Université de Montréal

Influence du chondroitin sulfate (CS) sur l'activité et l'expression de plusieurs isoformes du Cytochrome P450 et de la NADPH P450 réductase

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Ce mémoire intitulé :

Influence du chondroitin sulfate (CS)
sur l'activité et l'expression de plusieurs isoformes du Cytochrome P450
et de la NADPH P450 réductase

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ABSTRACT

In rabbits, an acute inflammatory reaction induced by the injection of turpentine causes a decrease in cytochrome P450 (CYP) isoforms activity and expression. Chondroitin sulfate (CS) is a Symptomatic Slow Acting Drug for OsteoArthritis (SYSADOA) that elicits anti-inflammatory effects. Since patients take CS over long periods, it was of interest to assess whether CS modulates the activity of cytochrome P450 isoforms. In order to determine the effect of CS on the cytochrome P450, CS was administered *in vivo* to two animal models, e.g. chronic intake of CS in control rabbits, and chronic intake of CS in rabbits with a CYP down-regulated by an inflammatory reaction (IR). We used six groups of five rabbits: three to assess the effect of CS on cytochrome P450, one without CS and two receiving orally about 20 mg/kg/day CS for 20 and 30 days; and the remaining three groups of rabbits received turpentine s.c. to generate an aseptic IR (AIR) 48 h before their sacrifice, e.g. days -2, 18 and 28, while exposed to CS for 0, 20 or 30 days, respectively.

In order to verify the presence of inflammation we measured the seromucoids in serum of rabbits with an AIR. Another marker of inflammation, e.g. nitric oxyde ($NO^{\cdot}$) production, was assessed in control hepatocytes (H_{cont}) and in hepatocytes from rabbits with an AIR (H_{infla}). In addition, the effect of CS on the nuclear translocation of NF-κB was studied by fluorescence in hepatocytes. Finally, in hepatocytes (both H_{cont} and H_{infla}) the CYP3A6, CYP1A2 and NADPH P450 reductase (NADPH) activity, expression and mRNA were measured. *In vitro*, the effect of different concentrations of CS, 4S-, 6S- and 4,6S-sulfated disaccharides of CS on the cytochrome P450 was documented.

Compared with control rabbits, 20 and 30 days CS did not affect the activity of CYP3A6 and CYP1A2. The AIR increased seromucoids from 8.4±1.6 mg/dl in controls to 95.1±5.7 ($p<0.05$), as

well as the nuclear translocation of NF-κB, and nitric oxide concentrations. The AIR reduced CYP3A6 activity by 62% and CYP1A2 activity by 54%, decrease associated to a reduction in protein expression and in mRNA, e.g. pre-transcriptional down-regulation.

The nuclear translocation of NF-κB was prevented by the administration of CS to rabbits with an AIR, moreover CS impeded the increase of the concentrations of nitric oxide; however CS did not prevent the increase in seromucoids. CS did not prevent the down-regulation of CYP1A2 produced by the inflammatory reaction.

CS prevented the time-dependent down-regulation of CYP3A6 in control rabbits and in rabbits with an inflammatory reaction. In this last group, CS restored the amounts of CYP3A6 protein to levels observed in control rabbits, however this increase was independent of the mRNA that remained very depressed. It is noteworthy that even if CS increased CYP3A6 protein, its activity was not recovered. CS did not affect NADPH activity or expression.

Finally, *in vitro,* CS, 4S-, 6S and 4,6S-sulfated disaccharides of CS did not change the activity and expression of the two isoforms of CYP, and of NADPH.

It is concluded that CS does not affect the activity or expression of CYP1A2, nor prevents CYP1A2 AIR-induced down-regulation. However, CS prevents the down-regulation of CYP3A6 time dependently and following the AIR but does not prevent the decrease of catalytic activity.

Keywords: cytochrome P450, NADPH-reductase, inflammation, chondroitin sulfate, osteoarthritis

RESUME

Le CS fait partie de la famille des SYSADOA (SYmptomatic Slow Acting Drugs for OsteoArthritis) et est utilisé par les patients avec de l'ostéoarthrose de façon chronique pour ses propriétés anti-inflammatoires. Étant donné que ces patients reçoivent d'autres médicaments, il était intéressant de documenter les effets du CS sur le cytochrome P450 et la NADPH-réductase (NADPH).

Pour cette étude, deux modèles ont été utilisés: des lapins témoins (LT) et des lapins avec une réaction inflammatoire (LRI) afin de diminuer l'activité et l'expression du CYP. Six groupes contenant chacun cinq lapins ont été utilisés: un groupe sans CS et deux groupes qui ont pris oralement dans l'eau approximativement 20.5 mg/kg/jour de CS pendant 20 et 30 jours; les lapins des trois groupes restants ont pris du CS comme décrit plus haut, mais ont reçu 5 ml sous-cutanées de térébenthine afin de produire une réaction inflammatoire aseptique (RIA) deux jours avant leur sacrifice, c'est-à-dire aux jours -2, 18 et 28. Les hépatocytes ont été isolés pour évaluer l'activité et l'expression du CYP3A6, CYP1A2 et NADPH et aussi le ARNm de ces protéines. *In vitro,* nous avons étudié l'effet de différentes concentrations de CS-disaccharides sulfatés, 4S, 6S, et 4,6S de CS, sur l'activité et l'expression du CYP1A2 et du CYP3A6. Pour documenter la présence de la réaction inflammatoire, nous avons mesure les mucoprotéines, dans le sérum des lapins avec une réaction inflammatoire. Aussi nous avons mesuré la présence de l'oxide nitrique (NO) chez les hépatocytes de lapins contrôles et chez les hépatocytes des lapins avec une réaction inflammatoire. La translocation nucléaire du NF-κB a été etudiée par fluorescence chez les hépatocytes.

Par comparaison aux lapins témoins, l'administration du CS pendant 20 et 30 jours n'affecte pas l'activité du CYP3A6 et du CYP1A2. La RIA a augmenté les mucoprotéines à 95,1±5,7 vs 8,4±1,6 mg/dl dans les lapins témoins ($p<0,05$). La RIA a diminué l'activité du CYP3A6 de 62% et l'activité

du CYP 1A2 de 54%. Le CS n'empêché pas la diminution du CYP1A2 produite par la RIA. Par ailleurs, le CS n'affecte pas l'activité ni l'expression de la NADPH.

La translocation nucléaire de NF-κB a été empêche par l'administration chronique de CS aux lapins avec RIA; en plus, la concentration de l'oxide nitrique n'a pas démontré une augmentation en présence de CS; par contre, CS n'empêche pas l'augmentation des séromucoïdes.

Au contraire, CS affecte la diminution du CYP3A6 en fonction de temps et secondaire à la RIA. Dans ce group, CS a rétabli le niveau des protéines du CYP3A6 observé dans le group de lapins témoins. Pourtant cette croissance été independante de mRNA qui garde un niveau trés bas. Le plus remarcable a été la manière dont CS a augmenté la protéine du CYP3A6, sans avoir rétabli l'activité de cet isoforme. Finalement, *in vitro,* CS et ses trois disaccharides sulfatés (4S, 6S et 4,6S) n'affectent ni l'activité ni l'expression de CYP1A2, CYP3A6 et de la NADPH.

En conclusion, l'administration chronique de CS n'affecte pas l'activité ni l'expression du CYP1A2, ou la diminution du CYP1A2 produite par la réaction inflammatoire. Le CS n'affecte pas l'activité ni l'expression du NADPH. Cependant, CS empêche la diminution du CYP3A6 en fonction de temps et secondaire à la RIA.

Mots-clés : cytochrome P450, NADPH-réductase, inflammation, chondroitin sulfate, ostéoarthrite.

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LIST OF ABBREVIATIONS

Δdi-4S/6S/4,6S: (1-4)-*O*-(D-glucopyranosyluronic acid)–(1-3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-4/6-sulfate)

AhR: Aryl hydrocarbon receptor

AIR: aseptic inflammatory reaction

ALD: alcohol dehydrogenase

AO: aldehyde oxidase

AP-1: activator protein

CAR: constitutive androstane receptor

CCAAT: cytidine-cytidine-adenosine-adenosine-thymidine

COX: cyclooxygenase

CPR: NADPH-cytochrome P450 reductase

CS: chondroitin sulfate

CYP: cytochrome P450

DFB: 3,4-difluorobenxyloxy-5,5-dimethyl-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one

DNA: deoxyribonucleic acid

EM: extensive metabolizers

ERK: extracellular signal-regulated kinases

FAD: flavin adenine dinucleotide

FGF: fibroblast growth factor

FLS: fibroblast-like synoviocytes

FMN: flavin mononucleotide

FN-f: fibronectin fragment

FXR: farnesoid X receptor

GAIT: Glucosamine/chondroitin Arthritis Intervention Trial

GR: glucocorticoid receptor

H_{CONT}: hepatocytes from control rabbits

H_{INFL}: hepatocytes from rabbits with an inflammatory reaction

HNF4: Hepatic Nuclear Factor 4

HRP: horse-radish peroxidase-conjugated secondary antibody

IFN: interferon

IKK: IκB kinase

IL: interleukin

IR: inflammatory reaction

IκB: inhibitor of κB

JNK: Jun N-terminal kinase

LPS: lipopolysaccharides

LXR: liver X receptor

MAPK: mitogen-activated protein kinases

MIP: macrophage inflammatory proteins

MMP: metalloproteinases

mRNA: messenger ribonucleic acid

MROD: methoxyresorufin O-demethylation

NADPH: nicotinamide adenine dinucleotide phosphate

NF-κB: nuclear factor kappa B

NO: nitric oxide

NOS: nitric oxide synthase

NSAIDS: non-steroidal anti-inflammatory drug

OA: osteoarthritis

OPG: osteoprotegerin

PGE: prostaglandin E

PI-3K: phosphatidylinositol- 3′-kinase

PM: poor metabolizers

PPAR: peroxisome proliferator-activated receptor

PRR: pattern recognition receptor

PXR: pregnane X receptor

RANKL: receptor activator of nuclear factor-kappa B ligand

ROS: reactive oxygen species

RXR: retinoid X receptor

S/DMOAD: structure/disease modifying anti-osteoarthritis drug

SAPK: stress-activated protein kinases

SNP: single nucleotide polymorphisms

SYSADOA: symptomatic slow acting drug in osteoarthritis

TGF: tumor growth factor

TIIR: turpentine-induced inflammatory reaction

TIMP: tissue inhibitors of metalloproteinases

TLR: toll-like receptor

TNF: tumor necrosis factor

TR: thyroid hormone receptor

USP: ubiquitin specific protease

VEGF: vascular endothelial growth factor

WME: William medium E

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I. INTRODUCTION

PART I: CYTOCHROME P450s

The purpose of this chapter is to review the main enzymes of the human cytochrome P450 (CYP) family and to understand its pivotal role in the metabolism of drugs.

1.1 *The metabolism*

The body is equipped with several mechanisms to ensure that the xenobiotics are effectively eliminated from the body. The small and non-polar molecules have a great affinity for membranes rendering them difficult to be eliminated. The role of metabolism is to promote excretion of these molecules by oxidizing a lipophilic and non-polar product in to a hydrophilic and polar one. Xenobiotic biotransformation is the principal mechanism for maintaining homeostasis during exposure of organisms to small foreign molecules and occurs predominantly in the liver, although biotransformation also occurs in the intestine, kidneys, lungs, placenta, nasal mucosa, and skin.

Generally, the reactions catalyzed by drug-metabolizing enzymes are divided into two groups, phase I and phase II reactions. Phase I reactions introduce a functional group that increases hydrophilicity and they can lead to either activation or inactivation of the drug. Phase I reactions are mediated by the cytochrome P450, flavin-containing monooxygenase, xanthine oxidase, prostaglandin H synthase, amine oxidase, alcohol dehydrogenase, aldehyde dehydrogenase, epoxide hydrolase, and esterase. Among of all these enzymes, the cytochrome P450s are by far the most common and the most important. Phase II reactions include glucuronidation, sulfation, methylation,

acetylation, glutathione conjugation, and amino acid conjugation. In general, these reactions, with the exception of methylation and acetylation, result in a large increase in xenobiotic hydrophilicity.

It is generally recognized that the expression of drug-metabolizing enzymes may be altered in response to development, aging, gender, genetic factors, nutrition, pregnancy, and pathophysiological conditions such as diabetes, long-term alcohol consumption, inflammation, and protein-calorie malnutrition.

1.2 *Taxonomy of the cytochromes p450*

The cytochrome P450 superfamily of enzymes comprises over 7700 known members, or distinct CYP gene sequences, across all organisms (http://drnelson.utmem.edu/CytochromeP450.html for latest count). The human genome encodes 57 different forms of CYP proteins, called isoforms or isoenzymes (Lewis, 2004; Guengerich, 2005). Of these, 15 or more are associated with drug and other xenobiotic metabolism in humans, including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP3A7 (Guengerich, 2003; Lewis, 2004). The nomenclature for CYP isoforms is derived from amino acid sequence similarity determined through gene sequencing. Usually, amino acid sequences with greater than 40% similarity are placed in the same family, designated by a number (e.g., CYP1), while those with greater than 55% similarity are grouped in the same subfamiliy, designated by a letter (e.g., CYP1A) (Danielson, 2002).

1.3. *Structure and mechanism of action*

The overall global structure of CYP enzymes is globular, composed of alpha and beta substructures, with several of these secondary motifs roughly coplanar to the prosthetic heme group (Danielson, 2002) (see Protein Data Bank for structures of CYPs: *http://www.rcsb.org)* (*Figure 1*). In eukaryote, the vast majority of these proteins are bound to the endoplasmic reticulum membrane.

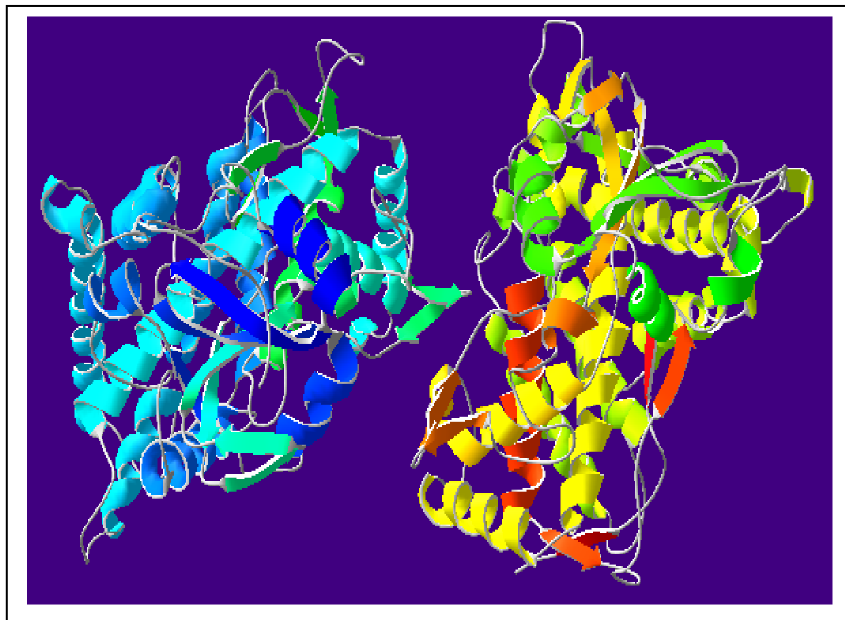


Figure 1 Secondary and tertiary structure of cytochrome P450.

Source: http://www.rcsb.org

Substrate reactivity can be altered by as little as a single amino acid difference, resulting in significant changes in substrate affinity and reaction regioselectivity and velocity (Danielson, 2002). The effects of such alterations may be observed in individuals possessing heritable genetic point mutations, or single nucleotide polymorphisms (SNPs), in their CYP genes, which may lead to reduced activity of the relevant isozyme (Parkinson, 2001). Interindividual allelic variations of this

nature can have undesirable pharmacological consequences, such as low blood clearance of a drug and exacerbation of toxic effects in poor metabolizers (Parkinson, 2001).

As a consequence, the CYP isoforms demonstrate differential affinity toward a myriad of potential substrates, as well as chemo- and region-selectivity toward reaction sites within these molecules. Active sites differences are found in substrate recognition sites (i.e., groups of amino acids in the active site that may determine reaction products by orienting the molecule via complementary chemical interactions) (Danielson, 2002).

1.4. *General Properties and Mechanism of Action*

The CYPs are moderately sized proteins having molecular weights that fall within the range of 48 to 53 kDa. The catalytic component of CYP is a heme cofactor, and the enzyme utilizes the redox chemistry of the Fe^{3+}/Fe^{2+} couple to activate molecular oxygen to oxidize and chemically modify drug molecules. The complete functional system also involves a second enzyme, cytochrome P450 reductase. Cytochrome P450 reductase is a 190-kDa protein that has both flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) as cofactors that serve to sequentially transfer reducing equivalents from reduced nicotine adenine dinucleotide phosphate (NADPH) to cytochrome P450. NADPH cannot reduce cytochrome P450 directly; the heme Fe^{3+} of CYP can only accept electrons in discrete single electron steps, whereas reduction by a hydride (H^-) ion from NADPH is a two-electron process. However, either the FMN or FAD cofactors of cytochrome P450 reductase can undergo a direct two-electron H^- reduction by NADPH and then transfer the electrons to CYP in single one-electron steps. O_2 is split into two oxygen atoms but only one atom is utilized in oxidizing the substrate (RH) while the second atom is reduced by two electrons to form water, in conformity with the following equation:

$$\text{CYP} + \mathbf{O_2} + \mathbf{RH} + \mathbf{2^{e-}} + \mathbf{2H^+} \rightarrow \mathbf{CYP} + \mathbf{ROH} + \mathbf{H_2O}$$

The cytochrome P450 catalytic cycle (1) is shown in greater detail in Figure 2.

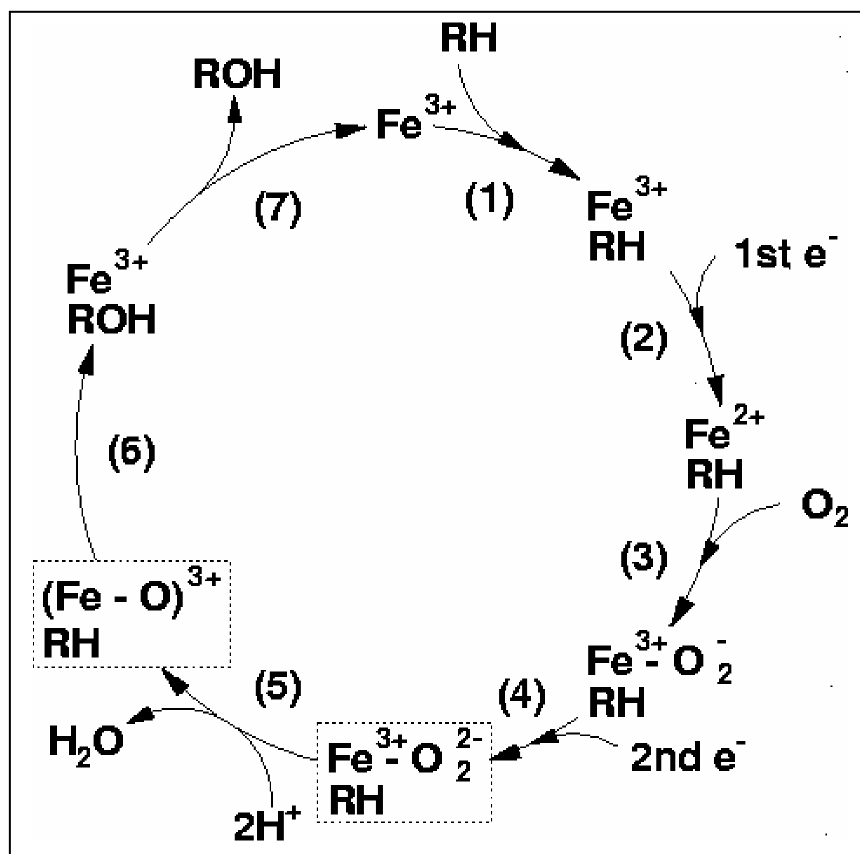


Figure 2. Catalytic cycle of cytochrome P450.

Source: http://www.tcm.phy.cam.ac.uk/~mds21

Step 1.

The substrate, RH, associates with the active site of the enzyme and perturbs the spin-state equilibrium. Water is ejected from the active site and the electronic configuration shifts to favor the high-spin form in which pentacoordinated heme Fe^{3+} becomes the dominant form-binding substrate. In this coordination state, Fe^{3+} is puckered out and above the plane in the direction of the sixth ligand site. The change in spin state alters the redox potential of the system so that the substrate-bound enzyme is now more easily reduced.

Step 2.

NADPH-dependent P450 reductase transfers an electron to heme Fe^{3+} to reduce it to heme Fe2+.

Step 3.

O_2 binds to Fe^{2+}, but can also dissociate. If it dissociates, the enzyme reverts to the heme Fe^{3+} resting state and generates superoxide radical anion in the process.

Step 4.

A second electron, via P450-reductase or in some instances cytochrome b5, is added to the system generating a heme-bound peroxide dianion formally equivalent to FeO^{2+}.

Step 5.

H^+ adds to the system generating a heme-bound hydroperoxide anion complex formally equivalent to heme $FeO_2H_2^+$.

Step 6.

A second H^+ is added. If H^+ adds to the inner oxygen of heme, $FeO_2H_2^+$ decoupling occurs, H_2O_2 is released, and the enzyme reverts to the heme Fe^{3+} form.

Step 7.

If the second H^+ adds to the outer oxygen of heme $FeO_2H_2^+$, water is formed and released. Residual heme FeO^{3+} bears an oxygen atom (oxene) complexed to heme Fe^{3+}, a species considered to be analogous to compound 1, the reactive intermediate of the peroxidases. Decoupling can again occur via a two-electron reduction of FeO^{3+} plus the addition of two protons. This generates a molecule of water and the heme Fe^{3+} resting state of the enzyme. The degree to which this process occurs depends on the relative rates of heme FeO^{3+} reduction versus oxygen atom transfer to the substrate as outlined in the next step (2).

Step 8.

An oxygen atom is transferred from heme FeO^{3+} to the substrate forming the oxidized product, thereafter the product is released, and the enzyme reverts to its heme Fe^{3+} resting state.

1.5. *Reactions catalyzed by cytochrome p450*

The cytochrome P450 enzyme superfamily, one of the most important drug-metabolizing enzyme systems in humans, is responsible for the oxidative metabolism of a large number of endogenous compounds and xenobiotics (Nebert, 2002). Few enzymes are more striking in both versatility and in sheer number of substrates than the cytochrome P450 enzyme system. CYP detoxify harmful xenobiotics or, in some instance, bioactivate them to reactive species, through biotransformation (Ortiz de Montellano, 2005; Parkinson, 2001). The ubiquitous presence of CYP enzymes, paired with their broad substrate selectivity, suggest that the biotransformations catalyzed by these enzymes were essential to an organism's ability to adapt to its environment. CYP enzymes catalyze the majority (>80%) of drug-related metabolism in humans (Guengerich, 2005), as well as the biosynthesis or catabolism of numerous endogenous substrates such as steroid hormones, eicosanoids, vitamine D, etc (Lewis, 2001). Common CYP mediated reactions are: alkyl hydroxylation, oxidation, dealkylation, epoxidation, and dehydrogenation.

1.6. *Variability and Polymorphism*

The area of pharmacogenetics (now also known as — or expanded to —"pharmacogenomics") was facilitated by the identification of the CYP enzymes involved in the drug metabolism phenotypes, and particularly by the development of molecular biology, which allows the precise characterization of genetic differences between individuals. The majority of the allelic differences are single nucleotide polymorphisms (SNPs), or single base changes. As anticipated from previous knowledge of pharmacoethnicity, many of these SNPs and polymorphisms show racial linkage. A

polymorphism is generally defined as a 1% frequency of an allelic variant in a population; below this frequency, the terms "rare genetic trait" or "rare allele" are applied or, in the case of a very detrimental allele, a mutant or "inborn error of metabolism." A nomenclature system has been set up for CYP alleles (using the suffixes *1, *2, *3... and is maintained by Oscarson at *http://www.imm.ki.se/Cypalleles/* (Ortiz de Montellano, 2005).

