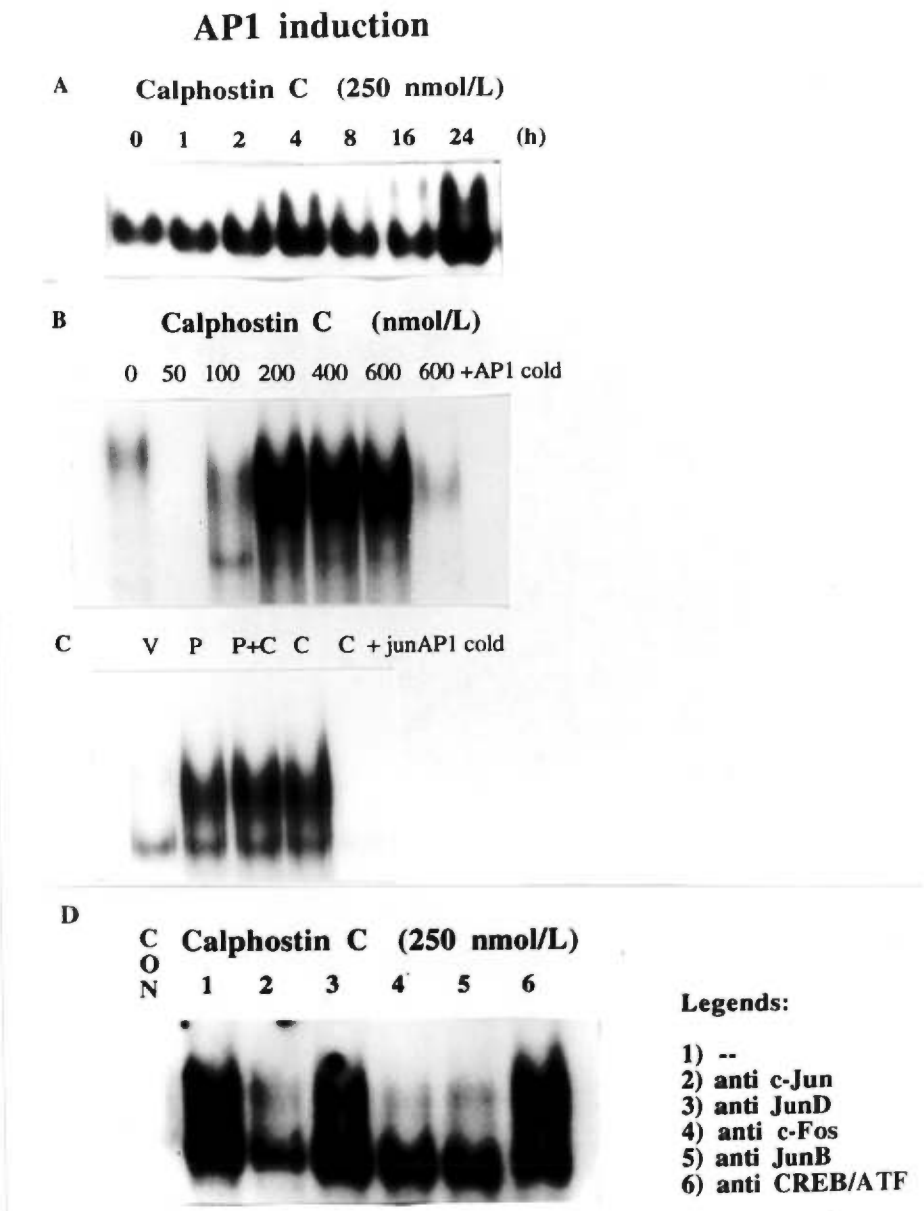

AP1 induction
A
Calphostin C (250 nmol/L)
0 1 2 4 8 16 24 (h)
B
Calphostin C (nmol/L)
0 50 100 200 400 600 600 +AP1 cold
C
V P P+C C C + junAP1 cold
D
CON
Calphostin C (250 nmol/L)
1 2 3 4 5 6
Legends:
1) --
2) anti c-Jun
3) anti JunD
4) anti c-Fos
5) anti JunB
6) anti CREB/ATF

**FIG. 3: Time course of CalC induction of members of the *c-jun* and *c-fos* families of protooncogenes.**

Serum-starved, confluent quiescent chondrocytes were incubated with 250 nmol/L of CalC for 0, 1, 2, 4, 8, 16, 24, and 48h as indicated. RNA was extracted using the TRIZOL reagent and analyzed by Northern blotting using specific probes for *c-jun*, *junB*, and*c-fos*. The ethidium bromide staining of ribosomal RNA is representative of multiple blots used to assess mRNA levels for the proto-oncogenes.

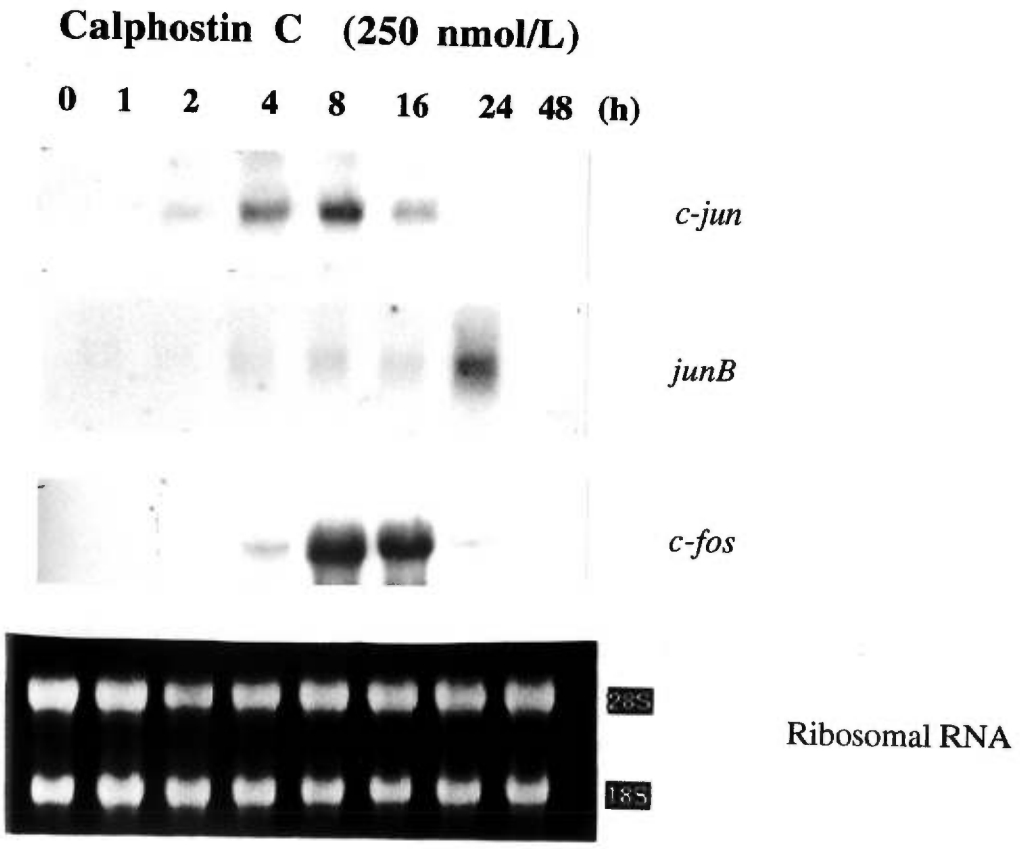

Calphostin C (250 nmol/L)
0 1 2 4 8 16 24 48 (h)
c-jun
junB
c-fos
28S
18S
Ribosomal RNA

**FIG. 4: Induction of c-jun promoter activity by CalC and PMA**

Cells were co-transfected by the calcium phosphate precipitation method with 0.5 μg each of a plasmid expressing ß-galactosidase (control of transfection efficiency) and the c-*jun*-CAT/AP-1 *jun*-CAT reporter plasmids (2 μg each) . Cells were activated with 250 nmol/L of CalC or 300 nmol/L of PMA for 20h. Cells were then lysed in buffer containing Triton X-100 and the lysates assayed for ß-galactosidase (colorimetry) and CAT activity by ELISA. The c-*jun*-CAT reporter plasmid was constructed by inserting a -79/+170 bp fragment from the promoter region of the human c-*jun* gene into the pBLCAT3 vector upstream from the CAT gene. A second reporter plasmid, identical to the previous one except that the AP-1 site (5' -GGTGACATCAT-3' ) was mutated to 5' -GGATCCACCAT-3' , was used to determine the role of the AP-1 site in the induction of c-*jun* promoter activity by CalC and PMA. Values represent the amount (in OD units) of CAT protein in cell extracts, and are the means of 3 determinations in duplicate. Intra and interassay coefficients of variation reached a maximum of 9% and 16%, respectively. Probability values (p) from Student's t-test: vehicle versus 250 nmol/L of CalC and 250 nmol/L of CalC + 300 nmol/L of PMA (*) was $< 0.001$. (**) vehicle versus 300 nmol/L of PMA, $p < 0.01$.

