JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2006, 57, Supp 5, 113–124
www.jpp.krakow.pl

R.M. BOTTING

INHIBITORS OF CYCLOOXYGENASES: MECHANISMS, SELECTIVITY AND USES

The William Harvey Research Institute, The John Vane Science Centre, St Bartholomew's and the London School of Medicine and Dentistry, Queen Mary, University of London, U.K.

The prostaglandins are lipid mediators, discovered in the 1930s by von Euler in Sweden and Goldblatt in the United Kingdom. They are made by the bifunctional enzyme, cyclooxygenase, which has both cyclooxygenase and peroxidase activities in the same molecule. Prostaglandins are involved in physiological functions such as protection of the stomach mucosa, aggregation of platelets and regulation of kidney function. They also have pathological functions such as their involvement in inflammation, fever and pain. Vane in 1971 elegantly showed that the pharmacological actions of aspirin and similar drugs were due to the inhibition of cyclooxygenase. Thus, aspirin-like drugs exert their anti-inflammatory, antipyretic and analgesic effects by inhibition of cyclooxygenase. In 1991, Simmons and his colleagues identified a second cyclooxygenase enzyme, designated cyclooxygenase-2, derived from a separate gene from cyclooxygenase-1. Cyclooxygenase-2 is upregulated by inflammatory mediators and forms prostaglandins which intensify the inflammatory response. Cyclooxygenase-1 is, therefore, a 'housekeeping' enzyme making prostaglandins, which are important for maintaining physiological functions and cyclooxygenase-2 makes prostaglandins which are important in inflammation. The discovery of cyclooxygenase-2 and the establishment of its structure led to the development of selective inhibitors of this enzyme, such as celecoxib and rofecoxib, with potent anti-inflammatory actions but with reduced gastrotoxic effects. A putative cyclooxygenase-3, has also been characterised and cloned. This enzyme is a product of the cyclooxygenase-1 gene, but retains intron 1 after transcription and translates into a cyclooxygenase enzyme with 34 additional amino acids. It is more sensitive to inhibition by paracetamol, aspirin and some other non-steroid anti-inflammatory drugs than cyclooxygenase-1 or cyclooxygenase-2. A cyclooxygenase enzyme induced in cultured cells by some non-steroid anti-inflammatory drugs is also more sensitive to inhibition by paracetamol than cyclooxygenase-2 induced by bacterial lipopolysaccharide

Key words: *prostaglandin, cyclooxygenase-1, cyclooxygenase-2, cyclooxygenase-3, non-steroid anti-inflammatory drugs, rofecoxib, celecoxib, lumiracoxib, valdecoxib, etoricoxib, paracetamol.*

INTRODUCTION

Among the many mediators of inflammation, the prostaglandins (PGs) are of great importance. They are released by almost any type of chemical or mechanical stimulus. The key enzyme in their synthesis is prostaglandin endoperoxide synthase (PGHS) or cyclooxygenase (COX) which possesses two catalytic sites. The first, a cyclooxygenase active site, converts arachidonic acid to the endoperoxide PGG_2. The second, a peroxidase active site, then converts the PGG2 to another endoperoxide, PGH_2. PGH_2 is further processed by specific synthases to form PGs, prostacyclin and thromboxane A_2. Of the PGs, PGE_2 and prostacyclin are the main inflammatory mediators. Cyclooxygenase activity has long been studied in preparations from sheep seminal vesicles and a purified enzymatically active COX was isolated in 1976 (1). We now know that COX exists in at least two isoforms, COX-1 and COX-2.

Over 35 years ago, Vane proposed that the mechanism of action of the non-steroid anti-inflammatory drugs (NSAIDs) was through the inhibition of PG biosynthesis (2; *Fig.1*) and there is now a general acceptance of the theory. 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 unique irreversible action of aspirin, other NSAIDs such as ibuprofen or indomethacin produce reversible or irreversible COX inhibition by competing with the substrate, arachidonic acid, for the active site of the enzyme.

