Cyclooxygenase-2 and its regulation in inflammation

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Introduction

There is no doubt that the identification of an inducible isoform of cyclooxygenase (here referred to as cyclooxygenase-2 or COX-2) has brought about a renaissance in prostanoid biochemistry, pharmacology and therapeutics. This area is now as vigorous as it was 20 years ago when thromboxane A\textsubscript{2} (TXA\textsubscript{2}) and prostacyclin (PGI\textsubscript{2}) were discovered\textsuperscript{1,2} and indeed is almost as active as that of nitric oxide (NO), with which it shares many features and correlations. One of the most important features of COX-2 is its close association with a variety of inflammatory mediators and its consequent description as the COX isoform involved in and responsible for many of the signs of inflammation. It is also generally accepted that COX-1 is the constitutive isoform involved in the physiological actions of prostaglandins (PGs) in the stomach and kidney, the inhibition of which leads to gastric ulceration and nephropathy as side effects of anti-inflammatory therapy with nonsteroidal anti-inflammatory drugs (NSAIDs).

The initial findings and many of the subsequent developments are based on the techniques of molecular biology\textsuperscript{3,4} and are often expressed in terms unfamiliar to many researchers already established in inflammation. Our purpose in this review is to summarize the progress made so far in characterizing the regulation of COX-2, to evaluate its role in inflammation and, as a consequence, to assess the utility of the selective inhibitors of COX-2. In order to establish the appropriate context for the analysis of regulatory mechanisms, we shall first consider the molecular biochemistry of COX-2 and its possible place in physiology. We shall also refer to work on COX-1 where necessary.

Molecular Biochemistry of COX-2

Although the early work on COX-2 utilized animal sources, information relating to the human form of this enzyme is steadily accumulating. Since the practical outcome of COX-2 research would be the more efficient alleviation of human inflammatory conditions, emphasis will be placed on results obtained with the human protein, along with data from animal sources wherever relevant. Here, for clarity and simplicity, the molecular biochemistry of COX-2 will be considered at three separate levels—its DNA, its RNA and the enzyme protein; further details of the molecular biology of COX-2 are available in two recent reviews.\textsuperscript{3,4}

DNA

The gene for COX-2 is located on chromosome 1 in both human and mouse cells\textsuperscript{5-8}. The small size of the COX-2 gene (7.5–9 kbp\textsuperscript{6,7,9}) is compatible with its inclusion in the group of inducible, immediate early genes, few of which are over 10 kbp in length.\textsuperscript{10} It is relevant here to note that the human gene for TXA\textsubscript{2} synthase is larger (75 kbp\textsuperscript{11}) and, like that for human PGI\textsubscript{2} synthase, is only weakly (two-fold) inducible.\textsuperscript{12,13} The COX-2 gene has ten exons, one less than that for COX-1.\textsuperscript{6,7} Overall, the descriptions from different groups, of human
genomic DNA for COX-2 are in agreement and show many similarities between the human gene and the corresponding murine gene, underlining the close relationship between species. The cDNA for human COX-2 was first derived from HUVEC cells. More recently cDNA prepared from a human line of erythroleukaemia cells (HEL cells) exhibited virtually an identical sequence with only two nucleotide differences.

In contrast to the similarity shown so far between species in the protein coding and 3'-flanking regions of COX-2 DNA, there are important species-related differences in the 5'-flanking region of the COX-2 gene, where the promoters and transcription factors bind. Whereas in the human gene there are putative binding sites for a variety of transcription factors including AP-2, SP-1, NF-IL6, NFκB and a cAMP responsive element (CRE) along with a TATA box and a TPA-response element in the first intron, the corresponding region of the mouse gene appears to lack a CRE, NFκB or NF-IL6 site, although the others are present. The rat gene which has over 80% identity with the mouse gene in this region also lacks CRE, NFκB or AP-2 sites and a TATA box but includes a site for NF-IL6. However more recent analysis of the mouse gene for COX-2 in an osteoblastic cell line has found an action of and sites for, NFκB; the same authors have also identified a NFκB binding site in the rat gene.

mRNA

Reflecting the similarity in cDNA for COX-2 across species, there is also considerable similarity in mRNA for COX-2, which at 4 kb is almost twice the size of that for COX-1 (2.8 kb) in all species so far examined. Since the enzyme proteins (COX-1 and COX-2) are very similar in size, just over 600 amino acid residues, most of the difference in mRNA for COX-1 and COX-2 is taken up by the extensive 3'-untranslated region in COX-2 mRNA. This region includes several copies of the Shaw-Kamen ‘instability’ sequence, the actual number varying between species from 14 to 18 in animals. In two examples of human mRNA, 17 and 22 copies were found. These sequences are characteristic of rapidly degraded RNA and have been found in the mRNA for other immediate early proteins. However such sequences do not occur in the mRNA for COX-1 in any species.

Estimates of the half-life of COX-2 mRNA vary with the cells studied and with the stimuli used for induction of the protein. In an endothelium-derived cell line (ECV304) with IL-1 as the inducing agent, COX-2 mRNA had a half-life of about 1 h. In the same system with transcription blocked, IL-1 was able to prolong the half-life of existing COX-2 mRNA to about 90 min, thus contributing also at a post-transcriptional stage to the overall induction of the enzyme. In an epithelial cell line (EGV6), COX-2 mRNA induced by the phorbol ester, TPA, had a half-life of 30 min. It appears that the ‘built-in’ instability of the COX-2 mRNA is an essential component of the regulation of this protein and hence of its activity.

Protein structure and function

The COX proteins are very similar, both between species and between isoforms, as they both carry out the same two separate catalytic functions, oxidation of arachidonate to PGG2 and reduction of peroxide, specifically that of PGG2 to PGH2. The differences in protein structure are small and chiefly outside what is considered to be the catalytically active site.

The two isoforms are almost identical in size, COX-1 is about 602 residues whereas COX-2 comprises 604 residues. The major differences in sequence are at the N terminal where COX-2 has 17 less amino acids in the signal peptide and at the C-terminal where COX-2 has 18 more residues than COX-1. The central parts of the proteins where the catalytic and substrate binding sites are located, are almost identical. The tyrosyl groups crucial for the oxidation and the histidines interacting with the haem group are all highly conserved as is the serine acetylated by aspirin.