The first characterization of a monogenic variability in a human drug-metabolizing CYP was the work of Smith with debrisoquine (Mahgoub, 1997) to which was added the work of Dengler and Eichelbaum on sparteine (Eichelbaum et al., 1979). This polymorphism was first described in the context of extensive metabolizers (EMs) and poor metabolizers (PMs). The debrisoquine polymorphism is now understood in terms of CYP2D6 and has been a prototype for research in this area.

Though CYP2D6 and CYP2C19 are often mentioned as displaying polymorphism, allelic mutants have been described in most human CYP isozymes involved in exogenous biotransformation (Smith et al., 1998). The study of CYP polymorphisms has been instrumental in discovering the substrate affinities of several isozymes; for example, the metabolism of S-mephenytoin was originally thought to be catalyzed exclusively by CYP2C9. However, kinetic studies of several variants of CYP2C9, derived from SNPs, showed that its relative contribution to S-mephenytoin biotransformation is negligible, being predominantly metabolized by CYP2C19 (Smith et al., 1998).

It should be pointed out that several of the CYPs can be down-regulated by cytokines, and the result has practical significance in the impairment of drug metabolism in individuals with colds or flu, or who have received vaccinations (Renton and Knickle, 1990).

1.7. *Human CYP Enzymes*

The following section briefly outlines important aspects of each family of human CYP. Members of three families of cytochrome P450, CYP1, CYP2, and CYP3 dominate human drug metabolism, and the primary property that distinguishes one CYP from another is the difference in the spectrum of activity displayed by each individual isoform in their ability to discriminate between substrates. Further details, on each of the 57 human CYPs, can be found elsewhere (Crivori and Poggesi, 2006; Danielson, 2002; Ekins et al., 2001; Lewis, 2001; Ortiz de Montellano, 2005; Parkinson, 2001).

1.7.1. CYP1 family

The CYP1A subfamily contains the two members, CYP1A1 and CYP1A2, which are involved in drug metabolism and have sparked considerable interest because they also seem to be associated with the metabolic activation of pro-carcinogens to mutagenic species.

- **CYP1A1**

In humans, of the two members, CYP1A2 is the major player while CYP1A1 is a relatively minor extrahepatic isoform associated with the oxidation of polycyclic aromatic hydrocarbons like benzo[a]pyrene. Similarly, in test rodent species CYP1A1 is responsible for the generation of toxic intermediates and carcinogenic metabolites (Miners and Mckinnon, 2000).

- **CYP1A2**

CYP1A2 has been implicated in the activation of procarcinogenic species such as aflatoxin B1, 2-acetylaminofluorene, and other arylamines. It tends to favour aromatic substrates, both heterocyclic aromatic substrates like caffeine and aromatic substrates like phenacetin (Miners and McKinnon, 2000). In the case of caffeine, CYP1A2 is the major isoform catalyzing the N-demethylation at the three *N*-methyl sites. In this regard, the 3-N-demethylation of caffeine to generate paraxanthine can serve as a particularly good *in vivo* indicator of the presence and activity of CYP1A2. Differences in CYP1A2 activity have clinical relevance. For instance, low CYP1A2 activity toward phenacetin favours a potentially toxic secondary pathway, deacetylation followed by quinoneimine formation and methemoglobinemia (Fischback and Lenk, 1985). High levels of CYP1A2 activity have also been associated with ineffectiveness of theophylline therapy (for asthma) (Kappas *et al*., 1978). Another concern is the co-carcinogenic effect. In this regard, there is some epidemiological evidence that high CYP1A2 activity (measured as *in vivo* caffeine metabolism) is associated with enhanced risk of colon cancer, although the effect was not seen in the absence of high *N*-acetyltransferase activity and high consumption of charbroiled meat (Lang *et al.*, 1994). Some drug interactions at the CYP1A2 level have been reported.

1.7.2. CYP2 family

The CYP2 family contains isoforms from at least five subfamilies, 2A, 2B, 2C, 2D, and 2E, which contribute significantly to drug metabolism.

- **CYP2A6**

The 7-hydroxylation of coumarin (Pelkonen et al., 2000) and the initial carbon hydroxylation of the α-carbon to the pyrrolidine nitrogen of nicotine, which upon further oxidation by aldehyde oxidase (AO) yields cotinine, are the defining metabolic activities associated with CYP2A6. CYP2A6 is also responsible for the stereospecific 3'-hydroxylation of cotinine to form *trans*-3'-hydroxycotinine (Nakajima et al., 1996), a major metabolite of nicotine in the human. CYP2A6 is polymorphic and its activity has a significant effect on smoking behaviour. People with reduced or deficient CYP2A6 levels demonstrate a significantly reduced dependency upon nicotine (Tyndale and Sellers, 2002). While CYP2A6 is the primary CYP responsible for nicotine metabolism, only a few other substrates have thus far been identified where CYP2A6 serves a similar role (Le Gal, 2003). The few that have been identified suggest that the active site of CYP2A6 favours small aromatic or heteroaromatic substrates, alkoxy ethers, and *N*-nitrosoalkylamines that are neutral or basic in character.

CYP2A6 expression has been reported to be induced during infection by (carcinogenic) liver flukes (Satarug et al., 1996) and down-regulated during infection by hepatitis A virus (Pasanen et al., 1997).

- **CYP2B6**

While generally accounting for significantly less than 1% of the total CYP present in human liver, CYP2B6 is also found in extrahepatic tissue, including brain, and it has been established as a major catalyst for the oxidation of several important drugs in current clinical use. For example, CYP2B6 catalyzes the 4-hydroxylation and the N-decholorethylation of the anticancer agents' cyclophosphamide and ifosfamide (Chang et al., 1993), respectively, the 4-hydroxylation of the anaesthetic agent propofol (Oda et al., 2001), and the methyl group hydroxylation of the antidepressant and antismoking agent bupropion (Hesse et al., 2000). The O-deethylation of 7-ethoxy-4-trifluoromethylcoumarin has been the favoured substrate to probe for CYP2B6 activity (Yuan et al., 2002), but recent evidence indicates that it is not as selective for CYP2B6 as one would hope because both CYP1A2 and CYP2E1 also catalyze this reaction. A much better indicator of CYP2B6 activity appears to be the N-demethylation of (*S*)-mephenytoin, particularly at higher concentrations of (*S*)-mephenytoin.

- **CYP2C9**

CYP2C9 is the most abundant isoform of the CYP2C subfamily (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) and one of the most extensively characterized of all the human CYPs. The active site has been explored with a variety of substrates, and computer-derived homology models that predict substrate affinity have been developed. The enzyme displays a distinct preference for acidic substrates with the defining substrates being warfarin, tolbutamide, and the nonsteroidal anti-inflammatory drugs (NSAIDS). Typical examples of the latter are flurbiprofen and diclofenac. In the case of warfarin, CYP2C9 stereoselectively catalyzes the 7-hydroxylation and 6-hydroxylation of (*S*)-warfarin to generate both (*S*)-7-hydroxywarfarin and (*S*)-6-hydroxywarfarin in a ratio of 3:1.

Together the two biologically inactive metabolites account for more than 80% of the clearance of (*S*)-warfarin from the body (Black et al., 1996). Since (*S*)-warfarin is responsible for most of the drugs anticoagulant activity [(*S*)-warfarin is five to eight times more potent an anticoagulant than (*R*)-warfarin], CYP2C9 effectively controls the level of anticoagulation by controlling the *in vivo* concentration of (*S*)-warfarin, a drug with a narrow therapeutic index. As a consequence, interference with CYP2C9 activity could be expected to have a major impact on anticoagulant response. Thus, if a second drug, in addition to warfarin, were present *in vivo,* and if the second drug were either a substrate and/or inhibitor of CYP2C9, a serious drug interaction could result. This indeed seems to be the case as a number of warfarin drug interactions have been shown to be caused by a second drug inhibiting CYP2C9 (Rettie et al., 1992), and the metabolic inactivation of (*S*)-warfarin as a direct consequence. In this regard, it is informative to note that while (*R*)-warfarin is not a substrate of CYP2C9, it is a reasonably potent inhibitor (Ki = 8 μM) of the enzyme, and does affect the elimination rate of (*S*)-warfarin (Km = 4 μM) when the drug is administered as a racemate, its normal mode of administration (Kunze et al., 1991). Therefore, while the two enantiomers of the drug have comparable affinities for the enzyme, one enantiomer is a substrate while the other is an inhibitor.

Hydroxylation of the benzylic methyl group of tolbutamide, the preferred site of oxidative attack by CYP2C9 (Veronese et al., 1991), generates hydroxytolbutamide. Hydroxytolbutamide is rapidly oxidized by other enzymes, presumably aldehyde oxidase and/or alcohol dehydrogenase (ALD), to form the major isolated metabolite, the benzoic acid analog.

The major CYP2C9-catalyzed transformation of (*S*)-flurbiprofen is formation of (*S*)-4`-hydroxyflurbiprofen (Tracy et al., 1995) and that of diclofenac is formation of 4`-hydroxydiclofenac (Leemann et al., 1993). A recently determined crystal structure of flurbiprofen-bound CYP2C9 indicates that the interaction of the carboxylate anion of flurbiprofen with a complex of hydrogen-

bonded residues, Arg-108, Asp-293, and Asn-289, orients the substrate for regioselective hydroxylation (Wester et al., 2004). Moreover, the identification of this anionic-binding site helps explain how CYP2C9, an enzyme that has a relatively large active site, is able to catalyze the regioselective hydroxylation of small molecules such as the NSAIDS with high catalytic efficiency.

- **CYP2C19**

While CYP2C19 is not a major human CYP, it does illustrate two features of this enzyme family that are worth highlighting. First, it is 91% structurally homologous with CYP2C9 and yet the two enzymes have distinct substrate selectivity (Rettie et al., 2000). It is not particularly active in metabolizing the substrates that characterize CYP2C9 nor does it favour anionic substrates. Defining substrates include the anticonvulsant, mephenytoin, and the proton-pump inhibitor, omeprazole, neither of which is a substrate for CYP2C9. This suggests that relatively limited structural changes can have profound effects on substrate selectivity despite the fact that all the CYPs utilize the same activated oxygen species. Indeed, a change as limited as a single amino acid in an enzyme that is comprised of as many as 500 amino acids can have a major effect. For example, the I359L allelic variant of wild-type CYP2C9 is much less effective in metabolizing (*S*)-warfarin, the pharmacologically active enantiomer of racemic warfarin. *In vitro,* kinetic analysis of CYP2C9 I359L indicated that the mutant metabolized (*S*)-warfarin with a fivefold lower V_{max} and a fivefold higher K_m than the wild-type CYP2C9 (Haining et al., 1996), suggesting that individuals who carried this mutant would be much more sensitive to the effects of the anticoagulant and require a much lower dose. This indeed has been found to be the case (Steward et al., 1997).

The second important feature of CYP2C19 is that it is the first isoform to illustrate the potential importance of mutant forms of the enzyme to therapeutic outcome using standard dosing. The 4-

hydroxylation of (*S*)-mephenytoin is the major metabolic pathway leading to the elimination and termination of the anticonvulsant activity of (*S*)-mephenytoin. CYP2C19 is the cytochrome CYP that catalyzes this metabolic transformation. However, in early studies the ability to metabolize mephenytoin seemed to vary within the population such that two distinct groups could be identified: extensive metabolizers and poor metabolizers. It turns out that a defective mutant form of CYP2C19 is carried by 4% of Caucasians but a full 20% of Asians. Thus, it is clear that if effective therapeutics is to be achieved, particularly with drugs with a narrow therapeutic index, knowledge of the metabolism of the drug and the enzymes and possible enzyme variants that control its metabolism is critical.

- **CYP2D6**

CYP2D6 can be considered a major contributor to the metabolism of a significant number of potent drugs used in human therapy, even though the amount of CYP2D6 present in human liver is generally less than 10% (Shimada et al., 1994) of the total amount of the cytochrome CYP present in human liver. From the perspective of substrate preference, CYP2D6 prefers basic substrates. Since most active central nervous system drugs are bases, it is hardly surprising that CYP2D6 plays an important role in the metabolic processing of these agents. A recent compilation listed 56 drugs where CYP2D6 is the primary or one of the major cytochrome CYP responsible for their metabolism (Zanger and Eichelbaum, 2000). Typical examples include the benzylic hydroxylation of the antidepressive agent, amitriptyline, the O-demethylation of the analgesic, codeine, the N-dealkylation of the antipsychotic, haloperidol, and the 4-hydroxylation of the antihypertensive, propranolol. In contrast, the antiarrhythmic agent, quinidine (also a base), is a potent (sub-μM) inhibitor of the enzyme. This fact illustrates that while the basic properties of quinidine insure that it has affinity for CYP2D6, affinity does not guarantee that the substrate will properly orient in the active site of the

enzyme with respect to the active oxidant, FeO^{3+}, for efficient metabolic transformation. Thus quinidine could be considered as a "silent substrate" of CYP2D6, i.e., a compound that is a highly effective inhibitor by virtue of its affinity for the enzyme but one that is a poor substrate by its failure to achieve an efficient catalytically susceptible orientation. Silent substrates are potentially important causes of drug interactions because if they are present *in vivo* with another drug whose metabolism is governed by an enzyme that they potently inhibit, an exaggerated pharmacological response would result (Uetrecht and Trager, 2007).

Like CYP2C19, CYP2D6 exhibits a common genetic polymorphism. In fact it was the first cytochrome P450 for which a genetic polymorphism was clearly established (Meyer and Zanger, 1997). Historically, the two drugs that defined the polymorphism and indicated that individuals within the European population could be categorized as either extensive metabolizers or poor metabolizers were the antihypertensive agent, debrisoquine, and the labor-inducing agent, sparteine. About 5–10% of this population was found to be poor metabolizers and has little capacity to convert either of these two drugs to their major metabolites, 4-hydroxydebrisoquine and 5-dehydrosparteine. While the clinical usefulness of both the drugs has been superseded by the development of better agents, they can still be effectively used as analytical tools to evaluate the catalytic activity of CYP2D6 *in vivo* in an individual or *in vitro* in a liver sample (Uetrecht and Trager, 2007).

- **CYP2E1**

Chronic exposure of rats to ethanol leads to enhanced cytochrome P450 activity. After discovery of the phenomenon, the enhanced activity was soon characterized as being primarily due to the induction of a single CYP. This enzyme was subsequently identified as CYP2E1 (Raucy and Carpenter, 2000). Later studies with the selective CYP2E1 substrate, chlorzoxazone, confirmed that

chronic ethanol ingestion also led to the selective induction of CYP2E1 in humans. Ethanol is both an inducer and substrate of CYP2E1. Indeed, CYP2E1 seems to be structurally geared to favour small volatile molecules such as ketones, aldehydes, alcohols, halogenated alkenes, and alkanes as substrates (Koop, 1992). Moreover, many of these same compounds, like ethanol, are inducers of the enzyme. A major mechanism by which this diverse group of compounds appears to initiate induction is by inhibiting normal enzyme degradation.

The apparent preference for small molecules suggests that CYP2E1 has a restricted active site. This simple observation is supported by the formation of aryl–iron complexes (Fe–Ar) in the reactions of human CYP2E1 with phenyldiazene, 2-naphthylhydrazine and *p*-biphenylhydrazine (Mackman et al., 1996).

Since a number of CYP2E1 substrates are industrial chemicals to which large numbers of people are exposed, induction has significant toxicological implications. It turns out that the structural properties of many CYP2E1 substrates can lead to the formation of chemically reactive metabolites upon enzyme-catalyzed oxidation. There is evidence that a number of these reactive metabolites are either carcinogenic or generate the expression of other toxicities. For example, chloroform is converted to phosgene, other halohydrocarbons can similarly be metabolized to acid chlorides or reductively transformed to reactive radicals, e.g., CCl_4 to $^{\bullet}CCl_3$, ethanol is converted to acetaldehyde, and alkenes are converted to epoxides, e.g., butadiene to butadiene monoepoxide. In addition, CYP2E1 generates methyl carbonium ion, a reactive methylating species capable of methylating DNA, subsequent to the *N*-demethylation of tobacco-generated nitrosoamines, e.g., *N*, *N*-dimethylnitrosoamine, to *N*-methylnitrosoamine, methyl carbonium ion, water, and nitrogen (Uetrecht and Trager, 2007).

1.7.3. CYP3 family

The CYP3 family is often considered the most important of the drug-metabolizing enzymes in humans, particularly CYP3A4, which metabolizes the majority of exogenous compounds that potentially enter the human body, in addition to a few endogenous substrates such as steroids (Lewis, 2001; Parkinson, 2001).

- **CYP3A4**

Out of all the cytochrome P450s involved in human drug metabolism, CYP3A4 could be considered to be the most important by virtue of the fact that at least 50% of marketed drugs that are metabolized by CYPs are metabolized by the CYP3A4 (Shimada et al., 1994). Generally, it is the most abundant CYP present in human liver, averaging 29% in a study that determined CYP content in 60 human liver samples. Like all CYPs, percent content of any specific CYP can vary between individuals. The variability of CYP3A4 between individuals can be as high as 20-fold (Wrighton and Thummel, 2000).

In addition to being the most abundant CYP in human liver, it is also the most abundant CYP in human intestinal mucosa averaging about 40% of what is found in liver. The high intestinal content of CYP3A4 can have a major effect on the bioavailability of orally administered drugs, because any orally administered drug must first pass through the intestinal mucosa before reaching the systemic circulation. Thus, a significant fraction of a first-pass metabolism effect might be due to the passage through the intestine and exposure to CYP3A4 before the drug reaches the liver via the portal vein, where it is again exposed to metabolism in the liver before it enters the systemic circulation (Uetrecht and Trager, 2007).

A number of substrates of CYP3A4 have been used as *in vivo* and/or *in vitro* markers of the enzyme activity to determine CYP3A4 content in human subjects or in liver or intestinal preparations. Examples include the N-demethylation of erythromycin, the ring oxidation of nifedipine, the 6*β*-hydroxylation of testosterone, and the 1-hydroxylation of midazolam. Out of these examples, the 1-hydroxylation of midazolam has properties that make it the method of choice, particularly as an *in vivo* probe. Midazolam is completely adsorbed, has a half-life of 60 to 90 minutes, the 1-hydroxylation process is specific to CYP3A4 at the concentrations used, and it appears not to be a substrate for *p*-glycoprotein, the efflux pump present in the intestinal mucosa. This means that, if desired, it would be possible to independently assess the CYP3A4 content in liver and intestine within a subject by simultaneously administering oral and intravenous doses (one dose being labelled with a stable or radioactive isotope to distinguish it from the other dose) of midazolam.

The implication of the effectiveness of CYP3A4 in catalyzing the biotransformation of so many drugs in current use implies that at least potentially clinically significant drug interactions might be associated with the use of these drugs. Clearly, one might expect to observe a drug interaction when a drug primarily metabolized by CYP3A4 is co-administered with another medication that is also either a substrate or inhibitor of this enzyme. In clinical practice, however, this turns out not to be the major problem that might have been expected. In order for a significant interaction to occur, the enzyme must be substantially inhibited and this generally requires a concentration of the inhibitor at the active site of the enzyme well in excess of its *K*i. For many inhibitors, the *in vivo* concentration achieved at the active site of the enzyme is less than its binding constant, i.e., *K*i. Thus, significant interactions generally arise from very potent competitive inhibitors, i.e., ones with a *K*i in the low micromolar or sub-micromolar region, or time-dependent inhibitors, i.e., ones that covalently modify the enzyme (Uetrecht and Trager, 2007). Also, variations in levels of CYP3A4 can cause clinical

problems when the therapeutic window is narrow. For instance, low cyclosporine levels will not prevent organ rejection during transplant but high levels can cause renal toxicity, so adjustment of the dose must be done very carefully (Yee et al., 1984).

The effect of disease on CYP3A4 has been considered. CYP3A4 expression appears to be decreased as a result of liver cirrhosis or cancer (El Mouelhi, 1984). CYP3A4 levels were also decreased in celiac disease and reversed by a change in diet (Lang et al., 1996).

The influence of herbal medicines on CYP3A4 represents an important issue in herb-drug interacions (Zhou et al., 2003). One of the most studied issues is St. John's wort, which induces CYP3A4 by virtue of being an agonist of PXR receptor (Moore et al., 2000). The induction of CYP3A4 by St. John's wort has been responsible for the loss of the effectiveness of oral contraceptives (Henderson et al., 2002).

Another issue is the inhibition of CYP3A4 by grapefruit juice and other fruit juices, first reported by Bailey (Bailey et al, 1990). The effect was rather specific for grapefruit and a few other citrus fruits (not orange), and warning labels now include this contraindication for many drugs (Greenblatt et al., 2001).

1.7.4. CYP4 family

The CYP4 family of isozymes is important in metabolizing endogenous fatty acids. Thus, this family typically binds substrates with a carboxylic acid moiety at the terminus of aliphatic chains and facilitates the ω-hydroxylation (and ω – 1 hydroxylation) of long chain fatty acids (Lewis, 2001). These enzymes do not play a major role in xenobiotic metabolism (Parkinson, 2001).

1.8. *NADPH-cytochrome P450 reductase*

It is well known that NADPH P450 reductase is a necessary component in the monooxygenase cycle and that high levels of the reductase promotes the catalytic activity of CYP-enzymes by supplying electrons to the CYP-cycle (Nakajima et al., 2002; Schenkman and Jansson, 2003; Wu et al., 2005).

Cytochrome P450-mediated microsomal electron transport is responsible for oxidative metabolism of both endogenous compounds, including fatty acids, steroids, and prostaglandins, and exogenous compounds ranging from therapeutic drugs and environmental toxicants to carcinogens. It is mediated by a multicomponent monooxygenase system, in which reducing equivalents from NADPH ultimately are transferred to molecular oxygen
(Shen & Kasper, 1993).

In its simplest form, the monooxygenase sytem consists of NADPH-cytochrome P450 reductase (CPR; NADPH-ferrihemoprotein reductase) and one of many cytochrome P450 isenzymes (Williams & Kamin, 1962; Phillips & Langdon, 1962). Both CPR and microsomal cytochromes P450 are integral membrane proteins, and CPR is one of only two known mammalian enzymes containing both

FMN and FAD as prosthetic groups. Other physiological electron acceptors of CPR include microsomal heme oxygenase (Schacter et al., 1972), and cytochrome b5 (Enoch & Strittmatter, 1979) and, although non-physiological, CPR is capable of transferring reducing equivalents to cytochrome c (Horecker, 1950).

CPR accepts a pair of electrons from NADPH as a hydride ion, with FAD and FMN being the port of entry and exit, respectively, and transfers these electrons one at a time to cytochromes P450. Cytochromes P450, in turn, use these reducing equivalents for the hydroxylation of a variety of substrates. The redox potentials of each flavin half-reaction in the native enzyme have been determined by potentiometric titrations (Iyanagi et al., 1974; Vermilion & Coon, 1978). The enzyme cycles between $1e^-$ and $3e^-$ reduced levels (or $2e^-$ and $4e^-$), with the one-electron-reduced semiquinone of the FMN being the highest oxidation state during catalytic turnover (Masters et al., 1965; Backes & Reker-Backes, 1988).

The enzyme has two functional domains, a hydrophobic N-terminal membrane-binding domain and a hydrophilic C-terminal catalytic domain that is comprised of several structural domains. The hydrophobic N-terminal domain (6 kD) serves to anchor the protein molecule to the endoplasmic reticulum and nuclear envelope (Figure 3) (Kasper, 1971), thus ensuring proper spatial interaction for electron transfer between the reductase and cytochromes P450.

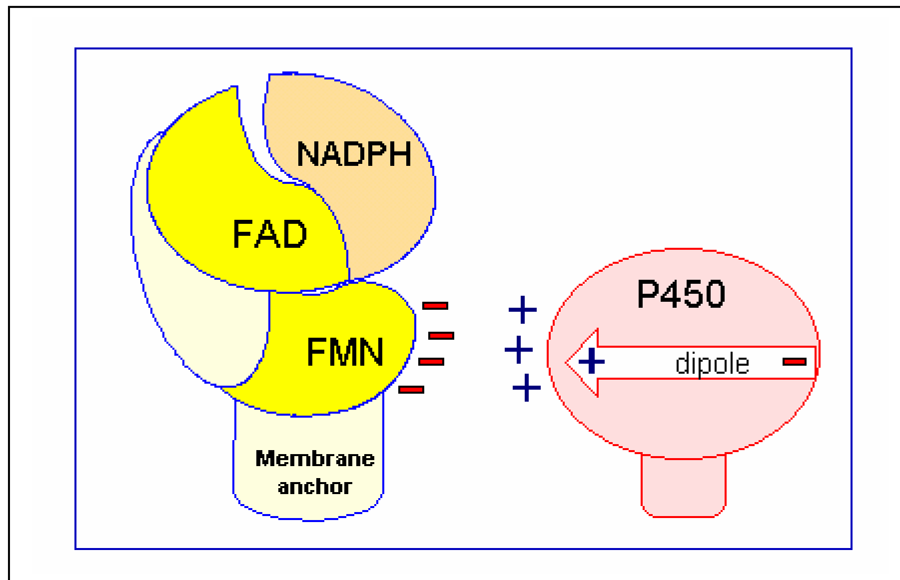


Figure 3. Electron transfer to cytochrome P450.