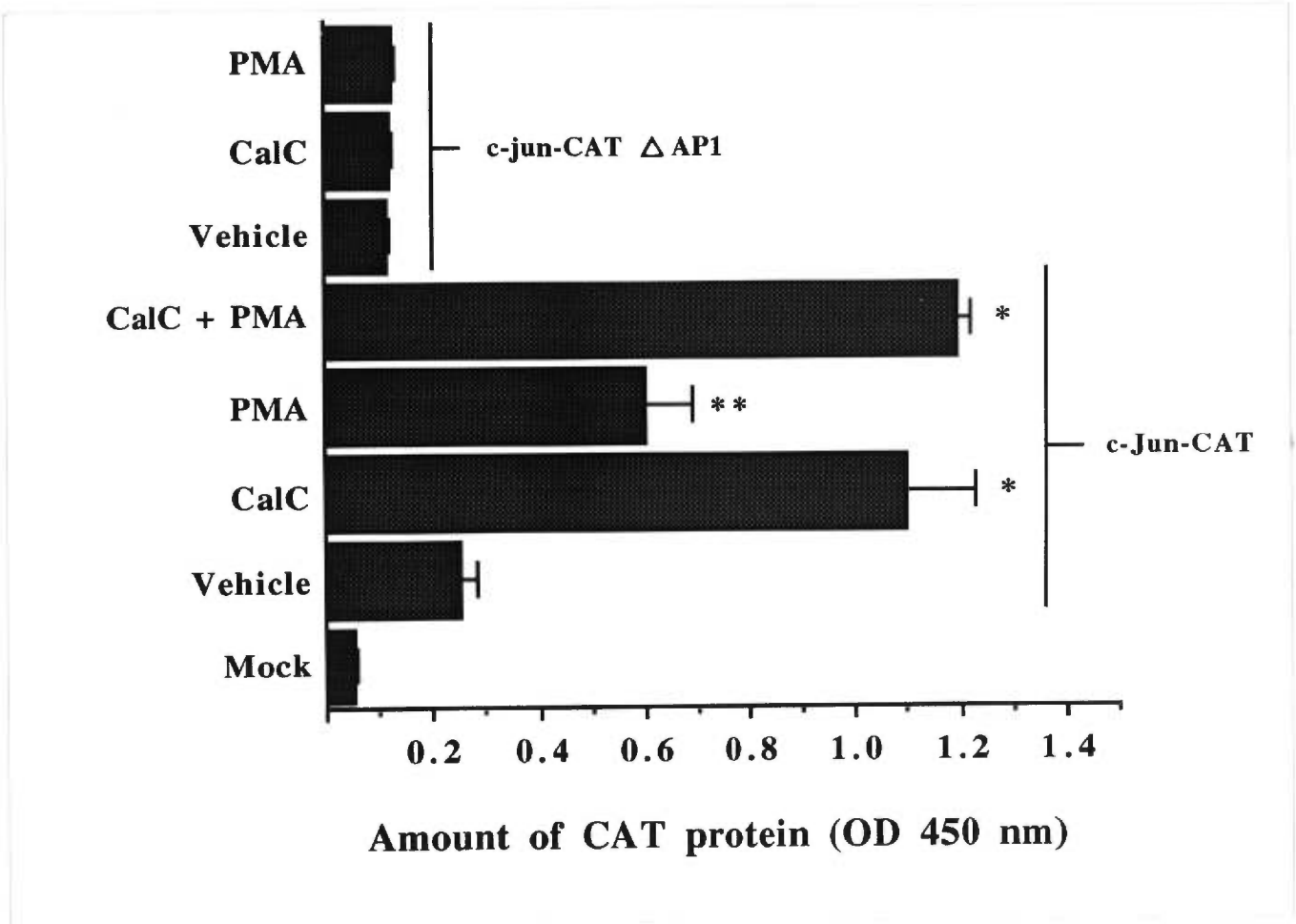
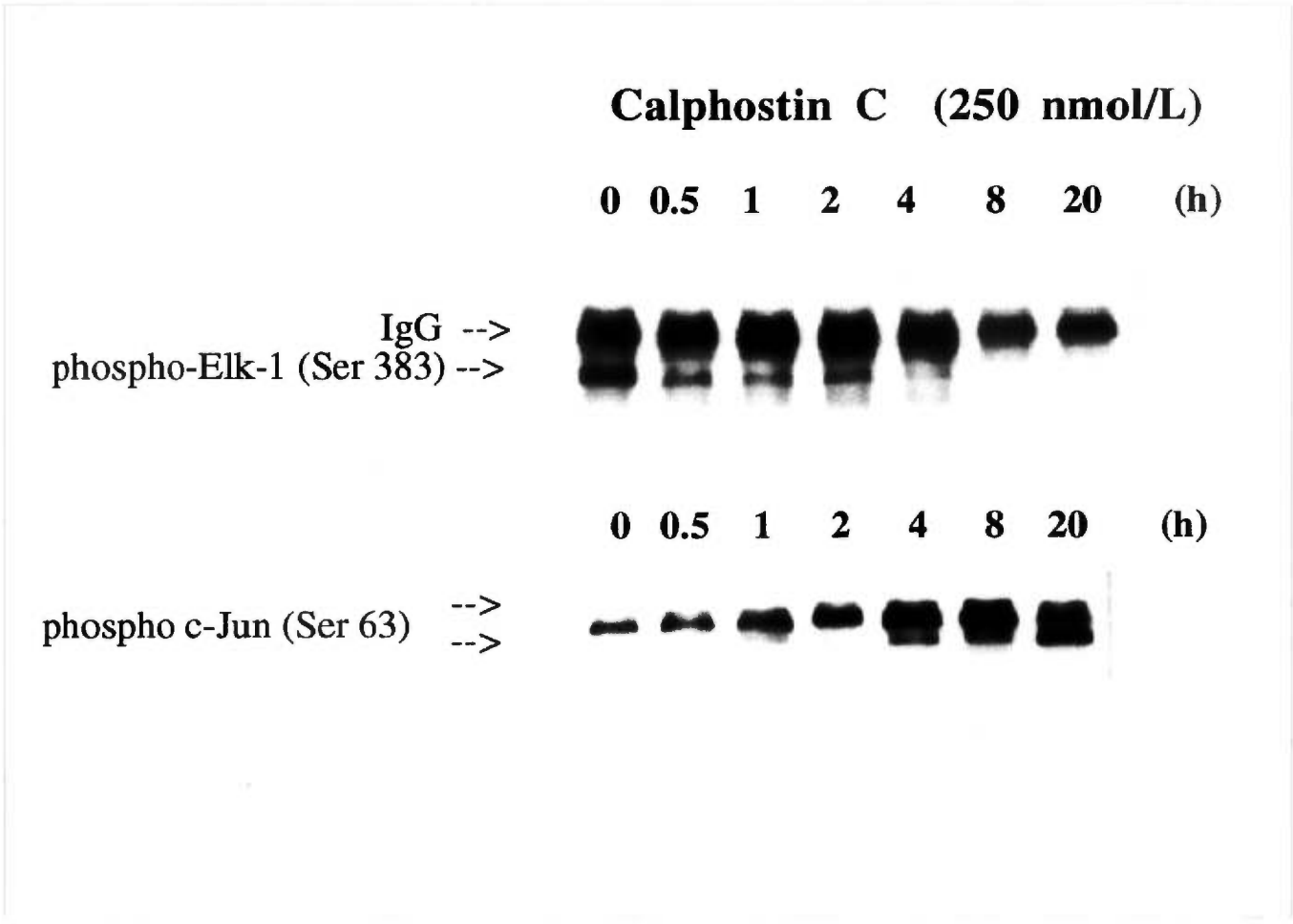

PMA
CalC
Vehicle
CalC + PMA
PMA
CalC
Vehicle
Mock
c-jun-CAT Δ AP1
c-Jun-CAT
*
**
*
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Amount of CAT protein (OD 450 nm)

**FIG. 5: Time course of CalC inhibition on mitogen-activated protein kinase (MAPK p42/44/ERK1,2) and stimulation of c-Jun N-terminal-stress activated protein kinase (JNK/SAPK).**

Serum-starved, confluent quiescent chondrocytes were incubated with 250 nmol/L of CalC for 0, 0.5, 1, 2, 4, 8, 20h. Cellular proteins were extracted into RIPA buffer (plus phosphatase and protease inhibitors) and analyzed for MAPK (p42/44)/ERK1,2 and JNK/SAPK activity as described in Materials and Methods. Specific substrates were an Elk-1 fusion protein, phosphorylated at ser 383 by ERKs and a c-Jun (1-89 a.a) GST fusion protein, phosphorylated by SAPK/JNK at ser 63 and ser 73. Detection of phosphorylated substrates was achieved using specific anti-phosphosubstrate antibodies.