(i) Substrate binding sites

There are important functional differences between the isoforms which suggest that the active site in COX-2 is larger or has a looser fit than that in COX-1. This has been deduced from various mutations at the serine residue, which is acetylated by aspirin in either enzyme, Ser 530 in COX-1 or Ser 516 in COX-2. (The different numbers for similarly placed residues in the two isoforms is due to the longer N terminal sequence in COX-1; the numbering for COX-1 is thus about 14 in advance of that for COX-2.) Mutation of serine to alanine in either isoform altered neither Km nor PG production but did confer protection against the irreversible inhibition caused by aspirin, since alanine cannot be acetylated. However, mutation of serine to asparagine (isosteric with acetylated serine) has a strikingly differential effect; the COX-1 mutant lost cyclo-oxygenase activity whereas the COX-2 mutant retained full activity.
and an unchanged $K_m$. Substitution with a larger amino acid, glutamine, abolished cyclo-oxygenase activity in both isoforms.28,29

Another indication of the larger active site in COX-2 may be drawn from the effects of aspirin on catalytic activity. This compound irreversibly inhibits the production of PGs by COX-1 or COX-2 through the acetylation of Ser 530 or Ser 516. Nevertheless, acetylated COX-2 but not acetylated COX-1, still oxidized arachidonic acid (AA) to an alternative product, 15-HETE.28–30 This finding would suggest that there is space for AA to bind to acetylated COX-2 close enough to the active site for oxidation to occur even though the orientation is not adequate for the full cyclo-oxygenase reaction to take place. Further support comes from the effect of another substitution of this serine in COX-2, with methionine; this leads to a ‘pseudo-acetylated’ form in which the mutant protein shows increased production of 15-HETE but with much reduced COX activity (5–20% of normal) and almost 200-fold increase in $K_m$.28,29 Clearly with this substitution both the binding affinity and the binding mode were affected.

These deductions from biochemical findings were compatible with the model of COX-1 structure derived from X-ray crystallographic analysis.31,32 The Ser 530 lies halfway along a tunnel leading up to the active site and it is relatively easy to imagine how the acetylation of Ser 530 would block access of substrates to the active site at the head of the tunnel. On the basis of the biochemical results for COX-2, one would assume that Ser 516 either lies further within the wall of the tunnel or that in COX-2 there is enough room for AA to ‘squeeze past’ the acetyl group and to bind close enough to the tyrosine-haem complex to allow oxidation to 15-HETE, but not to take up the configuration which leads to the cyclic endoperoxide (PGG$_2$).

Another possible factor is the residue on the opposite side of the tunnel, Ile523 in COX-1 which in COX-2 is substituted by Val509, one methylene group smaller than Ile. This location also provides the only difference between the active sites of the two isoforms. It is therefore possible that the extra methylene group of Ile 523 creates enough of a narrowing of the tunnel so that in combination with the acetyl group on Ser 530, access to the active site in COX-1 is essentially prevented. In COX-2, the presence of Val 509 would not only allow a wider range of fatty acid configurations to gain access as substrates in the normal enzyme but also provide less of a ‘choke point’ in the acetylated enzyme. Nevertheless, Val 509 was not important in binding of substrate as marked mutations at this site (Val to Lys or Glu) did not materially alter $K_m$ for AA of the COX-2 protein.33

An attempt to exploit the selectivity suggested by the larger substrate binding site in COX-2 had an unexpected outcome. It was argued that, as the acetylation of COX-2 still allows binding of AA to give 15-HETE, acylation with a larger acyl group should prevent any oxidation of AA by encroaching further into the binding area. In the event, the most potent analogue of aspirin was valeryl salicylic acid but it was a selective inhibitor of COX-1 with no inhibition of COX-2.34 The explanation for this result is still to be put forward.

It is important to note that in the COX mutant proteins and in the acetylated native COX, the peroxidase activity catalysed by an active site on the other side of the haem group from that involved in the formation of PGG$_2$ remains unaffected, demonstrating quite clearly the separation of these two activities within the same protein molecule.29,35,36

(ii) Inhibitor binding sites
Because COX-1 and COX-2 are catalytically and structurally almost identical, it is likely that if selective inhibitors of COX-2 are to be found then these would not bind to any catalytically relevant site which would be the same for both isoforms, but to some other region possibly unique to COX-2. However access to substrate must still be denied in order to inhibit COX-2 activity. This line of reasoning would explain why most of the selective COX-2 inhibitors so far disclosed are not carboxylic acids (as are most COX-1 inhibitors) but interestingly contain a different common grouping, the sulphonamide or sulphone group.

Support for this suggestion for different binding sites for the two types of inhibitors comes from another mutant of COX-1 in which Arg 120 was replaced with Glu.37,38 This positively charged residue (Arg 120) is located at the opening of the active site tunnel31 and was assumed to be the binding site for the carboxylic acid group in the substrate fatty acids. It was also assumed to provide a binding site for the ‘old’, carboxylic acid, non-selective, COX inhibitors. These assumptions were fully confirmed by the characteristics of the (Arg 120–Glu) mutant COX-1, which showed a 100-fold higher $K_m$ for AA and a much reduced susceptibility to the carboxylic acid inhibitors including indomethacin, flurbiprofen and diclofenac all of which did not inhibit the mutant enzyme at concentrations between 200- and 8000-fold.
higher than their IC$_{50}$ values for the wild-type enzyme. However two COX-2 selective compounds, the sulphone DuP 697 and a sulphonamide analogue, were more potent as inhibitors of the mutant enzyme. Although this increased potency is probably due to the decreased binding of substrate AA (DuP 697 is a competitive reversible inhibitor of the wild-type COX-1 enzyme), there was clearly no decrease in the efficacy of the sulphonamide compounds and hence no loss of their binding to the mutant protein. In the other report, the Arg 120–Glu mutant of ovine COX-1 showed no COX activity at all. Flurbiprofen binding was thus assessed with the Arg 120–Gln mutant which had 5% of the wild-type activity. This mutant did not show time-dependent inhibition and flurbiprofen's IC$_{50}$ value had increased from 5 µM (wild type) to 1 mM in the mutant. These results are probably adequate evidence for the importance of the Arg 120 residue in the binding of the 'old' non-selective inhibitors but the binding sites for the COX-2 selective inhibitors still remain to be determined, although two recent reports provide some clues to their location.