Source : http://www.uky.edu/Pharmacy/ps/porter/CPR_enzymology.htm

Chemical cross-linking and modification studies have shown that CPR contains multiple carboxylate groups, presumably contributed by the acidic amino acids aspartate and glutamate (Nisimoto, 1986). These charge groups pair with basic amino acids (lysines, arginines) on the various electron acceptor proteins. In addition, cytochrome P450 forms a dipole across the molecule, with the positive charge at the proximal face of the protein where the heme makes its closest approach to the surface (Hasemann et al., 1995). This is thought to be the surface most suitable for electron transfer from CPR. While electrostatic forces may serve to connect and orient the pair, hydrophobic forces contributed by nonpolar amino acids (leucine, tryptophan, valine, and others) may be responsible for bringing the two proteins close enough together for electron transfer (Inano and Tamaoki, 1985).

Site-directed mutagenesis studies have identified two clusters of acidic amino acids in the FMN domain of CPR (consistent with its role as the electron donor flavin) that, upon mutation to nonacidic

amino acids, disrupt the interaction with cytochrome P450 and cytochrome c (Shen and Kasper, 1995). The 3-dimensional structure of the reductase FMN domain-P450 BM3 complex (shown below in figure 4) supports the above model for interaction of these proteins (Servrioukova et al., 1999).

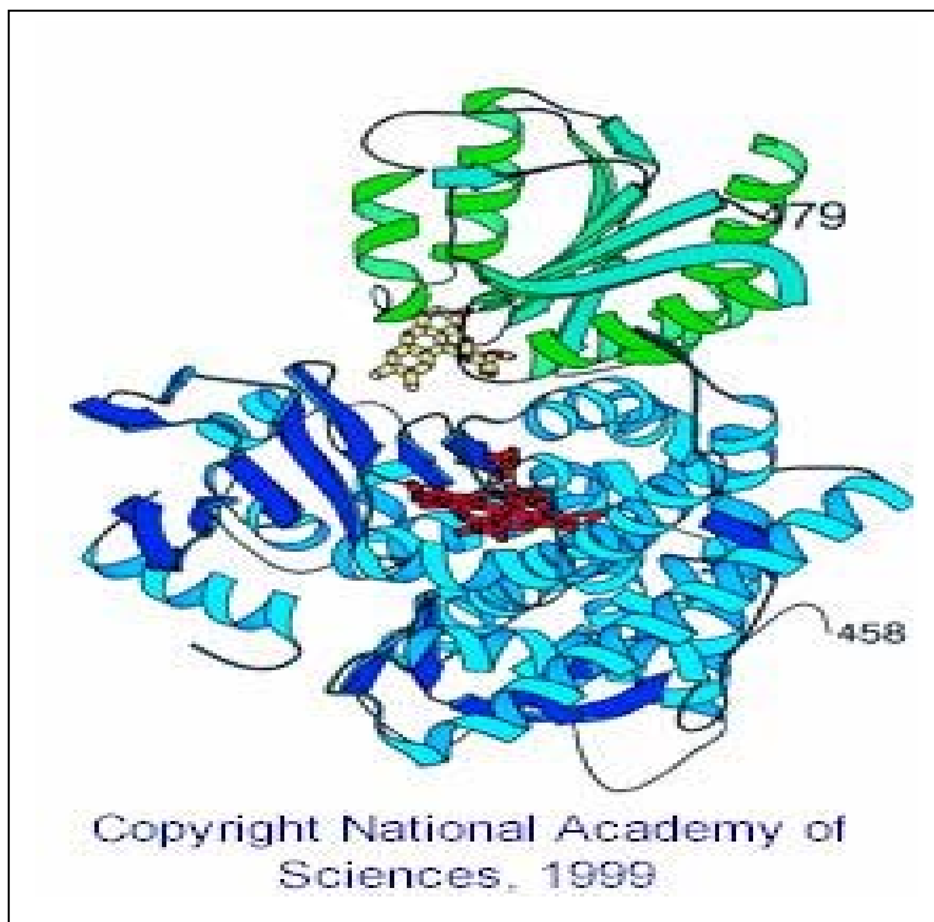


Figure 4. Three-dimensional structure of FMN domain of CPR

PART II: INFLAMMATION AND CYTOCHROME P450

Inflammation (Latin, *inflammatio*, to set on fire) is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue.

Inflammatory responses are complex, and occur in response to a number of pathological disorders, including infection, tissue damage, burns, trauma, tumors, and autoimmune disease. The entire process usually is initiated by the activation of inflammatory cells, such as macrophages or neutrophils, and is characterized by the release of cytokines, mediators, acute phase proteins, and hormones. The release of these agents results in the acute phase response, which includes the formation of a number of proteins. Because inflammation is a key component of many disease states, the alteration of drug biotransformation during any inflammatory process has to be taken into account in clinical therapeutics (Renton, 2001).

Samaras and Deitz have first documented that CYP was altered during activation of host defence mechanisms, in an abstract published in 1953 (Samaras and Deitz, 1953). They noted that the actions of pentobarbital were greatly exaggerated in rats that had been treated with a Tryptan blue particulate that had activated the immune system.

The concept that infections and inflammatory compounds could alter drug disposition originated 23 years later in the late Gil Mannering's laboratory at the University of Minnesota (Renton and Mannering, 1976). Agents such as tryptan blue dye, symosan, dextran sulphate, and latex beads have been assumed to lower CYP by activating an inflammatory response within the liver macrophages (Morgan, 1997). Inflammation in sites outside the liver, such as the administration of irritants,

adjuvants, or vaccines, has similar effects. In a classic model of inflammation, the subcutaneous administration of turpentine causes a significant decrease in a number of CYP-dependent reactions (Chindavijak et al., 1987; Morgan, 1989; Barakat & du Souich, 1996; El-Kadi et al., 1997; El-Kadi & du Souich, 1998; Bleau et al., 2000).

The first report of altered drug disposition in humans with an infection showed that the clearance of theophylline was diminished during upper respiratory tract infections caused by influenza or adenovirus (Chang et al., 1978). Several reports followed indicating that the use of theophylline in children with infections resulted in the accumulation of the drug in plasma to dangerous levels (Fleetham et al., 1978; Clarke & Boyd, 1978; Walker & Middlekamp, 1982; Greenwald & Koren, 1990). The accumulation of theophylline to levels in brain causing convulsions in several asthmatic children occurred during influenza A epidemic in Seattle (Woo et al., 1980; Kraemer et al., 1982). This effect of influenza on theophylline disposition has been reported by others. For instance Koren and Greenwald reported that routinely monitored theophylline levels tended towards the toxic range during influenza epidemics (Koren and Greenwald, 1985).

Changes in drug clearance have also been observed in disease states that involve an inflammatory response. Inflammation following surgical procedures reduced CYP3A4 activity as measured by the erythromycin breath test (Haas et al., 2003). The enzyme activity gradually diminished over a period of three days to produce a significant decrease at that time. The reduction in CYP3A4 activity at all times after surgery correlated with the concentrations of IL-6 present in blood. This study suggests that the presence of acute inflammation after elective surgery may impact on the metabolism and clearance of a large group of commonly used drugs.

2.1. Mechanisms of cytochrome P450 down-regulation

The down-regulation of CYP concentrations during infection and inflammation is almost certainly a complex and multifaceted process that involves a number of possible factors and the formation of a chain of mediators, leading to an eventual effect at the level of enzyme expression or function. Many of the conditions and immune modulators that alter CYP expression elicit a large number of effects that are mediated by a variety of mediators and intermediates. Although some have suggested that a common mediator may be involved, the diversity of agents down-regulating a variety of CYP isoforms would make this unlikely. There is much evidence to support the idea that the enzyme form and the "immune" activator determine the spectrum of response and its time course (Morgan, 1997).

2.1.1. Pro-inflammatory cytokines (Il-1, Il-6, IFN, TNF)

Since first suggested in 1976 that interferons could trigger a down-regulation of cytochrome P450 enzymes (Renton & Mannering; Leeson & Biedenback), it has been a common finding that the production and release of cytokines play a key role in the intermediate pathways and signal transduction that leads to the loss in cytochrome P450 (Morgan, 2001; Renton, 2001; Morgan, 1997; Renton and Knickle, 1990). Recombinant interferons of the three major classes have been shown to depress most isoforms of the cytochrome P450 in rodents (Parkinson, et al., 1982; Singh, Renton and Stebbing 1982; Calleja et al. 1998; Carelli et al. 1996).

Other cytokines including IL-1α, IL-1β, IL-2β, IL-6, TNF-α, and TGF-β have also been shown to mimic infection and inflammation by depressing several cytochrome P450 isoforms in rodents and in hepatocyte cultures (Fukuda et al. 1992; Clark et al., 1995; Sanne & Krueger, 1995; Barker et al,

1992; Wright & Morgan, 1991; Nadin et al, 1995). A recent review and tabular data collection indicates that many of the cytochrome P450 isoforms that are involved in steroid synthesis pathways in different mammalian species are modulated by cytokines (Herrmann et al., 2002). IL-6 appears to have a widely differentiated response on cytochrome P450 isoforms and has a dose dependent sensitivity often absent with other cytokines (Chen et al, 1992; Chen et al, 1994).

2.1.2. Nitric Oxide

Another mediator that has received much attention is nitric oxide (NO) that is produced by NOS2 in response to inflammation. There is no doubt that considerable amounts of NO is produced in mammalian species in response to inflammatory stimuli that could reduce cytochrome P450 activity by decreasing mRNA and protein levels, by altering the protein or by binding to the heme moiety (Liaudet et al., 2000). Although a number of studies have demonstrated that inflammation-mediated reduction of cytochrome P450 correlate with NO production, and that this decrease can be blocked by NOS inhibitors, other studies have shown that inhibition of NOS has no effect in modulating the effect of inflammation on cytochrome P450 isoforms (Hodgson & Renton, 1995; Monshouwer et al, 1996; Sewer & Morgan, 1997).

2.1.3. Oxidative Stress

Several other mechanisms underlying the down-regulation and/or the decrease in activity of cytochrome P450 enzymes have been suggested, including oxidative stress and the production of reactive oxygen mediators (Renton, 2001; Morgan, 1997). However, these mechanisms may play a role only for some specific cases and at specific levels of signal transductions pathways and appears to have a minor role in the widely reported down-regulation.

2.1.4. Decrease in Gene Expression

At the level of cytochrome P450 isoform expression itself, it is fairly well accepted that for most of the enzyme forms there is a decrease in the specific mRNA and subsequent protein synthesis during inflammation, infection or following the administration of cytokines (Renton, 2001; Morgan, 2001). For most cytochrome P450s the reduction in mRNA precedes the decrease in enzyme and its activity, and the entire process is following other pattern as that of enzyme induction processes (Renton, 2004). The down-regulation of cytochrome P450 isoforms involves an intracellular protein intermediate, assumed to be a transcription factor (Moochhala & Renton, 1991).

2.1.5. Intra-cellular Signalling Pathways

The response to cytokines is closely associated to the activation of transcription factors such as NF-κB or C/EBP (CCAAT-enhancer binding protein) (Morel & Barouki, 1998; Iber et al, 2000). In addition, the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) play a role in the inflammation mediated decrease in CYP2B and CYP3A (Van Ess et al, 2002; Sachdeva et al, 2003; Beigneux et al, 2002). From all evidence to date, it is clear that the

decrease of most cytochrome P450 isoforms during inflammatory responses involves a decrease in transcription following changes of the expression and regulation of a variety of transcription factors. Changes in specific transcription factors are targeted to specific cytochrome P450 isoforms. The apparent lack of evidence for the involvement of a single common transcription factor for all cytochrome P450 forms is likely a good explanation to account for the differential responses of the various enzyme families to diverse inflammatory stimuli (Renton, 2004).

2.1.6. Post Transcriptional Mechanisms

A few studies have suggested that for some cytochrome P450 isoforms inflammation mediated depression is partly due to post transcriptional mechanisms (Morgan, 1989; Delaporte and Renton, 1997). While it is clear that the decrease in cytochrome P450 isoforms can occur at post transcriptional stages, this mechanism only accounts for a small proportion of the reduction that occur during inflammatory responses and it appears to involve more frequently the CYP3A family. The vast majority of reports indicate that the down-regulation of most enzymes occurs at the level of transcription (Renton, 2004).

At the practical level more evidence continues to emerge indicating that drug clearance can be significantly changed in humans during disease states that involve an inflammatory component. With the recognition that most isoforms of cytochrome P450 are modified during periods of inflammation or infection, there may be a need to alter drug therapy during these periods and to be particularly vigilant at monitoring for untoward drug responses (Renton, 2004).

PART III: OSTEOARTHRITIS

The definition of osteoarthritis (OA) has evolved over the past two decades and now recognizes OA as a syndrome with a complex aetiology rather than as a single disease entity. Osteoarthritis can be defined as a gradual loss of articular cartilage, combined with thickening of the subchondral bone, bony outgrowths (osteophytes) at joint margins, and mild, chronic nonspecific synovial inflammation. The difference between physiologic aging of the cartilage and OA cartilage is not sharp. However, three cartilage stages can be identified: stage I, normal cartilage; stage II, aging cartilage; and stage III, OA.

3.1. Normal Cartilage

Normal cartilage has two main components. One is the extracellular matrix, which is rich in collagens (mainly types II, IX, and XI) and proteoglycans (mainly aggrecan). Aggrecan is a central core protein bearing numerous glycosaminoglycan chains of chondroitin sulfate and keratan sulfate, all capable of retaining molecules of water. The second component consists of isolated chondrocytes, which lie in the matrix. The matrix represents 95% of the cartilage and the chondrocytes only 5%. The matrix components are responsible for the tensile strength and resistance to mechanical loading of the articular cartilage (Klippel, 2008).

3.2. Osteoarthritic Joints

Osteoarthritic joints have abnormal cartilage and bone, with synovial and capsular lesions (Kenneth et al., 2003). Macroscopically, the most characteristic elements are reduced joint space, formation of osteophytes (protrusions of bone and cartilage) mostly at the margins of joints, and sclerosis of the subchondral bone (Figure 5).

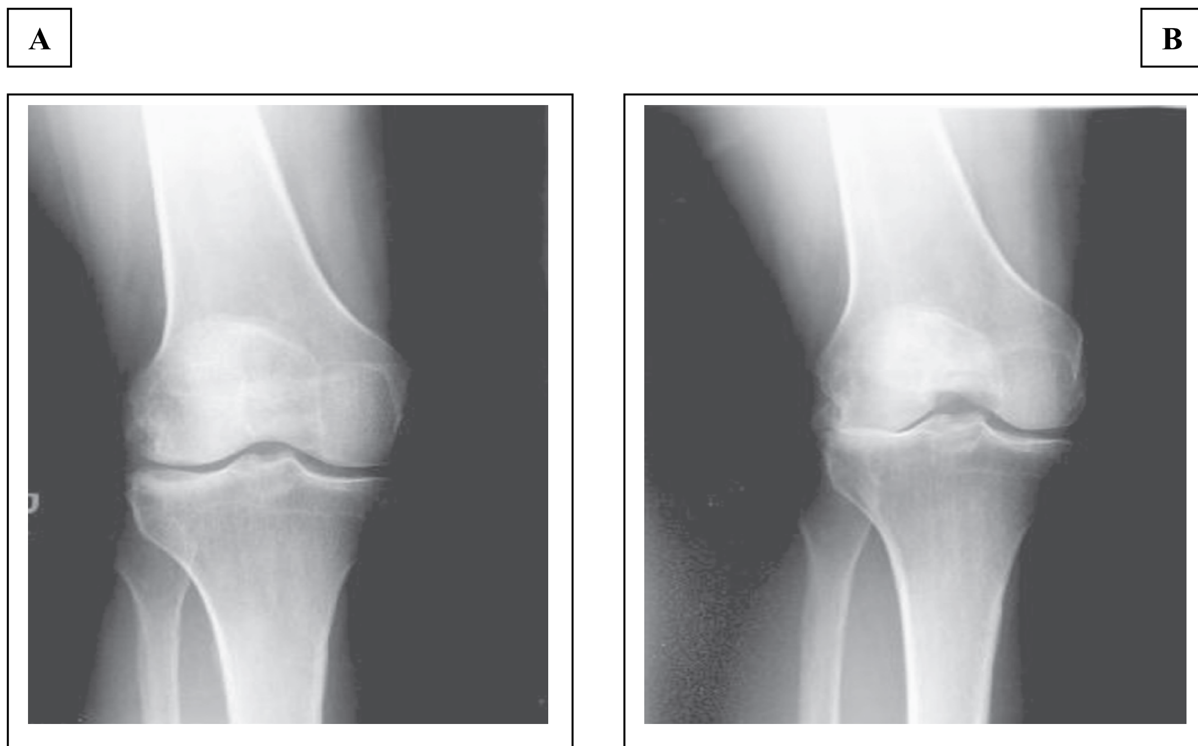


Figure 5. Standing anteroposterior (A) and standing flexed postero-anterior (B) views of the right knee.[a]

[a]In (A), no significant narrowing of the joint is identified although osteophytes and subchondral sclerosis, indicative of osteoarthritis, are evident. In (B), however, the standing flexed view demonstrates complete articular cartilage loss in the lateral compartment.

3.3. Enzymes Involved in Cartilage Degradation

The main proteinases involved in the destruction of the cartilage in OA are the matrix metalloproteinases (MMPs) (Cawston, 1998). There are at least 18 members of this gene family of neutral Zn^{2+} metalloproteinases. Because they are active at neutral pH, the MMPs can act on the cartilaginous matrix at some distance from the chondrocytes. They can be synthesized by chondrocytes and synoviocytes under the influence of cytokines.

The activities of MMPs are strictly controlled by stoichiometric inhibition with specific inhibitors, the tissue inhibitors of metalloproteinases (TIMP1-4). Therefore, the balance between the amounts of MMPs and TIMPs in cartilage determines if cartilage is degraded (Dean et al., 1989). MMPs produced by the chondrocytes and released into the extracellular matrix are activated by an enzyme cascade involving serine proteinases (plasminogen activator, plasminogen, plasmin), free radicals, cathepsins, and some membrane-type MMPs. This enzymatic cascade is regulated by natural inhibitors, including the TIMPs and the inhibitors of the plasminogen activator. MMP-13 is elevated in OA joint tissues, particularly in articular cartilage, and colocalizes with type II collagen cleavage epitopes in regions of matrix depletion in OA cartilage (Dean et al., 1989).

The other enzymes that can degrade type II collagen and proteoglycans are the cathepsins. They are active only at low pH and include the aspartate proteinases (cathepsin D) and cysteine proteinases (cathepsins B, H, K, L, and S) that are stored in chondrocyte lysosomes and released into the pericellular microenvironment. Glycosidases also may be important, because proteoglycans are very rich in carbohydrate chains. Although hyaluronidases are not present in cartilage, other glycosidases may contribute to the degradation of proteoglycans (Cawston et al., 1998).

3.4. Cytokines

Although OA has often classified as a non-inflammatory disease, numerous studies have shown that inflammatory cytokines provide essential biomechanical signals that stimulate chondrocytes to release cartilage-degrading enzymes. Pro-inflammatory cytokines synthesized by chondrocytes and synoviocytes bind to specific receptors on chondrocytes. These bound cytokines trigger the transcription of the MMP genes, and the genes' products are exported from the cell in an inactive form. It is generally accepted that IL-1 is the pivotal cytokine released during inflammation of the osteoarthritic joint (Jacques et al., 2006). Other cytokines are released, including chemokines (IL-8), growth-regulated oncogene (GRO) alpha, macrophage inflammatory proteins (MIP-1 alpha and MIP-1 beta). Some of these cytokines and chemokines may be regulatory [e.g., IL-6, IL-8, lymphocyte inhibitory factor (LIF)], or inhibitory (e.g., IL-4, IL-10, IL-13, IFN-γ). IL-1 receptor antagonist, IL-4, IL10, and IL-13 prevent the secretion of some MMPs, and may increase the synthesis of TIMPs. In a more general way, IL-4 and IL-13 counteract the catabolic effects of IL-1. Finally, IL-1 alters the quality of the cartilage matrix by decreasing the synthesis of type II and IX collagens, while increasing the synthesis of type I and type III collagens.

3.5. Lipid Mediators

The eicosanoids also can take part in chondrocyte activation (Goldring and Berenbaum, 2004). Prostaglandins, produced after activation of phospholipases A2, cyclooxygenases (mainly the cyclooxygenase-2 isoform) and prostaglandin synthases (mainly the microsomal prostaglandin E synthase-1) by proinflammatory cytokines, can favour the synthesis of MMPs by activating the cell via specific cellular or/and nuclear prostaglandin receptors (Goldring and Berenbaum, 2004). Among the eicosanoids, prostaglandin E_2 seems to be the main lipid mediator produced by synovial cells, chondrocytes, and subchondral osteoblasts and involved in cartilage degradation in OA.

3.6. Reactive Oxygen Species

Reactive oxygen species (ROS) play a crucial role in the regulation of a number of basic chondrocyte activities, such as cell activation, proliferation, and matrix remodelling. However, when ROS production exceeds the antioxidant capacities of the cell, an oxidative stress occurs, leading to structural and functional cartilage damages like cell death and matrix degradation (Henrotin et al., 2005).

Nitric oxide (NO) is a gas synthesized by way of the oxidation of L-arginine by the NO synthases (NOS). Chondrocytes produce large amounts of NO after up-regulation of the inducible NOS gene (iNOS or NOS2) by cytokines. Most *in vitro* studies indicate that NO is partly responsible for the blockade of glycosaminoglycan and collagen synthesis by IL-1, and may contribute to the activation of the latent forms of MMPs. NO may also mediate the IL-1–stimulated synthesis of *MMP* mRNA and protein, and may contribute to chondrocyte cell death by interfering with survival signals from the extracellular matrix. However, NO may have anabolic and anticatabolic effects in cartilage

under certain conditions. Therefore, the actual role of NO in the degradative process of OA is not clear (Abramson et al., 2001).

3.7. Matrix Degradation Products

The products of matrix degradation, such as fibronectin fragments, can activate chondrocytes through integrin-type receptors that will increase the synthesis of MMPs. These products can stimulate or activate other factors, such as catabolic cytokines, that amplify the damage. The damage, in turn, enhances the concentrations of the degradation products themselves, as in a positive feedback loop (Peters et al., 2005).

3.8. Mechanical Stress

Along with chemical mediators, biophysical mediators could also be directly involved in chondrocyte activation in OA. Compressive, but also shear and stretch, stresses occur on cartilage. Interestingly, there is considerable evidence that interactions between biomechanical factors and pro-inflammatory mediators are involved in the initiation and the progression of OA (Guilak et al., 2004). *In vivo* studies have shown increased concentrations of inflammatory cytokines and mediators in the joint in mechanically induced models of osteoarthritis. *In vitro* explant studies confirm that mechanical load is a potent regulator of matrix metabolism, cell viability, and the production of pro-inflammatory mediators such as NO and prostaglandin E_2. Chondrocytes have receptors for responding to mechanical stress and can respond to direct biomechanical perturbation by up-regulating synthetic activity or inflammatory cytokines, which are also produced by other joint tissues. Chondrocytes express several members of the integrin family, and these can serve as

receptors for fibronectin (alpha 5 beta 1), types II and VI of collagen (alpha 1 beta 1, alpha 5 beta 1, alpha 10 beta 1), laminin (alpha 6 beta 1), and vitronectin and osteopontin (alpha V beta 3). Some of these receptors are sensitive to prolonged changes in pressure (mechanoreceptors). Injurious static or dynamic compression stimulates depletion of proteoglycans and damage the collagen network and decreases the synthesis of cartilage matrix proteins, whereas low intensity dynamic compression increases matrix synthetic activity. Certain types of mechanical stress and cartilage matrix degradation products are capable of stimulating the same signalling pathways as those induced by IL-1 and tumor necrosis factor alpha (TNF-α). These signalling pathways involve cascades of kinases, including the stress-activated protein kinases (SAPKs), including the c-Jun N-terminal kinases (JNKs) and p38 MAP kinase, phosphatidylinositol- 3′-kinase (PI-3K), and IκB kinases, leading to the nuclear translocation of NF-κB. Because these signalling pathways may also induce the expression of the genes encoding these cytokines, it remains controversial whether inflammatory cytokines are primary or secondary regulators of the progressive cartilage destruction in OA (Yasuda et al, 2002; Loeser et al, 2005).