Calphostin C (250 nmol/L)
0 0.5 1 2 4 8 20 (h)
IgG -->
phospho-Elk-1 (Ser 383) -->
0 0.5 1 2 4 8 20 (h)
phospho c-Jun (Ser 63) -->
-->

# CALPHOSTIN C, A PROTEIN KINASE C INHIBITOR, UP-REGULATES COX-2 GENE EXPRESSION IN NORMAL HUMAN CHONDROCYTES.

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Running title: Cyclooxygenase-2 induction by Calphostin C.

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**ABSTRACT:**

Increased prostaglandin (PG) synthesis may be an important component in the pathogenesis of arthritic diseases and cyclooxygenase-2 (COX-2) is the first induced enzyme in the pathway that leads to prostanoid synthesis. Our aim was to elucidate the signal transduction pathways controlling COX-2 expression, and, in particular, the role of protein kinase C (PKC) with the aid of a specific pharmacological inhibitor calphostin C (CalC) and an activator, phorbol ester (PMA). COX-2 mRNA expression and COX-2 protein synthesis were stimulated in human chondrocytes treated with CalC in a complex kinetic pattern. We observed an initial stimulatory phase reaching a zenith at 8 hours, followed by a rapid decline with a nadir at 16h, and finally another rapid inductive phase with a maximum at 24 h. COX-2 expression remained elevated for up to 48 h. CalC exhibited a narrow dose-response profile with an EC50 of 75 nmol/L, maximum response at 200 nmol/L, and a sharp decline to near control levels at doses up to 600 nmol/L. This decline was not due to cellular toxicity as markers for cell death were not affected (e.g. Bcl-2, lamin).

The CREB/ATF transcription factors modulate COX-2 gene expression, in part, through CRE elements in the proximal promoter region. CalC modulated the cellular levels of phosphoCREB/ATF and CRE nuclear binding proteins, as judged by gel-shift, in a complex kinetic pattern similar to that of the COX-2 gene. We conclude that the COX-2 gene in human chondrocytes may be upregulated through increased gene transactivation by CREB/ATF transcription factors. Moreover, we show for the first time that CalC can influence, on a dose-dependent basis, signalling cascades other than PKC/PKC associated pathways .

**INTRODUCTION:**

Among the cellular responses activated by proinflammatory stimuli, increased prostaglandin synthesis may be of fundamental importance in the etiopathogenesis of many immune and inflammatory diseases (Robinson et al, 1975; Dayer et al, 1976; Mizel et al, 1986; Crofford, et al, 1994). Acting in a paracrine and/or autocrine fashion, $PGE_2$ can initiate and modulate cell and tissue responses involved in many physiological processes affecting essentially all organ systems (Wu, 1996; Samuelsson et al, 1978). The rate-limiting step in the formation of prostanoids is the conversion of phospholipid derived arachidonic acid to $PGH_2$ (which is rapidly converted to $PGE_2$) by cyclooxygenase (COX)[1] (DeWitt, 1991; Picot et al, 1994). Two forms of COX have been identified: a constitutive COX-1 and the inducible COX-2 (Kubuju et al, 1991; O'Banion et al, 1992; Appleby et al, 1994). They are integral, monotopic, endoplasmic-reticulum associated, homodimeric bifunctional enzymes that possess heme-dependent peroxidase and cyclooxygenase activity (Picot et al, 1994).

Regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels and the COX-2 gene (mRNA 4.1 kb) is rapidly induced by tumor promoters, growth factors, cytokines and mitogens in many cell model systems (Crofford, et al, 1994; Kubuju et al, 1991; O'Banion et al, 1992). The COX-2 message has an extensive 3' UTR having at least two distinct polyadenylation sites and 22 Shaw-Kamen 5' -$AUUU_n$-A-3' motifs associated with message stability (Appleby et al, 1994, Beelman and Parker, 1995). Sequence analysis of the 5' -flanking region has shown several potential transcription regulatory sequences, including a TATA box, a C/EBP motif, two AP-2 sites, 3 SP-1 sites, two NF-κB sites, a CRE motif, and an Ets-1 site (no AP-1 site) (Appleby et al, 1994). It is still unclear how the COX-2 gene is regulated by external stimuli in terms of signalling pathways, although the nuclear

transcription factors AP-1, CREB/ATF, c/EBP, and NK-kB are known to mediate many COX-2 gene activating stimuli (Inoue et al 1995 Yamamoto et al 1995). Xie and Herschman's (1995) results indicated that the signal pathway leading to src-induced COX-2 transcription involves both Ras/MEKK-1/JNK and Ras/Raf-1/ERK pathways and we have recently shown that serine/threonine phosphatases, like JNK phosphatase, play a major role in the control of COX-2 gene expression in human chondrocytes (Miller et al, 1998).

Calphostin C is a polycyclic hydrocarbon (perylenequinones) isolated from *Cladosporium cladosporioides* which binds specifically and with high affinity ($IC_{50}$=50 nmol/L) to the DAG site in the regulatory (unique) domain of PKC (Kobayashi et al, 1989; Bruns et al, 1991). The inhibitor has been used extensively and has proven to be a useful probe in the study of PKC-dependent signaling pathways and gene expression (Das and White, 1997; Xu and Clark, 1997). Interestingly, AP-1, known to induced by synthetic PKC activators (e.g. PMA), is also sensitive to CalC. Indeed, we recently showed that CalC could up-regulate AP-1 expression and synthesis, TRE binding, and AP-1 transactivation of the *c-jun* gene through the major TRE site in the proximal promoter region. Thus we believe our data have provided a rationale to explain some of the biochemical effects of CalC previously observed .

We report here, that while studying the role of PKC-α (major conventional isoform in chondrocytes) in the control of cyclooxygenase -2 (COX-2) gene expression in human chondrocytes, we observed that CalC surprisingly mimicked the stimulatory effects of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). When co-incubated, CalC and PMA produced additive effects in terms of COX-2 expression in human, phenotypically stable chondrocytes. We show that the complex temporal pattern of COX-2 induction by CalC is concomitant with the modulation of phosphoCREB/ATF proteins and CRE binding.

## EXPERIMENTAL PROCEDURES:

*Chemicals*

Diethylpyrocarbonate (DEPC), polyvinylpyrrolidone (PVP), Ficoll, salmon testes DNA, ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), sodium acetate, ethidium bromide (EtBr), pyrrolidine dithiocarbamate (PDTC) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Calphostin-C and PMA were the products of Calbiochem (San Diego, CA). Bio-Rad (Mississauga, Ont) supplied acrylamide, bis-acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), bromphenol blue, coommassie blue G-250, and glycerol. Dulbecco's modified Eagles' medium (DMEM), HEPES, heat-inactivated fetal calf serum (FCS), stock antibiotic/antimycotic mixture (10,000 units/ml of penicillin base, 10,000 μg/ml of streptomycin base, and 50 μg/ml of amphotericin-B), agarose and phenol came from GIBCO BRL (Gaithersburg, MD). Fisher-Scientific (Montréal, Québec) provided the TRIS (tris-(hydroxymethyl)-aminomethane), NaCl, $MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, isopropanol, acetic acid, methanol and ethanol.

