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COMMENTARY

Biochemical Pharmacology of Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT. Aspirin and conventional nonsteroidal anti-inflammatory drugs are nonselective inhibitors of cyclooxygenase-1 (COX-1) and COX-2 enzymes. Two classes of selective COX-2 inhibitors: (1) sulfonamides, such as L-745,337, and (2) tricyclic methyl sulfone derivatives, such as SC58125, have been developed. X-ray crystal structures of COX-1 and COX-2 have provided valuable information regarding the structural basis for their COX-2 selectivity. These compounds have less gastrointestinal complications in animal experiments. Their clinical efficacy and side-effects are being evaluated. Salicylate has very weak activity against either COX isoform and yet possesses anti-inflammatory actions. Recent studies indicate that it suppresses the expression of genes involved in inflammation. These activities may provide a plausible explanation for the pharmacological dilemma and, furthermore, may represent novel mechanisms for controlling inflammation. BIOCHEM PHARMACOL 55;5:543–547, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nonsteroidal anti-inflammatory drugs; cyclooxygenase; aspirin; salicylate effect on NF-κB; inflammation; aspirin; colon cancer

Since the discovery of SA† as an anti-inflammatory compound and the subsequent synthesis of SA and ASA (aspirin) over a century ago, several classes of structurally diverse compounds have become available for the treatment of human inflammatory disorders [1]. These compounds, collectively known as NSAIDs, share with ASA a common mechanism by which they exert their anti-inflammatory action. The pioneering work of Vane [2] and Smith and Willis [3] has provided convincing evidence that aspirin and other NSAIDs block the biosynthesis of inflammatory PGs, especially PGE_2. Their observations have been confirmed subsequently by many laboratories. Although it remains disputable whether inhibition of PGs is the only mechanism underlying the anti-inflammatory action of NSAIDs, it is generally accepted that inhibition of PGs is the major mechanism.

The key enzyme catalyzing the biosynthesis of prostaglandins is PGHS (also called COX). COX is a bifunctional enzyme, containing the COX activity that catalyzes the bisoxygenation of AA to form PGG_2 and a peroxidase activity, which catalyzes the reduction of PGG_2 to PGH_2 [4]. PGH_2 is converted to PGE_2 enzymatically and nonenzymatically. PGH_2 is also converted to PGI_2, TXA_2, $PGF_{2\alpha}$, and PGD_2 by specific enzymes, i.e. PGI synthase, TXA synthase, PGF synthase and PGD synthase, respectively. Aspirin and other NSAIDs inhibit the COX activity, thereby suppressing the biosynthesis of PGs, TXA_2, and PGI_2.

BIOCHEMICAL MECHANISMS OF COMMONLY USED NSAIDS

The biochemical mechanism by which aspirin inhibits COX activity has been elucidated extensively. It acetylates Ser-530 of the COX-1 enzyme (according to the ovine PGHS-1 sequence) [5]. Based on site-directed mutagenesis and crystallographic data [6, 7], acetylation of this serine residue, which is located near the COX active site, blocks the entrance of substrate AA from contacting with the active site residue Tyr-385 and the nearby heme ligand His-388. The inhibitory action of aspirin on COX activity is irreversible. SA has essentially no inhibitory action against COX activity and, yet, it has an anti-inflammatory action equipotent to that of aspirin. This pharmacological dilemma will be discussed in more detail later.

Most conventional NSAIDs are competitive inhibitors of arachidonic acid [8, 9]. Two types of inhibition have been noted: (1) time-independent inhibition in which the competitive inhibition occurs in a concentration-dependent manner (this type of inhibition is reversible), and (2)

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† *Abbreviations:* SA, salicylic acid; ASA, acetylsalicylic acid; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; PGE_2, prostaglandin E_2; PGHS, prostaglandin H synthase; COX, cyclooxygenase; AA, arachidonic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGI_2, prostaglandin I_2 or prostacyclin; TXA_2, thromboxane A_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGD_2, prostaglandin D_2; and PGIS, prostacyclin synthase.