3.9. Chondroitin sulfate

Chondroitin sulfate (CS) is a linear heteropolysaccharide chain of repeating disaccharide units of d-glucuronic acid and d-galactosamine sulfated at the C-4 and/or C-6 positions, covalently linked to proteins forming proteoglycans; the proteoglycan aggrecan contains hyaluronic acid instead of CS (Guilak et al., 2004). Joint cartilage consists of chondrocytes (5% of the volume) embedded in a matrix of fibrous collagen within a concentrated water proteoglycan gel. Damage of the structure collagen/proteoglycans and synovitis are the basis of osteoarthritis (Bhosale and Richardson, 2008).

While it is a prescription or over-the-counter drug in 22 countries, chondroitin is regulated in the U.S. as a dietary supplement by the Food and Drug administration. As a result, in chondroitin sulfate supplements, there are no mandatory standards for formulation, and no guarantee that the product is correctly labelled. This is not the case of Europe where there is a chondroitin sulfate formulation approved as a drug and considered as the reference product, with evidenced efficacy and safety demonstrated by clinical trials in osteoarthritic patients (Vergés and Castañeda-Hernández, 2004).

3.10. Chondroitin sulfate in osteoarthritis

Randomized clinical trials in patients with OA have shown that CS reduces pain and improves articular function (Uebelhart et al., 2004), reduces joint swelling and effusion, and prevents joint space narrowing of the knee (Uebelhart et al., 1998) and fingers (Rovetta et al., 2002) more effectively than placebo. According to these effects, CS has been classified as a symptomatic slow acting drug in osteoarthritis (SYSADOA). Because CS appears to slow down cartilage damage, CS has also been classified as a structure/disease modifying anti-osteoarthritis drug (S/DMOAD) (Uebelhart et al., 2004).

3.11. Effect of CS on articular cartilage

The complex clinical response to CS may tentatively be explained by the numerous effects that have been attributed to this drug. On the one hand, the decrease in pain and swelling may be explained by an anti-inflammatory effect of CS, probably through diverse mechanisms such as diminishing the expression of phospholipase A2 (PLA2) (Ronca et al., 1998), of cyclooxygenase-2 (COX-2), and the concentrations of prostaglandin E_2 (PGE_2) (Chan, 2005; Orth, 2002; Bassleer,

2002). Moreover, CS reduces in joints the concentrations of pro-inflammatory cytokines, such as TNF-α (Campo 2003) and IL-1β (Chou, 2005), as well as systemic and joint concentrations of NO (Orth, 2002; Chan, 2005) and of reactive oxygen species (ROS) (Campo, 2003).

There is evidence that in chondrocytes, CS diminishes IL-1β-mediated increase in MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 (Orth, 2002; Chan, 2005). On the other hand, it has been documented that hyaluronan and mixtures of low concentrations of CS and glucosamine were able to prevent the release of MMP-3 and MMP-13 triggered by fibronectin fragments (FN-f) (Homandberg et al., 2004; 2006). In subchondral bone, CS increases osteoprotegerin (OPG) and reduces the expression of receptor activator of nuclear factor-kappa B ligand (RANKL), effects that may result in the reduction of the resorptive activity (Tat et al., 2007).

In chondrocytes, CS diminishes ERK1/2 phosphorylation and abrogates the phosphorylation of p38MAPK induced by IL-1β; as a consequence, CS reduces IL-1β-induced NF-κB nuclear translocation. However, CS does not reduce IL-1β-induced AP-1 nuclear translocation. On the other hand, CS decreases nitroprusside-induced apoptosis of the chondrocytes which is associated to p38MAPK activation (Jomphe et al., 2008). In chondrocytes, chondroitin disaccharides sulphated at positions 4 and/or 6, (1-4)-*O*-(D-glucopyranosyluronic acid)–(1-3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-4/6-sulfate) (Δdi-4S, Δdi-6S and Δdi-4,6S) reduce IL-1β-induced NF-κB nuclear translocation to a similar extent as CS, e.g. Δdi-4S, Δdi-6S and Δdi-4,6S reduce NF-κB translocation (Figure 6).

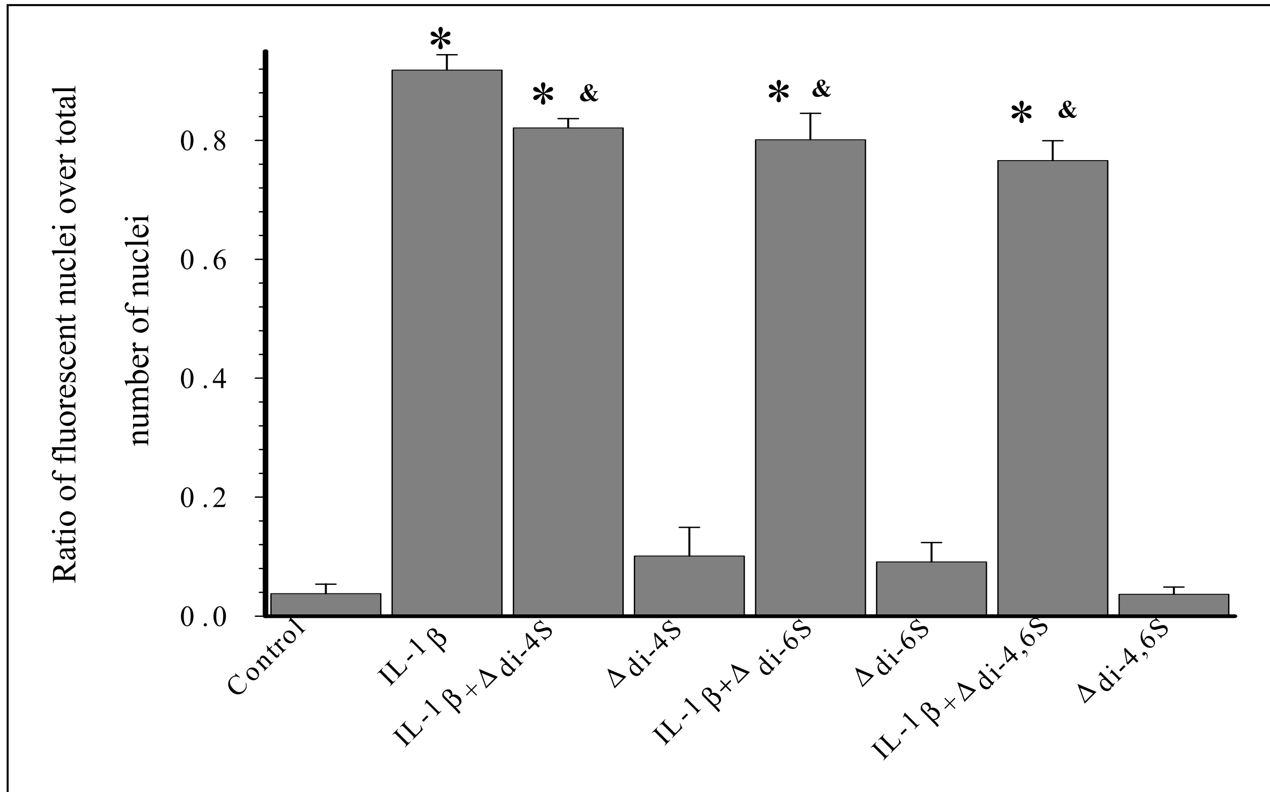


Figure 6. Effect of CS disaccharides sulphated in position 4 and/or 6 (Δdi-4S, Δdi-6S, Δdi-4,6S) on Il-1β-induced NF-κB nuclear translocation

It has been widely documented that IL-1β-induced increase in expression of MMP-3 (Liacini et al., 2002; Schulze-Tanzil, 2004; Sylvester et al., 2001), MMP-9 (Lianxu, 2006), MMP-13 (Liacini et al., 2002; Mengshol, 2000; Wada et al., 2006), COX-2 (Wada et al., 2006; Berenbaum et al. 2003), NOS2, IL-1β and TNF-α (Wen et al., 2006; Hanada et al. 2002) is mediated by the activation and nuclear translocation of NF-κB and AP-1. Moreover, there is evidence that the activation of phospholipase A2 requires the activation of p38MAPK and ERK1/2 (Berenbaum et al., 2003), and that the induction of RANKL expression requires the activation of ERK1/2 and PI-3K/Akt pathways (Tsubaki et al., 2007). Since the above mentioned effects of IL-1β and of NF-f are mediated by the activation of p38MAPK and of ERK1/2, and the nuclear translocation of NF-κB, it has been

proposed that the pleiotropic effects of CS are dependent, at least in part, by its ability to inhibit p38MAPK and ERK1/2 phosphorylation and NF-κB nuclear translocation (Iovu et al., 2008).

3.12. Effect of CS on the synovial membrane

Synovial tissue from patients with early osteoarthritis show activated fibroblast-like synoviocytes (FLS), macrophages, T lymphocytes, and mast cells infiltration (Benito et al., 2005). FLS release IL-1β, IL-6, IL-8, MMP-1, MMP-2, MMP-3, MMP-13, MMP-14, MMP-16, TIMP-1, RANKL, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF).

The role of NF-κB in the development of synovitis appears essential. In FLS, the production of IL-1β, IL-6, IL-8, MMP-1, and MMP-3, requires the activation and nuclear translocation of NF-κB (Xu, 2007; Lauder, 2007). Moreover, the activation of NF-κB increases FLS proliferation and transforms the phenotype of these cells to highly invasive cells with great motility and ability to secrete cytokines and MMP-13 (Li et al., 2006). Inhibition of the IκB kinase (IKK) complex impedes the phosphorylation of inhibitor of κB (IκBα) and as a consequence, prevents NF-κB activation. In synovial macrophages, inhibition of IKK diminishes IL-1β-induced production of IL-6; moreover, in rats with adjuvant-induced arthritis, intraarticular injection of a specific IKK-β inhibitor reduces arthritis activity and bone destruction; synovial inflammation is also decreased as documented by the reduction in synovial cellularity, TNF-α, IL-1-β concentrations, and reduction of the volume of the paw (Tas et al., 2006). These results were confirmed by administering in the articulation a dominant-negative form of IKK-β that reduced synovial cellularity by 50%, and diminished synovial concentrations of IL-1β, TNF-α and MMP-3 (Tas et al. 2006). These results provide evidence that activation and nuclear translocation of NF-κB is an important step in the development of synovitis.

There is little information about the effect of osteoarthritis treatment on synovitis manifestations, e.g. joint swelling and effusion. The multicenter, double-blind, placebo- and celecoxib-controlled Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) assessed the effect of CS and glucosamine alone or in combination on joint swelling and/or effusion in 1583 patients with mild to severe knee osteoarthritis (Clegg et al. 2006). The patients received 1200 mg of CS, or 1500 mg of glucosamine or both CS and glucosamine, or 200 mg of celecoxib or placebo, daily for 24 weeks. The study demonstrates that CS diminished the percentage of patients with signs of synovitis (joint swelling and effusion) from 28.3% at baseline to 12.4% at the end of 24 weeks of treatment. It is of interest that the beneficial effect of CS was observed in the patients with mild pain (WOMAC pain scores 125 - 300). In patients with moderate to severe pain (WOMAC pain scores 301 – 400) receiving CS, the percent of patients with swelling and/or effusion tended to decrease from 30.0% at baseline to 14.9% ($p = 0.3$) at the end of follow-up.

Further supporting that CS reduces the signs and symptoms of synovitis, a study showed that intra-articular injection of hyaluronate, a glycosaminoglycan with a mw of 8.4x105, to patients with rheumatoid arthritis improves local clinical symptoms, decreases synovial fluid, reduces prostaglandin E2 concentrations and diminishes pain (Goto et al. 2001).

Several animal studies demonstrate that CS reduces the signs and symptoms of synovitis. In DBA/1J mice with a type II collagen-induced arthritis, treated for 9 weeks with various dosages of CS, the infiltration of inflammatory cells, granulated tissue formation, proliferation of synovial lining cells, paw edema and destruction of articular cartilage were partially prevented by treatment with 1000 mg/kg/day of CS for 63 days (Omata et al. 2000). In dogs with unilateral carpal synovitis induced by injecting into the right radiocarpal joint chymopapain, prior treatment with CS reduces the extent of synovitis (Canapp et al., 1999). In rabbits with experimental OA, intra-articular

administration of N-acetylglucosamine elicited an anti-inflammatory effect and suppressed the synovitis (Shikhman et al. 2005).

All these studies strongly support that in animal models and in humans, glycosaminoglycans reduce the synovitis. The mechanism of action underlying the reduction of synovitis signs by CS and other glycosaminoglycans remains incompletely characterized. It has been reported that chondroitin sulfate disaccharide Δdi-6S reduces IL-1β-induced nuclear translocation of NF-κB by 67% in synoviocytes (Alvarez-Soria et al., 2005). This observation is in agreement with the effect of CS and its Δdi-4S and Δdi-6S disaccharides in chondrocytes, e.g. they reduce NF-κB nuclear translocation (Iovu et al., 2008). Since oral CS increases plasma concentrations of Δdi-4S and Δdi-6S (Volpi, 2002), it is conceivable that in humans, the decrease in synovitis signs produced by CS may be explained, at least in part, by the reduction in NF-κB nuclear translocation in synoviocytes and macrophages, with the subsequent diminution of activation of these cells and decrease in synovitis.

In summary, CS and/or the sulfated disaccharides appear to elicit an anti-inflammatory effect at the synovial membrane and chondrocytes levels. Possibly, CS and/or disaccharides reduce the inflammatory reaction by diminishing NF-κB nuclear translocation (Figure 7). In the chondrocytes, this effect is mediated by the inhibition of p38MAPK phosphorylation and to a minor degree ERK1/2 phosphorylation. Indeed, further studies are required to better characterize the precise mechanism of action underlying CS-induced improvement of synovitis.

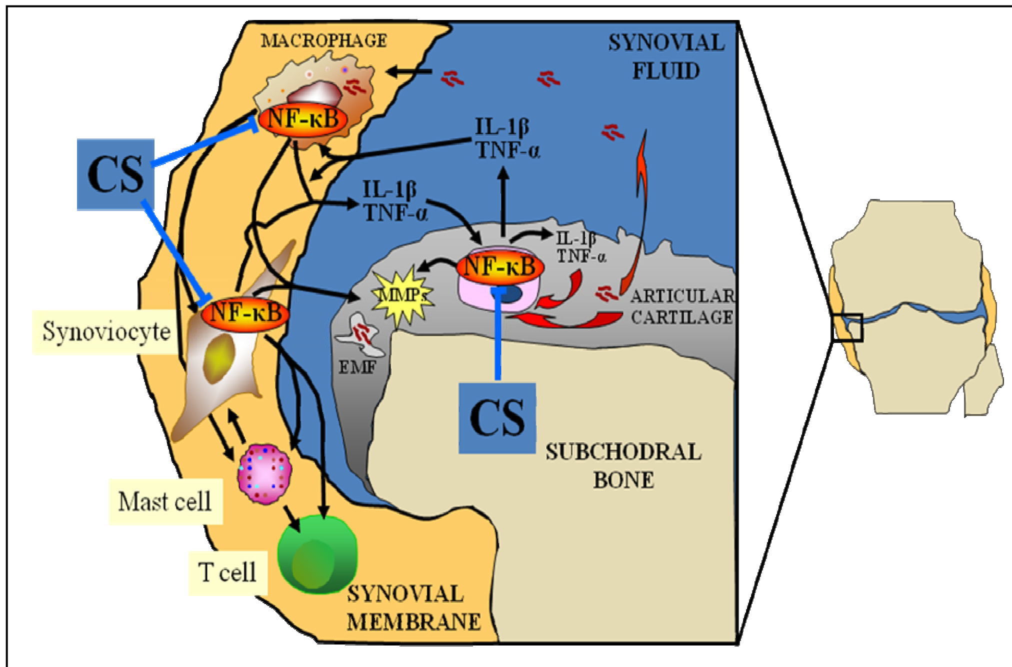


Figure 7. Diagram depicting the potential sites of effect of CS and/or its disaccharides[a]

Source: Iovu et al., 2008

[a]Local microtraumas produce EMFs and FN-f that activate chondrocytes by increasing the nuclear translocation of NF-κB in the chondrocytes, synovial macrophages and synoviocytes. NF-κB has a key role in the pro-inflammatory activation of chondrocytes, and synovial macrophages, mast cells, T-cells and synoviocytes and in the release of cytokines and MMPs that will sustain cartilage and subchondral bone destruction.

Conclusion

Patients with osteoarthritis are usually multi-medicated for the disease itself and other accompanying diseases, e.g. hypertension, diabetes, hyperlipidemia, etc. The lack of safe alternatives and the results of clinical studies that have not identified any significant side effects or overdoses of CS (Hathcock and Shao, 2007), makes this drug a one of the safest drugs for osteoarthritis.

The benefit of chondroitin sulfate in patients with osteoarthritis is likely the result of a number of effects including its anti-inflammatory activity, the stimulation of the synthesis of proteoglycans

and hyaluronic acid, and the decrease in catabolic activity of chondrocytes inhibiting the synthesis of proteolytic enzymes, nitric oxide and other substances that contribute to damage cartilage matrix and cause death of articular chondrocytes. A recent review summarizes data from relevant reports describing the biochemical basis of the effect of chondroitin sulfate on osteoarthritis articular tissues (Monfort et al., 2007). The rationale behind the use of chondroitin sulfate is based on the belief that osteoarthritis is associated with a local deficiency in some natural substances, including chondroitin sulfate.

Recently, new mechanisms of action have been described for chondroitin sulfate. In an *in vitro* study, chondroitin sulfate reduced the IL-1β-induced nuclear factor-kB (Nf-kB) translocation in chondrocytes (Jomphe et al., 2007). In addition, chondroitin sulfate has recently shown a positive effect on osteoarthritic structural changes occurred in the subchondral bone (Tat et al., 2007).

Since CS is taken by the patients for long periods of time, the questions are what does CS do on baseline CYP isoforms and, by the nature of its anti-inflammatory effect, does CS prevents the down-regulation of CYP isoforms during an inflammatory reaction?

II. HYPOTHESIS AND STUDY OBJECTIVE

Patients with infectious and/or inflammatory diseases may present transient decreases in the activity of the mixed function oxidase system (Chang et al., 1978; Sonne et al., 1985). In animals, a turpentine-induced inflammatory reaction reduces the activity of selected isoforms of the cytochrome P450 (Letarte & du Souich, 1984; Wright & Morgan, 1990; Parent et al., 1992). There is limited information about the mechanism of action underlying the changes in CYP activity and expression provoked by an inflammatory reaction secondary to the injection of turpentine (Barakat et al., 2001); although it is known that cytokines like IL-6 and IL-1β are the primary serum mediators and that the mechanism of action implies a pretranscriptional phenomena (Bleau et al., 2003). It is also known that IL-1β activates the nuclear translocation of NF-κB which binds to negative regulatory elements of cytochrome P450 genes impeding the transcription of the gene (Iber et al., 2000). On the other hand, chondroitin sulfate (CS) elicits an anti-inflammatory effect by a mechanism not fully understood. CS prevents IL-1β-induced p38MAPK and Erk1/2 phosphorylation and diminishes NF-κB nuclear translocation (Jomphe et al., 2008).

These observations led us to hypothesize that chronic intake of CS could prevent the effects of turpentine-induced inflammatory reaction on cytochrome P450 expression and activity.

The project's objective was to assess the effect of CS on the activity and expression of hepatic CYP in rabbits *in vivo* with and without a turpentine-induced inflammatory reaction, and secondly to assess the effect of CS on the activity and expression of hepatic CYP *in vitro*, along with the three sulphated disaccharides of CS (4S, 6S and 4,6S). The primary objective of this study is an exploratory one.

III. MATERIALS AND METHODS

EXPERIMENTAL PROTOCOL

The dose of chondroitin sulfate used in humans for the treatment of osteoarthritis is 800-1,200 mg orally per day or 11 to 17 mg/kg. The dose of CS administered to the rabbits was approximately 20 mg/kg/day (± 5 mg/kg/day) dissolved in the water to be drunk along the day. The effect of chronic intake of CS on the cytochrome P450 was tested with two models, control rabbits and in rabbits with an inflammatory reaction. Seven groups of five rabbits were used: one group was kept for 30 days in the animal facilities and used as control; three groups of normal rabbits were used to assess the effect of CS on cytochrome P450, one without CS and two receiving orally 20 mg/kg/day CS for 20 and 30 days. The remaining three groups received turpentine s.c. generating an aseptic inflammatory reaction (AIR) 48 h before their sacrifice, e.g. at days -2, 18 and 28, and were exposed to CS for 0, 20 or 30 days, respectively. Rabbits of all groups were sacrificed and the liver was removed to assess the activity, protein content and mRNA of CYP1A2, 3A6 and NADPH-reductase (Figure 8).

Before the sacrifice of the rabbits, blood was withdrawn (15 ml). Serum was obtained by leaving the blood at room temperature for at least 2 h, followed by centrifugation at 2500 rpm for 5 minutes. The serum was employed to assess the seromucoids according to the method described elsewhere (Parent et al., 1992).

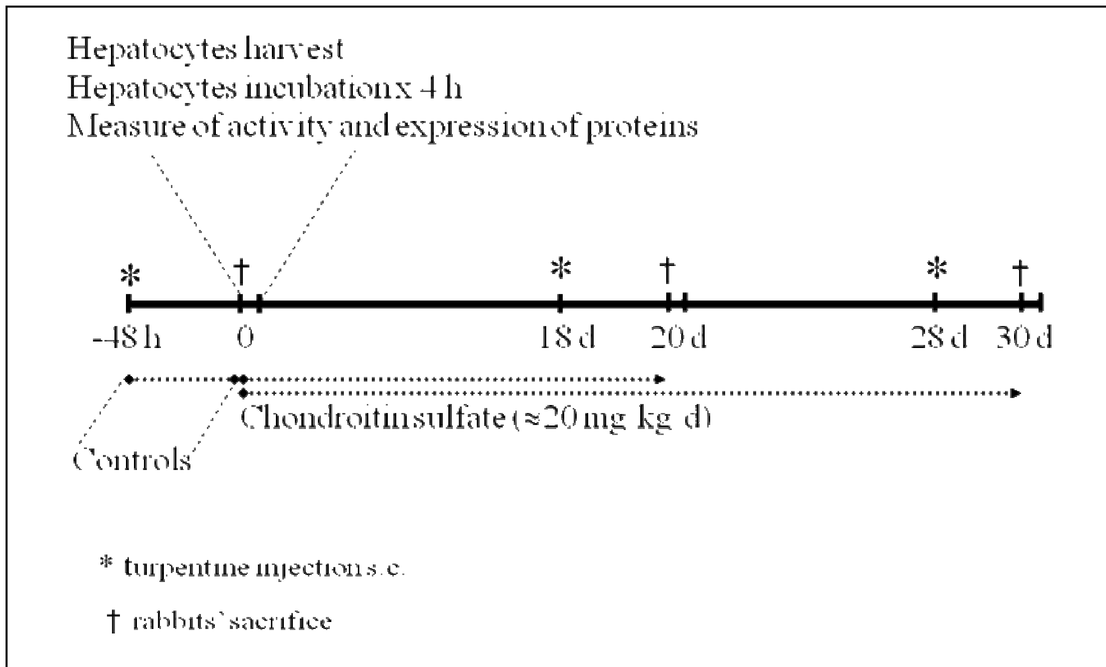


Figure 8. Protocol representation

In vivo studies

Male New Zeeland rabbits (2-2.3 kg) (Charles River, St-Constant, Qc, Canada) were housed in separate cages and fed water and chow *ad libitum* for at least seven days before experiments started. Seven groups of five rabbits each were used, of which three groups were controls and the remaining four received dissolved in the drinking water 20 mg/kg/day of CS orally for 20 or 30 days. Two of the control groups were used to assess the effect of the inflammatory reaction on the cytochrome P450; to this purpose, the rabbits of one control group had an aseptic inflammatory reaction caused by the subcutaneous (s.c.) injection of turpentine (volume injected 5 ml distributed at four sites of the back), and were sacrificed 48 h later; the rabbits of the second control group received saline s.c. and were sacrificed 48 h later (Figure 8.). Finally, the rabbits of the third control group were simply kept

in the animal facilities for 30 days to assess the effect of age and environment on the cytochrome P450. Of the four groups of rabbits receiving CS, two were exposed to CS for 20 days and two for 30 days. The rabbits of one group receiving CS for 20 days and one group receiving CS for 30 days received turpentine s.c. at day 18 and at day 28, respectively, while the rabbits of the remaining two groups exposed to CS received saline s.c., and all rabbits were sacrificed two days later, e.g. at days 20 or 30.