*Cell Culture*

Normal cartilage from tibial plateaus and femoral condyles were obtained at necropsy from the knee joint of human cadavers within 12 hours of death. To ensure that only normal tissue was used, cartilage specimens were examined thoroughly, both macroscopically and microscopically. Only those with neither lesions nor alterations were processed further. Human chondrocytes were released from articular cartilage by sequential enzymatic digestion as previously described (Di Battista et al, 1991). Tissue specimens were incubated at 37°C with filter-sterilized solutions of pronase or trypsin

(1 mg/ml, 1.5 h) and collagenase (1 mg/ml, 6 h) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and an antibiotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/mL amphotericin ß). Cell viability was determined by the trypan blue exclusion test. In order to maintain a terminally differentiated chondrocyte phenotype, primary cultures were seeded at high density, and were used at a confluent and stationary phase (1-3 days).

*Northern Blot Analysis of mRNA*

Total cellular RNA was isolated (1.2 x $10^6$ cells = 10-20 μg RNA) using the Trizol (Gibco) reagent. Generally, 10-15 μg of total RNA was resolved on 0.9% agarose-formaldehyde gel and transferred to Hybond-N™ nylon membranes (Amersham, Canada Ltd. Oakville, ON.) in 20 X SSC buffer, pH 7 by vacuum blotting. After prehybridization for 24 hrs, hybridizations were carried out at 50-57°C for 24-36 hrs, followed by high-stringency washing as previously described (Di Battista et al, 1994). Human COX-2 cDNA was used for hybridization (1.8 kb, Cayman Chemical Company, Ann Arbor, MI) and was cloned into the EcoRV site of pcDNA 1 (Invitrogen), released by PstI and XhoI digestion and labelled with digoxigenin (DIG)-dUTP by random priming. All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA) for semi-quantitative measurements, with the relative amount of test mRNA normalized to the level of 28S/18S rRNA (negative image of EtBr staining pattern of membrane). CalC modulates the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), actin and tubulin mRNA in a time dependent and, as such, they are ill-suited as controls.

*Preparation of cell extracts and Western blotting:*

Fifty-100 μg of cellular extract (in RIPA 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 sodium orthovanadate, and 1 mM NaF) from control and treated cells were subjected to SDS-PAGE through 10-12% gels (final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham). Following blocking (with 5% BLOTTO) and washing, the membranes were incubated for either 2 h at RT, or overnight at 4°C, with primary antibodies (see below) in TTBS containing 0.25% BLOTTO. Second anti-rabbit or anti-mouse antibody-HRP conjugates (1:2000 dilutions) were subsequently incubated with membranes for 1 h at RT, and then washed extensively for 30-40 min with TTBS, and a final rinsing with TBS at RT. Following incubation with an ECL chemiluminescence reagent (Amersham), membranes were prepared for autoradiography and exposed to Kodak X-Omat film, then subjected to laser scanning densitometry for semi-quantitative analysis. The antibodies used were a polyclonal anti-human COX-2 (Cayman Chemical Co., 1:5000 dilution); anti-CREB-1/CREB-1-phosphate and ATF-1/ATF-1-phosphate are products of New England Biolabs and were used at dilutions suggested by the manufacturer.

*Gel-retardation experiments*

Confluent control and treated chondrocytes in 4 well cluster plates (3-5 x $10^6$ cells/well) were carefully scraped into 1.5 ml of ice-cold PBS and pelleted by brief centrifugation as previously described (Ney et al, 1990). The cellular pellet was gently resuspended in 200-400 µL of ice-cold hypotonic lysis buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Pefabloc$^{TM}$, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 1 mM NaF and 1% Nonidet P-40. Cells were allowed to swell on ice for 10 min, vortexed vigorously for 10 s, and nuclei recovered by brief centrifugation at 3,000 x g for 60 s. The nuclear pellets were resuspended in 25 µl of high salt extraction buffer

containing 20 mM HEPES-KOH, pH 7.9, 0.42 M NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM EDTA, 25% glycerol, 0.5 mM Pefabloc$^{TM}$, and 10 µg/ml each of aprotinin, leupeptin and pepstatin, followed by incubation on ice for 45 min with intermittent vortexing. The nuclear extracts were recovered by centrifugation at 16,000 x g for 30 min at 4$^{o}$C and stored at -86$^{o}$C until used.

Double-stranded oligonucleotides containing consensus and promoter specific sequences (synthesized, annealed and purified by HPLC) were end-labeled with [g-$^{32}$P]ATP using T4 polynucleotide kinase (Pharmacia, Montreal, Quebec). The sense sequences of the oligos used were as follows: AP-1; 5'-CGC TTG ATG AGT CAG CCG GAA-3': NF-kB; 5'-AGT TGA GGG GAC TTT CCC AGG C-3': CRE-COX-2; 5'-AGA GAT TGC CTT TCG TCA GAG AGC TAG-3': SP-1; 5'-ATT CGA TCG GGG CGG GGC GAG C-3': AP2; 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3': NF-IL6-COX-2; 5'-CAC CGG GCT TAC GCA ATT TTT TTA A-3'. 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 15 µg of nuclear extract and 100,000 cpm of $^{32}$P-labeled oligonucleotide probe at 22$^{o}$C for 20 min in a final volume of 10 µl. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried and prepared for autoradiography.

*Statistical analysis*

All results are expressed as mean ± standard deviation of between 3 to 5 separate experiments. Statistical significance was assessed using the Student's t-test and significant differences were confirmed only when the probability was less than or equal to 5%.

**RESULTS:**

Calphostin C upregulates COX-2 mRNA expression and protein synthesis: Time and dose-response studies:

Dose-response studies conducted over a period of 24 h indicated that the effective concentration of CalC necessary for a 50% induction ($EC_{50}$) of COX-2 protein was 75 nmol/L ( Fig 1A and B) based on the analysis of densitometric scans. Maximal induction of COX-2 protein was achieved at 200 nmol/L but declined with increasing concentrations of CalC up to 600 nmol/L. This decline was not associated with cellular toxicity as markers (e.g. Bcl 2 and lamin cleavage) for cell death were unaffected (data not shown).

In time course experiments using 200 nmol/L of CalC, a complex pattern of COX-2 mRNA and protein was observed. An initial phase was characterized by a gradual increase in COX-2 protein (Fig. 2A and B) and COX-2 mRNA (Fig.3A and B) that peaked at 8 h and was followed by a precipitous drop in COX-2 protein and mRNA expression with a nadir at 16 h. A second stimulatory phase was observed beginning at 16h with the zenith at 24 h. This phase was stable for up to 48 h with only a small decline in COX-2 mRNA expression and protein synthesis between 24 and 48 h.

Calphostin C modulates phosphoCREB/ATF protein synthesis and CRE nuclear protein binding: Time-course studies:

Calphostin C, at 200 nmol/L, stimulated an initial increase in CREB/ATF phosphorylation followed by a gradual decrease that reached a nadir at 16 h (Fig. 4A and B). Between 16 and 24 h, renewed CREB/ATF phosphorylation was observed

continuing up to 48 h. A similar pattern was also observed for CRE nuclear binding proteins following gel-shift analysis (Fig. 5A and B). As we showed in a previous study, AP-1 is also up-regulated in a manner similar to CRE nuclear binding proteins but chondrocyte nuclear proteins recognizing either Sp-1, AP-2, NF-κB, or NF-IL-6 consensus oligos were unaffected by CalC (data not shown).

**DISCUSSION:**

We have identified signaling pathways regulating COX-2 gene expression in phenotypically stable human chondrocytes (Miller et al, 1998). Taken together, our data suggest that one major MAP kinase cascade, the Raf/MEKK/MEK/ERK pathway, is either not associated with COX-2 expression or negatively regulates the COX-2 gene. In contrast, a related MAP kinase cascade, the MEKK1/JNKK/JNK/SAPK pathway, strongly up-regulates COX-2 expression probably through the activity of JNK/SAPK which phosphorylates and activates critical transcription factors controlling COX-2 promoter activity. Furthermore, protein kinase A (PKA) activation with its associated pathways, was identified a positive regulatory factor of COX-2 gene expression. Parenthetically, upregulation of COX-2 by activation of PKA pathways was probably responsible for the Raf/MEKK/MEK/ERK pathway shutdown that we observed because PKA inhibits Raf kinase activity (Cook and McCormick, 1993). As a result of the activation of MEKK1/JNKK/JNK/SAPK and PKA pathways, two major transcription factor families were activated, namely AP-1 and CREB/ATF, respectively. This was verified by monitoring changes in protein phosphorylation and binding to cognate enhancer elements (gel shift). These latter transacting factors play a pivotal role in the transactivation of the COX-2 gene in human chondrocytes by binding to the CRE in the promoter region (Xie and Herschman, 1995; Inoue et al, 1994).

