

# CYCLOOXYGENASES 1 AND 2

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## ABSTRACT

Cyclooxygenase (COX), first purified in 1976 and cloned in 1988, is the key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid. In 1991, several laboratories identified a product from a second gene with COX activity and called it COX-2. However, COX-2 was inducible, and the inducing stimuli included pro-inflammatory cytokines and growth factors, implying a role for COX-2 in both inflammation and control of cell growth. The two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Protective PGs, which preserve the integrity of the stomach lining and maintain normal renal function in a compromised kidney, are synthesized by COX-1. In addition to the induction of COX-2 in inflammatory lesions, it is present constitutively in the brain and spinal cord, where it may be involved in nerve transmission, particularly that for pain and fever. PGs made by COX-2 are also important in ovulation and in the birth process. The discovery of COX-2 has made possible the design of drugs that reduce inflammation without removing the protective PGs in the stomach and kidney made by COX-1. These highly selective COX-2 inhibitors may not only be anti-inflammatory but may also be active in colon cancer and Alzheimer's disease.

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## INTRODUCTION

Cyclooxygenase (COX) or prostaglandin H<sub>2</sub> synthase (PGHS) is the enzyme that catalyzes the first two steps in the biosynthesis of the prostaglandins (PGs)

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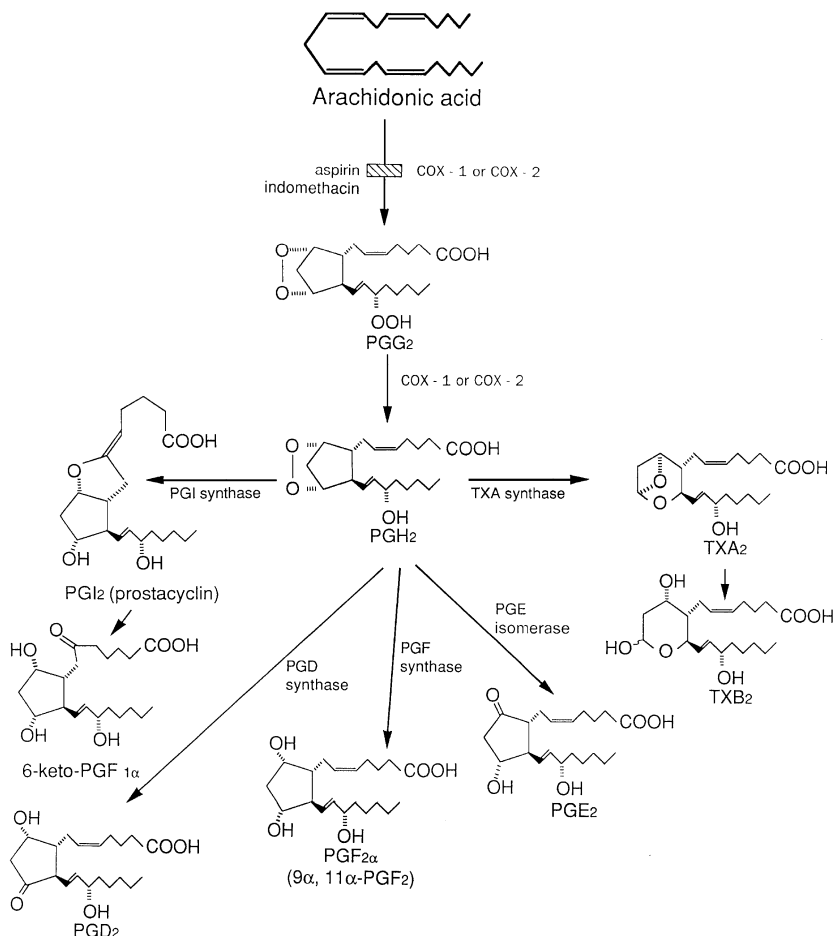


Figure 1 The arachidonic acid cascade.

from the substrate arachidonic acid (AA). These are the oxidation of AA to the hydroperoxy endoperoxide  $\text{PGG}_2$  and its subsequent reduction to the hydroxy endoperoxide  $\text{PGH}_2$ . The  $\text{PGH}_2$  is transformed by a range of enzymes and nonenzymic mechanisms into the primary prostanoids,  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGD}_2$ ,  $\text{PGI}_2$ , and  $\text{TXA}_2$  (Figure 1).

COX activity has long been studied in preparations from sheep seminal vesicles, and this enzyme was cloned by three separate groups in 1988 (1–3). The discovery of a second form of COX in the early 1990s was the most important

event in prostanoid biology in almost 20 years. Induction of this isoform, COX-2, by several stimuli associated with cell activation and inflammation assured the relevance of this finding to inflammatory disease in general. A clear sign of the therapeutic value of this discovery is that in the relatively short time of about five years, several highly effective anti-inflammatory agents and new therapeutic areas have become subjects for investigation. This review concentrates on those aspects in COX and PG research that have proved to be most relevant to the development of new anti-inflammatory drugs. The reader is referred for further reading to some recent reviews (4–7).