Forty-eight hours after the injection of turpentine or saline, a blood sample (15 ml) was withdrawn from the rabbit with a sterile Vacutainer Brand SST (Becton Dickinson, Mississauga, Ont., Canada). The severity of the inflammatory reaction was assessed by taking the rectal temperature and by measuring the concentration of seromucoids (Parent et al., 1992). All experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals and protocols approved by the Comité de déontologie de l'expérimentation animale of Université de Montréal.

Hepatocyte isolation and culture conditions

Male rabbits were housed in separate cages and fed water and chow ad libitum for at least 7 days before experiments started. The inflammatory reaction was provoked by local subcutaneous injections of turpentine distributed at four sites of the back of the rabbits (total volume injected 5 ml). The severity of the inflammatory reaction was assessed by taking the rectal temperature and by measuring the concentration of seromucoids (Parent et all., 1992).

Isolation and culture of primary rabbit hepatocytes was conducted according to the two-step liver perfusion method described by Seglen (1976), with minor modifications (El-Kadi et al., 1997).

The rabbits were anesthetised with 30 mg/kg of sodium pentobarbital. After a laparotomy, the cava and portal veins were cannulated; through the portal vein, the liver was perfused with 800 ml of

a solution containing 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 25 mM HEPES, 0.5 mM EGTA, 5.5 mM glucose and 0.067 mg/ml heparin, to clear blood from the liver. Thereafter, the liver was perfused with a solution containing 0.013% collagenase, 1 mM $CaCl_2$ and 0.25 mM trypsin inhibitor, until the digestion of liver was completed (the time will vary with the size of the liver). Complete digestion was indicated by softening and enlargement of the tissue.

When the perfusion was completed, the liver was removed and placed in a dish; using tissue scissors the capsule was gently thorn and the cells were separated in 100 ml medium containing 0.013% collagenase, 1 mM $CaCl_2$ and 0.25 mM trypsin inhibitor, 1 ml William medium E (WME) to which was added 10 % calf serum, 1% streptomycin/penicillin and 1.2 μM $MgSO_4$ at 37° C. The digested material was then filtered through a nylon membrane of 230 μm, followed by a second filtration through a nylon membrane of 80 μm.

The resulting cell suspension was then divided equally into four sterile centrifuge tubes of 50 ml on the ice, adding WME up to 50 ml. The cell suspension was washed by low-speed centrifugation (100 g for 5 min). The supernatant was discarded and cells were gently resuspended in approximately 20 – 30 ml WME. This stage was repeated three times.

Harvested cells were centrifuged on isodensity Percoll to isolate viable liver cells (over 95% viability as assessed by tryptan blue exclusion). Hepatocytes (3×10^6 in 3 ml of William's medium E supplemented with 10% calf serum) were plated in 12-well plastic culture plates (Corning; Fisher, Mississauga, Canada) coated with type I rat tail collagen. Cell culture was conducted under sterile conditions and maintained at 37° C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was changed 2h after plating, and thereafter the hepatocytes were incubated for an additional 4 hours before onset of the experiments.

In vitro studies

The objective of this series of experiments was to explore whether incubation of various concentrations of CS and its disaccharides with hepatocytes for 48 hours affected the activity and expression of CYP1A2, CYP3A6 and NADPH cytochrome P450 reductase (CPR).

Plated hepatocytes were kept for one hour at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air before their culture medium was changed and four different concentration of CS and its three disaccharides were added as follows:

- CS: 50, 100, 200, 450 µg/ml
- 4S-disaccharide: 10, 20, 40, 80 µg/ml
- 6S-disaccharide: 10, 20, 40, 80 µg/ml
- 4,6S-disaccharide: 10, 20, 40, 80 µg/ml

The hepatocytes were allowed up to 48 hours of incubation before assessing the activity and the amount of CYP1A2, CYP3A6 and NADPH-reductase. Proteins were assessed by Western blot analysis.

The chondroitin sulfate used in the present study is purified chondroitin 4&6 sulfate of bovine origin (Volpi and Maccari, 2005), the same was used in clinical studies (Uebelhart and col., 2004; Clegg and col., 2006). The chondroitin sulfate used may contain disulfated disaccharides that are not detectable or present in a minor concentrations (<0.1%).

Measure of Oxide Nitric ($NO^{\cdot}$) from hepatocyte culture supernatant

Nitric oxide was determined by measuring nitrite and nitrate in the culture media using a colorimetric method based on the Griess reaction (Nims et all., 1996).

To reduce nitrate to nitrite, samples were incubated at 37° C in the presence of 0.1 U/ml nitrate reductase, 50 µM NADPH and 5 µM FAD. Following nitrate reduction, to avoid any interference with the determination of nitrite, NADPH was oxidized by incubating the samples with 10 U/ml lactate dehydrogenase and 10 mM sodium pyruvate for 5 min at 37° C. The concentration of nitrite was assumed to reflect that of nitric oxide and the results are expressed as concentration of $NO^{\cdot}$ relative to total mg proteins.

Quantification of CYP3A6 activity

The activity of CYP3A6 was determined by measuring the ability of the hepatocytes to convert 3,4-difluorobenxyloxy-5,5-dimethyl-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one (DFB), a CYP3A probe, to 3-hydroxy-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one (DFH), its fluorescent metabolite (Chauret et all., 1992). After 4 hours of incubation, the growth media was removed and the cellular layer was rinsed twice with 300 µl of Krebs solution (9.6 g/L Krebs, 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM sodium bicarbonate, 12.5 mM HEPES of pH 7.4). Briefly, 60 µM of DFB were incubated for 20 min with the hepatocytes, and then 100 µl of the supernatant was transferred to a microtiter plate and quenched with an equal volume of acetonitrile containing 40% Tris buffer (0.05 M). The fluorescence metabolite DFH was measured at excitation and emission wavelengths of 360 and 440 nm, respectively, using a fluorescent plate reader (Victor2, 1420 Multilabel Counter, PerkinElmer Wallac, Gaithersburg, Maryland, USA).

Quantification of CYP1A2 activity

The activity of CYP1A2 was determined by measuring the methoxyresorufin O-demethylation (MROD) to resorufin in intact cells, as described by Van Vleet et al. (2002). Growth media was removed and cells washed twice with 300 µl of WME. After removal of WME, 3.3 µM methoxyresorufin in 300 µl of WME was added to the wells containing the hepatocytes and incubated for 10 min at 37° C. Thereafter, 100 µl of supernatant was added to 100 µl of a solution of perchloric acid/glycine and 5.4% K_2CO_3 (2:1, v/v). Resorufin production was measured fluorimetrically at excitation and emission wavelengths of 530 and 584 nm respectively, with a fluorescent plate reader (Victor2, 1420 Multilabel Counter, PerkinElmer Wallac, Gaithersburg, Maryland, USA).

NADPH-Reductase Assay

This assay measures the reduction of cytochrome c by NADPH-cytochrome c reductase in the presence of NADPH. The absorption spectrum of cytochrome c changes with its oxidation/reduction state. Upon reduction, a sharp absorption peak is observed at 550 nm. The reduction of cytochrome c is monitored by the increase of cytochrome c absorbance at 550 nm. The activity is measured with a spectrophotometer, using Soresen's phosphate buffer at room temperature; 1-4 milliunits of enzyme are added per assay. Samples from rabbit hepatocytes are assayed in the presence of potassium cyanide. The activity of the samples assayed is calculated using the following unit definition: one unit will reduce 1.0 µmole of oxidized cytochrome c in the presence of 100 µM NADPH per minute at pH 7.8 at 25ºC (Masters et Kamin, 1967).

$$\frac{Units}{ml} = \frac{\frac{\Delta A550}{\min} \times dil \times 1.1}{21.1 \times Enzvol}$$

$\Delta A_{550}/\min = \Delta A_{sample} - \Delta A_{blank}$

dil = the dilution factor of the original enzyme sample

Enzvol = volume of the enzyme sample (ml)

21.1 = extinction coefficient (ε^{mM}) for reduced cytochrome c

1.1 = reaction volume (ml)

BCA Protein Assay

Hepatocyte monolayers were frozen under liquid N2, and then stored at -80oC, for a latter utilisation. FOZ- buffer (with 1:1000 leupeptine, 1:100 sodium ortho-vandate and 1:1000 phenylmethanesulphonylfluoride) is added to six-well plates containing the hepatocytes. After 30 minutes incubation, the cells are scraped off and transferred in tubes for centrifugation (10 min at 13,000 rpm). The supernatant is stored at -80oC or it is used further for protein measurement, which is done by BCA Protein Assay as it follows:

1. Supernatant is diluted 1/70
2. Serial dilution for standard curve was performed using bovine serum albumin (BSA)
3. 25 µl of each standard and each sample was added to a 96-well microplate. Samples were assessed in triplicata.
4. Working reagent is prepared using a ratio of 1:20 (Reagent A: Reagent B)
5. 200 µl of working reagent was added to each well.
6. The microplate was placed in an incubator for 30 minutes at 37 ^{o}C.

7. Absorbance of each well was read with a microplate reader.
8. Amount of protein in each well was calculated by plotting a standard curve using Excel software.

Measure of the expression of CYP1A2, CYP3A6 proteins, and NADPH-reductase

The amount of CYP1A2 and CYP3A6 proteins in hepatocytes incubated for 4 hours was assessed by Western blot analysis. Proteins (60 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (7.5% polyacrylamide) at 120 V. Proteins were electrophoretically transferred to a nitro-cellulose membrane using a semidry transfer process (Bio-Rad, Hercules, CA, USA).

CYP1A2 was detected with a polyclonal anti-rabbit CYP1A1/2 antibody (1:100) incubated for 16 hours, and then visualized with an alkaline phosphatase conjugated secondary goat antibody (1:16666) using blue tetrazolium as the substrate.

CYP3A6 protein was detected with a monoclonal anti-rat CYP3A1 antibody (1:2000), with a cross-reactivity to rabbit CYP3A6, incubated for 16 hours; thereafter, an incubation of 1 hour follows with a horse-radish peroxidase-conjugated secondary antibody (HRP) diluted at 1:8000. Chemiluminescence was visualized by autoradiography with the kit ECL advance (GE Healthcare, Canada).

NADPH-reductase was detected with a monoclonal anti-mouse NADPH-reductase antibody (1:5000) incubated for 16 hours; thereafter, an incubation of 1 hour follows with a horse-radish peroxidase-conjugated secondary antibody (HRP) diluted 1:2000. Chemiluminescence was visualized by autoradiography with the kit ECL advance (GE Healthcare, Canada).

In each gel, 50 µg of proteins extracted from the same batch of H_{CONT}, with constant amounts of CYP1A2, CYP3A6 and NADPH-reductase were used as reference proteins. The assay was linear in the range of protein amounts assessed under the present experimental conditions. The intensities of the bands were measured with the software Un-Scan-It-Gel (Silk Scientific Inc., Orem, UT, USA), and the results are presented as the ratio of the experimental samples to the respective reference protein.

NF-κB nuclear translocation

The effect of CS on the nuclear translocation of NF-κB was assessed with hepatocytes cultured for 48 hr in 12-well plates containing 12 glass coverslips. Hepatocytes were incubated overnight with 250 µl of rabbit anti-p65 NF-κB antibody diluted 1:250. Secondary antibodies included the Alexa 488 goat anti-rabbit IgG (H + L) antibody 1:250 to visualize NF-κB. To visualize the nuclei, the glass coverslips were incubated with the blue fluorescent probe 4',6-diamidino-2-phenylindole (DAPI), 1 µl/ml, for 15 min., after which they were washed with water and mounted on slides in Vectashield for subsequent observation by epifluorescence microscopy, on a Nikon Eclipse TE-200 inverted microscope. Images of immunofluorescent labelling were acquired using a Hamamatsu Orca-II digital cooled CCD camera and an Inovision workstation using the Isee software (Inovision Corporation, Raleigh, NC, USA).

Nuclear translocation of NF-κB, determined by immunofluorescence, was expressed as the ratio between the numbers of nuclei stained with secondary goat anti-rabbit IgG anti p65 NF-κB antibodies over the total number of nuclei marked with the DAPI probe. In each experiment, a minimum of five fields were examined and assessed, and the average value recorded.

Purification of total RNA from hepatocytes

The protocol for purification of total RNA was done according to RNeasy Mini Kit Qiagen bench protocol. Hepatocytes were plated in 6-well plastic culture plates (Corning; Fisher, Mississauga, Canada) coated with type I rat tail collagen. Cell culture was conducted under sterile conditions at 37° C in a humidified atmosphere containing 95% air and 5% CO_2 for 4h. After incubation, the plates were washed with 500 µl of PBS 1X, then trypsin-EDTA 0,25% was added to detach the cells from plate, and medium (containing serum to inactivate the trypsin) was added. A centrifugation was performed for 5 min at 300 x g to recover the cells in the pellet, and the supernatant was carefully removed by aspiration. The pellet was kept at -80 ºC, for a later use or was used directly in the procedure. B-mercaptoethanol (β-ME) was added to Buffer RLT before use, to produce the lysis. The lysate was homogenized by passing it at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe; 1 volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. At this time DNase digestion was performed, for eliminating genomic DNA contamination. The whole volume was transferred to an RNeasy spin column and centrifuged for 30 s at 13,000 rpm; the flow-through was discarded. Next, RW1 buffer was added to the RNeasy spin column followed by a centrifugation at 13,000 rpm for 30s to wash the spin column membrane. Five hundred µl Buffer RPE was added to the RNeasy spin column and centrifuged for 30 s at 13,000 rpm to wash the spin column membrane; the flow-through was discarded. This procedure was repeated twice. To elute the RNA, the RNeasy spin column was placed in a new 1.5 ml collection tube, and 20-25 µl RNase-free water was added directly to the spin column membrane. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide to visualize RNA under ultraviolet light. The samples were kept to -80ºC until the next

utilisation. Real-time RT-PCR was performed by the Genomic platform of the IRIC to quantify mRNA expression in hepatocytes.

Briefly, total RNA was isolated using Trizol according to manufacturer's protocol (RNeasy Mini Kit Qiagen bench protocol). RNA was treated with DneaseI (Invitrogen) before cDNA synthesis. Reverse transcription of total RNA was performed using the MMLV-RT and random hexamers according to manufacturer's protocol (Invitrogen).

Gene expression levels (CYP3A6 and NADPH P450 reductase) were measured by custom primers and TaqMan probes using the online version of PrimerQuest software *(http://scitools.idtdna.com/Primerquest/)*. Default parameters for real-time PCR were used to select the best primers and probes. In order to amplify only the cDNA, primers were located in different exons or in the splicing junction between two exons. PCR reactions were performed using 2 µL of cDNA samples (10-40 ng), 5 µL of the TaqMan PCR Master Mix (Applied Biosystems), 10 pmol of each primer, and 5 pmol of the TaqMan probe in a total volume of 10 µL.

The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed to an initial step of 10 minutes of 95ºC, followed by 50 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. All reactions were run in triplicate and the average values were used for quantification. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as endogenous control. The relative quantification of target genes was determined by using the ΔΔCT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to the endogenous control gene (GAPDH) ($\Delta CT = Ct_{target} - Ct_{GAPDH}$) and compared with a calibrator (control Sca^+Lin^- cells): $\Delta\Delta CT = \Delta Ct_{Sample} - \Delta Ct_{Calibrator}$. Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula $RQ = 2^{-\Delta\Delta CT}$.

Oligos Sequence

NADPH-Cyt P-450 reductase

Primer a = ACAAGACCTACGAGCACTTCAACG

Primer b = AAGTCCTCCTCCAGGTTTGCATCA

Probe = AGCGCATCTTCGAGCTGGGCAT

CYP3A6

Primer A = AGCACTGGACTGAGCCTGATGAAT

Primer B = AACCTCATGCCAAGGCAATTTCGG

Probe = CCGCCCTGAAAGGTTCAGTAAGAAGA

GAPDH

Primer A = GGCATTGCCCTCAATGACCACTTT

Primer B = GTGGTTTGAGGGCTCTTACTCCTT

Probe = ACGAATTTGGCTACAGCAACAGGGTGGT

Statistical analysis

All results are reported as means ± S.E. The comparison of the results from the various experimental groups and their corresponding controls was carried out using a two-way analysis of variance (ANOVA) followed by "Holm-Sidak Method". The differences were considered significant when $P < 0.05$. The analysis was done by the means of SigmaStat software 3.11.

Materials

Percoll gradient, William's medium E, calf serum, type I rat tail collagen, trypsin inhibitor, NaCl, KCl, KH_2PO_4, EDTA, EGTA, glucose, heparin, N-2-hydroxyethylpiperazine-n '-2-ethanesulfonic acid, pH 7.2 (HEPES), trypsin inhibitor, glycine, T RIS tampon, sulphanilamide, N-NEDA, methoxyresorufine, blue tetrazolium, sodium orthovanadate, Krebs-Henseleit, and Temed were purchased from Sigma (Oakville, Ontario, Canada). NaCl, KCl, KH_2PO_4, $CaCl_2$, sodium pyruvate, acetonitrile, methanol, DMSO and 6, 24 and 96 well-plates were purchased from Fisher Scientific Canada (Ottawa, ON, Canada). The foetal calf serum (FCS) and penicillin/streptomycin were purchased from Gibco (Invitrogen cell culture, Burlington, ON, Canada). The type IV collagenase was purchased from Worthington Biochemicals (Lakewood, NJ, USA). The turpentine was purchased from Recochem (Montréal, QC, Canada). The DFB and DFH were generously donated by Merck Frost Canada (Kirkland, QC, Canada). Monoclonal anti-rat CYP 3A1 antibody and polyclonal anti-rabbit CYP 1A1/2 antibody was purchased from Oxford Biochemical Research (Oxford, MI, USA). Monoclonal anti-mouse NADPH-reductase antibody and the horse-radish peroxidase-conjugated secondary antibody (HRP) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Nitrate reductase and lactate dehydrogenase were

purchased from Roche Diagnostics (Mannheim, Germany). Rabbit anti-p65 NF-κB antibody was acquired from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Chondroitin sulfate and its derived disaccharides were provided by Bioibérica (Barcelona, Spain).

IV. RESULTS

CYTOCHROME P450 AND NADPH-REDUCTASE ACTIVITY

1 - Effect of an inflammatory reaction on CYP1A2, CYP3A6 and NADPH-reductase activity

The first step of this study was to determine if the inflammatory reaction would cause the down-regulation of the cytochrome P450 isoforms CYP1A2 and CYP3A6, and would the effect NADPH-reductase activity. The activity of CYP1A2, CYP3A6 and NADPH-reductase in hepatocytes obtained from the rabbits with an aseptic inflammatory reaction was compared with those activities from control rabbits. Results are reported as arbitrary units of activity divided by the amount of proteins (mg). As indicated in table 1, the activity of CYP1A2 decreased by 56% ($p<0.05$), and the activity of CYP3A6 was reduced by 63% ($p<0.05$); the inflammatory reaction did not change the activity of NADPH-reductase.

Activity (Arbitrary units/mg protein)	$\mathbf{H_{cont}}$	$\mathbf{H_{infla}}$
NADPH-reductase	33 ± 3 (n=5)	29 ± 7 (n=5)
CYP1A2	633 ±131 (n=5)	276 ± 77* (n=5)
CYP3A6	1565 ± 263 (n=5)	570 ± 174* (n=5)

*Table 1. NADPH-reductase, CYP1A2, CYP3A6 activity assessed in hepatocytes from control rabbits (H_{cont}) and in hepatocytes from rabbits with an inflammatory reaction (H_{infla}). *p <0,05 compared with H_{cont}*

2 - Effect of the administration of CS for 20 days on the activity of CYP1A2, CYP3A6 and NADPH-reductase

In the control rabbits receiving CS for 20 days, the activity of hepatic CYP1A2 tended to increase. On the other hand, following 20 days of intake of CS, the activities of CYP3A6 and NADPH-reductase tended to decrease ($p>0.05$).

Treatment of rabbits with chondroitin sulfate for 20 days did not prevent the reduction in activity of CYP1A2 and CYP3A6 produced by the inflammatory reaction.

As indicated in table 2, the inflammatory reaction diminished the activity of CYP1A2 by 73% ($p<0.05$), and that of CYP3A6 by 67% ($p<0.05$). In rabbits with the inflammatory reaction, intake of CS for 20 days did not affect the activity of NADPH-reductase.

Activity (Arbitrary units/mg protein)	**$H_{cont}CS_{20days}$**	**$H_{infla}CS_{20days}$**
NADPH-reductase	23 ± 3 (n=5)	20 ± 2 (n=5)
CYP1A2	920 ±171 (n=5)	246 ± 90* (n=5)
CYP3A6	1030 ± 270 (n=5)	340 ± 65* (n=5)

*Table 2. NADPH-reductase, CYP1A2, CYP3A6 activity assessed in the hepatocytes from control rabbits (Hcont) and in the hepatocytes from rabbits with an inflammatory reaction (Hinfla) following the administration of CS for 20 days. *p <0,05 compared with Hcont*

3- Effect of the administration of CS for 30 days on the activity of CYP1A2, CYP3A6 and NADPH-reductase

Since the intake of CS for 20 days did not affect the activity of the isoforms of the CYP, it was of interest to document whether a prolonged intake of CS, eg. for 30 days, would modify the effect of the inflammatory reaction.

In control rabbits, after the intake of CS for 30 days, the activity of CYP1A2 was not modified although the activity of CYP3A6 tended to decrease ($p>0.05$). In rabbits with the inflammatory reaction, the intake of CS for 30 days did not prevent the decrease of the activity of CYP1A2 and CYP3A6. The activity of NADPH-reductase was not affected by the experimental conditions. As indicated in table 3, in rabbits administered CS for 30 days, the inflammatory reaction decreased the activity of CYP1A2 by 48% ($p<0.05$) and that of CYP3A6 80% ($p<0.05$).

Activity **(Arbitrary units/mg protein)**	$H_{cont}CS_{30days}$	$H_{infla}CS_{30days}$
NADPH-reductase	18 ± 1 (n=5)	15 ± 2 (n=5)
CYP1A2	410 ±37 (n=5)	213 ± 43* (n=5)
CYP3A6	827 ± 112 (n=5)	159 ± 30* (n=5)

*Table 3. NADPH-reductase, CYP1A2, CYP3A6 activity assessed in hepatocytes from control rabbits (Hcont) and in hepatocytes from rabbits with an inflammatory reaction (Hinfla) following the administration of CS for 30 days. *p <0,05 compared with Hcont*

There is no significant change in activity of NADPH-reductase due to inflammation, when compared with control rabbits after 30 days of CS intake. Contrary there is a significant decrease of this activity when compared with control rabbits with no CS intake. It is important to observe that there is a tendency to decrease as a function of time, for the activity of NADPH-reductase and CYP3A6, and CS does not exibit a protective role.