In both, mutants of COX-2 have been generated with Val 509 being changed to Ile, as in COX-1, and, in the mutants, selective COX-2 inhibitors were much less potent and less capable of causing the time-dependent inhibition of COX-2, characteristic of wild-type COX-2. However in one report, the COX-2 selective inhibitors (nimesulide, DuP697, NS398, SC58125) were still able to bind and inhibit presumably on a 'reversible' basis. The 'old' NSAIDs were unaffected by this mutation. Here the Val/Ile substitution appears to be crucial in determining the activity and possibly selectivity of inhibitors of COX-2. The possible effects of this substitution have already been discussed above in terms of substrate access but it is less easy to visualize the crucial influence of a methylene group in the binding of strongly polar sulphone/sulphonamide compounds such as the selective COX-2 inhibitors. The crystal structure of human recombinant COX-2 was described at a recent meeting and although the full report is not yet available some details relevant to this point have emerged. As expected the crystal structure of COX-2 is almost identical to that of COX-1. However for COX-2, two conformations appear to be possible for the binding of inhibitors, one in which inhibitor binds to both Arg 120 and Tyr 355 (see Ref. 38) as polar sites (the closed conformation) and the other (open conformer) in which inhibitor binding excludes Arg 120. Since mutants of Arg 120 do not change binding of COX-2 selective inhibitors, the open conformation would appear to be the most likely form of COX-2 bound to a selective inhibitor. However these changes are at the mouth of the substrate tunnel and involve polar residues not the non-polar Val 509 which has such striking effects on inhibitor efficacy. It may thus be necessary to reassess the role of another polar residue, Glu 524 (in COX-1). This negatively charged amino acid is close enough in the crystal to form a salt bridge with the positively charged Arg 120. Although Glu 524 was not important for enzymatic activity in COX-1, it, along with Tyr 355 could provide polar binding sites in COX-2, alternative to Arg 120. This residue Glu 524/510 is also immediately adjacent to the Ile 523/Val 509 and it may be that the crucial effects of the Ile/Val substitution on inhibitor binding are actually to alter the configuration of the next residue, Glu 524/510. Clearly we need more information before the binding site for COX-2 selective inhibitors and the nature of the conformational change associated with their action are fully elucidated. (See Note Added in Proof).

The largest difference between the two proteins is the C-terminal extension (18 amino acids) and the lack of the 17 residues at the N-terminus in COX-2. Although the C-terminus in COX-1 and probably in COX-2, is also distant from the active site, it clearly exerts a considerable influence on the function of the enzyme as site-directed mutations at the extreme C-terminal of recombinant COX-1 had unexpectedly strong effects on activity. Frustratingly, in COX-1 crystals, the X-ray analysis appears to extend reliably only to residue 586 of the 600 total residues in the ovine protein. Although the full report more information on this region may be presented. Mutants of COX-2 with alterations in this region will be invaluable in this analysis.

Another feature of inhibitor interaction with the COX proteins is the time-dependent irreversibility of some compounds (apart from aspirin). This varies between inhibitors and isoforms and is discussed further below but one aspect is relevant here. An inference from time-dependent and irreversible inhibition is that there may be chemical reaction between inhibitor and enzyme, for instance the formation of a Schiff base. These interactions and subsequent conformational changes in protein structure are different from acetylation by aspirin as all oxidation of AA is prevented, i.e. there is no formation of 15-HETE as in acety-
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The selective, time-dependent inhibition of COX-2 would imply that conformational changes in COX-1 derive from interactions different from those that inactivate COX-2. It is important to note that neither reversible nor irreversible inhibitors affect the peroxidase activity of the proteins which continue to function normally in the presence of the inhibitors. Another important consequence of the conformational rearrangement of the enzyme protein with inhibitor could be that the structure of COX-1 crystallized with inhibitor could be different from that of enzyme crystallized with substrate, as already recognized.

(iii) Intracellular location and function

The C-terminal region may also be especially important in securing the protein to the endoplasmic membranes. For both COX-1 and COX-2, the most commonly suggested location is in the membrane of the endoplasmic reticulum (ER) with an additional locus for COX-2 on the nuclear membrane. Results from the crystallographic analysis suggested that the enzyme is bound to the ER by a sequence of short helical stretches of the molecule which probably only interact with one-half of the lipid bilayer, i.e., there is no transmembrane structure. This description may now need to be modified. Ren et al. using antibodies specific for particular sequences in COX-1 protein (including the C-terminus, the active site and glycosylation sites), together with selective lysis of cellular membranes have suggested that the C-terminus may cross the ER membrane to the cytoplasm, while agreeing with a luminal location for the rest of the molecule. Such a model would be compatible with the crystallographic analysis where the C-terminus beyond Arg 586 was not resolved and other results attributing importance to amino acid residues distant from the active site. Although analogous mutations in COX-2 have not yet been assessed, these findings re-emphasize the importance of the structural analysis of the C-terminal region of the proteins.

Inflammation

Two separate reports of COX-2 knock-out mouse strains (null mice) have appeared and in each a model of acute inflammation (oedema following AA applied to the ear) was used to test responses in the null mice. Both agreed that
the null mice had normal ear oedema in response to AA and the interpretation of this result was that COX-1, still present, was able to generate the PGs required. Two other models of acute inflammation (PMA-ear oedema and carrageenan paw oedema) also gave normal results in null mice. The only model tested which failed in COX-2 null mice was one of LPS-induced hepatotoxicity which depends on induction of COX-2 in macrophages and/or Kupffer cells. The implications of these experiments in mice lacking COX-2 are that where COX-1 is normally present (ear skin, paws) this isoform will substitute for the missing COX-2; where COX-1 is absent or at very low levels, as in macrophages, the inducing agent fails to generate the usual response.

Reproduction and development

An essential role for COX-2 in embryonic development would be deduced from the severe morphological defects and consequent functional failures in the kidneys of COX-2 null mice, which lead to their early death (some at about 8 weeks and most by 6 months). A similarly absolute requirement for COX-2 in female fertility must be deduced from the failure of null female mice to ovulate. It is important to realize that these failures in null mice must represent a highly localized generation of COX products as the nature of the products from COX-2 is the same as those derived from COX-1 and COX-1 is still fully active in the COX-2 null mice.

A strong linkage between isoform and physiological function was further supported by the results from COX-1 knock-out mice. Here the null mice are healthy, without developmental defects and with no ovulatory changes. However null pups carried by a null mother were mostly (> 90%) born dead; either a heterozygous mother or some heterozygous pups restored viability of the whole litter to normal. Clearly the COX-2 present in the COX-1 null mice cannot provide the PGs needed for foetal survival but COX-1 present in some littermates will ensure survival of all foetuses. Again the separate events in reproduction appear to have differing absolute requirements for COX isoforms.