## BIOCHEMISTRY AND CRYSTAL STRUCTURE OF COX-1 AND COX-2

### *Biochemical Comparisons*

The inducible enzyme COX-2 is very similar in structure and catalytic activity to the constitutive COX-1. The biosynthetic activity of both isoforms can be inhibited by aspirin and other nonsteroid anti-inflammatory drugs (NSAIDs) (8). The inhibition by aspirin is due to the irreversible acetylation of the COX site of PGHS, leaving the peroxidase activity of the enzyme unaffected. In contrast to this irreversible action of aspirin, other NSAIDs such as ibuprofen or indomethacin produce reversible or irreversible inhibition by competing with the substrate AA for the active site of the enzyme. Both isoforms have a molecular weight of 71 K and are almost identical in length, with just over 600 amino acids, of which 63% are in an identical sequence. However, the human COX-2 gene at 8.3 kb is a small immediate early gene, whereas human COX-1 originates from a much larger 22-kb gene. The gene products also differ, with the mRNA for the inducible enzyme being approximately 4.5 kb and that of the constitutive enzyme being 2.8 kb (4, 5). The three-dimensional X-ray crystal structure of human or murine COX-2 (9, 10) can be superimposed on that of COX-1 (11); the residues that form the substrate binding channel, the catalytic sites, and the residues immediately adjacent are all identical except for two small variations. In these two positions, the same substitutions occur: Ile in COX-1 is exchanged for Val in COX-2 at positions 434 and 523 (the residues in COX-2 are given the same number as their equivalent amino acids in COX-1).

In spite of this structural identity, there are clear biochemical differences between the isoforms in substrate and inhibitor selectivity. For example, COX-2 will accept a wider range of fatty acids as substrates than will COX-1 (4). Thus, although both enzymes can utilize AA and dihomo- $\gamma$ -linolenate equally well, COX-2 oxygenates other fatty acid substrates, such as eicosapentaenoic acid,  $\gamma$ -linolenic acid,  $\alpha$ -linolenic acid, and linoleic acid more efficiently than does

COX-1. Also, COX-2 acetylated by aspirin on Ser 530 will still oxidize AA but to 15-HETE, whereas similarly acetylated COX-1 will not oxidize AA at all (12–14). In addition (see below), inhibitors will differentiate between COX-2 and COX-1 with over 1000-fold selectivity (6, 15).

### *Structural Comparisons*

The biochemical differences between the two isoforms of the enzyme have been attributed to the changes resulting from the Ile/Val substitutions mentioned above. Supporting evidence is strongest from the work on COX-2-selective inhibitors; mutation of Ile 523 to Val in the COX-1 protein allows COX-2-selective inhibitors to bind and inhibit PGH<sub>2</sub> formation without altering the  $K_m$  for AA (16), and the reverse mutant of COX-2 in which Val 523 is exchanged for Ile shows inhibitor binding and selectivity profiles comparable to those of wild-type COX-1 (17, 18). The structural basis for this has been shown clearly in the crystal analyses of COX-2, which have used either the human (9) or the murine protein (10), each bound to a nonselective COX-1 or a selective COX-2 inhibitor. The smaller size of Val 523 allows the inhibitor access to a side pocket off the main substrate channel in COX-2—access that is denied sterically by the longer side chain of Ile in COX-1. Selective inhibitors of COX-2 do not bind to Arg 120, which is used by the carboxylic acid of the substrate AA and by the COX-1-selective or -nonselective NSAIDs, all of which are carboxylic acids (19, 20).

Kurumbail et al (10) have suggested that the other Val substitution in COX-2, at residue 434, also contributes to the opening of the side pocket. However, it is more likely that this variation controls the substrate selectivity of COX-2. This residue is closer to the acetyltable Ser 530, and the smaller bulk of Val could provide additional space in this region of the substrate channel to allow a greater range of substrates in native COX-2 and, in acetylated COX-2, to allow AA to “squeeze” past the acetylated Ser 530 into the catalytic site. Support for this suggestion can be deduced from the results obtained with the recently described double mutant of COX-1 with Ile523Val and His513Arg, which exhibited increased binding of selective COX-2 inhibitors and decreased binding of COX-1 inhibitors, as predicted, but did not show increased 15-HETE production after acetylation with aspirin (16).

Another striking structural difference between the isoforms, but of unknown significance, is the absence of a sequence of 17 amino acids from the N terminus and the insertion of a sequence of 18 amino acids at the C terminus of COX-2 in comparison to COX-1 (4, 5). This accounts for the different numbering for the analogous residues in the two isoforms (e.g. the acetyltable serine is Ser 530 in COX-1 but Ser 516 in COX-2). The C-terminal insert in COX-2 does not alter the last four amino acid residues, which in both proteins form the signal

for attachment to the membrane of the endoplasmic reticulum (ER) (21, 22). However, COX-2 is located on the nuclear membrane as well as on the ER (23–25), while COX-1 is found attached only to the membranes of the ER. The reason for this selective localization may lie in the different sequence of the C terminus. It is relevant that in the X-ray structural analysis of either isoform, the three-dimensional structures of the last 18 C-terminal residues in COX-1 and the last 30 residues in COX-2 were not resolved, implying a marked flexibility in this region of the proteins even in the crystalline form (10, 11).

Although emphasis has been placed here on the differences between isoforms, the extensive overall structural and biochemical similarity between COX-1 and COX-2 must be reiterated. Both use the same endogenous substrate, AA, and form the same product by the same catalytic mechanism. Their major difference lies in their pathophysiological functions.

## PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF COX-1 AND COX-2

Chronic inflammation is an excellent example of a disease that represents a malfunction of normal host defense systems. Thus, rather than classifying PG biosynthesis into physiological and pathological, it may be better to use the classification applied to the COX isoforms: either constitutive or induced. COX-1 activity is constitutive, present in nearly all cell types at a constant level; COX-2 activity is normally absent from cells, and when induced, the protein levels increase and decrease in a matter of hours after a single stimulus (4, 5).