4A - Effect of 30 days stay in the animal facilities on CYP1A2, CYP3A6 and NADPH-reductase activities

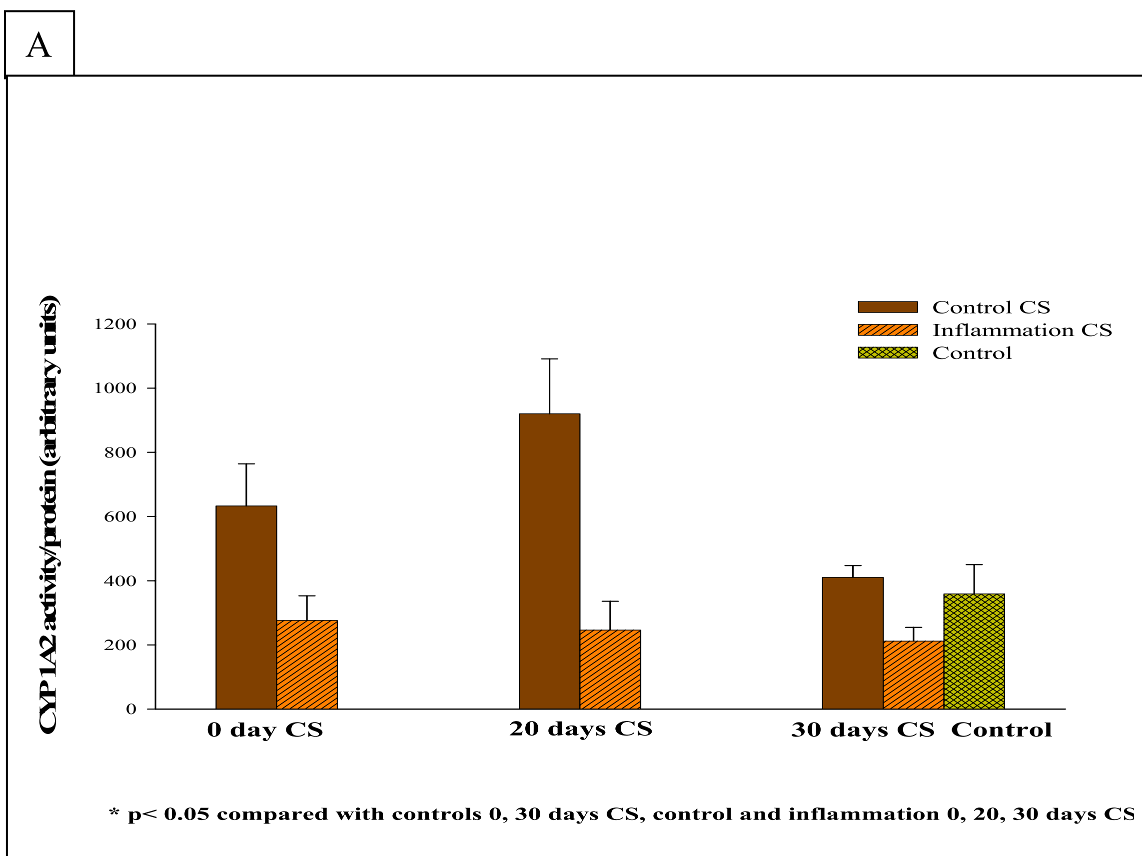
Since in the the previous two study groups, the rabbits were kept for 30 days in the animal facilities, a control group of rabbits kept for 30 days in the animal facilities without any other intervention was necessary. Therefore, the effect of 30 days in the animal facilities on the activities of CYP1A2, CYP3A6 and NADPH-reductase was assessed.

The activity of CYP1A2 for the group of rabbits without CS intake, kept 30 days in the animal facilities is not significantly decreased when compare with control 0 days CS. The only difference observed is the significant increase in the activity of CYP1A2 for control group with 20 days intake of CS, but overall the activity of CYP1A2 does not seams to be influenced by the time the rabbits spent in the animal facilities (figure 9-A).

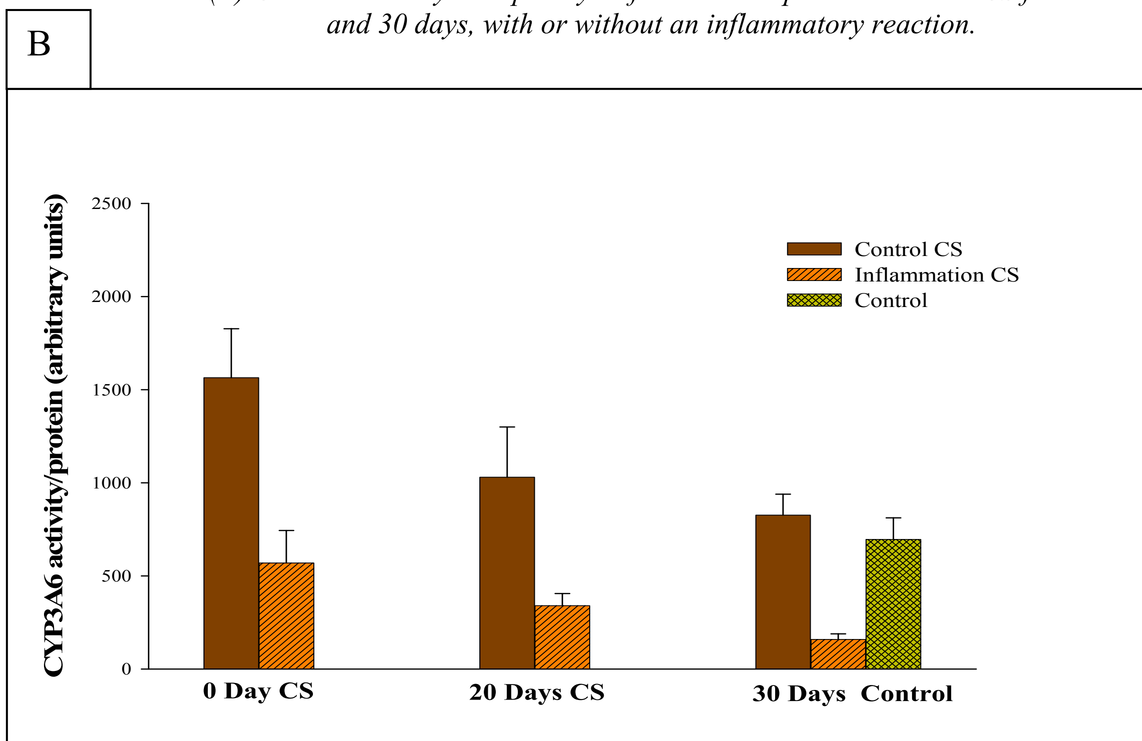
The activity of CYP3A6 remains depressed as a function of time. The group of rabbits kept in animal facilities for 30 days register the lowest activity of CYP3A6, but comparable with that of control group with 30 days of CS intake (figure 9-B).

The activity of NADPH-reductase shows a significant decrease for group of rabbits kept for 30 days in animal facilities, when compared with control group 0 days, but is still comparable with that of control group 30 days CS (figure 9-C).

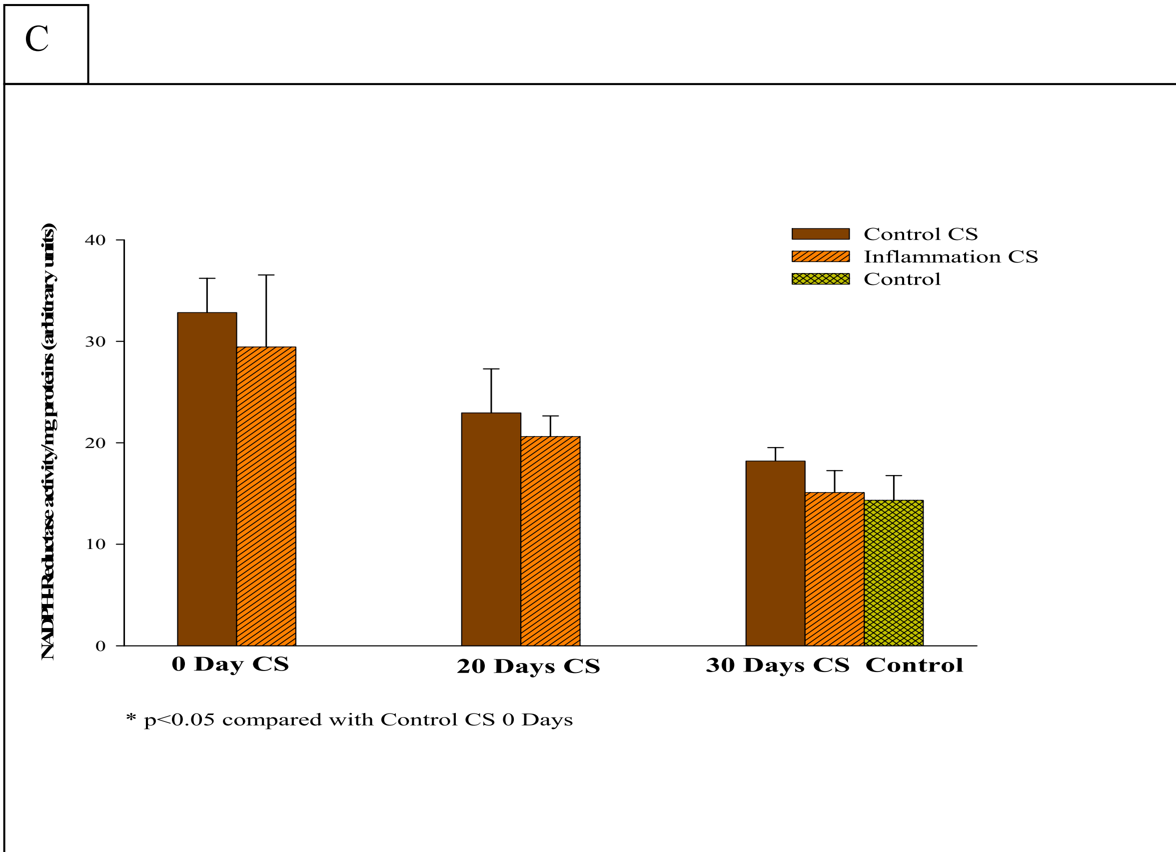
Figure 9. (A) CYP1A2 activity in hepatocytes from rabbits pre-treated with CS for 20 and 30 days, with or without an inflammatory reaction.



(B) CYP3A6 activity in hepatocytes from rabbits pre-treated with CS for 20 and 30 days, with or without an inflammatory reaction.



(C) NADPH-reductase activity in hepatocytes from rabbits pre-treated with CS for 0, 20 and 30 days, with or without an inflammatory reaction.



CYTOCHROME P450 AND NADPH-REDUCTASE EXPRESSION

1- Effect of the inflammatory reaction on the expression of CYP1A2, CYP3A6 and NADPH-reductase

Proteins from group of rabbits without CS administration, with and without an AIR, were subjected to Western blotting analyses, to confirm the differential expression of CYPs and NADPH-reductase (Figure 10). Compared with control rabbits at Day 0, CYP1A2 protein expression decreased by 0.69 ± 0.150 ($p<0.05$) and CYP3A6 protein expression decreased significantly by 0.78 ± 0.07 ($p<0.05$) 48 hours after the production of turpentine-induced inflammatory reaction. There was no change in the protein expression of NADPH-reductase following an aseptic inflammatory reaction.

2- Effect of the administration of CS for 20 days, on the expression of CYP1A2, CYP3A6 and NADPH-reductase

Proteins from gruop of rabbits with CS intake for 20 days, with and without and AIR were subjected to Western blotting analyses, to confirm the differential expression of CYPs and NADPH-reductase (Figure 10). CS administered for 20 days to the control group of rabbits prevents the time-dependent decrease of CYP3A6 protein expression, when compared with control group without CS intake (figure 11-A). Contrary, it does not exhibit the same effect on the CYP1A2 protein expression, since it is significantly decreased when compare with control group 0 days CS. NADPH-protein expression shows no significant diference for group of rabbits with 20 days of CS intake.

The administration of CS for 20 days before producing the turpentine-induced inflammatory reaction did not prevent the decrease in protein expression of CYP1A2 (0.33± 0.06). On the other hand, after 20 days of CS intake, decrease in CYP3A6 protein expression produced by the aseptic inflammation was less apparent, but still lower ($p<0.05$) than that observed in control rabbits at Day 0 (0.55 ± 0.06). The protein expression of NADPH-reductase showed no change after 20 days of CS intake.

3- Effect of the administration of CS for 30 days, on the expression of CYP1A2, CYP3A6 and NADPH-reductase

Proteins from group of rabbits with CS intake for 30 days, with and without and AIR were also, subjected to Western blotting analyses, to confirm the differential expression (Figure 10). CYP1A2 protein expression is significantly decreased in group of rabbits with 30 days of CS intake, when compared with control group without CS intake (figure 11-B). Contrary, the administration of CS seems to stabilize CYP3A6 protein expression at levels comparable with control group without CS intake (figure 11-A). There is no decrease of NADPH-reductase protein expression in the group of rabbits with 30 days of CS intake, when compared with control group without CS intake (figure 11-C). The administration of CS for 28 days before producing the turpentine-induced inflammatory reaction and for the next 48 hours did not prevent the decrease in protein expression of CYP1A2 (figure 11-B). Interestingly, after 30 days of CS intake, CYP3A6 protein expression in rabbits with a turpentine-induced inflammatory reaction was similar to the values estimated in control rabbits receiving CS for 30 days, e.g. 0.85 ± 0.18 vs 0.88 ± 0.10, respectively. In other words, after 30 days of CS, the inflammatory reaction did not diminish CYP3A6 expression (figure 11-A). NADPH-reductase protein expression showed a step-wise diminution time-dependent that could not be prevented by the administration of CS (figure 11-C).

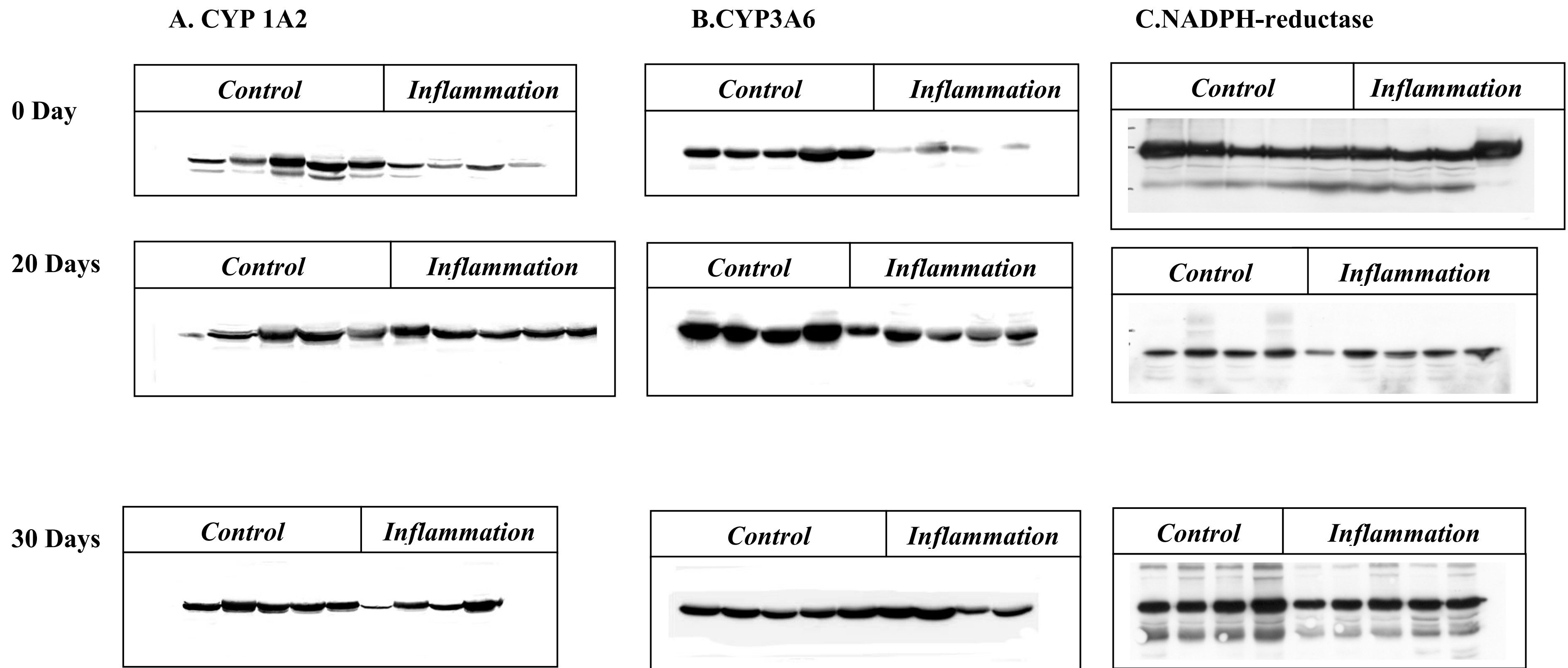


Figure 10. Protein expression at O Days, 20 Days and 30 Days for (A) CYP1A2, (B) CYP3A6, (C) NADPH-reductase.

4- Effect of 30 days stay in the animal facilities on protein expression

Compared with control rabbits at 0 Days, e.g. after only seven days of acclimatization, the protein expression of CYP1A2 in rabbits kept for 30 days in the animal facilities was significantly decreased, but similar with that observed at rabbit group with 30 CS intake (figure 11-B). The protein expression of CYP3A6 in group of rabbits kept in animal facilities for 30 days is decreased significantly when compared with control group without CS or rabbits group with 30 days CS intake (figure 11-A).

The expression of NADPH-reductase did not change as a result of keeping rabbits for 30 days in the animal facilities (there is no statistically significant change) (figure 11-C).

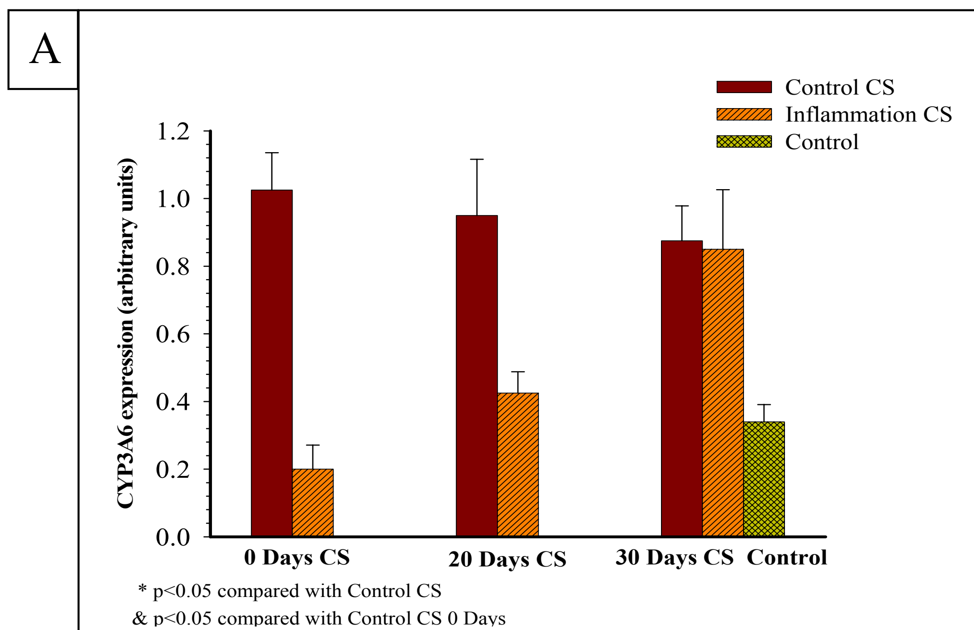
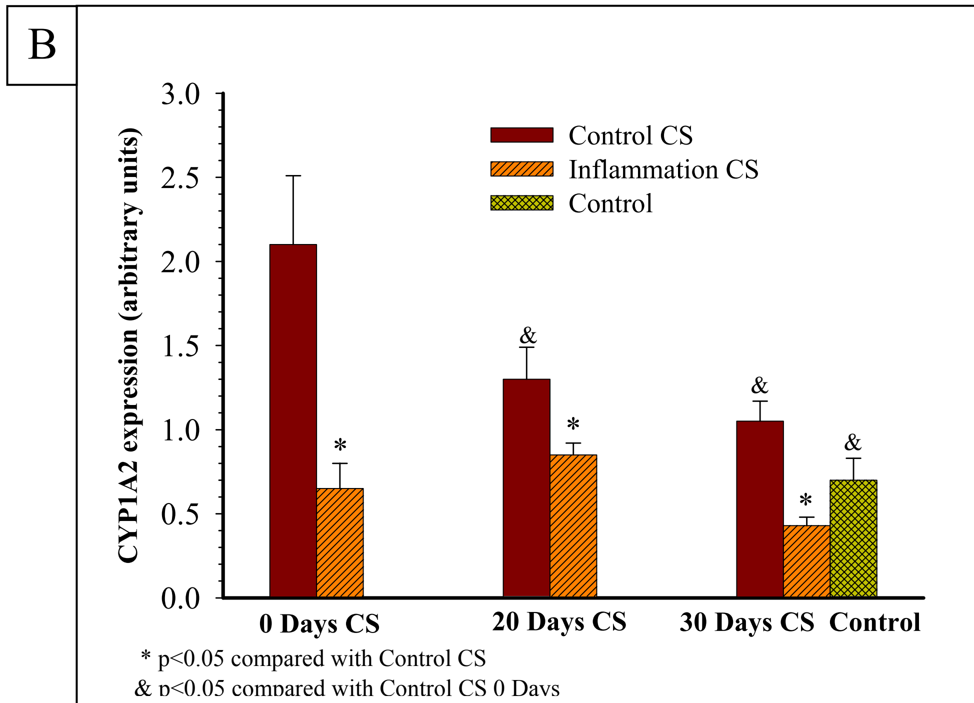
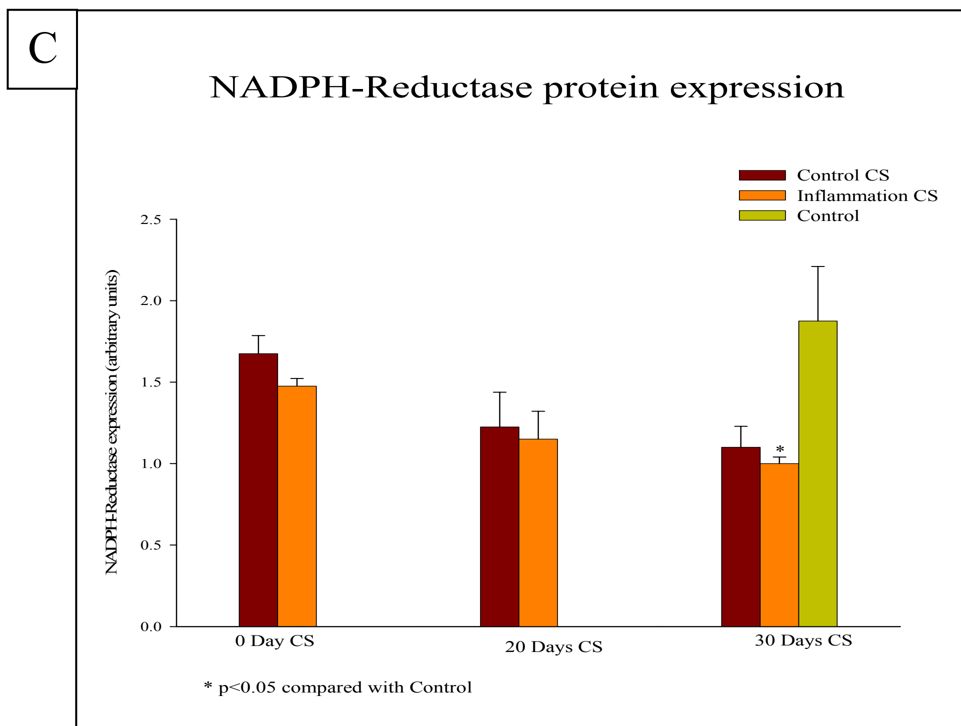


Figure 11. *Effect of the administration of CS for 20 and 30 days on (A) CYP3A6 expression in hepatocytes from rabbits with or without an inflammatory reaction*



(B) CYP1A2 expression in hepatocytes from rabbits with or without an inflammatory reaction.



(C) NADPH-reductase expression in hepatocytes from rabbits with or without an inflammatory reaction.

5 - Effect of CS on mRNA of CYP3A6

Contrary with the effect observed on the activity of CYP3A6, but similar with the CYP3A6 protein expression, the mRNA of CYP3A6 remains stable as result of time (figure 12-A), in group of rabbits with 20 days and 30 days of CS without inflammation . The intake of CS for 20 and 30 days does not prevent the decrease of *CYP3A6* mRNA in rabbits with aseptic inflammation, though not statistically significant because of variability. Data on mRNA of CYP1A2 in not available, due to laboratory technical problems.