time-dependent inhibition in which, after the initial competitive inhibition, these NSAIDs presumably cause active site structural changes to facilitate irreversible (or slowly reversible) inhibition. This phase of inhibition takes minutes to accomplish and, hence, is time dependent. Most potent NSAIDs, such as indomethacin and flurbiprofen, belong to this group of inhibitors. The nature of the time-dependent structural changes in the active site pocket of COX has not been elucidated, however, despite the availability of an X-ray crystal structure of COX-1 complexed with these NSAIDs, because the active conformation of COX-1 has not been solved [7]. The crystallographic structure of COX-1 complexed with flurbiprofen indicates that the aromatic ring of this compound is under Tyr-385 and the carboxyl group of this compound forms a bridge with Arg-120. The substrate channel is otherwise lined with hydrophobic residues. Once flurbiprofen enters the channel, its interaction with this charged residue and Tyr-355 develops a constriction at the channel entrance, preventing arachidonate from entering into the active site channel. It may be speculated that these two residues are involved in channel constriction by other NSAIDs.

DIFFERENTIAL INHIBITION OF COX ISOZYMES BY NSAIDS

Conventional NSAIDs are confronted with common and serious side-effects, notably gastrointestinal bleeding and renal dysfunction [10]. These side-effects are considered to be caused by the inhibition of COX-1 physiological functions. The relative risk for gastrointestinal bleeding varies among currently used NSAIDs, but even compounds in the low risk group still have unacceptable side-effects. Hence, there has been an active search for "better" NSAIDs that have high anti-inflammatory efficacy with minimal side-effects. Several compounds, such as meloxicam [11], flosulide (CGP 28238) [12], DuP 697 [13], and NS-398 [14], were found to have improved pharmacological profiles in animal models, but their mechanism of action was unclear when they were developed. It is now well recognized that their improved pharmacological profiles are due to their selective inhibition of COX-2.

COX was initially purified from ram seminal vesicles by Hemler and Lands [15] and Miyamoto *et al.* [16]. It is a membrane-bound enzyme. The purified enzyme in detergent was a homodimer with a subunit molecular mass of 70 kDa. cDNA was cloned from ram seminal vesicle by DeWitt and Smith [17], Merlie *et al.* [18] and Yokoyama *et al.* [19]. Its coding region contains 1800 nucleotides encoding a 600-amino acid (aa) protein with a 24-aa signal peptide. Its human counterpart encodes a 599-aa protein with a 23-aa signal peptide [20]. Sequence comparison between human and ovine COX-1 cDNA reveals a >90% sequence homology. It was suspected from pharmacological and kinetic studies that an isoform of COX might exist. This isoform, now well recognized as COX-2, was cloned from phorbol ester-induced NIH 3T3 fibroblasts by Herschman and coworkers [21] and from Src oncogene-induced chick embryo fibroblasts by Simmons and coworkers [22]. Both cDNAs code for a 604-aa protein. COX-2 cDNA was subsequently cloned from human endothelial cells, monocytes, and other tissues [23, 24]. The human COX-2 cDNAs from various cell and tissue sources are very similar. Comparison of murine, human, and avian COX-2 cDNA sequences reveals a closer similarity between murine and human than between murine or human and avian cDNAs. However, the cDNAs from all these species encode a 604-aa protein with conservation of COX tyrosine active site and histidine heme ligand. When compared with COX-1, COX-2 has a shorter signal peptide and an 18-aa insert near the C-terminus, which is absent in COX-1.

A major difference between COX-1 and COX-2 is their inducibility. COX-1 is constitutively expressed in most mammalian tissues, whereas COX-2 is not constitutively expressed except in neurons and gastric mucosa [25, 26]. Recent studies indicate that COX-1 expression is up-regulated by phorbol esters and cytokines, but the magnitude of stimulation is about 2-fold over the based level [27]. In contrast, COX-2 is highly inducible, and its maximal level of induction in cells is 10- to 1000-fold higher than the maximally stimulated COX-1 levels. Hence, COX-2 induction is accompanied by a burst of large quantities of PG synthesis for a relatively short duration. COX-2 expression starts at about 1 hr, reaches a maximal level at 3 hr, and subsides at 6 hr after induction. COX-2 has been shown to be induced by cytokines in inflammatory cells (for a review, see Ref. 28). Its expression has been shown to be induced in inflammatory tissues in several animal models [29, 30] and in human rheumatoid arthritis inflammatory tissues [31]. These studies lead to the suggestion that COX-2 induction plays a critical role in inflammation. COX-1 expression, on the other hand, is important in cytoprotection and maintaining physiological functions.