Protein kinase C (PKC) mediates the induction of COX-2 gene expression by phorbol ester (e.g. PMA) and probably does so by phosphorylating, besides AP-1, the c/EBP family of transcription factors. The latter family can form heterodimers with CREB/ATF proteins and, as such, can recognize CRE element in the COX-2 promoter (Inoue et al 1995). This phenomenon was indentified in vascular epithelial cells and it is not known if other cell types react to PMA stimulation with increases in c/EBP protein synthesis.

We designed the present set of experiments to elucidate the role of PKC in human articular chondrocytes in terms of COX-2 expression. Prostaglandins derived from COX-2 activity, have profound effects on cartilage metabolism and integrity and so is of interest in the context of arthritic diseases. CalC binds not to the catalytic region of PKC, as most inhibitors do, but to the regulatory domain thereby increasing specificity immeasureably (Kobayashi et al, 1989; Bruns et al, 1991). In a companion study, CalC mimicked the action of PMA by stimulating AP-1 synthesis while inhibiting the translocation of the prinicipal chondrocyte PKC isoenzyme, PKC-α. Furthermore, CalC induced transactivation through an AP1 site in the *c-jun* promoter. Although CalC has been shown to induce *c-jun* and *c-fos* mRNA in other cell types (Gamou and Shimizu, 1994; Freemerman et al, 1996), to our knowldge this is the first demonstration that the inhibitor can induce AP-1 activation.

To these observations we now show that CalC can modulate CREB/ATF synthesis. We remarked that the pattern of simulation closely matched that of COX-2 induction suggesting a temporal, if not mechanistic, relationship between CREB/ATF phosphorylation and CRE binding and COX-2 expression. On closer inspection and analysis, AP-1 also responded to CalC in a complex biphasic pattern similar to CREB/ATF and COX-2. It is tempting to speculate that both these factors mediate CalC induction of COX-2 but this will have to await COX-2 promoter studies.

There are reports which link the expression and synthesis of COX-2 with cellular toxicity and apoptosis (Tsujii and DuBois 1995) although the mechanism for this is poorly understood. With cell types in culture, CalC can induce cell toxicity since, in the presence of light, the molecule can react with molecular oxygen to form free radicals. The cell membrane is sensitive to free radicals and is rapidly breeched causing cell death. In human glioma cells, CalC induces a process akin to apoptosis (Ikemoto et al, 1995; Freemerman et al, 1996). Ikemoto et al showed that the inhibitor (100 nmol/L) blocked PKC activity within 2-8h and then stimulated $Ca^{++}/Mg^{++}$-

dependent endonuclease activity after 16 to 24h , a harbinger of DNA fragmentation and apoptosis. In addition,the authors observed a down-regulation of the protooncogene Bcl-2 (anti-apoptotic) expression and synthesis prior to cell death. Human chondrocytes are more resistant to CalC than glioma cells to the extent that, even at concentrations up to 600 nmol/L, we observed no cellular toxicity. Cell extracts of human chondrocytes revealed that the cells produce relatively large amounts of Bcl-2 and that the levels are unaffected by CalC treatment. Furthermore, nuclear lamins were not cleaved into patterns normally observed when cells undergo apoptosis. As such, in human chondrocytes, induction of COX-2 is not necessarily associated with cell death.

In summary, CalC can induce COX-2 synthesis and expression probably through activation of transcriptional mediators like CREB/ATF and AP-1. The cellular and molecular mechanisms regulating the complex pattern of CalC-induction of COX-2 await elucidation but the induction of COX-2 was PKC-independent. New molecular targets for CalC in human chondrocytes remain to be identified.

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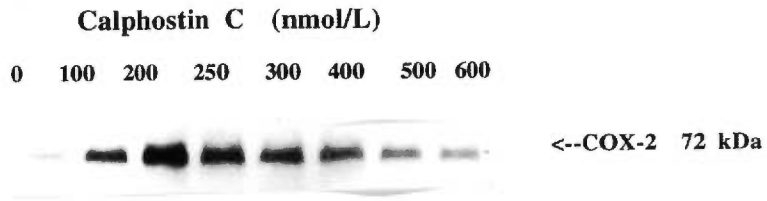
**LEGENDS:**

FIG. 1: Dose-response pattern of Calphostin C (CalC) stimulation of COX-2 protein synthesis in human articular chondrocytes.

Normal human chondrocytes were seeded at high density (1.2 x $10^6$ cells per well) in DMEM supplemented with 10% FCS and an antibiotic mixture (culture medium). Confluence (stationary phase) was reached in 1-3 days. The cells were serum starved for 24h and then incubated for 24 h in serum free culture medium with 0, 100, 200, 250, 300, 400, 500, and 600 nmol/L of CalC for 24 hours. Cellular proteins were extracted as described in the *Materials and Methods* section and analyzed by Western blotting using a specific anti-COX-2 antiserum (1:5000 dilution). A representative blot is shown in (A). (B) Bar diagram of densitometric scanning of multiple Western blots and values, expressed as arbitrary optical density (OD) units, represent the mean of 3 determinations. Data are expressed as mean ± standard deviation (SD). ANOVA analysis, control (O) is CalC 100-6–nmol/L, $p < 0.001$.

A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<--COX-2 72 kDa

B

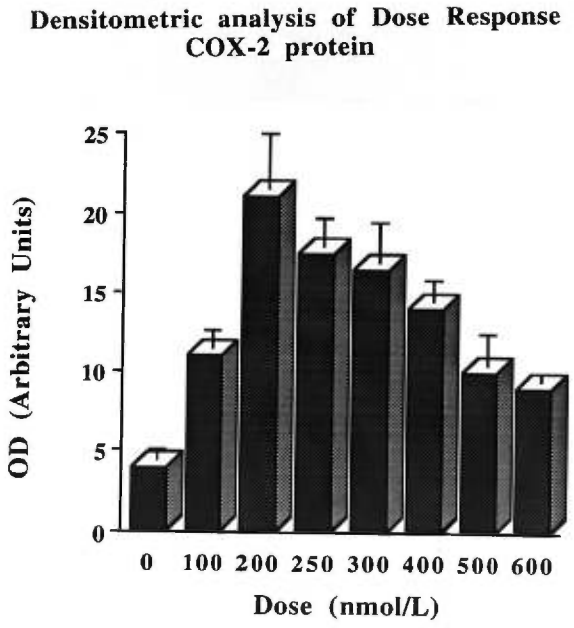
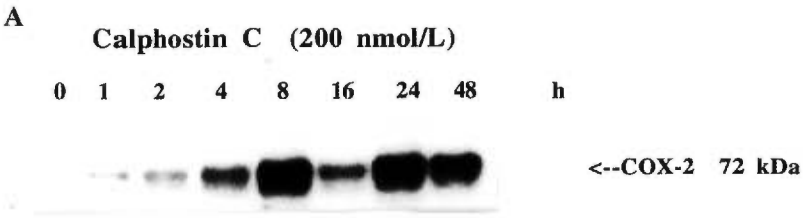

Densitometric analysis of Dose Response
COX-2 protein
OD (Arbitrary Units)
25
20
15
10
5
0
0 100 200 250 300 400 500 600
Dose (nmol/L)

FIG. 2: Time course of CalC induction of COX-2 protein synthesis.

Normal chondrocytes were seeded at high density and confluence was reached in 1-3 days. The cells were then serum starved for 24h and then incubated with 200 nmol/L of CalC for 0, 1, 2, 4, 8, 16, 24 and 48 h. Cellular proteins were extracted as described in the *Materials and Methods* section and analyzed by Western blotting using a specific anti-COX-2 antiserum (1:5000 dilution). A representative blot is shown in (A). (B) Bar diagram of densitometric scanning of multiple Western blots and values, expressed as arbitrary optical density (OD) units, represent the mean of 5 determinations. Data are expressed as mean ± standard deviation (SD). $P<0.0001$ for 8, 24, 48h vs 16h. T-test.


