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Activation of peroxisome proliferator-activated receptor isoforms and inhibition of prostaglandin H_2 synthases by ibuprofen, naproxen, and indomethacin

Maisa S. Jaradat[a], Buanus Wongsud[b], Srichan Phornchirasilp[b], Shamina M. Rangwala[c], Gamal Shams[c] Melissa Sutton[d], Karl J. Romstedt[d] Daniel J. Noonan[e] Dennis R. Feller[a,c,*]

[a]*Department of Pharmacology, National Center for Natural Products Research, School of Pharmacy, 303 Faser Hall, P.O. Box 1848, The University of Mississippi, University, MS 38677-1848, USA*

[b]*Department of Pharmacology, Mahidol University, Bangkok 10400, Thailand*

[c]*Division of Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA*

[d]*Department of Biology, Capital University, Columbus, OH 43209, USA*

[e]*Department of Biochemistry, University of Kentucky, Lexington, KY 40506, USA*

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Abstract

A series of nonsteroidal anti-inflammatory drugs (NSAIDs) [*S*(+)-naproxen, ibuprofen isomers, and indomethacin] were evaluated for their activation of peroxisome proliferator-activated receptor (PPAR) α and γ isoforms in CV-1 cells co-transfected with rat PPAR α and γ, and peroxisome proliferator response element (PPRE)-luciferase reporter gene plasmids, for stimulation of peroxisomal fatty acyl CoA β-oxidase activity in H4IIEC3 cells, and for comparative inhibition of ovine prostaglandin endoperoxide H synthase (PGHS)-1 and PGHS-2 and arachidonic acid-induced human platelet activation. Each drug produced a concentration-dependent activation of the PPAR isoforms and fatty acid β-oxidase activity, inhibition of human arachidonic acid-induced platelet aggregation and serotonin secretion, and inhibition of PGHS-1 and PGHS-2 activities. For PPARα activation in CV-1 and H4IIEC3 cells, and the stimulation of fatty acyl oxidase activity in H4IIEC3 cells, the rank order of stereoselectivity was *S*(+)- ibuprofen > *R*(−)-ibuprofen; *S*(+)-ibuprofen was more potent than indomethacin and naproxen on these parameters. On PPARγ, the rank order was *S*(+)-naproxen > indomethacin > *S*(+)-ibuprofen > *R*(-)-ibuprofen. Each drug inhibited PGHS-1 activity and platelet aggregation with the same rank order of indomethacin > *S*(+)-ibuprofen > *S*(+)-naproxen > *R*(-)-ibuprofen. Notably, the *S*(+)-isomer of ibuprofen was 32-, 41-, and 96-fold more potent than the *R*(-)-isomer for the inhibition of PGHS-1 activity, human platelet aggregation, and serotonin secretion, respectively. On PGHS-2, the ibuprofen isomers showed no selectivity, and indomethacin, *S*(+)-ibuprofen, and *S*(+)-naproxen were 6-, 27-, and 5-fold more potent as inhibitors of PGHS-1 than PGHS-2 activity. These results demonstrate that the mechanisms of action of NSAIDs on these cell systems are different, and we propose that the pharmacological effects of NSAIDs may be related to both their profile of inhibition of PGHS enzymes and the activation of PPARα and/or PPARγ isoforms.

Keywords: Ibuprofen isomers; Naproxen; Nonsteroidal anti-inflammatory drugs; Peroxisome proliferator-activated receptors; Prostaglandin endoperoxide H synthases

1. Introduction

NSAIDs possess anti-inflammatory, analgesic, and antipyretic activity [1,2]. Aspirin, ibuprofen, naproxen, and ketoprofen are available over the counter in the United States and there are over 20 additional prescription NSAIDs [3]. Naproxen and carprofen are the only NSAIDs sold worldwide as the *S*-enantiomer [3]. They are used to treat chronic inflammatory diseases such as rheumatoid and osteoarthritis, and pain from dysmenorrhea or muscle sprain.

* Corresponding author. Tel.: +1-662-915-7330; fax: +1-662-915-5148.

E-mail address: dfeller@olemiss.edu (D.R. Feller).

Abbreviations: AA, arachidonic acid; ACO, acyl-CoA oxidase; COX; cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PGHS, prostaglandin endoperoxide H synthase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; and CoA coenzyme A.