Analysis of differential inhibition of COX-1 versus COX-2 by conventional NSAIDs reveals that most NSAIDs nonselectively inhibit both isozymes [32], and that there is a reverse correlation between COX-2 selectivity and gastrointestinal complications [33]. Compounds exhibiting a higher COX-2 selectivity (a higher COX-1 IC_{50} to COX-2 IC_{50} ratio) have fewer gastrointestinal complications. Diclofenac and miloxicam belong to this group. By contrast, indomethacin, naproxen, aspirin, and ibuprofen preferentially inhibit COX-1 (low COX-1/COX-2 IC_{50} ratio) and have a higher risk for gastrointestinal complications.

NS-398, flosulide, and DuP 697 exhibited anti-inflammatory action with no gastrointestinal side-effects at very high doses when administered to inflammatory animal models [12–14]. It was subsequently shown that they act primarily on COX-2 and have very weak anti-COX-1 action. These compounds have served as prototypes for developing more potent COX-2 selective inhibitors. Availability of COX-2 selective inhibitors should be useful in determining the importance of COX-2 in inflammatory

response, testing the relation between differential COX inhibition and toxicity, and developing better and safer NSAIDs.

SELECTIVE COX-2 INHIBITORS

Biochemical Mechanisms

Two classes of selective COX-2 inhibitors have been developed: (1) sulfonamides, including NS-398, flosulide, and L-745,337, and (2) tricyclic methylsulfone derivatives including DuP 697, SC58125, and SC57666. SC58125 [30, 34] and L-745,337 [35, 36] have been evaluated extensively and have begun clinical trials. Like the prototypic compounds NS-398 and DuP 697 from which they were derived, respectively, SC58125 and L-745,337 are potent specific inhibitors of COX-2 (IC_{50} in the nanomolar range) and have no inhibitory action on COX-1 at millimolar concentrations [30, 34–36]. Animal experiments confirm their selective anti-inflammatory actions without gastrointestinal or renal complications. The fact that these COX-2 selective inhibitors are effective in suppressing acute and chronic inflammation in animal models lends further credence to the notion that COX-2 induction is a key step in the development of inflammation.

It has been shown that NS-398 and DuP 697 inhibit equipotently both COX-1 and COX-2 activities when added to purified enzymes for 15 sec [8]. However, their inhibition of COX-2 is time dependent, and incubation of purified COX-2 with either agent for a longer period of time causes a time-dependent inactivation of the enzyme activity [8, 9]. In contrast, inhibition of COX-1 activity by NS-398 or DuP 697 is time independent [8, 9]. It also has been shown that the time-dependent inactivation of COX-2 by either agent is virtually irreversible [8]. These results indicate that COX-2 selective inhibitors differ from nonselective inhibitors by being able to induce conformational changes after binding to the active site channel of COX-2. The conventional NSAIDs, such as indomethacin, exert time-dependent inactivation of COX-1 and COX-2 and, therefore, are not selective for COX-2 [8, 9]. The nature of time-dependent irreversible inactivation of COX-2 by selective COX-2 inhibitors is not known. However, experimental data suggest that it does not involve covalent modification at the active site [8].

Recent crystallographic analysis of COX-2/selective COX-2 inhibitor complexes has provided a plausible explanation for selective COX-2 inhibition, despite structural similarities between COX-1 and COX-2 [7, 37, 38]. As mentioned above, COX-1 and COX-2 share about 60% sequence identity, and the active site residues are highly conserved. Comparison of COX-2 crystallographic structure with COX-1 structure shows expected similarities of the backbone structure as well as the heme pocket and unliganded and substrate binding channel [7, 37, 38]. Kurumbail *et al.* [38] analyzed the structures of liganded murine COX-2 and complexes with flurbiprofen, indomethacin, and a selective COX-2 inhibitor, SC558. They showed that besides the hydrophobic active site channel that is similar to the channel of COX-1, COX-2 has a side channel branched out from the active site channel in which the bulky isoleucine at position 523 of ovine COX-1 is replaced with valine. This substitution makes the COX-2 branched channel accessible to the sulfonamide side chain of SC558. The selectivity of SC558 is considered to result from the sulfonamide moiety that binds in this branched pocket. This pocket is more restricted in COX-1 and is unoccupied in COX-2 complexed with nonselective inhibitors such as indomethacin [38]. Two other residues, Val-434 and Arg-513, are also important in facilitating the binding of the sulfonamide moiety to this side pocket [38]. The selectivity of the methylsulfonyl derivatives has not been evaluated by crystallography but is considered to be governed by a similar mechanism.