A
Calphostin C (200 nmol/L)
0 1 2 4 8 16 24 48 h
<--COX-2 72 kDa

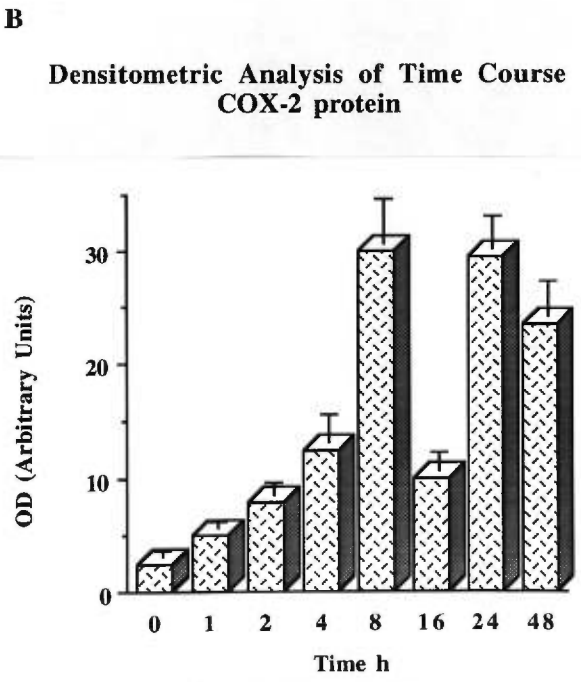

B
Densitometric Analysis of Time Course
COX-2 protein
OD (Arbitrary Units)
0
10
20
30
0 1 2 4 8 16 24 48
Time h

FIG. 3: Time course of CalC induction of COX-2 mRNA expression.

Normal chondrocytes were seeded at high density and confluence was reached in 1-3 days. The cells were serum starved for 24h and then incubated with 200 nmol/L of CalC for 0, 1, 2, 4, 8, 16, 24 and 48 h. Total RNA was extracted as described in the *Materials and Methods* section and 10 μg analyzed by Northern blotting using a specific human COX-2 cDNA probe. A representative blot is shown in (A) along with the negative image of EtBr staining pattern of 28S/18S rRNA fixed to the membrane. (B) Bar diagram of densitometric scanning of multiple Northern blots and the values, expressed as arbitrary optical density (OD) units normalized to the amount of 28S rRNA (COX-2 mRNA /28S rRNA), represent the mean of 5 determinations. Data are expressed as mean ± standard deviation (SD). $P<0.0001$ for 8, 24, 48h vs 16h. T-test.

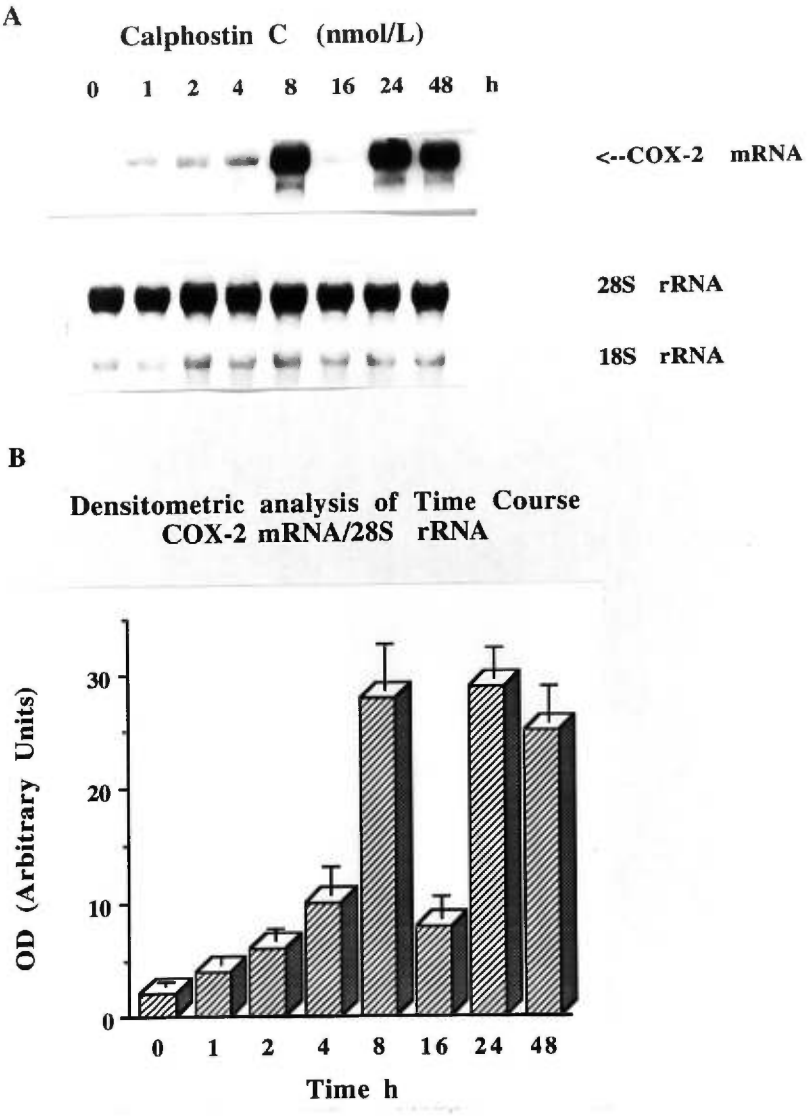
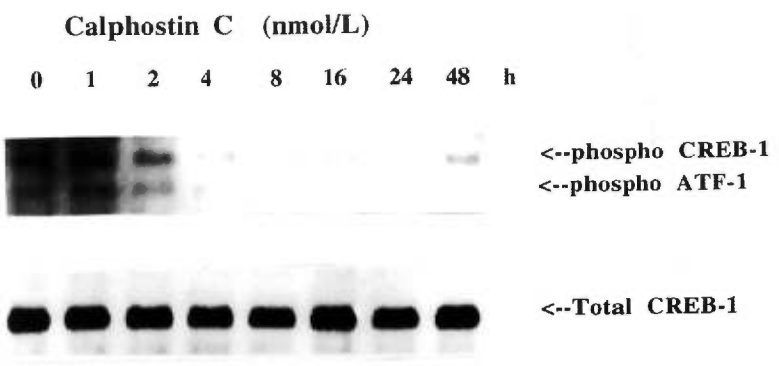

A
Calphostin C (nmol/L)
0 1 2 4 8 16 24 48 h
<--COX-2 mRNA
28S rRNA
18S rRNA
B
Densitometric analysis of Time Course
COX-2 mRNA/28S rRNA
OD (Arbitrary Units)
30
20
10
0
0 1 2 4 8 16 24 48
Time h

FIG. 4: Time course of CalC induction of CREB-1/ATF-1 protein phosphorylation.

Normal chondrocytes were seeded at high density and confluence was reached in 1-3 days. The cells were then serum starved for 24h and then incubated with 200 nmol/L of CalC for 0, 1, 2, 4, 8, 16, 24 and 48 h. Cells were washed with ice-cold PBS and then solubilized in 100 µl of boiling SDS sample buffer. A total of 100 µg of the whole cell extracts was resolved by PAGE and subjected to Western blotting using specific antiserum to total CREB-1 (constitutive) and phosphoCREB-1/ATF-1. A representative blot of phosphoCREB-1/ATF-1is shown in (A) along with a representative blot of total CREB-1. (B) Bar diagram of densitometric scanning of multiple Western blots and values, expressed as arbitrary optical density (OD) units normalized to the amount of constitutively produced CREB-1, represent the mean of 4 determinations. Data are expressed as mean ± standard deviation (SD). $P < 0.001$, 0 or 1h vs 8 or 16h. T-Test.