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It is believed that the molecular basis for the therapeutic actions of these NSAIDs is through inhibition of PGHS activity, which inhibits the production of pro-inflammatory prostaglandins. There are two PGHS enzymes called PGHS-1 and PGHS-2 [4,5], which catalyze the conversion of AA to prostaglandin endoperoxide G_2 and H_2 (PGG_2 and PGH_2) by dioxygenase and peroxidase reactions, respectively. The two PGHS isozymes have different patterns of expression. PGHS-1 is expressed in most tissues and is probably involved in the production of prostaglandins involved in cellular housekeeping functions, such as coordinating the actions of circulating hormones and regulating vascular homeostasis [6,7]. In contrast, PGHS-2 is rapidly, but transiently, induced in a variety of cell types following inflammation [8] or treatment with mitogens, growth factors, cytokines [9], and tumor promoters including the action of phorbol esters in fibroblasts [10] and lipopolysaccharides in monocytes and macrophages [11]. Prostaglandins produced through the action of PGHS-2 may be specifically involved in cellular differentiation and proliferation [7]. PGHS-1 had been proposed initially to be the pharmacological target of aspirin and NSAIDs [12,13]; however, evidence suggests that PGHS-2 may be the target of NSAIDs acting in their anti-inflammatory capacities [14–17]. The development of selective PGHS-2 inhibitors has proven useful for the treatment of inflammation and has reduced adverse ulcerogenic effects on the gastrointestinal tract [18–20].

A recent report has indicated that NSAIDs activate and bind to PPAR isoforms, α and γ [21]. The PPAR isoforms are members of the nuclear receptor superfamily of ligand-dependent transcription factors, and there are three isoforms named α, γ, and δ in various species [22]. The PPAR α and γ isoforms have been shown to play a role in the regulation of inflammatory processes in the body, and they have also been shown to be activated by several NSAIDs and AA and its metabolites [21,23]. Based upon the regulation of pro-inflammatory mediators and angiogenesis, a greater role for PPAR α and γ in the anti-inflammatory actions of NSAIDs has been suggested by Gilroy and Colville-Nash [20].

The PPARα isoform is expressed primarily in tissues that are involved in the metabolism of fatty acids. The functions of PPARα in the liver are to degrade fatty acids and detoxify various xenobiotics primarily by the ω-hydroxylation and β-oxidation pathways. Leukotriene B_4 is a potent chemotactic agent that coordinates and amplifies the inflammatory response, and has been shown to be an activating ligand for the transcription factor PPARα. Since these receptors regulate the oxidative degradation of fatty acids, AA, prostaglandins, and leukotrienes [24], a feedback mechanism has been proposed. In this regard, Devchand *et al.* [23] provided evidence that the catabolism of leukotriene B_4, a proinflammatory eicosanoid, to an inactive metabolite is mediated through its activation of PPARα. A recent report suggests that activators of PPARα improve insulin sensitivity without adipocyte differentiation, and may have value in the treatment of diabetes [25].

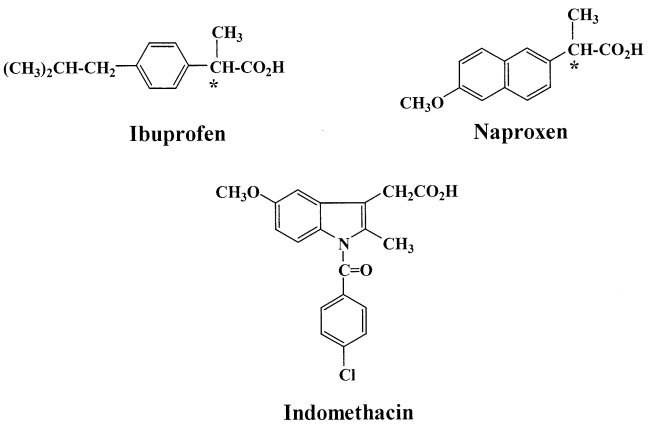

Fig. 1. Chemical structures of ibuprofen, naproxen, and indomethacin. An asterisk indicates the presence of a chiral carbon atom.

PPARγ activation has been demonstrated to regulate adipocyte differentiation and glucose homeostasis [24]. In addition, activation of this PPAR isoform inhibits the expression of inducible nitric oxide synthase, gelatinase B, and scavenger receptor A genes in response to synthetic ligands, and opposes the actions of several cytokines including tumor necrosis factor-α, interferon-γ, interleukin-1 and -6, and transforming growth factor-β [26–28]. Recently, Staels *et al.* [29] showed that PPARγ activation inhibits the induction of PGHS-2. These data strongly suggest that PGHS-2 may be pivotal in processes of inflammation and/or mitogenesis, and that activation of the PPAR isoforms may contribute to the anti-inflammatory activity of NSAIDs.