Advantages and Potential Problems

Selective COX-2 inhibitors have been hailed to have a potential as "better aspirin" (and NSAID) [39], primarily based on the premise that they do not perturb COX-1-related physiological functions and, hence, will not cause side-effects that are caused by nonselective COX inhibitors. Furthermore, it has also been presumed that the primary role of COX-2 induction is in the pathogenesis of inflammation. In view of recent experimental data, these presumptions need to be reconsidered. Recent studies have shown that COX-2 is constitutively expressed in neurons and gastric epithelial cells and may be important in neural transmission and gastric protection. Furthermore, COX-2 induction in vascular endothelium may play an important role in vasoprotection. On the other hand, experimental data from COX-1 and COX-2 gene knockout mice provide somewhat contradictory information. For example, COX-1-deleted mice did not develop gastric lesions and, unexpectedly, were more resistant to inflammatory challenges than control mice [40]. COX-2-deleted mice, on the other hand, were more susceptible to inflammatory challenges [41, 42]. These results raise questions as to whether the physiological and pathological roles of COX-1 and COX-2 can be as clearly defined as presumed previously. They also raise concerns as to whether selective COX-2 inhibitors may have unexpected inadvertent effects. These issues await the results from human clinical trials.

SALICYLATE PHARMACOLOGICAL DILEMMA

Salicylate is a weak, reversible, competitive inhibitor of COX activity. For practical consideration, it has no anti-COX activity. Its anti-inflammatory action cannot be explained by inhibition of COX-1 and/or COX-2. Aspirin is deacylated rapidly in human blood, and its chronic anti-inflammatory action is derived from salicylate. Despite lacking anti-COX activities, salicylate exerts substantial anti-inflammatory actions and has considerable inhibitory effects on PG biosynthesis in intact cells and animals [43,

44]. These observations led us to postulate that salicylate alters the expression of COX isozymes. We showed, prior to the discovery of the COX-2 isoform, that sodium salicylate and aspirin equipotently suppressed the steady-state level of 2.7 kb COX mRNA in cultured human umbilical vein endothelial cells [44]. Seventy kilodalton COX protein levels and COX activities were suppressed by salicylate in accord with the reduction of the mRNA levels [44]. Our recent preliminary data suggest that sodium salicylate suppresses COX-2 and COX-1 expressions in cultured cells as well as in mice treated with lipopolysaccharide *in vivo* (Wu *et al.*, unpublished data).

Sodium salicylate at millimolar ranges suppresses NF-κB-mediated transcriptional activation by inhibiting IκB degradation [45]. Expression of a number of inflammatory molecules requires NF-κB. It has been suggested that this may be a mechanism by which salicylate exerts its anti-inflammatory action. For example, sodium salicylate has been shown to down-regulate the expression of a chemokine gene [46], the inducible nitric oxide synthase gene [47], and tumor necrosis factor-induced signal transduction [48]. Interestingly, suppression of inducible nitric oxide synthase expression by salicylate is independent of NF-κB [47]. A recent study has further shown that salicylate suppresses gene transcriptional activation by AP-1, and this effect is correlated with reduced intracellular pH [49]. Taken together, the recent studies indicate that salicylate is capable of suppressing the expression of genes involved in inflammation. However, the salicylate concentrations required to suppress these gene transcriptions are millimolar concentrations, which exceed the therapeutic concentrations of salicylates, generally in the range of 10^{-4}–10^{-6} M. Hence, the pharmacological action of salicylate may not be explained by its effects on NF-κB or AP-1. We postulate that salicylate achieves its anti-inflammatory action by suppressing COX-2 transcription at therapeutic concentrations. Since COX-2 has a rapid turnover, suppression of its expression would represent the most effective way of inhibiting PG biosynthesis. This hypothesis is being tested in my laboratory. Salicylate has been reported to induce heat shock protein gene expression by enhancing binding of heat shock transcription factor to DNA [50, 51]. The relation of this observation to the anti-inflammatory action of salicylate is unclear at the present time. However, when considered collectively, these recent studies suggest a common mechanism by which salicylate modulates gene expression. How to relate this mechanism to its anti-inflammatory action is a subject of intense investigation.

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