A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0 1 2 4 8 16 24 48 h
<--phospho CREB-1
<--phospho ATF-1
<--Total CREB-1

B

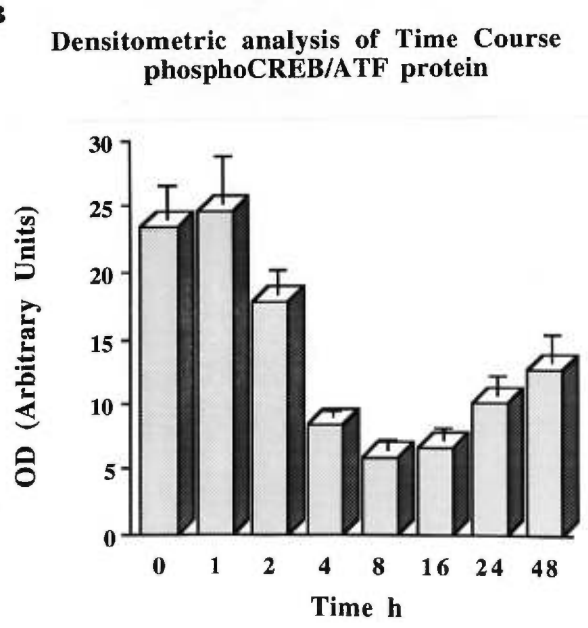
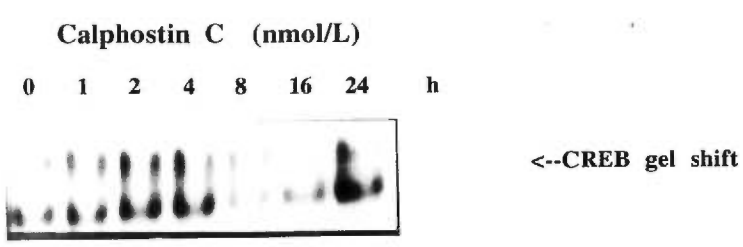

Densitometric analysis of Time Course
phosphoCREB/ATF protein
OD (Arbitrary Units)
30
25
20
15
10
5
0
0 1 2 4 8 16 24 48
Time h

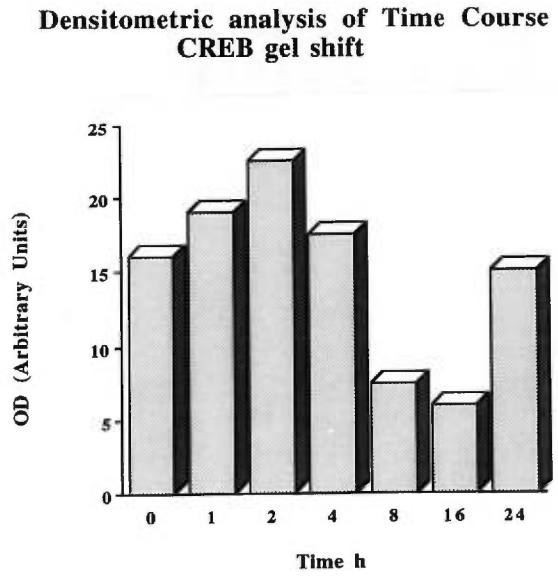
FIG. 5: Time course of CalC induction of CRE nuclear binding proteins.

(A) Normal chondrocytes were seeded at high density and confluence was reached in 1-3 days. The cells were then serum starved for 24h and then incubated with 200 nmol/L of CalC for 0, 1, 2, 4, 8, 16, and 24 h. Cells were washed with ice-cold PBS, scraped into 1.5 ml of PBS, and pelleted. Nuclei were then isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer. Fifteen to thirty μgs of nuclear protein were incubated with $^{32}$P-labeled CRE COX-2 sequence and mobility gel shifts were performed as described in the *Materials and Methods* section. (B) Representative bar diagram of densitometric analysis of gel shift.

A


Calphostin C (nmol/L)
0 1 2 4 8 16 24 h
<--CREB gel shift

B


Densitometric analysis of Time Course
CREB gel shift
OD (Arbitrary Units)
25
20
15
10
5
0
0 1 2 4 8 16 24
Time h

# CHAPTER THREE: DISCUSSION AND CONCLUSION

## 3.1 DISCUSSION

While studying the role of PKC-α, the major conventional isoform of the enzymes in chondrocytes, with respect to the control of COX-2 gene expression in normal human chondrocytes, we observed that CalC mimicked the stimulatory effects of the PMA. According to those observations, this research project was aimed at elucidating the signal transduction pathways controlling COX-2 expression in human chondrocytes with CalC, and in particular, the role of PKC. Also to determine whether PKC is implicated in the regulation of AP-1 expression and synthesis in human chondrocytes.

The previous studies in our laboratory identified some potential signaling pathways involved in regulation of COX-2 gene expression in human chondrocytes (Miller *et al.*, 1998). This was achieved with the use of specific serine/threonine phosphatase (type 1 and 2A) inhibitors, namely OKA, which provided a cellular enviroment favouring an increased level of protein phosphorylation. One major MAP kinase cascade, the Raf/MEKK/MEK/ERK pathway, was inhibited by treatment with the phosphatase inhibitors while another, the MEKK/JNKK/JNK/SAPK pathway, was fully activated. In addition, PKA activity was stimulated, which was probably responsible for Raf/MEKK/MEK/ERK pathway shutdown because PKA inhibits Raf kinase activity (Cook and McCormik, 1993). Two major transcription factor families were activated as a result of the activation of MEKK1/JNKK/JNK/SAPK and PKA pathways, namely AP-1 and CREB/ATF, respectively. PKC was not activated by okadaic acid treatment, but PMA, a potent activator of PKC, strongly increased AP-1 and COX-2 synthesis. In order to resolve this conudrum, we designed the present set of experiment to elucidate the role of PKC with the use of CalC, a specific inhibitor of PKC.This inhibitor has been used extensively and has been proven to be a useful probe in the study of PKC-dependent

signaling pathways and gene expression (Das and White, 1997; Xu and Clark, 1997). Furthermore, it has been cited for its potential as a useful drug in cancer treatment and various forms of arthritis because it is pro-apoptotic in many cell types (Lee *et al.*, 1995; Yang *et al.*, 1995). A biochemical rationale for the action of CalC in this regard remains to be clarified although proto-oncogene expression (e.g. c-jun) has been implicated (Gamou *et al.*, 1995; Freemerman *et al.*, 1996).

### 3.1.1 CalC inhibits PMA-induced PKC-α translocation in human chondrocytes

AP-1 is phosphorylated by a group of proline-directed mitogen-activated MAP, called JNK/SAPK (Karin, 1995). Depending on the cell type, the activity of JNK may be controlled by $Ca^{2+}$ and PKC-dependent pathway (Su *et al.*, 1994). Twelve PKC isoforms have been identified and classified into two groups based on their structure and cofactor regulation (Nishizuka, 1995). Conventional PKCs: α, two alteratively spliced variants βI βII, and γ. Next group is the novel protein kinase Cs: δ, ϵ, η, θ, and μ. Maximizing the activity of conventional isoforms of PKC is dependent on the presence of $Ca^{2+}$, phospholipids (principally phosphatidylserine, ) and DAG, which in turn are generated in the cell by a diversity of transduction mechanisms promoting lipid hydrolysis (Newton, 1995).

A useful tool in identifying and characterizing PKC regulation is PMA, a phorbol ester that activates PKC. It is a potent inducer of PKC by virtue of its affinity for the DAG site in the regulatory domain of the enzyme (Newton, 1995). Diacylglycerol and phorbol esters serve as hydrophobic anchors to recruit PKC to the membrane; they cause a dramatic increase in the enzyme' s membrane affinity.

At first, by using specific antibodies (conventional PKC-α, βI, βII, and γ), we

determined that PKC-α is the major conventional identifiable isoform in human chondrocytes. PMA induced a time-dependent depletion of PKC-α protein from the cytosolic fraction to membrane fraction in human chondrocytes. We have shown that CalC inhibits PKC activity in vitro and in vivo (DiBattista *et al.*, 1996) and in this study, we showed that the inhibitor blocks cytoplasmic to membrane translocation of PKC-α in human chondrocytes in culture, which means PKC pathway is shutdown by CalC in chondrocytes. Although there were trace levels of PKC-βI, but we have no evidence of its contribution to cellular PKC activity or if it sensitive to CalC.

The use of CalC with cells in culture have lead to observations of cell toxicity. Specially in the presence of light, the molecule acts with molecular oxygen to form free radicals. This can lead to membrane destruction and cell death. Other studies have demonstrated an effect of CalC akin to apoptosis, particularly in human glioma cells (Freemerman *et al.*, 1996). Our cultures of human chondrocytes are more resistant to CalC than glioma cells to the extent that, even at concentration up to 600 nMol/L, the cells remained intact (data not shown). Cellular toxicity, however was dependent on how long the cells are exposed in the presence of light. For example, if the cells were exposed in the presence of the light over 30 minutes, the cellular toxicity became more evident.