Implications of results from knock-out mice

The possibility of compensatory changes suggested for inflammation in COX-2 knock-out mice could also explain why in COX-1 null mice there was no gastric ulceration or NSAID-type nephropathy. Both these effects would have been predicted from the known effects of aspirin and other NSAIDs in normal animals. However in COX-1 null mice the compensation was not due to increased amounts of COX-2 activity as gastric tissue from the null mice synthesized less than 1% of the normal amount of PGs. The most likely alternative compensation would be from NO; this mediator is, like PGI2, a vasodilator and ulceration is associated with vasoconstriction in the gastric microcirculation. Like COX, both constitutive and induced NOS can be expressed in endothelium. One predictable consequence of this alternative pathway would be that NOS inhibitors would be ulcerogenic in the COX-1 null mice, whereas they are not noticeably so in normal animals.

If the knock-out mice show that COX-1 and COX-2 have separate and important physiological roles in reproduction and COX-2, unlike COX-1, plays an essential part in foetal development, what do the knock-out mice tell us about their pathological importance in inflammation? One conclusion is that there is a clear need for COX-2 in certain forms of inflammation, perhaps all those related to the actions of LPS. However the logical deduction from the other results is that COX-2 is not relevant to the inflammatory models used for many years to screen for anti-inflammatory compounds. On the basis of the knock-out results alone, several major pharmaceutical companies are wasting time, effort and money in searching for selective COX-2 inhibitors; these compounds will not decrease inflammation nor will they affect the incidence of NSAID-induced gastric ulcers as that effect is not connected with the presence or absence of COX-1 activity. In contrast to these logical deductions, there is a considerable body of empirical experimental evidence clearly demonstrating both the anti-inflammatory efficacy of selective COX-2 inhibitors and their decreased ulcerogenicity when compared with the more COX-1 selective inhibitors. This paradox is not without hope of resolution; there are still many relevant measurements to be made in the null strains—levels of COX-1 or COX-2 activity, of PLA2 activity, the effect of selective inhibitors and many others—and when these results are gathered, another synthesis of the apparently opposing views may be possible.

Regulation of COX-2 Activity

It is now clear that the activity of COX-2, as expressed by the synthesis of PGs, is normally controlled through the synthesis of the protein.
Several of the transcription factors effective on the COX-2 gene are known to be stimulated by inflammatory cytokines. Other cytokines and corticosteroids can alter the half-life of the inherently unstable mRNA, either increasing or decreasing translation into protein. For the protein itself, one option is to control the provision of substrate arachidonic acid (AA), although the most obvious regulator of activity would be a selective COX-1 or COX-2 enzyme inhibitor. All these possibilities, considered in more detail below, are summarized in Fig. 1.

Regulation by inflammatory factors

A great variety of agents, mostly derived from inflammatory situations, have been used to induce COX-2 activity. The most frequently used inducing stimuli are IL-1 and lipopolysaccharide (LPS; used here as a synonym for bacterial endotoxin) and not as might have been expected the inflammatory stimuli often used for in vivo models, carrageenin^{46-71} or zymosan^{72}. These and other stimuli used with human cells are listed in Table 1.

(i) Cytokines

Although IL-1 might be considered a model stimulus for the local induction of COX-2 in arthritis and LPS for the cardiovascular effects of the systemic induction of COX-2, these two stimuli are, in vivo, closely related as the effects of LPS are consequent on the production of TNFα and IL-1 from a range of cell types.

![Diagram of PG synthesis regulation](image)
Table 1. Stimuli known to induce COX-2 mRNA, protein or activity in human cells

| Stimuli       | Reference                        |
|---------------|----------------------------------|
| LPS           | 17, 76, 99, 108, 128, 130, 191–193|
| TNF, IL-1     | 89, 193, 194                     |
| EGF           | 88, 89, 91, 200                   |
| PDGF          | 200, 201                         |
| TGFα, β       | 88, 89, 91                       |
| Phorbolester  | 17, 148, 157, 202–204            |
| Pregnancy     | 142, 203, 205, 206               |
| Parturition   | 207–212                          |

The references given are restricted to cells and tissues of human origin and to papers published in 1994, 1995 and early 1996. These are given as a guide to the range of systems (stimuli, cells, tissues or in vivo) used and not as a complete list of all the work on COX-2.

Differences between the effects of LPS and IL-1 in cultured cells will clearly depend on the ability of the cell line to release IL-1 in response to LPS; such differences are less likely in vivo or ex vivo where a wide range of cell types have been exposed to the inducing agent. However as at least two cytokines, IL-10 and IL-4, decrease induction (see below), their synthesis in vivo following IL-1 or LPS treatment could modify the final level of COX-2 activity attained.

In endothelial cells COX-2 is induced readily by LPS, in some cases through the release of TNFα, PDGF and other cytokines. However the initial stage of this process, the binding of LPS to the cell membrane, is still unclear as endothelial cells do not express the particular LPS-binding membrane proteins (CD 14) that may be used as receptors on leukocytes, a major cell type responding to LPS with induction of COX-2. There are some indications that soluble forms of CD 14 are involved in the mediation of responses to LPS in endothelial cells.

Whereas most cytokines so far studied increase induction of COX-2, there are examples of inhibition by cytokines. Two interleukins, IL-10 and IL-4, already known to antagonize other effects of 'pro-inflammatory' cytokines, decreased COX-2 levels in monocytes stimulated by LPS or Con A, but in mast cells IL-10 potentiated, whereas IL-4 still inhibited, induction of COX-2 by c-kit ligand and IL-1. It is possible that this discrepancy in the effects of IL-10 is related to the cell types involved; more studies would be needed to define such a selectivity.

The TGF proteins, TGFα and TGFβ, present conflicting results for analysis. As might be expected from its mitogenic activity, TGFα was able to stimulate COX-2 production in epithelia and to increase PGE2 output in human amnion cells and in osteoblasts. TGFβ synergized with IL-1 or TNFα to increase PG output, perhaps because TGFα can also induce IL-1 receptors.

Although TGFβ potentiates the induction of COX-2 caused by phorbol ester in fibroblasts, it had no effect when given alone. In macrophages, the same cytokine inhibited the induction of COX-2 by LPS, more in keeping with its general anti-inflammatory profile. The apparent divergence of the effects of TGFβ may be more readily rationalized on the basis of cell types; for an overall anti-inflammatory and wound-healing effect, it would be reasonable to de-activate leukocytes and to stimulate fibroblasts at the same time. TGFβ also affects NOS induction in a range of cells but here the majority of the results show a suppression of iNOS synthesis. The down-regulation of two major components of the inflammatory process would contribute importantly to the anti-inflammatory action of this cytokine.