The PGHS enzymes are proposed as the target site of aspirin and other NSAIDs [7,17]. However, the inhibition of PGHS isozymes may not be the only mechanism of pharmacological action for this class of compounds [3,20]. Our hypothesis is that the anti-inflammatory actions of NSAIDs are dependent upon an interaction with more than one cellular target, and our approach is to examine the degree of selectivity of common NSAIDs (ibuprofen isomers, *S*(+)-naproxen and indomethacin) on PGHS enzymes and PPAR isoforms. In this paper, we have determined if these NSAIDs (Fig. 1). selectively activate PPAR isoforms and peroxisomal fatty acid β-oxidation, and have also established their comparative abilities to inhibit PGHS-2 and PGHS-1 enzymes. The ibuprofen isomers were used to determine the stereo-dependency for the inhibition or activation of these cellular targets. The effects of these agents on human platelet PGHS-1-mediated aggregatory and secretory responses were correlated with the inhibitory activities against the PGHS-1 enzyme. We also examined the functional effects of these NSAIDs on endogenous PPARα and peroxisomal ACO activity in H4IIEC3 cells.

2. Materials and methods

2.1. Materials

Ibuprofen isomers were obtained from Spacer Inc. *S*(+)-Naproxen was a gift from Popat N. Patil (Division of Pharmacology, The Ohio State University). The plasmid preparation kit was purchased from Qiagen, and the luciferase assay kit (Luclite) was purchased from Packard. Assay kits for ovine PGHS-1 and PGHS-2 were obtained from Oxford Biomedical Research, Inc. and the Cayman Chemical Co., respectively. These enzymes were purified from ram seminal vesicle. Hematin and AA were purchased from the Sigma Chemical Co. and NuChek Preps, respectively. All chemicals were of reagent grade.

2.2. Vector constructions

The plasmid pRSrPPAR was constructed by directionally cloning the rat PPARαcDNA sequence into the *Kpn*I/*Bam*HI sites located downstream from the RSV-LTR promoter in the previously described pRSV mammalian expression plasmid [30]. Response element plasmids were generated by inserting the synthetic oligonucleotides representing the regulatory AB site sequence on the fatty ACO gene [31] into the *Xho*I site 5′ of the thymidine kinase (tk) promoter in the previously described pBLtk-luciferase vector [32]. The construction of the pPPREaP2-tk-luc PPARγ reporter is described by Yuan [33]. A 518 bp *Eco*RI/*Xba*I fragment extending from -5.4 kb to -4.9 kb in the promoter of the mouse adipocyte fatty acid binding protein gene was generated from mouse genomic DNA using polymerase chain reaction (PCR) technologies and the primers 5′-GAATTCCAGCAGGAATCAGG-3′ and 5′-TCTAGAAG-GAAAACCAGGG-3′. This fragment, containing previously reported PPARγ-specific response element motifs [34], was subcloned into the *Xho*I site located 5′ of the tk minimal promoter in the previously described pBLtk-luciferase(luc) reporter vector [32] to generate pPPREaP2-tk-luc. The integrity of all constructs was verified by DNA sequencing.

2.3. Activation of PPAR isoforms in H4IIEC3 and CV-1 cells

Conditions for the handling and incubation of drugs with these cells were as described previously [35]. H4IIEC3 cells (500,000/well, 48 microtiter plate) and CV-1 cells (40,000/well, 96 microtiter plate) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum and were plated 24 hr prior to transfection at 50% confluency. Transfection of a peroxisome proliferator response element-luciferase reporter (PPRE-luc) plasmid gene (5 μg/100 μL cell suspension) in H4IIEC3 cells, and of both PPAR (2.5 μg/100 μL cell suspension) and PPRE-luc (5 μg/100 μL cell suspension) gene constructs in CV-1 cells, was performed by electroporation (Square electroporator model T820, at 190 V for H4IIEC3 cells and at 150 V for CV-1 cells for 70 msec and 1 pulse). After 24 hr, the medium was replaced, and drugs were added. After incubation with drugs for 48 hr, the cells were lysed, and the luciferase activity was determined in a luminometer (Packard Instrument Co.). The data obtained for ligands were normalized to percent response of control cells for PPARγ and of 30 μM ciprofibrate for PPARα.