The main reason for labeling COX-1 and COX-2 as physiological and pathological, respectively, is that most of the stimuli known to induce COX-2 are those associated with inflammation, for example, bacterial lipopolysaccharide (LPS) and cytokines such as interleukin (IL)-1, IL-2, and tumor necrosis factor (TNF)- $\alpha$ . The anti-inflammatory cytokines, IL-4, IL-10, and IL-13, will decrease induction of COX-2, as will the corticosteroids (4, 6, 26). The physiological roles of COX-1 have been deduced from the deleterious side effects of NSAIDs, which while inhibiting PG biosynthesis at inflammatory sites, also inhibit constitutive biosynthesis. Thus, COX-1 provides PGs in the stomach and intestine to maintain the integrity of the mucosal epithelium and its inhibition leads to gastric damage, hemorrhage, and ulceration.

### *The Stomach*

In most species, including humans, cytoprotective PGs in the stomach are synthesized by COX-1, although small quantities of COX-2 are also expressed constitutively (27). It has always been assumed that the cytoprotective role of PGs (e.g. prostacyclin; PGI<sub>2</sub>) in the stomach is largely due to their vasodilating

properties, enhancing mucosal blood flow. Thus, PGs produced by COX-1 confer protection on the epithelial cells of the crypts of Lieberkühn in the ileum of irradiated mice. Radiation injury results in a decrease in the number of surviving crypt stem cells. These numbers were further reduced by the administration of indomethacin to the irradiated mice but not of a selective COX-2 inhibitor. Since the presence of COX-1 was demonstrated in the epithelial cells of the crypts of nonirradiated mice and in the regenerating crypt epithelium of irradiated animals, PGs produced by COX-1 are the most likely to promote crypt stem cell survival and proliferation (28). The increased mucosal damage caused by indomethacin is also likely to be due to inhibition of COX-1. Interestingly, COX-2 mRNA levels were raised in human gastric adenocarcinoma tissues compared with those in normal specimens of gastric mucosal tissue. COX-1 mRNA levels were not elevated in the carcinoma (29).

### *The Kidney*

PGs do not maintain normal renal blood flow, but PG production becomes important in maintaining blood flow of the compromised kidney (30). Maintenance of normal kidney function is dependent on PGs both in animal models of disease states and in patients with congestive heart failure, liver cirrhosis, or renal insufficiency. Patients are therefore at risk of renal ischemia when PG synthesis is reduced by chronically administered NSAIDs. Those kidney cells that synthesize PGs contain mostly COX-1, but low levels of COX-2 mRNA have also been detected (31). Cultured rat mesangial cells increase their production of PGI<sub>2</sub> and PGE<sub>2</sub> after induction of COX-2 with cytokines or growth factors (32). The PGI<sub>2</sub> formed by mesangial cells may directly stimulate renin secretion as a feedback control for inhibition of salt reabsorption. Up-regulation of COX-2 expression has been observed in the macula densa, following salt deprivation (31).

### *The Platelet*

In the platelet, the only isoform detectable is COX-1, and loss of AA-induced platelet aggregation is not only a well-established side effect of NSAID treatment, but also the therapeutic aim of the “half an aspirin a day” prophylaxis against thromboembolic disease (33). This prophylaxis is achieved through inhibition of COX-1, which leads to decreased production of thromboxane A<sub>2</sub> (TXA<sub>2</sub>). Prostacyclin production in endothelial cells is also decreased, but the COX-1 there regenerates so that PGI<sub>2</sub> synthesis is reestablished. However, platelets do not form new enzyme, and TXA<sub>2</sub> synthesis is irreversibly inhibited for their lifetime of 8–10 days in the circulation. In addition, aspirin acetylates COX-1 of the platelets in the presystemic circulation before it reaches the general circulation. As it passes through the liver, up to 50% of the aspirin is

deacetylated and it becomes diluted further when joining the rest of the venous blood. In humans, aspirin blocks COX activity in platelets within an hour of oral administration (8). This results in inhibition of platelet function for several days after a single dose of aspirin. Dose regimens from 25 to 325 mg a day have been suggested, but a consensus of opinion now recommends 75 mg a day (34).

### *Gestation and Parturition*

PGs are important for inducing uterine contractions during labor. NSAIDs such as indomethacin will thus delay premature labor by inhibiting this production of PGs (35). Expression of COX-1 is much greater than that of COX-2 in fetal hearts, kidneys, lungs, and brains, as well as in the decidual lining of the uterus (35,36). Constitutive COX-1 in the amnion could also contribute PGs for the maintenance of a healthy pregnancy (37). In human amnion cells, human chorionic gonadotrophin stimulates the expression of the COX-1 gene and increases mRNA and COX-1 protein levels (38).

Both COX-1 and COX-2 are expressed in the uterine epithelium at different times in early pregnancy and may be important for implantation of the ovum and in the angiogenesis needed for establishment of the placenta (39). PGs originating from COX-2 may play a role in the birth process, since COX-2 mRNA in the amnion and placenta increases substantially immediately before and after the start of labor (36). Glucocorticoids, EGF, IL-1 $\beta$ , and IL-4 all stimulate COX-2 production in human amnion cells (40,41), and glucocorticoids can cause premature labor in pregnant sheep, possibly by inducing progesterone-metabolizing enzymes that reduce progesterone levels below those needed to maintain pregnancy (42). Preterm labor could be caused by an intrauterine infection resulting in release of endogenous factors that increase PG production by up-regulating COX-2 (41). Selective inhibitors of COX-2 reduce PG synthesis in isolated fetal membranes and should be useful in delaying premature labor without the side effects of indomethacin (35).