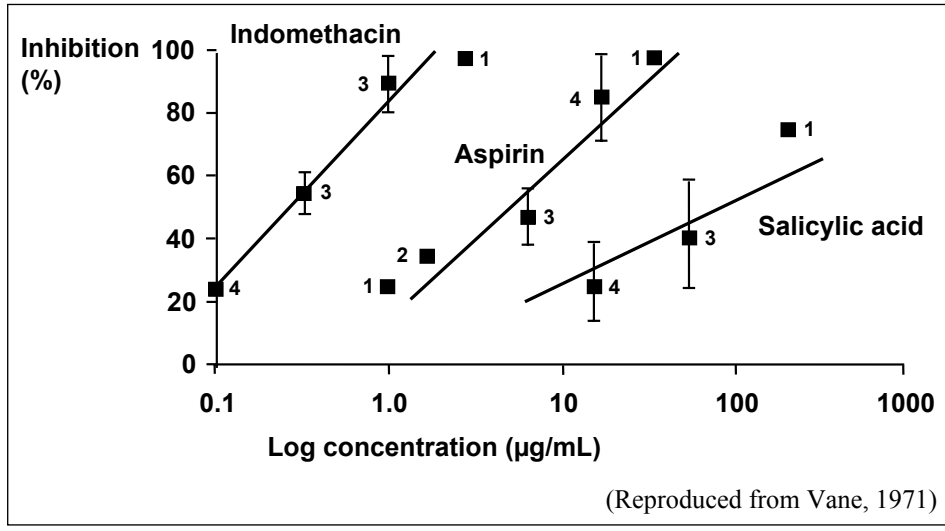

Fig. 1. Inhibition of cyclooxygenase activity in homogenate of guinea pig lung by nonsteroid anti-inflammatory drugs. Indomethacin, aspirin and salicylate inhibited prostaglandin production by guinea pig lung homogenate in a dose-dependent manner. Indomethacin was the most potent and salicylate the least, which parallels the therapeutic anti-inflammatory potency of these drugs. Narcotic analgesics such as morphine did not cause inhibition. (Reproduced from Vane, 1971 with permission).

The inhibition of PG synthesis by NSAIDs has been demonstrated in a wide variety of systems, ranging from microsomal enzyme preparations, cells and tissues to whole animals and man. For instance, the concentration of PGE_2 is about 20 ng/ml in the synovial fluid of patients with rheumatoid arthritis (3). This decreases to zero in patients taking aspirin, a good clinical demonstration of the effect of this drug on PG synthesis. Over the last three decades, many new drugs such as piroxicam, flurbiprofen, diclofenac, naproxen, ibuprofen and various others have reached the market based on COX enzyme screens.

Comparison of COX-1 and COX-2

The constitutive isoform, COX-1, has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin which when released by the vascular endothelium is anti-thrombogenic (4) and when released by the gastric mucosa is cytoprotective (5). It is also COX-1 in platelets that leads to thromboxane A_2 production, causing aggregation of the platelets to prevent inappropriate bleeding (6). During the 1970s and 1980s, there were various clues in the literature suggesting that there may be a second COX enzyme. As early as 1972, Smith and Lands (7) and Flower and Vane (8) speculated on the existence of isoenzymes. The breakthrough came from biologists outside the field of prostaglandins. Simmons and his colleagues were studying immediate early genes and discovered a gene encoding a second form of COX, induced by *v-src*, serum or phorbol esters in chicken embryo fibroblasts (9, 10). The human COX-2 gene is 8.3 kb in length, similar to the COX-2 gene of mouse and chicken but smaller than the 22 kb human COX-1 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 (11, 12). However, both enzymes have a molecular weight of 71 kDa with just over 600 amino acids, of which 63% are in an identical sequence. The inhibition by the glucocorticoids of the expression of COX-2 is an additional aspect of the anti-inflammatory action of the corticosteroids. The levels of COX-2, normally very low in cells, are tightly controlled by a number of factors including cytokines, intracellular messengers and availability of substrate (13).