In group of rabbits with 20 and 30 days of CS intake the mRNA of NADPH-reductase remains similar with that of control rabbits without CS intake (Day 0). In rabbits with turpentine-induce inflammatory reaction, the mRNA expression of NADPH-reductase was similar to that estimated in control rabbits at Day 0; however, after 20 days of CS intake in rabbits with aseptic inflammation mRNA of NADPH-reductase tends to decrease (figure 12-B). In group of rabbits with AIR and 30 days of CS intake, mRNA of NADPH-reductase tends to further decreased although not statistically significant because of variability.

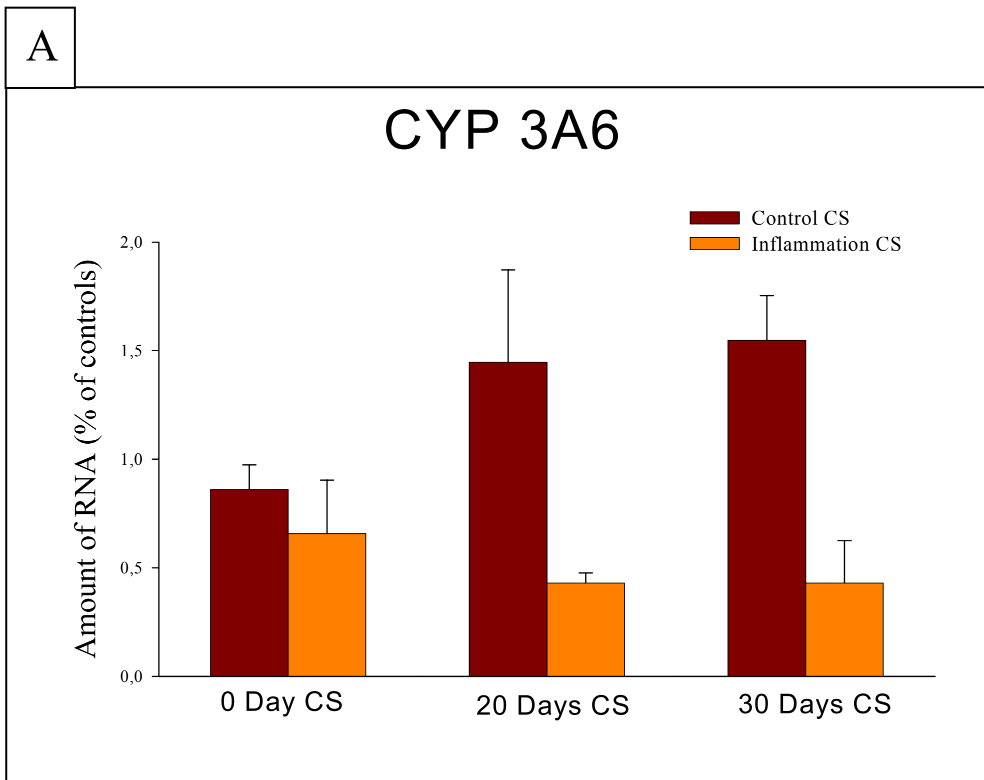
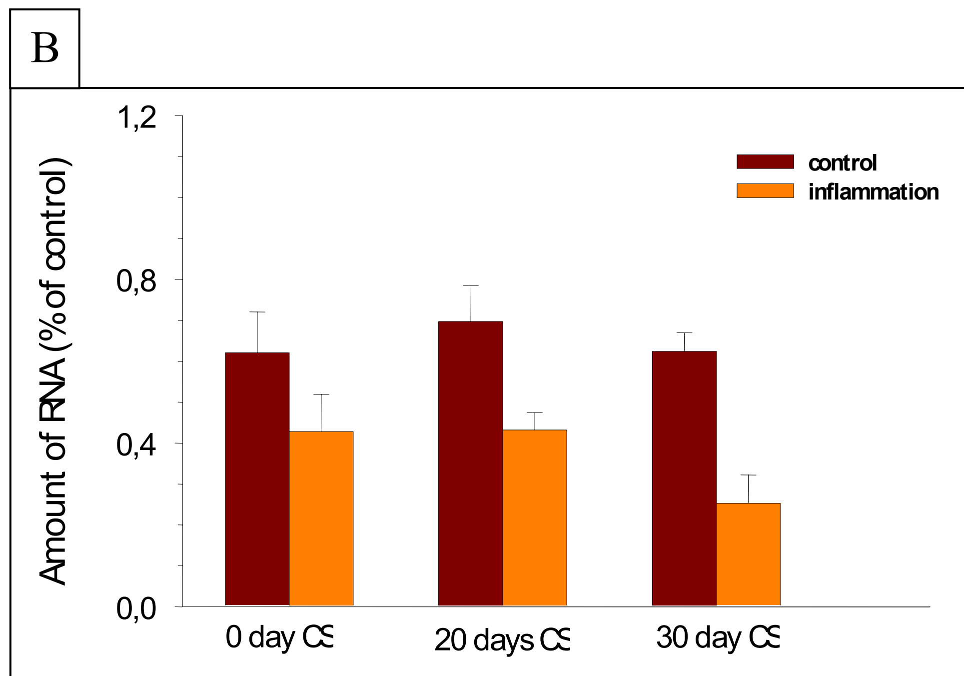


Figure 12. (A) CYP3A6 mRNA (expressed as percentage of controls).



(B) NADPH-reductase mRNA (expressed as percentage of controls).

MARKERS OF INFLAMMATION

The estimates of seromucoid concentrations *in vivo,* as well as nitric oxide ($NO^•$) concentrations in the supernatant of the 4 hours incubation of rabbit hepatocytes, and NF-κB nuclear translocation in the hepatocytes were used as biomarkers of systemic and cellular inflammation and also to evaluate the effect of CS on the inflammatory reaction.

1- *Seromucoid concentration*

At day 0, in control rabbits (n = 5), the concentration of seromucoid was 8.35 ± 1.55 mg/dl, and in rabbits with a turpentine-induced inflammatory reaction (n = 5), 48 hours after the injection of turpentine, the concentration of seromucoid was tenfold greater ($p<0.05$ compared with values in control rabbits) (Table 4), strongly supporting that the injection of turpentine did produce an inflammatory reaction. The administration of CS to control rabbits for 20 (n = 5) or 30 days (n = 5) did not influence seromucoid concentrations. Similarly, in rabbits with a turpentine-induced inflammatory reaction, after 20 (n = 5) or 30 days (n = 5) of CS intake, seromucoid concentrations remained increased and similar to the values determined in rabbits not exposed to CS at day 0.

Table 4 Seromucoid concentrations in serum from control rabbits and rabbits with a turpentine-induced inflammatory reaction (TIIR) at Day 0 and following the intake of approximately 20 mg/kg of chondroitin sulfate (CS) for 20 and 30 days.

Seromucoids (mg/dl)	**Day 0**	**Day 20 + CS**	**Day 30 + CS**
Control rabbits	8.4 ± 1.6	15.1 ± 2.0	9.5 ± 1.1
Rabbits with TIIR	95.1 ± 5.7*	93.5 ± 2.8*	92.2 ± 9.8*

* $p<0.05$ compared with values of control rabbits

2- *Nitric oxide concentrations*

In the supernatant of hepatocytes harvested from control rabbits, the concentrations of $NO^{\bullet}$ tended to increase in groups taking CS for 20 and 30 days (Table 5). Compared with hepatocytes from control rabbits at Day 0, the turpentine-induced inflammatory reaction *in vivo,* increased the release of $NO^{\bullet}$ from the hepatocytes *in vitro* ($p<0.05$). However, compared with control rabbits exposed to CS, in the rabbits with an inflammatory reaction and receiving CS for 20 and 30 days, the concentrations of $NO^{\bullet}$ were not increased by the inflammatory reaction.

Table 5 Nitric oxide (NO•) concentrations in hepatocyte's culture media from control rabbits and rabbits with a turpentine-induced inflammatory reaction (TIIR) at Day 0 and following the intake of approximately 20 mg/kg of chondroitin sulfate (CS) for 20 and 30 days.

$NO^{\bullet}$ (µM)	**Day 0**	**Day 20 + CS**	**Day 30 + CS**
Control rabbits	1.26 ± 0.18	3.02 ± 0.90	1.68 ± 0.38
Rabbits with TIIR	2.10 ± 0.29*	2.69 ± 1.13	1.48 ± 0.35

* $p<0.05$ compared with values of control rabbits

3- ***Nuclear translocation of NF-κB in hepatocytes***

At day 0, around 15% of hepatocytes harvested from control rabbits showed nuclear fluorescence, indicating a relatively small NF-κB nuclear translocation (Figure 13).

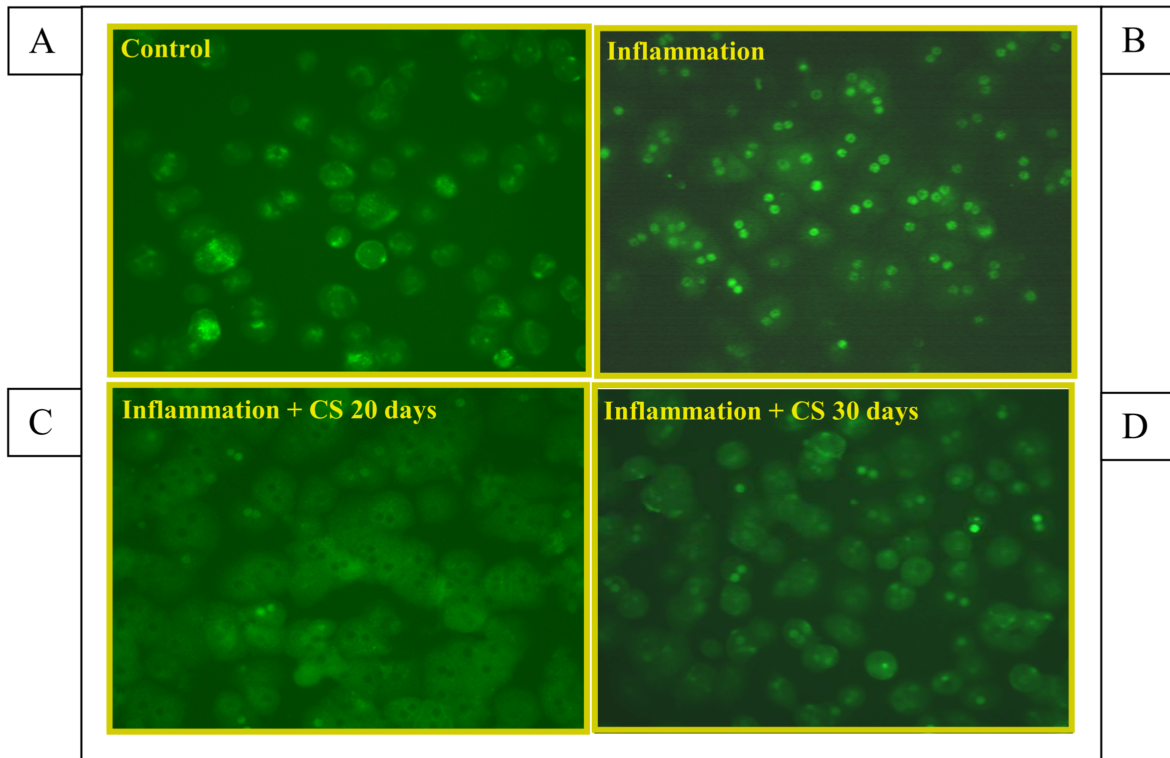


Figure 13. Fluorescent micrographs representing p65–NF-κB immunopositive nuclei in hepatocytes.

(A) in control hepatocytes, nuclei do not show immunofluorescence that is concentrated in the cytoplasma.

(B) NF-κB immunopositive nuclei activated by inflammation.

(C) hepatocytes exposed to chondroitin sulfate for 20 days and inflammation, showing a reduction in nuclei immunofluorescence by reference to panel B.

(D) hepatocytes exposed to chondroitin sulfate for 30 days and inflammation, showing a reduction in nuclei immunofluorescence by reference to panel B. Scales bar in panel A (20 µm).

In hepatocytes from control rabbits kept for 30 days in the animal facilities, NF-κB nuclear translocation was very similar to that observed at Day 0. In the hepatocytes from rabbits with a turpentine-induced inflammatory reaction at Day 0, NF-κB nuclear translocation was increased fivefold ($p<0.05$) compared with hepatocytes from control rabbits. In control rabbits, the intake of CS for 20 or 30 days did not influence NF-κB nuclear translocation. It is noteworthy that in hepatocytes from rabbits with a turpentine-induced inflammatory reaction, CS prevented the increase in NF-κB nuclear translocation triggered by the turpentine-induced inflammatory reaction (Figure 14).

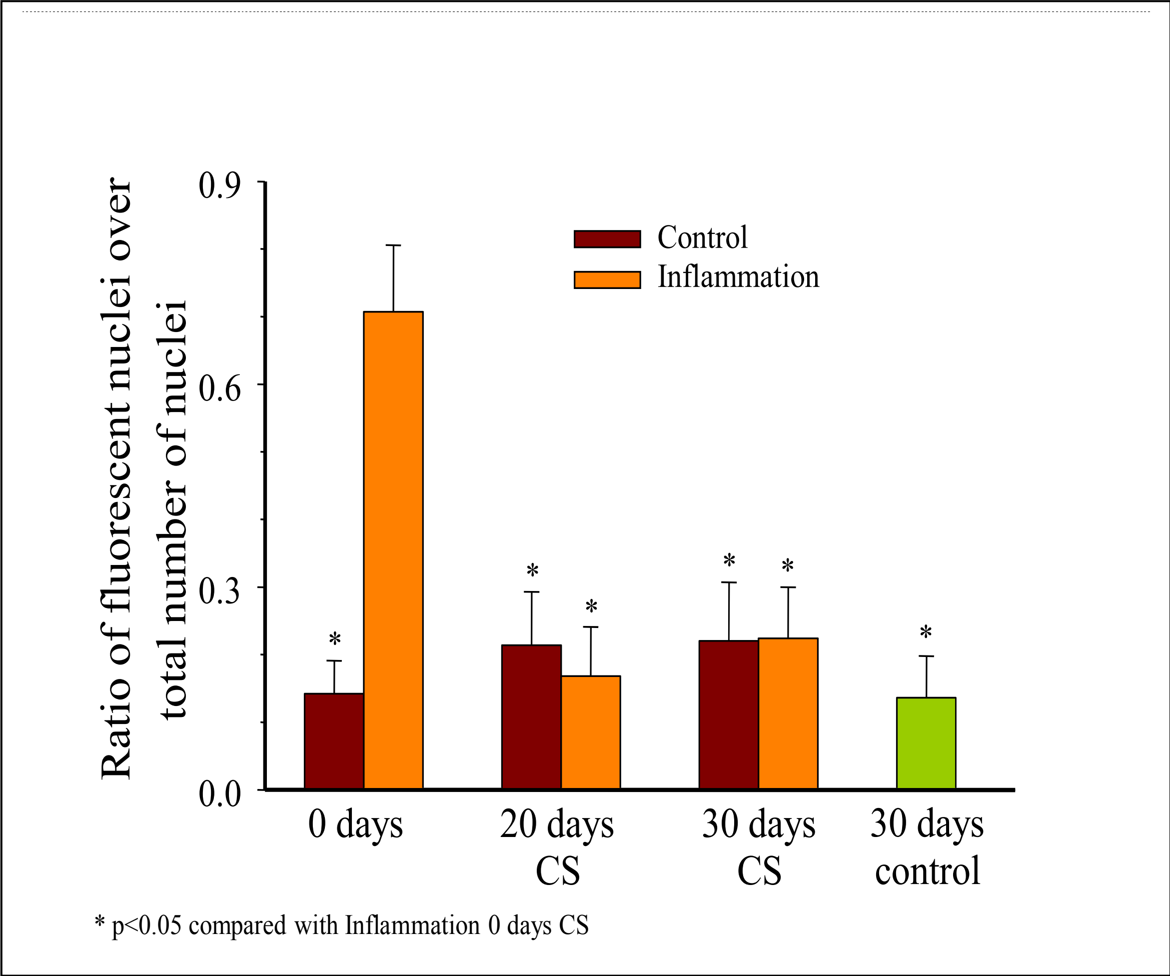


Figure 14. Effect of chondroitin sulphate (CS) on NF-κB nuclear translocation in rabbits hepatocytes, in vivo.

4- *Effect of CS and the ∆di-4S, ∆di-6S and ∆di-4,6S disaccharides on CYP1A2, CYP3A6 and NADPH-reductase activity and expression in vitro*

Incubation of 10, 20, 40, 80 µg/ml of ∆di-4S, ∆di-6S and ∆di-4,6S and 50, 100, 200, 450 µg/ml of CS with hepatocytes harvested from control rabbits (n = 3) for 48 hours did not affect the activity or the expression of CYP1A2, CYP3A6 and CPR (Figure 15).

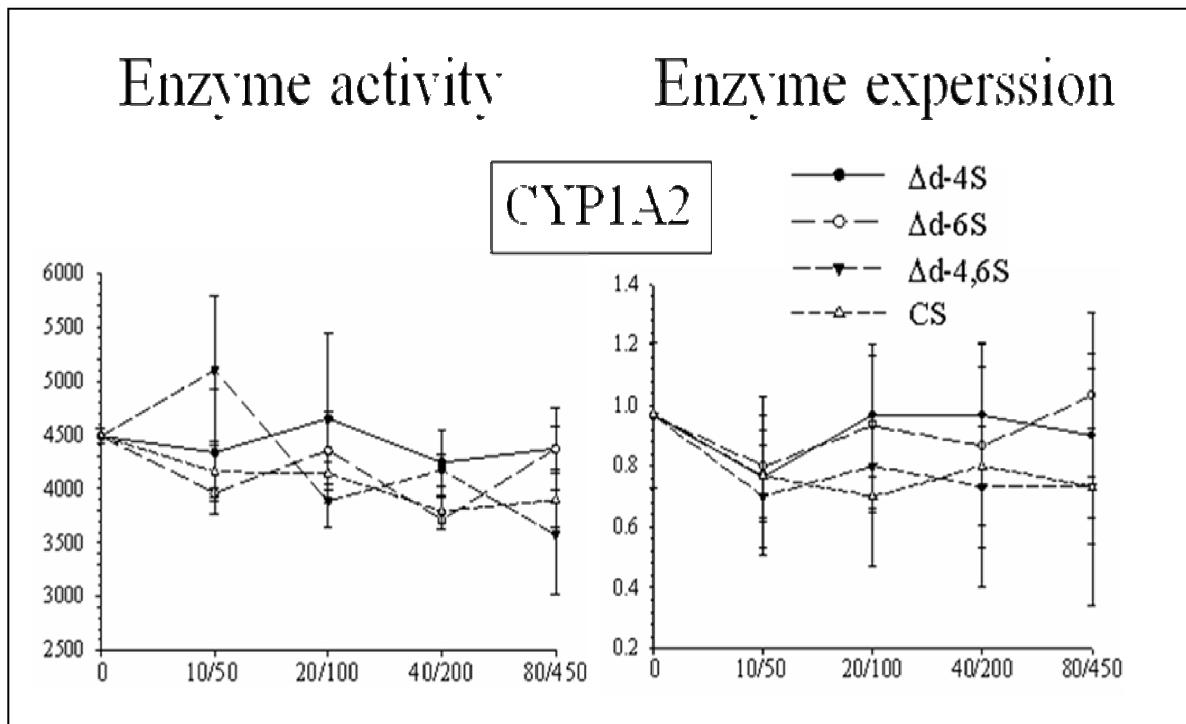
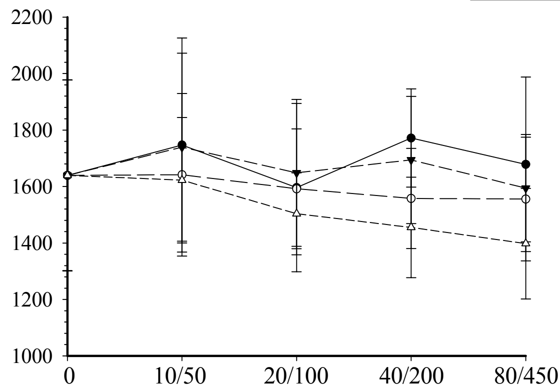
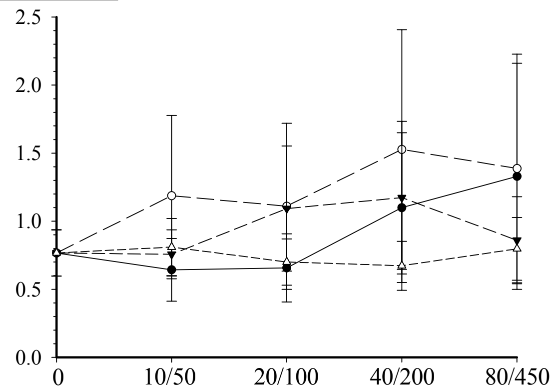


Figure 15. Activity and expression of CYP1A2, 3A6, and NADPH-reductase in the presence of different concentrations of CS and its disaccharides (∆di-4S, ∆di-6S and ∆di-4,6S), in vitro.

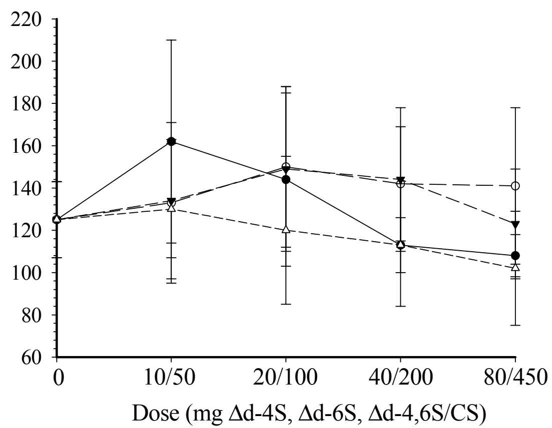
Enzyme activity Enzyme expression

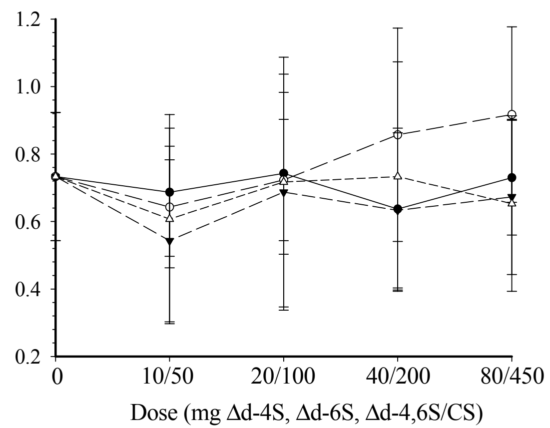
CYP3A6





NADPH-reductase





V. DISCUSSION

Patients with osteoarthritis may receive CS for long periods of time (Zhang et al., 2008). Osteoarthritis is frequently associated with other diseases such as obesity, diabetes, hypertension, hyperlipidemia and cardiovascular diseases and consequently, the patients with osteoarthritis may be poly-medicated (Caterson et al., 2004; Bray and Bellanger, 2006). Therefore, the question whether CS may be responsible of drug-drug interactions may be raised. More specifically, since CS is an anti-inflammatory agent, two questions can be raised: a) does CS modify the biotransformation of drugs under control conditions, and b) does CS modulate the down-regulation of CYP enzymes produced by an inflammatory reaction and so may regulate the biotransformation of drugs.

Turpentine-induced inflammatory reaction and markers of inflammation in this model

Turpentine oil-induced local irritation is a model of inflammation, which results in the recruitment of inflammatory cells and the up-regulation of IL-6 (Sheikh et al., 2006). In the model of turpentine-induced inflammatory reaction, IL-6 and in smaller amounts IL-1β are produced locally, at the site of turpentine oil injection, released into the blood and responsible forthe acute phase reaction triggered in the liver; the acute phase reaction as well as cytokines modulate the activity and expression of CYP (Sheikh et al., 2007). Apparently, IL-6 plays an important role in the down-regulation of several CYP isoforms as demonstrated by a study conducted with IL-6 transgenically deficient mice, e.g. the turpentine-induced inflammatory reaction did not depress CYP3A1 in mice IL-$6^{-/-}$ (Siewert et al., 2000). In a study done by Bleau et al. (2000) it was reported that IL-6 and IL-1β are the serum mediators that down-regulate CYP3A6 which are released *in vivo* by the turpentine-induced inflammatory reaction in rabbit.