### *Gene Deletion Studies*

In contrast to the analysis based essentially on the effects of inhibition of COX-1 with NSAIDs in adults are the results from molecular biology's equivalent of ablation, the gene knockout. Mice with the COX-1 gene disrupted lack mRNA, protein, and enzyme activity of COX-1 (43). Their platelets are unresponsive to AA, but they exhibit no gastric or intestinal ulcers nor any renal dysfunction. The absence of spontaneous gastric bleeding or erosions in these knockout mice leads to the conclusion that other cytoprotective mechanisms, such as the synthesis of nitric oxide or calcitonin gene-related peptide (CGRP), take over in the absence of the PGs. The lack of renal pathology confirms previous findings that NSAIDs only cause dysfunction in already compromised kidneys.

However, PGs synthesized by COX-1 are apparently essential for the survival of fetuses, since the majority of offspring born to homozygous COX-1 knockout mice did not survive (43).

The COX-2 knockout strain of mouse yielded similarly unexpected results (44, 45). These knockout mice showed unchanged responses to acute experimental inflammation induced by AA or phorbol ester. The female mice were infertile, for they did not ovulate. Furthermore the COX-2 knockout mice had serious renal developmental deficiencies postpartum and a consequently short life span. These results would imply a constitutive role for COX-2 in ovulation, of which there is already some indication (46), but would appear to deny the relevance of COX-2 to inflammation, in contrast to the ample evidence of the presence of COX-2 protein and the effects of COX-2 inhibition on inflammatory events. However, in the tests reported, responses of the ears of the mice to AA or phorbol ester were measured between one and four hours later and could well have been due to PGs formed by COX-1. These studies also raise the possibility of the teratological potential of COX-2 inhibition. The questions raised by the discrepancies between the results from knockout mice and the predictions from the behavior of normal mice need to be resolved (6, 47, 48), but they do suggest a general caution in the interpretation of results from knockout models as indications of the physiological roles of mediators in adult animals.

## COX-1 AND COX-2 IN THE CNS

COX-1 is distributed in neurones throughout the brain, but it is most prevalent in forebrain, where PGs may be involved in complex integrative functions, such as modulation of the autonomic nervous system and sensory processing (49, 50). COX-2 is expressed constitutively in only a few organs and one of those is the brain. This expression is restricted to certain parts of the CNS, notably the cortex, hippocampus, hypothalamus, and spinal cord (50, 51). It is the predominant isoform in the brains of neonate pigs (52) and in the spinal cord of the rat (53), while human brain tissues contain equal amounts of mRNA for COX-1 and COX-2 (54).

### *Nerve Transmission*

The most interesting feature of COX-2 in the CNS is that the enzyme is up-regulated by normal or by abnormal (convulsive) nerve activity (49). Furthermore, COX-2 protein or mRNA was detected in neurones as well as in the nonneuronal cells of the CNS (49–51). These findings suggest a role for PGs in CNS transmission and raise the possibility that selective COX-2 inhibitors may modulate CNS function. This would be especially relevant for those COX-2 inhibitors that lack an acidic group and may, therefore, pass the blood brain



barrier. The major PGs in the CNS of most mammalian species—including humans, monkeys, and rats—are PGE<sub>2</sub> and PGD<sub>2</sub>. In neonatal rats, PGD<sub>2</sub> synthase was found in the neurons of the brain, but in adult animals, the neuronal enzyme had disappeared, and PGD<sub>2</sub> synthase was now located in the nonneuronal cells lining the CNS (meninges and choroid plexus) and also within the cerebral spinal fluid (CSF), as postnatal development progresses (55). Thus in the adult rat, the neurones express COX-2 and synthesize PGH<sub>2</sub>, but the subsequent metabolism to PGD<sub>2</sub> must take place in the nonneuronal cells or in the extracellular space. The unusual formation of this important sleep-inducing PG needs more evaluation before its functional importance can be assessed.

### *Fever*

It has been postulated that PGE<sub>2</sub> produced in the organum vasculosum laminae terminalis (OVLT) generates neuronal signals that activate the thermoregulatory center in the preoptic area of the anterior hypothalamus, which is situated close to the OVLT (56). PGE<sub>2</sub> synthesis is stimulated by cytokines such as IL-1, which are released by the actions of pyrogens such as LPS. Although the expression of COX-2 in the CNS is increased after LPS, the induction is not in neurones but in the endothelium of cranial blood vessels and in the microglia (57, 58). Furthermore, COX-2 in rat telencephalic neurones is induced by LPS but inhibited by urethane anesthesia without modifying the febrile response to LPS (59). Thus, it is clear that PGE<sub>2</sub> involved in the febrile response derives from COX-2 induced in nonneuronal cells, probably endothelial cells of the blood vessels perfusing the hypothalamus.

### *Hyperalgesia*

Another apparently central effect of PGs, considered to be mediated peripherally, is pain or, more accurately, hyperalgesia. Although the thalamus and other higher nuclei of the CNS associated with pain pathways are not rich in constitutive or induced COX-2, the spinal cord may be where the nociceptive process is most influenced by COX-2. For some time, it has been known that during inflammatory pain, PGs are generated at the peripheral terminals of sensory neurones and cause hyperalgesia (60, 61). This is accompanied by production of pro-inflammatory cytokines (IL-1, IL-8, and TNF- $\alpha$ ) and most probably by induction of COX-2 in inflammatory cells, if not in the nerve terminals themselves (61–63). Intrathecal administration of PGE<sub>2</sub> into conscious rats or mice induced hyperalgesia (64, 65). Moreover, ibuprofen, aspirin, ketorolac, indomethacin, or NS398 were potent analgesics in the formalin test when given intrathecally to rats (66, 67), suggesting an additional role for PGs in nociceptive processing in the spinal cord in this model of analgesia. More directly, an increase in mRNA (68) or immunoreactive staining (53) for COX-2, but

not for COX-1, in rat spinal cord resulted from inflammatory injury to the paw. However the rise in COX-2 mRNA was transient, lasting about 6–12 h, whereas inflammation and hyperalgesia persisted for several days (68). The effect of a noninflammatory stimulus—repetitive, low-frequency electrical stimulation of the sural nerve—which normally leads to a potentiation of the withdrawal reflex, was also susceptible to inhibition by indomethacin and SC58125, a selective COX-2 inhibitor, implying a mediation of PGs generated by COX-2 in another spinal response to a nociceptive stimulus (69). In a preliminary report, COX-2 was found to be localized to discrete laminae of rat spinal cord, in neuronal and nonneuronal tissue (53). It will be important to establish whether COX-2 in the spinal cord is in neurones or in nonneuronal cells and also which compartment shows induction in hyperalgesic conditions.