Garavito and his colleagues (11) have determined the three dimensional structure of COX-1, providing a new understanding for the actions of COX inhibitors. Each dimer of COX-1 comprises three independent folding units: an epidermal growth factor-like domain, a membrane binding motif and an enzymatic domain. The sites for peroxidase and COX activity are adjacent but spatially distinct. The conformation of the membrane-binding motif strongly suggests that the enzyme integrates into only a single leaflet of the lipid bilayer and is thus a monotopic membrane protein. Three of the helices of the structure form the entrance to the COX channel and their insertion into the membrane could allow arachidonic acid to gain access to the active site from the interior of the bilayer. The

COX active site is a long, hydrophobic channel and Garavito et al. (11) provided evidence to suggest that some of the NSAIDs, such as flurbiprofen, inhibit COX-1 by excluding arachidonate from the upper portion of the channel. Tyrosine 385 and serine 530 lie at the apex of the long active site. Aspirin irreversibly inhibits COX-1 by acetylation of the serine 530, thereby excluding access of arachidonic acid to the active site of this enzyme (11). The S(-) stereoisomer of flurbiprofen interacts via its carboxylate group with arginine 120, thereby placing the second phenyl ring within Van der Waal's contact of tyrosine 385. There may be a number of other sub-sites for drug binding in this narrow channel.

The three dimensional structure of COX-2 has also been published (12). It closely resembles the structure of COX-1, except that the COX-2 active site is slightly larger and can accommodate larger structures than those which are able to fit the active site of COX-1. A secondary internal pocket of COX-2 contributes significantly to the larger volume of the active site of this enzyme, although the central channel is also bigger by 17%. Although aspirin acetylates serine 516 in COX-2, this does not prevent an altered metabolism of arachidonic acid to 15(R)-hydroxy-eicosatetraenoic acid (15(R)-HETE) (14). Selectivity for COX-2 inhibitors depends on the replacement of histidine 513 and isoleucine 523 of COX-1 with the smaller arginine and valine respectively. This replacement removes the restriction at the mouth of the secondary side channel and allows access for the more bulky selective COX-2 inhibitors (15). A second replacement of isoleucine 434 with a smaller valine at the apex of the active site of COX-1 creates additional extra space in the COX-2 channel for the larger selective inhibitors of COX-2.

COX-1 performs a 'housekeeping' function to synthesise PGs which regulate normal cell activity. The concentration of the enzyme largely remains stable, but small (2- to 4-fold) increases in expression can occur in response to stimulation with hormones or growth factors (16, 17). Normally, little or no COX-2 is found in resting cells but its expression can be increased dramatically after exposure of cells to bacterial lipopolysaccharide (LPS), phorbol esters, cytokines or growth factors. However, 'constitutive' levels of COX-2 have been detected in some organs such as the brain and kidney. The induction of COX-2 generates $PGF_{2\alpha}$ to contract the uterus at the end of pregnancy to initiate birth.

Inhibitors of COX-1 and COX-2

Individual NSAIDs show different potencies against COX-1 compared with COX-2 and this explains well the variations in the side effects of NSAIDs at their anti-inflammatory doses. Drugs with low potency against COX-1 and therefore a lower COX-2/COX-1 activity ratio, will have anti-inflammatory activity with fewer side effects on the stomach and kidney. Garcia Rodriguez and Jick (18), Langman *et al.* (19) and Henry *et al.* (20) published a comparison of epidemiological data of the gastric side effects of NSAIDs. Piroxicam and indomethacin were amongst those with the highest gastrointestinal toxicity. These

drugs have a much higher potency against COX-1 than against COX-2 (21). Thus, when epidemiological results are compared with COX-2/COX-1 ratios, there is a parallel relationship between gastrointestinal side effects and COX-2/COX-1 ratios. COX-2/COX-1 ratios provide a useful comparison of relative values for a series of NSAIDs tested in the same system. However, the COX-2/COX-1 ratio for a particular NSAID will vary according to whether it is measured on intact cells, cell homogenates, purified enzymes or recombinant proteins expressed in bacterial, insect or animal cells. It also varies when measured in different types of cells derived from various species.