### 3.1.2 Up-regulation and transactivation of AP-1 by CalC in normal human chondrocytes

To activate or repress transcription, transcription factors must be located in the nucleus, bind to DNA, and interact with the basal transcription apparatus. AP-1 is a DNA sequence-specific transcriptional activator and an important mediator of cellular response to growth factors, serum, and tumor-promoting phorbol esters (Angel *et al.*, 1988). AP-1 is a dimeric protein consisting of c-Jun (c-Jun, Jun-B Jun-D) and c-Fos (Fra-1, FosB, c-

Fos) family members that enhances gene transcription in promoters harbouring the so-called phorbol ester response element (TRE) having the consensus sequence TGA (C/G) TCA (Curran and Franza, 1988). With careful control of the concentration of CalC used, we demonstrated for the first time that CalC acts on signaling pathway independent of PKC to induce AP-1. Maximum induction of AP-1 activity was reached at a CalC concentration of 250 nmol/L, the major up-regulation phase was at 24 hours. According to our observations, up-regulation of AP-1 was the major CalC inducible transcription factor in the human chondrocytes, so that a PKC-independent pathway should exist for AP-1 stimulation. Recent studies suggest that CalC can activate glycogen synthase kinase-3 (GSK-3) A431 at concentrations that inhibit PKC; CalC is inhibitory of GSK-3 at higher concentrations (Lee *et al.*, 1995). The latter data provided us with interesting posibilities since GSK-3 can indeed phosphorylate c-Jun (Boyle *et al.*, 1991). However, the kinase phosphorylates a threonine residue (239) in a domain of c-Jun that inhibits its ability to transactivate target promoters (Boyle *et al.*, 1991). Furthermore, GSK-3 is cytoplasmic making it an unlikely candidate for a physiological nuclear c-Jun protein kinase (Hunter and Karin, 1992). On the other hand, co-expression of GSK-3$\alpha$ or $\beta$ with c-Jun decreases its ability to transactivate an AP-1-dependent reporter gene (deGroot *et al.*, 1992), suggesting that GSK-3 may play a role in regulating c-Jun in vivo, although whether this occurs via direct c-Jun phosphorylation is unclear. We also studied NF$\kappa$B transcription factor activity with CalC in human chondrocytes, no stimulation was observed, despite the fact such as an effect has been demonstrated in other cell types (Schmidt *et al.*, 1995). Treatment of chondrocytes with CalC did not significantly increase binding of nuclear extracts to CREB, c/EBP, SP1, OCT-1 or AP-2 consensus oligonucleotides.

Some of the most important kinases regulating AP-1 DNA binding and transactivating ability are the MAP kinases, specifically JNK/SAPK, FRK, and possibly ERK1,2 (Karin, 1995). They are capable of phosphorylating c-Jun and family members at

the correct Ser residue (s) in the transactivation domain of the protein (Kallunki *et al.*, 1996). The activity of these latter kinases are controlled by upstream kinases such as MEKK and JNNK/SEK (Cobb and Goldsmith, 1995). Our observations demonstrated that CalC increases AP-1 DNA binding and transactivating ability by stimulating JNK/SAPK activity; ERK 1 and 2 were inhibited by CalC.

### 3.1.3 Synthesis and expression of COX-2 and certains proto-oncogenes by stimulation of calphostin C in normal human chondrocytes

We demonstrated that CalC causes a time-dependent increase in COX-2 synthesis and mRNA expression in human chondrocytes. A complex kinetic pattern was discovered with an initial phase reaching a zenith at 8 hours, followed by a rapid decline with a nadir at 16 hours, and finally another rapid inductive phase with a maximum at 24 hours. CalC exhibited a narrow dose-response profile with an EC50 of 75 nmol/L, maximum response at 200 nmol/L, and a sharp decline to near control levels at dose up to 600 nmol/L. These results were similar to those observed for GSK-3 (Lee *et al.*, 1995) where low concentrations of CalC were stimulatory and higher dose, inhibitory. It is conceivable that the stability of COX-2 mRNA is affected at higher concentrations of CalC or certain phosphatases are activated. Indeed, CalC, mimicking okadaic acid, can potently up-regulate COX-2 synthesis and mRNA expression in human chondrocytes suggesting that the inhibition of PKC activity may inhibit phosphatase action but at lower does.

Although AP-1 complexes are often composed of gene products of c-jun and c-fos, other members of the latter proto-oncogene families may also be present. We identified AP-1 complexes by mRNA and gel shift analysis of CalC treated chondrocytes. CalC up-regulated the expression of c-jun, c-fos and JunB protooncogene mRNA expression. There was no similar up-regulation of JunD or Fra-1 mRNA. Then we used specific antibodies

to c-Jun, JunB, JunD, c-Fos and CREB/ATF in super-gel shift studies and revealed that AP-1 complexes were probably c-Fos/c-Jun, c-Fos/JunB or c-Jun/JunB dimers as the AP-1 shift patterns were displayed by these respective antibodies and not by JunD or CREB/ATF antibodies. The up-regulation of c-jun expression is generally thought to occur through a TRE element (AP-1) in the proximal promoter region of the c-jun gene (Angel *et al.*, 1988). CalC would appear to activate the MEKK1/JNKK/JNK/SAPK cascade in human chondrocytes, and this would be consistent with what is known about the phosphorylation and stimulation of the transactivation capacity of c-Jun. JNK activity was apparent after 1-2 h but was dramatically increased at 8 h. We detected an increase in AP-1 binding activity after a few hours, which preceded the increased expression of c-jun, consistent with an autoactivational process. Presumably, resident nuclear AP-1 complexes are phosphorylated/activated followed by c-jun gene transactivation (Angel *et al.*, 1988). In contrast, JunB is not efficiently nor productively phosphorylated by JNK/SAPK, (whose activation is induced by CalC), nor does JunB efficiently transactivate the collagenase promoter via a TRE element (Kallunki *et al.*, 1996). JunB homodimers do not bind well to cognate regulatory elements (Ryseck *et al.*, 1992); thus, if JunB plays a role in COX-2 expression, it is likely the result of heterodimerization. The induction of c-fos mRNA by CalC was perhaps quantitatively more pronounced than the c-jun family members . The promoter of c-fos contains a transcriptionally critical CRE and serum response element (SRE). SRE binds the transcription factor Elk-1, which is phosphorylated by ERK kinases. However, we demonstrated here that this cascade is inactivated by CalC treatment. Probably CalC up-regulated c-fos expression through the CRE, since AP-1 complexes can bind th CRE. Our transfection studies indicated tha CalC can transactivate a c-jun-CAT promoter construct through an AP-1 site, since a mutation in the sequence completely abogated the response. The TRE site in the c-jun promoter differs by one base pair from the consensus TRE and, though it has a high affinity fot conventional AP-1 dimers, c-Jun/ATF2 dimers are more efficiently bound (Gupta *et al.*,

1995). We have no evidence that CalC can stimulate the synthesis and phosphorylation of CREB/ATF family members and, in fact preliminary data in our laboratory seem to suggest an inhibitory pattern with respect to CREB and ATF, at least on a short-term basis. However, conventional AP-1 complexes can still potently transactivate through the TRE site in a manner quite similar to c-Jun/ATF2. It is not difficult to contemplate AP-1 in the transactivation of the COX-2 gene, even in the absence of canonical or recognizable AP-1 elements. Because besides CRE binding, AP-1 was the only other significant transactivating factor detected in our gel shift assays that could potentially recognize regulatory elements (e.g., CRE) positively identified in the promoter region of the COX-2 gene.

## 3.2 CONCLUSION

We may conclude that, in human chondrocytes, the COX-2 gene is up-regulated by CalC. The inhibition of PKC results in a shutdown of MEKK1/MEK1/ERK cascade. By contrast, there is a concomitant activation of the other proline-directed MAP kinase pathway, namely, MEKK1/JNKK/SAPK/JNK. CalC can induce AP-1 synthesis and transactivation of AP-1 sensitive target gene in human chondrocytes independent of PKC-$\alpha$ inhibition. There is circumstantial evidence in this study to implicate a Fos kinase as well, given the prominence of c-Fos in the AP-1 complexes. The accumulated AP-1 factors probably consisted, in part, of c-Fos/c-Jun, c-Jun/JunB, and c-Fos/JunB dimers.

Indeed, CalC, mimicking okadaic acid, an inhibitor of phosphatase, can potently up-regulat COX-2 in human chondrocytes, suggesting that the inhibition of PKC activity may inhibit phosphatase action, one that is dependent perhaps on phosphorylation by PKC. Based on the evidence of this study, it is logical to hypothesize that CalC activation of MEKK1/JNKK/SAPK/JNK pathway may involve a mechanism by which a JNK phosphatase is inhibited, resulting in increased phosphorylation and activation of MEKK1/JNKK/SAPK/JNK pathway. Further studies are necessary to understand the mechanism by which the proline-directed MAP kinase pathway is activated by CalC.

We used the normal human chondrocytes as a cellular model to study the signal transduction pathway of the COX-2 gene expression. This knowledge will certainly benefit for better understanding the COX-2 gene expression in OA and RA.

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