## COX-2 IN THE NUCLEUS

Another possible function of COX-2, but not of COX-1, stems from its perinuclear location (22–25). The traditional view of the PGs is as extracellular and intercellular messengers acting on (the G-protein-linked, seven transmembrane type) cell membrane receptors and implying an export of PGs from the cell for their function to be realized. Indications for an intracellular function of PGs, which for many years were considered indirect (70), have been strengthened by the demonstration that PGJ<sub>2</sub> derivatives are potent ligands for the nuclear peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) (71, 72). This receptor is in the retinoid X receptor (RXR) heterodimer nuclear receptor family, a diverse group including the retinoid and thyroid nuclear receptors, all acting as transcription factors for genomic DNA (73). An action on a nuclear receptor might account for the cytostatic effects of PGJ<sub>2</sub> in transformed cell lines (74) and, in another context, form the link between COX-2 activity and the progression of precancerous epithelial cells to fully malignant phenotypes (see below) (75).

Another intriguing finding was the increase in Bcl-2 protein following COX-2 transfection and action in epithelial cells (76). The amount of this protein was decreased by treatment with a NSAID (sulindac), which also increased the apoptotic rate in the cell cultures. Although the link between PG production and Bcl-2 synthesis has not been elucidated, it is clear that in these cells, PGs synthesized by COX-2 influence another nuclear event, apoptosis. The pleiotropic character of the PGs and thus of COX was illustrated by the contrasting results in chick embryo fibroblasts where NSAIDs, in admittedly high concentrations, induced COX-2 protein and apoptosis (77). Whatever the final resolution of these paradoxes, the effects of PGs on cell growth and death have a topological justification in the perinuclear locus of COX-2.

## COX-2 AND INFLAMMATION

The discovery and characterization of COX-2 have answered some long-standing puzzles and created new and fascinating problems in biology. They have also solved one problem in therapeutics—how to suppress inflammation without the side effects of the present range of NSAIDs. These side effects—gastrointestinal ulceration and bleeding, renal damage, and platelet dysfunction—were accepted as inevitable consequences of the inhibition of COX activity required to prevent synthesis of PGs in inflammatory conditions such as rheumatoid or osteo-arthritis. Now with COX-2 clearly associated with inflammation but not with the physiological synthesis of PGs, selective inhibitors of COX-2 offered the possibility of inhibition of inflammatory PGs without affecting PGs generated by COX-1 in the stomach, kidney or platelet: “an aspirin without ulcers.” This possibility has generated a great deal of effort and a considerable degree of success in pharmaceutical research.

### *Selective Inhibition of COX-2*

The design of selective inhibitors would logically follow from the extensive structural analysis of the two isoforms of COX. However, the first generation of selective COX-2 inhibitors came from animal models in which compounds were sought that were potent anti-inflammatory agents with minimal side effects on the stomach. Nimesulide, etodolac, and meloxicam were discovered in this way, and they have all reached the market. Now, we know that they are selective for COX-2 rather than COX-1 (Table 1). Interestingly, the highly selective COX-2 inhibitors now in Phase III clinical trials were developed from the molecular variations of nimesulide, which has been marketed for 20 years (78). Nimesulide had been regarded as an aberrant example of an NSAID with good in vivo potency in inflammatory models but weak inhibition in vitro of the COX preparation available at the time. This preparation was derived from seminal vesicles and was almost certainly pure COX-1. Later evaluation of nimesulide, etodolac, and meloxicam (Figure 2) against COX-1 and COX-2 preparations confirmed their selectivity for COX-2 in contrast to the nonselective or COX-1-selective NSAIDs such as diclofenac, indomethacin, piroxicam, or naproxen. The selectivity ratios of inhibition for these COX-2 inhibitors were highly variable (Table 2), largely as a result of the variety of experimental conditions used in the assays (6), but always ranged from 10- to 100-fold selectivity for COX-2. Large-scale clinical trial results of one of this group (meloxicam) clearly show that severe gastric damage is significantly less than that caused by diclofenac or piroxicam, reinforcing the whole concept (79).

The newer compounds specifically designed by medicinal chemists as COX-2 inhibitors, such as SC58125 and L-745,337 (Figure 2), are more selective,