Assessment of selectivity

To examine the relative inhibition of COX-1 compared to COX-2 for various NSAIDs, the drugs were tested on cultured bovine aortic endothelial cells for inhibition of COX-1, and on cultured J774.2 macrophages induced with LPS, for inhibition of COX-2. This provided a ratio of two IC_{50} values against the two enzymes and it was possible to measure COX-2/COX-1 ratios for a large number of anti-inflammatory drugs (22, *Table 1*). Aspirin was highly selective for COX-1 compared to COX-2 with an activity ratio of 166 and thus with a relatively high propensity for causing gastric damage.

In 1994, the human whole blood assay superseded other measurements of COX-1 and COX-2 inhibition (23). This method consists of separating a human

Table 1. IC_{50} values (μg/ml) of NSAIDs on COX-2 or COX-1 activity in intact cells

NSAID	COX-2	COX-1	Ratio $\frac{\text{COX-2}}{\text{COX-1}}$
Tolmetin	7	0.04	175
Aspirin	50	0.3	166
Ibuprofen	15	1	15
Acetaminophen (IC_{30})	20	2.7	7.4
Diclofenac	0.35	0.5	0.7
Naproxen	1.3	2.2	0.6
Celecoxib	0.34	1.2	0.3
Rofecoxib	0.84	63	0.013

Based on data from Mitchell et al. 1993

Table 2. Inhibition of COX-1 and COX-2 by NSAIDs in the WHRI blood / A549 cell assay

	COX-1 (IC_{50} μM)	COX-2 (IC_{50} μM)	Ratio
Ibuprofen	5.05	12	2.4
Ketoprofen	0.078	0.19	2.4
Diflunisal	14	35	2.5
Indomethacin	0.018	0.048	2.7
Aspirin	1.2	5.2	4.3
Tolmetin	0.36	1.9	5.3
Flurbiprofen	0.048	0.58	12
Naproxen	0.82	14	17

Increasing COX-1 selectivity ↓

Data obtained from Warner et al. 1999

blood sample into separate suspensions of platelets and monocytes. Drugs are tested immediately for their ability to inhibit thromboxane synthesis by platelet COX-1, while monocytes are tested after COX-2 is induced by LPS for 18 hours. This method was adapted at the William Harvey Research Institute (WHRI), using cultured A549 cells previously treated with LPS, so that the activity against COX-2 could be measured at the same time as that against COX-1 (24, *Table 2*). Aspirin, by the WHRI modified human whole blood assay method also demonstrated selectivity for COX-1 and its COX-2/COX-1 activity ratio was estimated as 4.3.

Selective COX-2 inhibitors

Selective inhibitors of COX-2 were introduced in 1999. The first NSAIDs to be introduced as selective COX-2 inhibitors were celecoxib (Celebrex) and rofecoxib (Vioxx). In place of the carboxyl group of the non-steroid anti-inflammatory acids, the structure of celecoxib contains a sulfonamide group and that of rofecoxib contains a methylsulfone. The sulphur-containing phenyl rings of these drugs bind into the side pocket of the cyclooxygenase catalytic channel of COX-2 but interact weakly with the active site of COX-1 (25). Thus, they are potent inhibitors of COX-2 and weak inhibitors of COX-1. When the selectivity is estimated by the human whole blood assay, celecoxib has a selectivity of 7.6 and rofecoxib a selectivity of 35 in favour of COX-2 (26). Second generation

selective COX-2 inhibitors have now been developed with higher selectivities for COX-2. The successor to celecoxib, valdecoxib (Bextra) has a selectivity ratio of 30 and etoricoxib (Arcoxia), the successor to rofecoxib, has a selectivity of 106 (26). Parecoxib, an injectable pro-drug of valdecoxib is also available for the treatment of acute pain (27) and lumiracoxib, is currently in development (28) and has undergone clinical trials (29). Lumiracoxib differs in structure from other coxibs. It is a phenyl acetic acid derivative instead of a sulfonamide or sulfone, and compared to non-selective NSAIDs causes no cardiovascular side effects and less gastrointestinal complications (29).