(iii) PGs

The PGs themselves are inducing agents, as directly shown in osteocyte cultures or by inference from the effects of COX inhibitors. In osteoblastic cell (MC3T3) or calvarial cultures, PGE2, PGF2α, PGD2 and iloprost (a stable analogue of PGI2) all induced COX-2 mRNA and protein and indomethacin decreased COX-2 induction. Exogenous PGE2 also stimulated COX-2 expression in mouse macrophages treated with LPS. In these cells and in two other cell systems, rat epithelial cells stimulated with TGFα and phorbol ester and in human PMNs stimulated with LPS, COX inhibitors (indomethacin or sulindac sulphide) decreased COX-2 induction. The positive feedback implied in these systems contrasts sharply with the negative feedback loops more usually found in inflammatory conditions where PGE2 and other agonists that raise cAMP actually decrease cytokine secretion from macrophages and lymphocytes. Indeed the breaking of this loop with COX inhibitors (NSAIDs) in chronic inflammation is believed to increase cytokine production and subsequent degradation of joint cartilage while relieving pain and oedema; thus symptomatic benefit is undermined by a continuing or even accelerated disease process.

(iii) Free radicals and nitric oxide

Another totally different class of compounds associated with inflammatory situations are the reactive oxygen intermediates or oxygen derived free radicals (ODFR), such as superoxide...
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anion (O$_2^-$) and the hydroxyl radical (OH$^-$). A role for these species in the induction of COX-2 has also been proposed$^{107,108}$ These highly reactive chemical species are present at sites of inflammation, leukocytes generate ODFR in the phagocytic process (the oxidative burst) and NO is another free radical, present during inflammation, synthesized by iNOS and capable of interacting with the ODFR to increase free radical actions. Thus, there are many opportunities for interactions between these radicals and COX-2. In rat mesangial cells following LPS, IL-1 or TNFα, radical scavengers (thioureas) or other antioxidants inhibited the usual increase in COX-2 mRNA, COX-2 protein and PGE$_2$ synthesis$^{107}$ The effects were selective in that induction of other proteins (chemokines) by LPS or of COX-2 by other stimuli (phorbol esters, serum) were not similarly inhibited. At present, the actual mechanism of the interaction between the ODFR and the transcription of the COX-2 gene is not fully known, although ODFR are known to activate NFκB,$^{109,110}$ one of the transcription factors active on the COX-2 gene. However, in the rat the COX-2 gene may not exhibit a binding site for this transcription factor.$^{19,21}$

Another free radical associated with inflammation is nitric oxide (NO) and its effects on COX activity remain difficult to summarize briefly. Both enzymes, COX and NO synthase (NOS), have inducible isoforms, which are induced in inflammatory situations by the same cytokines and both genes belong to the family of immediate early genes. Thus interaction between these two enzyme systems is highly likely; the difficulty arises from the outcome of that interaction. There was potentiation of PG output in the presence of NO,$^{111-113}$ apparently by direct interaction of NO with the haem in COX protein,$^{116}$ although this interpretation has been questioned.$^{117,118}$ The opposite effect, inhibition of PG output by NO, has also been observed in macrophages.$^{119}$ Action of PGs on NO output is less frequently encountered$^{120,121}$ although such effects might be deduced from the interactions of raised cAMP with NO output.$^{122-124}$ In one case a truly reciprocal relationship, with PGs inhibiting NO output and NO inhibiting PG output, by which a constant level of vasodilator tone may be maintained has been described in human saphenous vein.$^{125}$ This last example is unlikely to involve induced forms of either enzymes and is perhaps less relevant to the present discussion but it does illustrate the range of interactions possible between these enzymes. The most valid conclusion would seem to be that there is no generalization and that each situation with its particular combination of species, cell type and stimulus has to be evaluated individually.

(iv) Contribution of phospholipases

A potential source of confusion in the interpretation of experiments involving COX-2 induction is the simultaneous induction of PLA$_2$ activity, most frequently with IL-1 as inducing agent.$^{86,126-132}$ Increased PLA$_2$ activity implies increased provision of free AA which could be as easily available to COX-1 as to COX-2. Thus the final effect, increased synthesis of PGs, is not necessarily due solely to increased COX-2. One prediction from this co-induction is that the effectiveness of inhibitors of COX-2 could depend on the level of PLA$_2$ induced at the same time and that level could vary with the nature of the major inducing agent (IL-1, PAF or TNF) and with the cell type involved. The localization of the human gene for cytosolic PLA$_2$ to the same chromosomal region as the gene for COX-2 (1q25), raises the possibility of coordinate regulation of these enzymes.$^{133,134}$

(v) Effects of corticosteroids

The major inhibitory mechanism affecting COX-2 induction, both in terms of experimental results and of pathophysiological relevance is the action of corticosteroids, most frequently demonstrated with dexamethasone (Dex). In many conditions, susceptibility of the production of PGs, enzyme protein or mRNA to inhibition by Dex is used as clear evidence for COX-2 induction. This simple conclusion is however clouded by other actions of Dex including the inhibition of the induction of PLA$_2$,$^{132}$ and inhibition of PLA$_2$ activity itself via lipocortin.$^{135,136}$ For instance over 20 years ago it was shown that the output of PGs from freshly isolated lungs from untreated guinea-pigs, conditions in which COX-2 induction should be minimal, was decreased by infusions of Dex.$^{137}$ There is nevertheless clear evidence for decreased induction of both COX-2 protein and mRNA in the presence of Dex.$^{25,93,138-141}$

There are also a few examples of corticosteroids increasing PG production. The mRNA for COX-2 but not that for COX-1 was increased 20-fold in human amnion cell cultures exposed to Dex for 16 h.$^{142}$ Interestingly, oestradiol and progesterone did not increase COX-2 mRNA but cortisol did. More typically, in human decidual tissue, Dex and progesterone were inhibitory.$^{143}$ As with other examples of corticosteroid inhibition of mRNA, the mechanism of action of Dex on COX-2 induction is poorly elucidated; either increased degradation of an already short-lived
mRNA or decreased transcription are possible contributors to the overall affect.