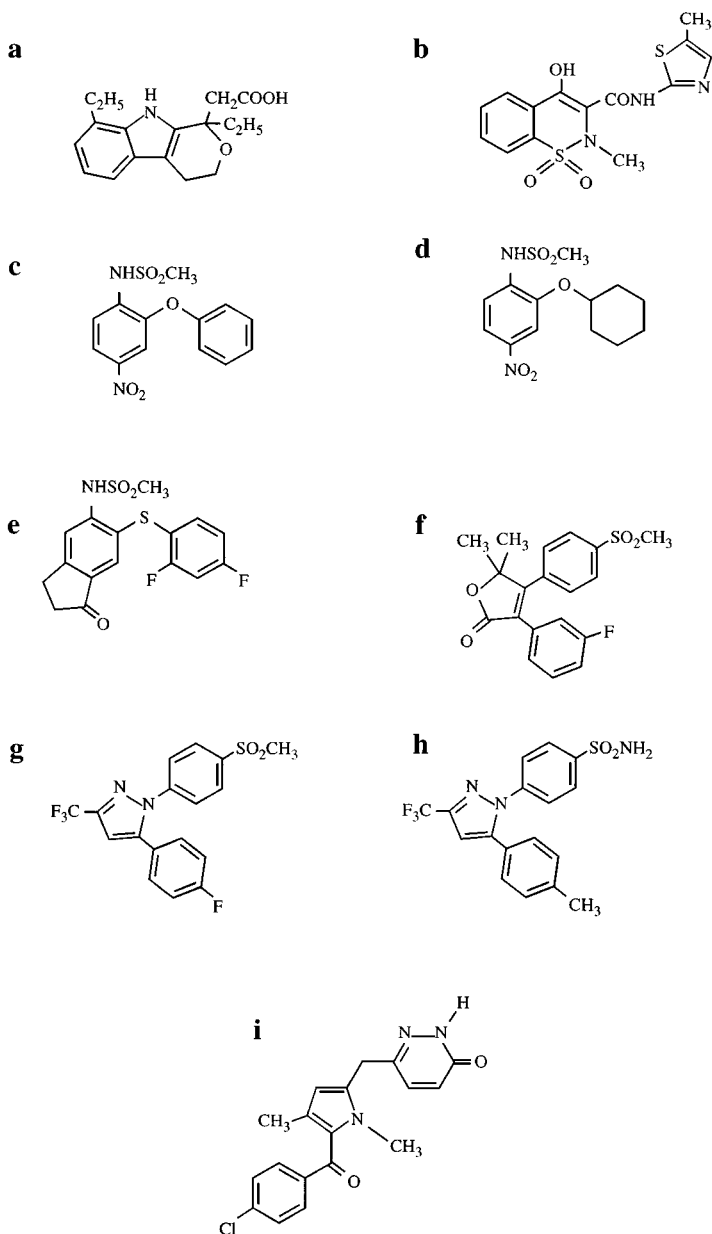


Figure 2 Chemical structures of some selective COX-2 inhibitors. (a) Etodolac; (b) Meloxicam; (c) Nimesulide; (d) NS398; (e) L-745,337; (f) DFU; (g) SC58125; (h) Celecoxib; (i) RS57067000.

**Table 1** Comparison of nonsteroid anti-inflammatory drugs for their selectivity towards COX-1 or COX-2

| Drug                          | IC <sub>50</sub> COX-1<br>( $\mu$ M) | IC <sub>50</sub> COX-2<br>( $\mu$ M) | Ratio IC <sub>50</sub><br>COX-2/COX-1 | System                      |
|-------------------------------|--------------------------------------|--------------------------------------|---------------------------------------|-----------------------------|
| <b>Nonselective for COX-2</b> |                                      |                                      |                                       |                             |
| Piroxicam                     | 0.0005                               | 0.3                                  | 600                                   | Cultured animal cells (110) |
| Aspirin                       | 1.67                                 | 278                                  | 166                                   | Cultured animal cells (110) |
| Indomethacin                  | 0.028                                | 1.68                                 | 60                                    | Cultured animal cells (110) |
| Diclofenac                    | 1.57                                 | 1.1                                  | 0.7                                   | Cultured animal cells (110) |
| 6-MNA <sup>a</sup>            | 278                                  | 187                                  | 0.67                                  | Human whole blood (111)     |
| <b>Selective for COX-2</b>    |                                      |                                      |                                       |                             |
| Etodolac                      | 34                                   | 3.4                                  | 0.1                                   | Human whole blood (111)     |
| Meloxicam                     | 4.8                                  | 0.43                                 | 0.09                                  | Human whole blood (111)     |
| Nimesulide                    | 9.2                                  | 0.52                                 | 0.06                                  | Human whole blood (111)     |
| SC58125                       | 38.7                                 | 0.27                                 | 0.007                                 | Human whole blood (111)     |
| NS398                         | 16.8                                 | 0.10                                 | 0.006                                 | Human whole blood (111)     |
| L-745, 337                    | 369                                  | 1.5                                  | 0.004                                 | Human whole blood (111)     |
| Celecoxib                     | 15                                   | 0.04                                 | 0.003                                 | Human enzymes (80)          |
| DFU                           | >50                                  | 0.04                                 | <0.001                                | Human enzymes (81)          |

<sup>a</sup>6-MNA, 6-methoxy-2-naphthyl acetic acid, the active metabolite of nabumetone.

with several 100-fold selectivity for COX-2 (Table 1). The constant molecular motifs in this range of compounds are the absence of a carboxylic group and the presence of a sulphonamide or sulphone moiety (Figure 2). Extensive structure-activity analyses of selective COX-2 inhibitors have been undertaken by the pharmaceutical industry, and some of these have already been reviewed (15).