The Celecoxib Long-Term Arthritis Safety Study (CLASS) in 8000 patients for 6 months demonstrated a lower incidence of ulcer complications in patients receiving celecoxib than those on ibuprofen or diclofenac. However, in patients taking aspirin as well as celecoxib the incidence of ulcers was no better than in the comparator group (30). Moreover, when the trial was continued for 12 months, there was no difference in the number of gastric adverse events between the celecoxib and comparator NSAIDs treated patients (31).

The 'Vioxx' Gastrointestinal Outcomes Research (VIGOR) Trial (32) comparing rofecoxib with naproxen in 8000 patients for 9 months reported fewer serious gastrointestinal adverse events with rofecoxib. Patients who were taking aspirin were excluded from the trial. However, the incidence of myocardial infarction was higher among patients in the rofecoxib group than among those in the naproxen group (0.4% versus 0.1%). The higher incidence of heart attacks may be a general risk factor for all selective COX-2 inhibitors. Recent trials investigating the efficacy of celecoxib (33) and rofecoxib (34) in preventing colorectal adenomas demonstrated a higher incidence of cardiovascular events, including myocardial infarction, in the drug-treated group compared to the placebo. It has been suggested that selective COX-2 inhibitors prevent the synthesis of the anti-thrombotic prostaglandin, prostacyclin, by endothelial cells while leaving unopposed the action of the pro-thrombotic thromboxane in platelets (35). Recent epidemiological studies show that non-selective NSAIDs in anti-inflammatory doses also increase the risk of myocardial infarction (36). This may be difficult to explain since the majority of these NSAIDs have a greater selectivity for COX-1 than for COX-2, which is the basis for their gastrotoxicity and for their anti-thrombotic action.

In 2004, Merck Frosst withdrew rofecoxib from the market and is currently involved in litigation with alleged victims of cardiovascular adverse events who were treated with rofecoxib. Selective COX-2 inhibitors are now required to carry a warning of the risk of cardiovascular side effects. Valdecoxib causes severe skin rashes as well as possible myocardial infarctions and the FDA has recommended that its sales should be suspended (37). Valdecoxib and parecoxib are associated with an increased incidence of cardiac events if used for pain after coronary artery bypass grafting (38).

Other Cyclooxygenases

Paracetamol belongs to a group of drugs known as antipyretic analgesics which also includes its precursor, phenacetin, aminopyrine and dipyrone. Most of these drugs have fallen out of use because of their toxicity for leukocytes, but dipyrone can still be obtained in some countries. Paracetamol is a weak inhibitor of isolated COX-1 and COX-2 and its mechanism of action has not yet been resolved. Similarly to other drugs in this group, it has only weak anti-inflammatory effects. In 1972, Flower and Vane (8) postulated the existence of a COX in dog brain more sensitive to inhibition with paracetamol than the COX in rabbit spleen. More recently, Simmons and his colleagues characterised and cloned a COX enzyme in dog brain which, unlike COX-1 and COX-2, was sensitive to inhibition with paracetamol, (39). This was a splice variant of COX-1 termed by the authors, COX-3, which consists of a COX-1 mRNA that retains intron-1. In dogs, intron-1 is 90 nucleotides in length and represents an in frame insertion into the portion of the COX-1 open reading frame encoding the N-terminal hydrophobic signal peptide.