Regulation via intracellular signalling

Tyrosine kinases play important roles in the intracellular signalling pathways for COX-2 induction in a range of cells; in endothelial cells, epithelial cells and macrophages this was demonstrated with tyrosine kinase inhibitors and in mesangial cells by measurement of protein tyrosine phosphorylation. The oncogene v-src encoding a tyrosine kinase is by itself enough to cause COX-2 induction in T cells. Whereas the induction by EGF probably utilized the receptor tyrosine kinase, the kinases used by other inducing agents have not yet been clearly identified. However the inhibition by tyrosine kinase inhibitors such as erbstatin and genistein of both COX-2 and iNOS induction offers a new mode of anti-inflammatory action, which would have the advantage of not acting on constitutive enzyme activities. Such an effect may contribute to the anti-inflammatory properties of leflunomide which was shown to inhibit the EGF-stimulated tyrosine kinase and the Src tyrosine kinases and the synthesis of PGE2 induced by LPS in human leukocytes.

Another signalling pathway, via protein kinase C (PKC), also appears to be involved but here the net effects of stimulating PKC activity are more variable. In most cases, increased PKC activity was associated with induction of COX-2 but in alveolar macrophages, inhibition of PKC with staurosporine caused COX-2 induction. Another confusing factor is the well-established down-regulation of PKC on continued stimulation by phorbol esters. In the present context this was illustrated by the stimulation of COX-2 mRNA by 5HT (mediated via 5HT2 receptors and PKC activation) or by short exposure to phorbol ester. However pre-incubation with phorbol ester followed by 5HT, abolished induction of COX-2 mRNA.

These kinase pathways are not exclusive and in mesangial cells with 5HT as stimulus both PKC and tyrosine kinase mediate the induction of COX-2 mRNA. In human skin fibroblasts after IL-1 stimulation, PKC appeared to be the major protein kinase with only minor contributions from PKA or tyrosine kinases. However in ovarian tissue both PKA and PKC were activated during LH and GnRH stimulation of COX-2. Other intracellular second messengers identified in osteoblasts include cAMP and PLC, stimulated by PGE2 or iloprost (a stable analogue of PGL2) and PGF2α respectively.

Regulation by substrate

One of the continuing controversies in the analysis of COX action centres on the influence of different sources of substrate on activity. There is a clear difference between the utilization of endogenous and exogenous AA in that the supply of endogenous substrate is controlled by enzymes outside the COX cascade, chiefly by the balance between phospholipid hydrolysis and re- or transacylation. The levels of endogenous substrate can be increased and PG output consequently stimulated in a number of cells and tissues (without induction of COX-2) by agents such as thrombin, histamine, bradykinin, PAF or the crosslinking of IgE receptors. This stimulation is characteristic of short duration, less than 30 min and there is no doubt that endogenous substrate is utilized by COX-1 to increase PG synthesis. The experiments in which COX-2 utilizes endogenous substrate are characteristically of longer duration (6–24 h) and entail incubation of cells with an inducing agent such as LPS, TPA or PDGF which is present throughout the incubation. The PGs accumulated over these longer periods are increased many-fold in the presence of the inducer molecule, relative to those in control incubations. Most of the inducing agents will increase phospholipase action as well as inducing COX-2 protein. Again there is no doubt that COX-2 can utilize endogenous substrate to form PGs.

The problems appear with the use of exogenous substrate. Addition of exogenous AA (10–50 μM) to systems containing COX-1 leads to increased output of PGs. However in some preparations where both COX-1 and COX-2 are present, the induced enzyme appears to contribute no additional amount of PGs over that seen with COX-1 alone, leading to the proposition that COX-2 does not utilize exogenous AA. Two comments are relevant. First, this 'inaccessibility' of exogenous AA is not a universal finding; in a variety of cells, induction of COX-2 did lead to an increase in PG output from exogenous substrate. Second, the use of exogenous AA involves incubation for short times, typically 10 min, compared with 6 h incubations to show COX-2 induction. Within 10 min over 100 ng PGE2/ml was generated, whereas over 6 h there was accumulation of only about 20 ng/ml. An equally plausible explanation would be that although COX-2 was induced several-fold, the actual amounts of COX-2 protein were still low relative to the amount of COX-1 present. It is thus not possible to come to a definite conclusion on the
selective accessibility of exogenous substrate to either isoform on the basis of these experiments.

A variation of this hypothesis was the conclusion drawn from work with only endogenous substrate in mast cells. Here release of PGD2 and COX-2 protein were induced by incubating the mast cells with a mixture of cytokines. Incubation in this medium for 5–10 h increased PGD2 output many-fold to about 5–10 ng/ml. ‘Acute’ stimulation of PGD2 biosynthesis by the crosslinking of IgE receptors in these cells was not increased until after 24 h incubation with the cytokine mixture, by which time the COX-2 protein had fallen again to near normal levels. There was certainly no increase in the ‘acute’ release of PGD2 at the time of peak COX-2 induction. From this the authors concluded that the induced COX-2 did not have access to the increased AA released during IgE stimulated PGD2 synthesis and that only COX-1 was utilized, even when COX-2 had been induced, to form PGs subsequent to IgE stimulation. A comparison of the times over which these experiments were performed shows that the IgE stimulation provided about 5 ng PGD2/10^6 cells in 10 min whereas the cytokine stimulation took 10 h to provide: 10 ng/10^6 cells. On this basis the induced COX-2 would provide about 0.2 ng in 10 min; this contribution would be rather difficult to detect against the total of 5 ng produced. The authors may be correct in postulating different coupling of stimuli to the isoforms but the experimental results are not an adequate test of their hypothesis. Indeed in a more recent paper from this group, the ‘stimulus selective’ linkage of COX-1 or COX-2 action has been replaced by a ‘time selective’ hypothesis.

If distinct pools of substrate for COX-1 and COX-2 do exist they are more likely to be defined on a spatial basis than on a simple endogenous/exogenous substrate criterion. Indeed if COX-2 shares the general three-dimensional structure proposed for COX-1 with the membrane anchors defining the entrance to the active site tunnel which guides AA cleaved from the adjacent membrane up to the oxidative site of the enzyme, it is not immediately obvious how one isoform could favour exogenous AA over the freshly hydrolysed product of the underlying ER membrane. However this model of COX action would assume a phospholipase in close proximity to the COX protein and a more likely basis of selectivity is in the phospholipase activated to supply endogenous AA. Although most emphasis has been placed on PLA2 in this context, the action of either PLC or PLD can also give rise to free AA. Different ligands for cell membrane receptors will activate these phospholipases differentially. Furthermore, each phospholipase has its own substrate selectivities and the distribution of the phospholipids is not uniform throughout all membranes. There is also evidence for a selective locus of COX-2 in the nuclear membrane, apart from the location in the ER demonstrable for both isoforms. Thus a combination of which phospholipase is activated by which stimulus and which phospholipid is closest to a particular COX protein might appear to give a degree of selectivity between isoforms in terms of the substrate.