Kourylko et al. (2006) showed that the serum of rabbits with a turpentine-induced inflammatory reaction, containing IL-6 and IL-1β as serum mediators, down-regulate CYP3A6 through the JAK-Erk1/2-NF-κB pathway. In aseptic as well as septic inflammatory reacion, NF-κB is a central mediator of inflammation, responsible for signals leading to a variety of cellular responses, including the induction of pro-inflammatory genes that would finally lead to the release of pro- and anti-inflammatory cytokines and of diverse enzymes (Figure 16).

Sterile tissue damage will release heat-shock proteins 60 and 70, and also breakdown products of tissue matrix, which will activate pattern recognition receptors (PRRs) and other receptors in immune and other tissue cells, as well α5β1-integrin, toll-like receptor (TLR), CD36 and CD44. The activation of these receptors will trigger the activation and nuclear translocation of the nuclear factor κB (NF-κB) in immune cells, epithelial cells of the liver, endothelial cells or locally in the tissue. Nuclear translocation of NF-κB will induce the expression of pro- and anti-inflammatory cytokines and of enzymes, such as inducible nitric oxide synthase-2 (NOS-2), cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs) that will perpetrate the inflammatory reaction (Dumais et al., 2008).

Because several of these conditions are similar to the inflammatory conditions of osteoarthritis (detailed in the Introduction of this thesis), we selected the turpentine-induced inflammatory model to study the impact of long term CS administration, focusing on major drug metabolizing enzyme.

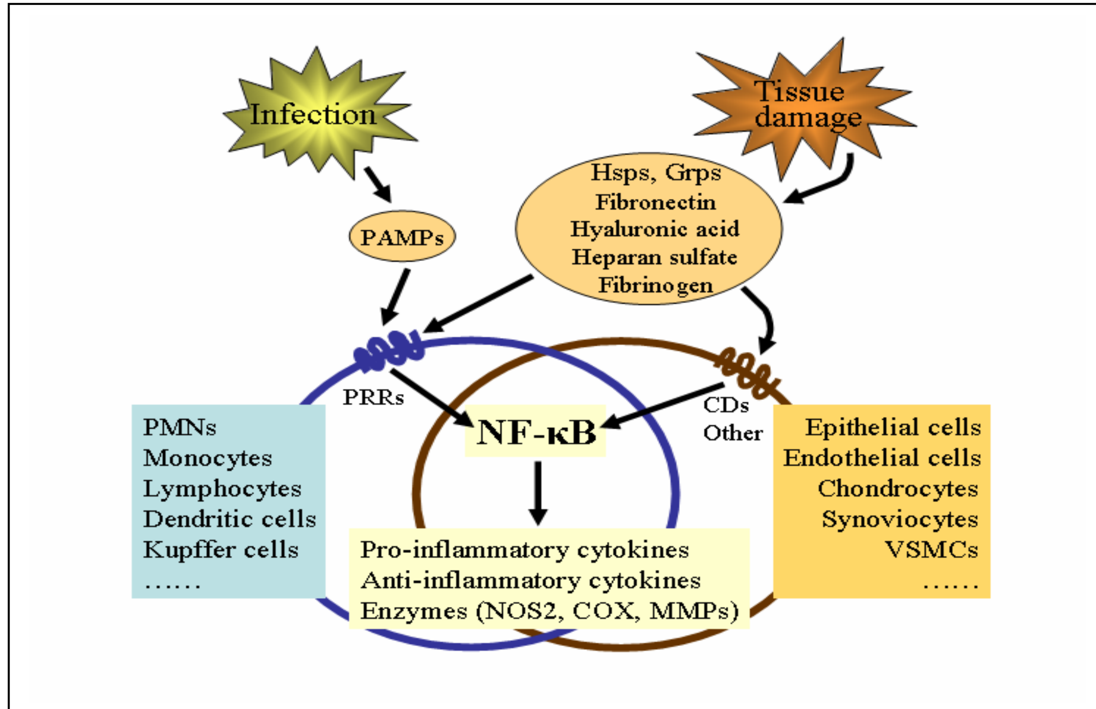


Figure 16. Production of an inflammatory reaction. Infections by means of pathogen-associated molecular patterns (PAMPs) activate the pattern recognition receptors (PRRs), including toll-like receptor (TLR), in polymorphonuclear cells (PMNs) and other immune cells.

The present study demonstrates that *in vivo*, in rabbits with a turpentine-induced inflammatory reaction, CS prevents the increase in NF-κB nuclear translocation in hepatocytes of rabbits with an AIR taking CS for 20 and 30 days. A possible explanation for this finding could be the partial inhibition of p38MAPK and to a minor extent Erk1/2 that could finally reduce NF-κB nuclear translocation. This explanation is supported by the fact that *in vitro*, CS diminishes NF-κB nuclear translocation in chondrocytes activated by IL-1β (Jomphe et al., 2008). Since NF-κB is activated by IL-1β and not by IL-6 (Leeman et al., 2008), we may postulate that even if IL-1β is produced in smaller amounts, these low concentrations are enough to trigger the activation of NF-κB in our *in vivo* model of inflammation.

Nitric oxide (NO) is induced by an inflammatory stimulation, secondary to the induction of inducible nitric oxide synthase (NOS2) in hepatocytes (Liaudet et al., 2000). In our study, NO was significantly increased in hepatocytes from the group of rabbits with a turpentine-induced inflammatory reaction. NO can bind to the heme of CYP enzymes and inhibit them by reversible or irreversible mechanisms (Wink et al., 1993; Vuppugalla and Mehvar, 2004) and therefore, NO may be one cause of enzyme inhibition observed in inflammatory states (Veihelmann et al., 1997; Kourylko et al., 2006). However, a large number of studies have shown that the down-regulation of the majority of hepatic, drug-metabolizing CYP proteins and mRNAs are not affected by deletion of the NOS2 gene or by inhibitors of NOS enzymes (Aitken et al., 2006), supporting the notion that NO may not be responsible for the down-regulation of CYP enzymes. In other words, there is a NO-independent pathway responsible for the down-regulation of CYP enzymes. In the present study, the production of NO is used as evidence of the presence of a systemic inflammatory reaction, since the production of NO could not be entirely accounted for the changes seen in the regulation of CYPs, observed in the present study.

In rabbits with a turpentine-induced inflammatory reaction, the increase of NO is prevented by the intake of CS for 20 and 30 days. Moreover, CS prevented NF-κB activation and therefore, we might speculate that the expression of NOS-2 should also be reduced (Iovu et al., 2008; Jomphe et al., 2008), and consequently the production of NO might be prevented.

The concentration of seromucoids represents another marker of inflammation used in our study. Seromucoids are a crude precipitation fraction of glycosilated proteins such as α_1-acid glycoprotein, α_1-cysteine proteinase inhibitor and hemopexin (Price et al., 1961). Interleukin-6 by regulating the synthesis of acute phase proteins in human hepatocytes increases α_1-acid glycoprotein levels (Castell et al., 1988; Lyoumi et al., 1998; Ling et al., 2004). In the present study, seromucoids concentrations

were constantly increased in the presence of turpentine-induced inflammatory reaction and that despite the administration of CS for 20 or 30 days. This is in agreement with the belief that the anti-inflammatory effects of CS are associated to the IL-1β mediated effects (Chan et al., 2005; Jomphe et al. 2008; Legendre et al., 2008). We may postulate that CS did not interfere with the regulation of seromucoids which are IL-6-dependent and these markers were used only as an evidence of a strong systemic inflammation.

Down-regulation of CYP enzymes by TIIR

The aseptic inflammatory reaction reduced CYP3A6 activity by 62% and CYP1A2 activity by 54%, consequence of a decrease of protein expression of CYP3A6 by 78% and that of CYP1A2 by 69%. This outcome confirms the results already reported by numerous studies (Chindavijak et al., 1987; Morgan, 1989; Barakat & du Souich, 1996; El- Kadi et al., 1997; El-Kadi & du Souich, 1998; Bleau et al., 2000) showing that in a classic model of inflammation, the administration of turpentine causes a significant decrease of several CYP isoforms.

The CYP isoforms CYP3A6 and CYP1A2 are both down-regulated by the turpentine-induced inflammatory reaction even though their regulation is mediated by different signalling pathways. The decrease of the expression of CYP enzymes by an inflammatory reaction involves transcriptional and post-transcriptional mechanisms. Transcriptional regulation of the expression of CYP enzymes depends upon the expression of nuclear receptors, the binding of the nuclear receptors to the DNA-binding domain, the availability of coactivators/enhancers, and the presence of repressors (Dumais et al., 2008). Differences in nuclear receptors, coactivators/enhancers and repressors may thus explain the different results observed for the two CYP isoforms characterized in the present study. In addition, the cytokine released and activated have a preferential effect on the nuclear receptors

modulating each of the two CYP isoforms. For example, IL-1β down-regulates CAR (Assenat et al., 2004), RXR-α, PPAR-α, PPAR-γ, LXR-α, and the coactivators SRC-1, PGC-1α and PGC-1β (Kim et al., 2007), HNF-4 (Krajewski et al., 2007), GR (Liu, 2002), TR (Kwakkel et al., 2007) and, to a minor extent, PXR and FXR (Geier et al., 2005). Moreover, IL-1β down-regulates AhR and Arnt (Wu et al., 2006). In human hepatocytes, IL-6 rapidly decreases the expression of *PXR* and *CAR* mRNAs (Teng and Piquette-Miller, 2005), whereas, it does not affect the expression of *FXR* mRNA (Kim et al., 2003).

NADPH-reductase in rabbits with a turpentine-induced inflammatory reaction in absence and in presence of CS intake

The present study shows that *in vivo* NADPH-reductase activity decreases as a function of time, since the activity of NADPH-reductase of rabbits kept in animal facilities for 30 days is reduced significantly when compared with rabbits at 0 day. The addition of CS does not modulate this time-dependent decrease in activity of NADPH-reductase.

The protein expression of NADPH-reductase does not share the same tendency as observed with the activity, since the protein from the group of rabbits kept for 30 days in animal facilities is not decreased. Protein expression of NADPH-reductase is in concordance with mRNA expression that is stable over time and is not affected by the administration of CS or by the inflammatory reaction. The mechanism underlying the time-dependent decrease in NADPH-reductase activity remains unknown.

The effect of an inflammatory reaction on the expression and activity of NADPH-reductase, a rate-limiting step enzyme of CYP catalytic cycle, remains poorly characterized. The administration of lipopolysaccharides (LPS) diminishes NADPH-reductase activity, but in agreement with the

present results, turpentine-induced inflammatory reaction does not diminish NADPH-reductase activity (Morgan, 1998).

Effect of CS on CYP1A2 and CYP3A6

The administration of CS for 20 and 30 days elicits a slightly different effect on the two isoforms targeted in our study.

The expression of CYP1A2 in the group of rabbits kept in the animal facilities for 30 days are decreased when compared with control rabbits at Day 0. Moreover, there is a significant decrease in the expression of CYP1A2 in the groups of rabbits with a turpentine-induced inflammatory reaction, when compared with control groups. The administration of CS for 20 and 30 days did not prevent the time-dependent or the inflammatory reaction-induced down-regulation of CYP1A2.

Compared with control rabbits at Day 0, the activity of CYP3A6 in control rabbits decreases as a function of time when the rabbits are kept for 30 days in the animal facilities. The intake of CS partially impeded the time-dependent decrease in activity of CYP3A6 in control rabbits yet completely prevented the time-dependent decrease in CYP3A6 expression.

The administration of CS for 20 or 30 days before producing the turpentine-induced inflammatory reaction did not modulate the inflammatory reaction-dependent decrease in CYP3A6 activity. However, after 20 days of CS intake, the turpentine-induced inflammatory reaction-induced decrease in CYP3A6 protein expression was less apparent, but still lower ($p<0.05$) than that observed in control rabbits at Day 0. Interestingly, after 30 days of CS intake, in rabbits with a turpentine-induced inflammatory reaction CYP3A6 protein expression was similar to the expression estimated in control rabbits at Day 0 and control rabbits receiving CS for 30 days.

In control rabbits kept for 30 days in the animal facilities, the decrease in CYP3A6 activity might be explained in first place, by the decrease in protein expression and secondarily, by the decrease in NADPH-reductase activity, that is an obligatory redox partner for it as role of electron transfer and conformational changes of CYP3A6 (Yamaguchi et al., 2004; Zhang et al., 2007).

Effect of CS on CYP3A6 mRNA

Compared with control rabbits at Day 0, in rabbits with the turpentine-induced inflammatory reaction, *CYP3A6* mRNA decreased by 23%, contrasting with the expression of CYP3A6 that decreased by 81%. It is noteworthy that in the group of rabbits with a turpentine-induced inflammatory reaction receiving CS for 20 or 30 days *CYP3A6* mRNA remained decreased whereas CYP3A6 protein expression increased progressively to reach values similar to those observed in control rabbits. The mechanisms underlying the increase in protein expression without changes in the mRNA might be explained at least in part by a post-transcriptional down-regulation. Several mechanisms might be implicated. The first one might be a change in CYP3A6 protein by reactive oxygen species or by hydroperoxy free fatty acids and subsequent degradation via the proteasome. The second mechanism might be the presence of microRNA that may block the translation of *CYP3A6* mRNA. Finally, protein O-glycosylation at serine or threonine-residues may reduce protein expression.

A major mechanism for CYP3A6 protein degradation is protein ubiquitination and subsequent proteolytic degradation by S26-proteasome (Correia et al., 2005). The ubiquitin proteasome pathway involves two discrete and successive processes: the first is tagging of substrates by covalent attachment of multiple ubiquitin molecules, and the second is degradation of ubiquitinated proteins by the 26S proteasome complex (Jiang and Beaudet, 2004; Karin et al, 2000). Polyubiquitin

modification is responsible for targeting proteins for degradation by the proteasome, or to eliminate misfolded proteins or to terminate protein function. In addition of that, the attachment of ubiquitin in the form of multiple monomers or polymers of different topologies, leads to differences in the function of the modified protein through these topological variants (Lang et al., 2008). The tagging action of ubiquitin consists of concerted action of several enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin carrier or conjugating enzyme) and E3 (ubiquitin-protein ligase) leading to recognition by the 26S proteasome, a holoenzyme complex composed of a core 20S catalytic particle and a 19S regulatory particle (Jiang et al, 2004; Ravid and Hochstrasser, 2008).

Ubiquitination and successive protein degradation is modulated by deubiquitination enzymes (Amerik and Hochstrasser, 2004) and also, by NF-κB that up-regulates the expression of ubiquitin conjugating enzyme, ubiquitin-protein ligase and of the 26S proteasome (Wyke nad Tisdale, 2005; Tisdale, 2007). There is evidence that overactivity of ubiquitin-proteasomal system comes in response to diverse inflammatory reactions as well as pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6 and TNF-α) (Dehoux et al., 2003; Li et al., 2003; Maelstrom et al., 2007; Al-Majid and Waters, 2008). In contrast, deubiquitinating enzymes are decreased by inflammatory reactions and pro-inflammatory cytokines (Haimerl et al., 2008).

Zangar and col. (2002) have suggested that the CYP3A4 ubiquitination could occur in the absence of CYP3A4 substrates to modulate the amount of protein required. Indeed, CYP3A substrates are known to stabilize CYP3A protein *in vivo*, and *in vitro* in primary culture rat hepatocytes (Watkins et al., 1986; Eliasson et al., 1994; Zangar et al., 2002). On the other hand, CYP3A4 ubiquitination occurs in presence of cellular stress, when enzymes generate reactive oxygen species and/or reactive metabolic products that attack the CYP heme and/or the protein moiety that damage the enzyme structurally and functionally (Correia et al., 2005).

In the present study, both conditions are present in rabbits with a turpentine-induced inflammatory reaction: the absence of any known substrates for CYP3A6, along with an increased hepatic oxidative stress (Proulx and du Souich, 1995; El-Kadi et al., 2000). The ubiquitination of CYP3A6 with subsequent degradation by proteasome is a very attractive explanation to the present results.

The mechanism underlying the up-regulation of CYP3A6 by CS might be explained by the fact that CS has an antioxidant effect (Canas et al., 2007); however this hypothesis is not supported by the fact that even in presence of CYP3A6 up-regulation, the enzyme's activity did not increase, suggesting that the protein remained changed. Alternatively, CS could affect the ubiquitin-proteasome system through several mechanisms. As was mentioned earlier in this section, NF-κB can modulate the ubiquitin-proteasome pathway. In the present study, the nuclear translocation of NF-κB is prevented by CS, and as a consequence, CS might have down-regulated intracellular concentrations of ubiquitin conjugating enzyme, ubiquitin ligase, and of the 26S proteasome.

Secondly, CS and other glycosaminoglycans may modulate the ubiquitin-proteasome system affecting directly the ubiquitination process. Preliminary evidences are brought by Shin and col., (2006) showing that glycosaminoglycans may be able to regulate the expression of deubiquitination enzymes, since the ubiquitin specific protease (USP17) contains the putative hyaluronan/RNA binding motifs, suggesting that glycosaminoglycans modulate its expression. Moreover, the glycosaminglycan heparin inhibits casein kinase 2, an enzyme involved in phosphorylation of ubiquitin-conjugating enzyme, needed for its activation (Block et al., 2001). This indirect evidence supports the presumption that CS could have increased the expression CYP3A6 by diminishing the 26S-proteasome. Indeed, this hypothesis is very attractive and further studies are required.

On the other hand, there is compelling evidence that CS inhibits multiple proteases (Monfort et al., 2008). Matrix metalloproteinases (MMPs) are effectively down-regulated by CS in cultured chondrocytes in various studies (Wang et al., 2002; Monfort et al., 2005). Further studies are needed to demonstrate the effect of CS on other proteases of the ubiquitin proteasome pathway, which could have a role in the CYP3A6 protein stability.

There is recent evidence that protein ubiquitination is modulated by O-GlcNAc glycosylation, reaction that could be a protective signal against proteasomal degradation both by modifying target substrates and/or by inhibiting the proteasome itself (Guinez et al., 2008; Vosseller et al., 2001). In a study performed by Guinez et al. (2008), glucosamine was used to increase glycosylation of proteins, leading to the stability of proteins against proteasomal degradation. Glucosamine (besides other substrates) is a source for N-acetylglucosamine (Ju et al., 2008; Kuo et al., 2008), responsible for O-glycosylation (Medzihradszky, 2008). On the other hand, N-acetylgalactosamine is one of the monomers of chondroitin sulfate disaccharides (Lamari et al., 2006; Medzihradszky, 2008), suggesting that CS could enhance protein glycosylation; actually, N-acetylgalactosamine glycosylates ubiquitin ligase, reduces its activity and therefore reduces proteasome degradation (West et al., 2004). It is noteworthy that multiple CYP enzymes can undergo glycosylation with loss of activity but protection against proteolysis (Lee et al., 1998; Aguiar et al., 2005). This last mechanism could also be proposed to explain the post-transcriptional increase in CYP3A6 by CS.

There is an additional recently described mechanism for the regulation of gene expression at the post-transcriptional level – microRNAs or miRNAs, a family of short noncoding RNA, that block mRNA translation or reducing mRNA stability, thus affecting protein expression (Ambros, 2004).

In a study done by Takagi et al. (2008) it was demonstrated that miR-148a negatively regulated human PXR post-transcriptionally, through a recognition element 3`-UTR. Interestingly, this

recognition element is also present in the CYP3A4 mRNA. The expression of several CYP enzymes is more likely to be regulated by miRNA, such as CYP1A2, CYP1B1, CYP2B6, and CYP2S1 (Ingelman-Sundberg et al., 2007).

Despite growing knowledge on miRNA biology, little is known about the transcriptional regulation of miRNA, also on the mechanism of regulation of miRNA gene expression itself. It is known that miRNA are deregulated during certain conditions like cancer (Kanellopoulou et al, 2008). There are some data showing that the expression of miR-146 and miR-155 is associated to the activation and nuclear translocation of NF-κB (Taganov et al., 2006; Rai et al., 2008). Two studies highlighted the importance of miRNA-mediated regulation of translation in neuronal synapses (Ashraf et al., 2006; Schratt et al., 2006). In such context, miRNAs provide an ideal way to regulate rapidly and specifically protein synthesis. It has been suggested that miRNAs enhance rapid protein degradation (Kanellopoulou et al., 2008). There is evidence suggesting that miRNAs play an important role in the regulation of immune functions and inflammation (Sonkoly et al., 2008). Indeed, endotoxin increases the expression of numerous miRNAs as do IL-1β and IL-6 (Tili et al., 2007; Meng et al., 2008; Perry et al., 2008).

An alternative explanation to the post-transcriptional effect of CS on CYP3A6 expression might be that CS, by preventing NF-κB activation and nuclear translocation produced by turpentine-induced inflammatory reaction, may reduce miRNA posttranscriptional down-regulation of CYP3A6. Indeed further studies are needed to confirm this hypothesis.

The observed effect of CS on the stability of CYP3A6 protein expression as a function of time comes to support the hypothesis that glycosylation of protein leds to its stability accordingly to Guinez and coll. (2008), Zhang and coll. (2003).

Effect of CS and the ∆di-4S, ∆di-6S and ∆di-4,6S disaccharides on CYP1A2, CYP3A6 and NADPH-reductase in vitro

To support the results obtained with the study conducted *in vivo*, e.g. that CS does not affect activity and expression of CYP1A2 and CYP3A6 in control rabbits, several concentrations of CS and its disaccharides were incubated for 48 hours with hepatocytes harvested from control rabbits. The data obtained imply that CS or its disaccharides do not elicit a direct effect on the activity and expression of CYP1A2, CYP3A6 and NADPH-reductase, accordingly with the data obtained form *in vivo* studies. These results are in concordance also, with the data obtained from surveys on patients using dietary supplements and concurrent use of medication, since no significant drug-drug interactions have been reported in patients taking CS and other drugs (Wold et al, 2005).

VI. CONCLUSION

This study is the first one that shows in vivo that NF-κB nuclear translocation was prevented by the administration of CS, in the presence of an aseptic inflammatory reaction. Moreover CS impedes the increase of the concentration of nitric oxide. Seromucoids does not share a similar pattern, since CS does not prevent the increase in their level.

The present study shows that CS elicits a different effect on CYP1A2 and CYP3A6, in the sense that CS does not affect CYP1A2 in control and in rabbits with a turpentine-induced inflammatory reaction. On the other hand, CS appears to reduces post-transcriptional degradation of CYP3A6, although the increase in protein is not accompanied by enhanced activity, suggesting that CS prevents the degradation of the inactive enzyme. Finally, the administration of CS did not affect the stability of NADPH-reductase activity and protein expression.

In vitro studies added to this project were undertaken in the effort to answer the question whether chondroitin sulphate and its degradation products contribute to the effects observed *in vivo*. The strait result was that CS, 4S-, 6S and 4,6S-sulfated disaccharides of CS did not change the activity and expression of the two isoforms of CYP, and of NADPH.

CYP3A4 is a very important enzyme, since in the organism more than 50% of drugs biotransformed are metabolized by this enzyme. The extrapolation of the effect of CS on CYP3A6 to human CYP3A4 suggests that the administration of CS to humans would potentially prevent the down-regulation of inactivated CYP3A4 under certain conditions like the presence of an inflammatory reaction, the presence/absence of CYP3A4 substrates, oxidative damage etc, but that would not affect the biotransformation of other drugs taken by polymedicated patients.

There are other models of inflammation (like the administration of LPS) where there is a slightly different interplay of the circulating cytokines and where the results of this study could not be extrapolated entirely.

One observation that needs to be further explored is that the levels of CYP3A6 mRNA at 0 Days and 30 Days do not necessarily reflect protein expression. Additional studies are needed to determine which, if any, of the potential mechanisms described here, are responsible for the changes in CYP3A6 protein stability suggested by this study. However, mRNA measurements are generally good indicators for proteins that are induced rather than constitutively expressed, since the former ones are not likely subjected to proteasomal degradation (Robert B, 1996)

Further studies should be done to elucidate the exacte role played by CS in the post-transcriptional degradation of CYP3A6, and its effect on the ubiquitin-proteasome system. Also, it shoud be of great interest to further explore the dynamics of different proteasome subunits on CYP3A4, alone and in combination with selective proteasome inhibitors.

The interesting effects of CS observed in this study does call for further exploration, while supporting its use in the clinic, since there are numerous clinical studies showing no severe adverse effects to patients who are taking this natural health product.

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