When expressed in insect cells, this variant produces enzyme protein containing the encoded intron-1 sequence. The activity of the protein is preferentially inhibited by analgesic antipyretic drugs such as paracetamol and may explain the therapeutic actions of this class of compounds (*Table 3*). However, although intron-1 is of similar size in all species, it is out of frame in humans and rodents. Thus, it would require additional mechanisms such as the

Table 3. IC_{50} (μM) values for COX inhibition by antipyretic analgesics

Drug	COX-1	COX-2	COX-3
Acetaminophen	>1000	>1000	460
Aminopyrine*	>1000	>1000	688
Antipyrine	>1000	>1000	863
Dipyrone	350	>1000	52
Phenacetin	>1000	>1000	102
Aspirin	10	>1000	3.1
Diclofenac	0.035	0.041	0.008
Ibuprofen	2.4	5.7	0.24
Indomethacin	0.010	0.66	0.016
Caffeine	>1000	>1000	>1000
Thalidomide	>1000	>1000	>1000

All assays were carried out in the presence of 30μM arachidonic acid.
*4-dimethylaminoantipyrine.

Reproduced from Chandrasekharan et al, 2002

use of alternative splice sites, ribosomal frameshifting or RNA editing to make a functional protein (39 - 42). However, COX-1 variant protein has been identified in both mouse and human tissues (39, 43 - 45), although the candidate protein identified by Qin *et al.* (44) is not selectively inhibited by paracetamol. An alternative mechanism has been proposed to explain the actions of paracetamol; that its inhibitory activity on COX depends on the level of peroxides in the target cells, since high levels of peroxide (such as exist in inflammatory tissues) abolish the action of paracetamol (46, 47). This hypothesis has not been conclusively proved.

An inducible COX-2 enzyme sensitive to inhibition with low concentrations of paracetamol was described in 1999 (48). Cultured J774.2 mouse macrophages when incubated with diclofenac for 48 hours expressed a COX-2-like activity which was more sensitive to inhibition by paracetamol than LPS-induced COX-2. In addition, LPS-induced COX-2 was a membrane-bound enzyme whereas COX-2 induced with diclofenac existed in the cytoplasm of the cells. If such a COX-2 variant is identified in vivo, it may explain some therapeutic actions of paracetamol which cannot be attributed to inhibition of a variant of COX-1.

CONCLUSIONS

The prostaglandin field has been one of the most exciting areas of research in recent times. More than seventy years after the marketing of aspirin, John Vane established its mechanism of action. Just twenty years later, with the increase in research into the properties of cyclooxygenase, a second isoenzyme, COX-2, was revealed by Daniel Simmons in 1991. In the eight years that followed, the eagerly awaited selective inhibitors of COX-2 reached the market, which alleviated the symptoms of inflammatory disease without damaging the stomach. The discovery of COX-2 inhibitors generated great enthusiasm and competition to design more effective drugs. However, increasing selectivity for COX-2 also increased toxicity, since the anti-thrombotic prostacyclin is formed by COX-2 and inhibiting its synthesis precipitated heart attacks. The problem of this side action has not yet been resolved. Is it possible to obtain the benefit of low gastrotoxicity and avoid the danger of a heart attack? Perhaps lumiracoxib has provided the solution (see above). However, it now appears that by taking any NSAID, patients risk experiencing a heart attack.

A third cyclooxygenase, COX-3, has also been postulated, based on the sensitivity of a COX-1 variant from dog brain to paracetamol. However, when the COX-3 mRNA of mouse or human is translated into an enzyme protein, the intron-1 insertion is out of frame. Thus, the mechanism by which conversion of mRNA occurs into active enzyme protein in mouse and human requires further investigation. When this question is resolved, the mechanism of action of a drug even older than aspirin may be clarified.

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Received: September 15, 2006
Accepted: October 2, 2006

Author's address: R.M. Botting, Ph.D. The William Harvey Research Institute, The John Vane Science Centre, St Bartholomew's and the London School of Medicine and Dentistry, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, U.K. Fax: 00 44 208 677 9671; E-mail: r.m.botting@qmul.ac.uk