Selective Inhibition of COX-2

One disadvantage of regulating COX-2 by interference with the processes of transcription, translation or intracellular signalling pathways is that, at present, selectivity of effect is low. This is well recognized for the corticosteroids which will prevent induction of many proteins apart from COX-2 and could be equally true for antagonists of or interference with, transcription factors such as NFkB or NF-IL6 or for the inhibitors of tyrosine kinase. Logically the most selective effect would be attained by inhibition of the enzyme protein and this consideration coupled with the effectiveness of COX inhibitors already known has led to an extensive search for new inhibitors with a selective action on COX-2. Particularly, such selective agents should be free of the most significant side effects associated with COX-1 inhibition, gastric ulcers and nephropathy.

Assessment of selectivity

The initial stages of this search were concerned with assessment of the selectivity of the known NSAIDs or COX inhibitors and very soon established one major difficulty in the analysis, a marked variation in the selectivity ratio (IC50 for COX-1/IC50 for COX-2) for any given compound; a high ratio representing selectivity for COX-2 inhibition. For instance, ratios for indomethacin ranged from 20 to 0.1. This variability is due to variation, at every level, in the experimental conditions of the assays. Different types of cell are used, derived from different species, as whole cells, homogenates, purified extracts or recombinant proteins expressed in bacterial, insect or animal cells. Further variation is introduced in the time of pre-incubation with inhibitor, the concentration of exogenous substrate or the use of endogenous substrate. The
last three factors contribute significantly to the marked differences in selectivity. For instance with 10 min pre-incubation, indomethacin at 1.6 μM completely abolished COX-2 activity whereas COX-1 activity suffered only 50% inhibition. However, with no pre-incubation, indomethacin had an IC₅₀ of 13.5 μM for COX-1 whereas for COX-2 the IC₅₀ was over 1 000 μM. Clearly two very different selectivity ratios would be calculated from IC₅₀ values obtained under these two experimental conditions. Time-dependent inhibition of COX-2 but not of COX-1 by NS 398 was the major reason for its selectivity with human recombinant enzymes. A similar differential time-dependency was reported for CGP28238, another COX-2 selective inhibitor. This feature is not the sole determinant of selectivity as several COX-1 inhibitors also show time-dependent inhibition of either isoenzyme.

Another source of variability with important practical consequences is the nature of the enzyme system used, i.e. in whole cells, homogenates or purified enzymes. Many groups have used human platelets as a source of COX-1 and a variety of cells (renal mesangial cells, macrophage cell lines, peripheral blood monocytes) stimulated with IL-1 or LPS to provide COX-2. The IC₅₀ values for indomethacin for whole cell preparations vary but are always lower than those reported for cell free preparations. This is true also for selective inhibitors; the IC₅₀ for CGP28238 against COX-2 was 15 nM in whole cells but 750 nM even after prolonged preincubation with purified enzyme. Similarly, increased potencies (lower IC₅₀) have been noted for aspirin, ibuprofen and even salicylate in whole cells compared with values obtained in broken cell or purified enzymes. There are no obvious explanations for this phenomenon. Preferential concentration of the inhibitor in lipid of the ER membrane to give a locally higher concentration than in the bulk solution would not explain why inhibitory potency is lost in broken cells as crude homogenates after centrifugation would contain enzyme still attached to fragments of ER. One possibility is that in whole cells the concentration of free AA is kept low by restraining phospholipase activity (through low intracellular calcium, for instance) and increasing re- or trans-acylation into lipid. Thus, the initial binding of the inhibitor to the enzyme in whole cells takes place with little or no competition from the substrate AA, allowing a maximal inhibitory effect. In a broken cell preparation the calcium concentration is much higher than the normal intracellular level, lipase activities and concentrations of AA could thus be higher, providing more competition for the binding of inhibitor. Even the time dependent irreversible inhibitors will show some reversal of inhibition when exposed to high AA concentrations.

The value of a model system must lie in its ability to predict compounds with selective COX-2 inhibition in vitro and one test of that value is to assess the compounds already known to exhibit such activity alongside those NSAIDs with the worst side effect profiles. Thus compounds such as NS398, SC58125 and CGP28238 must be clearly separated from NSAIDs such as piroxicam, azapropazone or ketoprofen. Most model systems achieve this separation and will probably be efficient screens for selective COX-2 inhibitors. However, it must be remembered that over the last 25 years, reliance on in vitro screening with COX purified from ram seminal vesicle (now known to be almost entirely COX-1) must have led to the rejection of many COX-2 selective inhibitors before they could be tested in vivo and a similar absolute reliance on a single in vitro screen for COX-2 could lead to similar mistakes.

Progress in development of selective inhibitors

There were at least two examples of possible selective COX-2 inhibitors in the literature; both exhibited a good anti-inflammatory effect in vivo with little or no inhibition of the standard COX preparation from ram seminal vesicle together with low ulcerogenic activity. The first of these compounds, nimesulide, was patented over 20 years ago and has been available latterly in several European countries as an over-the-counter, non-prescription analgesic and anti-inflammatory agent. The other, CGP28238, is closely related in structure (see Fig. 2) and was first reported in 1989. A third close relative, NS398, was described many years later as a selective inhibitor of COX-2.

Subsequent development has led to DuP 697, SC 58125 and L-745,337 with many other similar compounds less extensively studied. The most striking feature of this new generation of NSAIDs is that none of them are carboxylic acids, like the ‘old’ NSAIDs, and all have the sulphone or sulphonamide grouping. The simplest (and perhaps simplistic) inference from this is that the selective COX-2 inhibitors bind to a site that is different from that used by the ‘old’ NSAIDs and, as proposed earlier, would suggest a structure unique to the COX-2 protein which, at first sight, is most likely to be the 18-amino acid.
FIG. 2. Chemical structures of selective inhibitors of COX-2. All the compounds shown have exhibited inhibitory selectivity towards COX-2 in a range of systems (see Table 2). The sulphonamide grouping is present in all except meloxicam in which it forms part of a cyclic structure. None of these compounds has a carboxylic acid grouping characteristic of the ‘older’ NSAIDs.