**Table 2** Inhibition of COX-1 and COX-2 by NSAIDs in different systems

| System                    | COX-2/COX-1 Ratio |            |             |            |
|---------------------------|-------------------|------------|-------------|------------|
|                           | Indomethacin      | Nimesulide | Etodolac    | Meloxicam  |
| Cultured animal cells     | 22 (112)          | 0.05 (122) |             | 0.33 (113) |
|                           | 60 (110)          | 0.1 (123)  |             | 0.8 (110)  |
|                           | 30 (113)          |            |             |            |
|                           | 6 (114)           |            |             |            |
| Human recombinant enzymes | 1.3 (115)         | 0.2 (118)  | ~0.001 (81) | 0.003 (81) |
|                           | >75 (116)         | 0.02 (124) | 0.09 (127)  | 0.01 (118) |
|                           | 2.3 (14)          | 0.16 (125) | 0.8 (116)   |            |
|                           | 9 (117)           | 0.01 (81)  |             |            |
|                           | 3.5 (118)         | 0.02 (120) |             |            |
| Human whole blood cells   | 0.51 (119)        | 0.19 (120) | 0.1 (127)   | 0.09 (111) |
|                           | 12.5 (120)        | 0.06 (111) |             |            |
|                           | 2.9 (121)         | 0.07 (126) |             |            |

However, now that the binding sites for the selective inhibitors in COX-2 have been described in detail and the three-dimensional structure of the enzyme protein clearly established, modern molecular modeling techniques should be able to design de novo compounds binding with high affinity but without the sulphonamide or sulphone group. The compound RS57067000 (Figure 2) may be the first of this class of selective COX-2 inhibitor (9).

### *Anti-Inflammatory Drugs in Clinical Development*

Both celecoxib (80) and MK-966, derived from 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H-furanone) (DFU) (see Figure 2) (81) are in Phase III clinical trials for rheumatoid and osteo-arthritis. So far, both compounds are reported to be effective against inflammation and to cause no gastrointestinal or renal problems. For instance, in healthy volunteers, MK-966 administered daily at 250 mg for 7 days (which is 10 times the anti-inflammatory dose) produced no evidence of gastric damage, as determined by endoscopic examination (82). Celecoxib given for 7 days to volunteers also provided no evidence of gastric damage (83). At 1 g orally, MK-966 had no influence on ex vivo platelet aggregation. Both celecoxib and MK-966 were effective analgesics in humans for moderate to severe pain following dental surgery (84, 85). In animal studies, selective COX-2 inhibitors showed good antipyretic activity within the anti-inflammatory dose range (81, 86). These selective inhibitors and others still under development are also likely to be tested clinically in two other conditions: colon cancer and Alzheimer's disease.

It is clear that much of the pharmacological profile predicted for the highly selective COX-2 inhibitors has been realized, with little or none of the side effects associated with inhibition of COX-1. However, these compounds have been used in a relatively small number of carefully selected patients with high levels of monitoring, as is inevitable in the early stages in their development. The selective inhibitors are aimed at a mass market—approximately  $15 \times 10^{12}$  tablets of aspirin are consumed annually worldwide—and side effects or toxicities of these new compounds will take time and much patient exposure to emerge.

Nevertheless two possible side effects have already been identified, each with serious implications. One concerns wound healing, expressed primarily as the healing of gastrointestinal lesions induced by NSAIDs or other causes. The relevance of COX-2 to wound healing and the associated angiogenesis could reflect the activation of inflammatory cells such as macrophages and the secretion of growth factors such as TGF- $\beta$ , both conditions known to favor COX-2 induction (6, 87). In rats, COX-2 mRNA and enzyme protein were present in gastric lesions induced by alcohol or acetic acid. Treatment with NS-398, a

selective COX-2 inhibitor, during the acute stage of the damage-delayed healing, although the same treatment in normal rats did not by itself induce ulcers (88). The use of NSAIDs in postoperative analgesia is growing and this would be an obvious extension of use for COX-2 inhibitors (84, 85). Any indication that surgical wound healing might be hindered by the postoperative analgesic would be a serious disincentive to its use.

The other possible side effect is based on the results from the COX-2 knockout mice (44, 45). Particularly, no assessments in animals or in humans of possible effects of COX-2 inhibitors on fetal development or on female fertility have yet been published. Although it could be argued that most female patients with rheumatoid arthritis are postmenopausal and thus at no real risk, some evaluation of potential risk in other possible uses such as postoperative analgesia must be made. Such questions become even more important if the COX-2 inhibitors are to be used prophylactically in the two other conditions, colon cancer and Alzheimer's disease, discussed below.

## FUTURE THERAPEUTIC APPLICATIONS FOR COX-2 INHIBITORS

### *Cancer*

Colorectal cancer is a major form of cancer in the Western world; for instance, in the United States it is the next most important cause of cancer deaths after lung cancer. It has been estimated that 50% of those over 70 years old have colorectal adenomata and about 10% of those will progress to cancer (89). The initial evidence for the involvement of COX in colorectal cancer was epidemiological; from more than 10 studies since 1988, a negative correlation emerged between the chronic ingestion of NSAIDs and incidence of colorectal cancer (90–92). In young patients with familial adenomatous polyposis (FAP), a condition in which many colorectal polyps develop spontaneously with eventual progression to tumors, a small trial of sulindac (a nonselective NSAID) showed a significant decrease in number and size of polyps during treatment (93). These indications that COX activity was somehow involved in the progress leading to colorectal cancer were given a crucial scientific basis by the demonstration that COX-2 and not COX-1 was increased in samples of either malignant tissue from colorectal cancer or from polyp tissue from patients (94). A mutant *Apc* mouse is accepted as a model of FAP in humans in which comparable intestinal polyposis develops spontaneously. The number of polyps in these mice was strongly reduced either by treatment with a selective COX-2 inhibitor or by the deletion of the COX-2 gene (95).

In cultures of rat epithelial cells, transfection with COX-2 cDNA and the consequently increased PG synthesis decreased the apoptotic rate. This rate was restored to normal by inhibition of PG production by sulindac (76). Essentially similar results were obtained in vivo with chemically induced colon cancers in rats (96). A diet containing sulindac halved the incidence of adenomacarcinoma and doubled the apoptotic index in the tumor tissue. In an extension of the earlier work in FAP, sulindac caused regression of sporadic adenomatous polyps in 11 out of 15 patients and in 13 out of 20 polyps (97). This is an encouraging result, as most colorectal cancers are sporadic rather than hereditary in origin, but further and larger studies are clearly needed to establish a clinical case for therapy of colorectal adenomatous polyps with COX-2 inhibitors.