In spite of all the reservations outlined above, the search for selective COX-2 inhibitors has been remarkably successful. To some extent this reflects the efficacy of the exemplar compound, nimesulide, but all the subsequent developments exhibit the predicted properties. They all have good selectivity in vitro with pure enzymes or in cell systems with ratios of IC$_{50}$

insert at the C-terminus. It is important to note that neither the 'old' nor the new selective COX inhibitors affect the peroxidase activity of the proteins, implying that the binding of COX-2 inhibitors, like that of COX-1 inhibitors, does not disturb the three-dimensional structure of the protein on the 'other' side of the haem ring. 29,35,36
favouring COX-2 in all assays and some values are shown in Table 2. Furthermore they exert anti-inflammatory activity in a range of models, acute and chronic, as well as anti-pyretic and analgesic activities and at these doses there is little or no gastric ulceration. Clearly, it is possible to achieve selective inhibition of COX-2 and now it seems only a matter of refining the effective structures to combine the highest selectivity with the best pharmacokinetics and provide compounds for clinical evaluation.

One result of the selectivity of COX inhibition coupled with a better understanding of the conditions in which COX-2 can be induced is that it may now be reasonable to use COX-2 inhibitors in therapeutic areas such as endotoxin shock or asthma where the 'old' NSAIDs were ineffective. The greater potency against COX-2 together with lack of toxicity on stomach and kidney could allow a reduction in PG output via the induced enzyme while allowing the 'beneficial' output from constitutive COX-1. Another potential therapeutic area of considerable promise for COX-2 inhibitors is suggested by the negative correlation between colon cancer and NSAIDs; recently aspirin was shown to reduce the risk of colorectal cancer by almost half. The crucial observations were that the COX-2 isoform was present only in malignant tissue and conferred resistance to apoptosis, implying an important role for COX-2 in neoplastic growth.

The side effects or toxicity of COX-2 inhibitors are not easy to predict; certainly those of the 'old' NSAIDs should be absent, by definition. From the evidence of the knock-out mice, the major toxicities will be on the reproductive system, on fertility and foetal development. Whereas decreased fertility, as long as it is reversible, may not be an unacceptable side effect, developmental defects, especially in the kidney, would most certainly prevent the use of COX-2 inhibitors during pregnancy and thus extensive testing for possible teratogenic effects would be required.

**Summary**

Elucidation of the regulation of COX-2 provides an instructive example of the interaction between molecular biology and applied pharmacology. The basic science of the identification of the isoforms and the stimuli for induction was rapidly transformed into a new and powerful therapeutic concept, NSAIDs without the usual side effects. We now know a great deal about COX-2 from the gene to the crystal structure of the protein, its substrate sites and its intracellular location, much more than about many other enzymes of pharmacological importance. However in one significant aspect this encouraging utilization of molecular biology has failed; for all our knowledge, the design of selective inhibitors has not been based on a careful study of the structure of the protein and its interactions with substrate but, as in the past, on chemical variations of a molecular structure, found empirically to be effective. Moreover, deductions based on the knock-out mice would deny much of the equally empirical evidence correlating inflammation with COX activity.

Nevertheless it was molecular biology that disclosed the important place of COX-2 in reproduction, that raised new possibilities for

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**Table 2. Selective COX-2 inhibitors**

| Compound       | IC₅₀ for COX-2 (nM) | IC₅₀ COX-1:2 | Anti-inflammatory doses (mg/kg) | Ulcerogenic potential in vivo (mg/kg) | References |
|----------------|---------------------|--------------|--------------------------------|--------------------------------------|------------|
| Nimesulide     | 13                  | 13           | 3                              | 0.2                                  | 100        | 213, 214 |
| CGP 28236      | 15                  | 5000         | 2                              | 0.05                                 | 30         | 171, 177 |
| NS 398         | 100                 | 260          | 1                              | 5                                    | > 1000     | 44, 215  |
| SC 58125       | 50                  | > 2000       | 2                              | –                                    | > 600      | 180      |
| L 745337       | 23                  | > 400        | 2                              | –                                    | > 30       | 181      |
| Meloxicam      | 2                   | 3            | 3                              | 0.1                                  | 2.5        | 216, 217 |
| DuP 897        | 10                  | 80           | 0.03                           | 0.2                                  | > 400      | 45, 179  |
| Indomethacin   | 6                   | 0.03         | 1.5                            | 0.1                                  | 8          | 215, 216 |
| Piroxicam      | 175                 | 0.03         | 2.7                            | 0.6                                  | 1.1        | 216, 217 |

*These values were obtained in vitro with whole cells, purified native or recombinant enzymes.

*IC₅₀ for COX-2 and COX-1 at 1:2 ratio gives some indication of selectivity.*

*IC₅₀ for COX-1:COX-2 which gives some indication of selectivity in vitro.*

*IC₅₀ for COX-1:COX-2 which gives an estimate of in vivo selectivity.*

*These values represent the threshold dose for gastric damage or the highest dose at which damage was still absent.*

*Values shown are ED₅₀ or ED₉₀ doses in each model.*

*Bearing in mind the diversity of test systems used, the important values in the table are not the absolute potencies of the compounds in any given test system but the ratios, firstly of IC₅₀ COX-1:COX-2 which gives some indication of selectivity in vitro (see text for further comment) and then the ratio of the ED₅₀ in chronic inflammation and the ulcerogenic dose, giving an estimate of in vivo selectivity. Note that the last two compounds in the table (shown in italics) are examples of the 'old, non-selective' NSAIDs. In fact these were quite selective for COX-1, as shown by the values for the IC₅₀ ratios.*
the physiological role of COX-1 and that elucidated the correlation between COX-2 and neoplastic growth. In this last context, there is an intriguing possibility for which there is no direct evidence yet but which is entirely assessable with molecular biological techniques, namely that COX-2, like the products of other immediate-early genes, has effects on gene transcription and/or translation that do not entail the oxidation of AA to PGs. In all these roles and in the new ones to come, the analysis of regulatory mechanisms, physiological, pathophysiological and pharmacological, will remain central to scientific and clinical progress.

Note Added in Proof

The X-ray crystal structure of human COX-2 described by M. Browner et al. is to appear in Nature (Structural biology) in November 1996. Two further descriptions, of murine COX-2 by R. G. Kurumbail et al. and of the human protein by B. M. McKeever et al., were given at a meeting in Vienna in September 1996 and have been submitted for publication. All three reports include discussions of the binding sites for selective inhibitors of COX-2.

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