The progress from the deletion or mutation of the *Apc* gene, the initial step in the development of colorectal cancer, to the final malignant phenotype comprises at least seven genetic events (89). Clearly, the induction, expression, and activity of COX-2 is an essential step subsequent to loss of the *Apc* protein (75). Loss of *Apc* protein should decrease apoptosis, as the PGs produced by COX-2 appeared to do in cell lines and in vivo (76, 96). It may be that the survival of epithelial cells beyond normal lifetimes allows the malignant phenotype to develop.

Whatever the final mechanism, the hitherto unlikely proposition that "aspirin prevents cancer" is now seen to have a foundation in experimental fact at least for one type of tumor. Furthermore, it raises the strong possibility that COX-2 inhibitors could be used to decrease the incidence of colorectal cancer in genetically susceptible subjects, without causing gastrointestinal damage of their own. If this were successful, the prophylactic treatment could be extended to others on the basis of age alone, rather like the presently established aspirin prophylaxis against thromboembolic disease. This lateral development of COX-2 has provided new hope for prevention and perhaps even treatment of colorectal cancer and a new therapeutic use for COX-2 inhibitors, which is being pursued actively by the pharmaceutical industry (98).

Human gastric and breast tumors also express higher levels of COX-2 protein than surrounding normal tissues (29, 99). Piroxicam suppressed the growth of human cultured breast cancer cells (100), while sulindac sulfide reduced cancer incidence and the number of cancers per rat in experimental mammary carcinoma induced with 1-methyl-1-nitrosourea (101). Thus, gastric and breast tumors in humans may also be susceptible to treatment with selective COX-2 inhibitors.

### *Alzheimer's Disease*

The correlations between COX, PGs, and Alzheimer's disease were, as for colorectal cancer, initially epidemiological. Several case-control studies between 1988 and 1995 disclosed a significantly reduced odds ratio to almost half of



the normal risk for Alzheimer's disease in those taking NSAIDs as anti-inflammatory therapy (102–104). A report published in April 1997 confirmed the previous findings of an inverse correlation between the severity or incidence of Alzheimer's disease and the ingestion of NSAIDs, with ibuprofen as the most frequently used compound, probably reflecting its availability without prescription (105). In this study, paracetamol use was separated from that of the NSAIDs and was shown to be without benefit.

In all these analyses, the mechanisms proposed are essentially anti-inflammatory and reflect the recognition of inflammatory events and components in the Alzheimer's disease lesions (102, 103, 106). At the site of the plaques, along with the  $\beta$ -amyloid protein, there are activated microglia, complement fragments, release of cytokines, and other classical signs of inflammation. A crucial finding is that  $\beta$ -amyloid is capable of activating microglia. Although the NSAID would not be expected to modify the abnormal metabolism of  $\beta$ -amyloid, they could reduce the response of microglia to the protein. The neuronal damage in Alzheimer's disease may be due more to the inflammatory reaction with the consequent free radical and protease release than to the presence of amyloid per se. Thus, inhibition of inflammation may delay or even abort the loss of neurones consequent on amyloid deposition.

Rat microglia stimulated with LPS express COX-2 (107), and human microglia may respond similarly. With this additional source of COX-2, it was surprising that the total COX-2 content of brain tissue from Alzheimer's disease patients was in fact lower than normal (108). One explanation of this is that in these samples, necessarily from late-stage disease, the loss of neurones and their COX-2 outweighed the increased COX-2 in activated microglia. There may be a detectable increase in the total COX-2 content earlier in the disease process. The lack of a good animal model for Alzheimer's disease has undoubtedly delayed analysis of its causes.

A major benefit of the new selective COX-2 inhibitors could be early treatment in asymptomatic, but genetically at risk, subjects, which could result in a delaying or even preventing the clinical disease. Such treatment with the existing NSAIDs with their propensity to cause gastric damage and platelet malfunction has already been shown to have low compliance (109) and would always be difficult to justify in the asymptomatic subjects targeted. Selective COX-2 inhibitors should, however, enable this prophylactic action of decreased PG synthesis to be fully realized with a minimum of side effects.

## CONCLUSIONS

The years since the identification of COX-2 have been exciting and intriguing as well as frustrating and disappointing—in short, typical of scientific progress immediately after a breakthrough. The powerful techniques of molecular biology

have rapidly provided (a) detailed knowledge of the COX-1 and COX-2 proteins, from their linear sequence to their three-dimensional structure, and (b) an extensive description of the gene, its possible transcription factors, and its mRNA. The gene disruption techniques have yielded evidence for the unexpected involvement of either isoform in physiological processes, as well as evidence conflicting with that from pharmacological observations of the effects of COX inhibition.

Although the first generation of selective inhibitors (meloxicam, nimesulide, and etodolac) were discovered by animal screening, the next generation will surely owe more to techniques based on molecular modeling within the active site of the COX proteins. The therapeutic potential of COX-2 inhibition will ensure the continuing development of the basic biology of these proteins, of better inhibitors, and of more effective clinical applications. The beginning of the next millenium may indeed see that not only does aspirin cure cancer but that its siblings will prevent Alzheimer's disease and modify reproductive fertility with a minimum of side effects. Whatever the outcome, there are still many important and interesting questions to be asked and answers to be given about the two (and perhaps more) isoforms of COX.

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