2.4. Peroxisomal ACO assay

H4IIEC3 cells (100,000/well, 24 microtiter plate) were grown as described above for 24 hr. Agents were added to cells for 48 hr, and the ACO assay was performed by the fluorometric method of Walusimbi-Kisitu and Harrison [36] as reported previously [37]. The data obtained for ligands were normalized to the percent response of 30 μM ciprofibrate.

2.5. COX activity

AA-induced PGHS-1 and PGHS-2 activities were assayed by monitoring oxygen consumption with an oxygen electrode [38]. A closed microelectrode system was used to measure oxygen consumption in the presence of AA (10 μM) and various drug concentrations. Incubation mixtures contained enzyme (60–120 U), hematin (10 μM), and phenol (1 mM) in a final volume of 600 μL. In general, no more than 10% of the oxygen was consumed with AA in the absence and presence of drugs. Drug inhibitions of oxygen consumption were determined as a percent inhibition of the initial slope value obtained with AA alone. For data analysis, a computer interfaced with the oxygen electrode and a data acquisition system called Workbench software were used for the collection of 5 data points/sec to monitor oxygen consumption, and initial rates were calculated over 10-sec periods by linear regression using customized software [38].

2.6. Platelet aggregation and serotonin secretion studies

Platelet aggregation was performed according to the turbidometric method of Born [39] as modified by Mustard *et al.* [40]. Aggregation was quantified using a dual channel aggregometer with constant stirring of samples at 1100 rpm. The aggregometers were interfaced to a microcomputer, and data were collected, analyzed, and displayed graphically using original software [41].

PRP samples (0.35 mL) were incubated for 1 min at 37° prior to the addition of inducers to initiate aggregation. All inhibitors of platelet aggregation were added to PRP during the 1-min time period, and AA was then added to produce aggregation. In all experiments, the minimal concentration of AA (150–300 μM) that produced irreversible aggregation was used. Aggregation responses were measured as the

percent of maximal light transmission against PPP over 4 min.

For serotonin release studies, [^{14}C]serotonin (0.2 μCi/0.5 mL) was added to PRP, and the labeled serotonin was sequestered into dense granules during a 20-min incubation at room temperature. This allows sufficient time for maximal uptake of labeled serotonin, which is stored in a non-metabolic pool in dense granules [42]. Following aggregation, serotonin secretion was measured after centrifuging the platelet sample in a microfuge for 2 min at room temperature. A 0.1-mL aliquot of the supernatant was mixed into 2 mL of scintillation fluid (Thrift-Solv), and the samples were counted in a liquid scintillation spectrometer.

The background counting rate (BKG CPM) with no secretion present was determined from the supernatant of unstimulated samples after centrifugation (16,000 *g* for 2 min at room temperature) and represented less than 10% of the total count. The percent secretion of label was calculated using the following formula:

$$\% \text{ Secretion} = \frac{(\text{sample CPM} - \text{BKG CPM}) \times 100}{(\text{total CPM} - \text{BKG CPM})}$$

where CPM = counts per minute [43].

2.7. Statistical analyses

Comparisons of means were analyzed for differences using Student's *t*-test at the 1 or 5% level of significance.

3. Results and discussion

The demonstration of NSAID activation of the PPAR class of nuclear receptors is very limited. In the report of Lehmann *et al.* [21], NSAIDs were found to activate PPARα and PPARγ isoforms; however, these experiments used only fixed concentrations of the NSAIDs. Of the compounds used in the present study, indomethacin, *S*(+)-naproxen, and *racemic* [(*RS*-mixture)] ibuprofen are used clinically [3]. The structures of the drugs used in this study are given in Fig. 1. The results of the present study demonstrate that several NSAIDs produced concentration-dependent activations of these PPAR isoforms. Fig. 2 shows that the rank order of PPARα activation in rat hepatoma (H4IIEC3) cells was *S*-ibuprofen > *R*-ibuprofen > indomethacin > *S*-naproxen. The same stereoselective order of activity for the ibuprofen isomers (*S* > *R*) existed for activation of PPARα in CV-1 cells, whereas indomethacin and *S*-naproxen were inactive (Fig. 3). On the other hand, the order of activation of PPARγ was *S*-naproxen > indomethacin > *S*-ibuprofen > *R*-ibuprofen (Fig. 4), which indicates that a different rank order of potency exists with these NSAIDs for the activation of the two PPAR isoforms. The ibuprofen isomers were examined for their stimulation of ACO activity in H4IIEC3 cells (Fig. 5), which contain

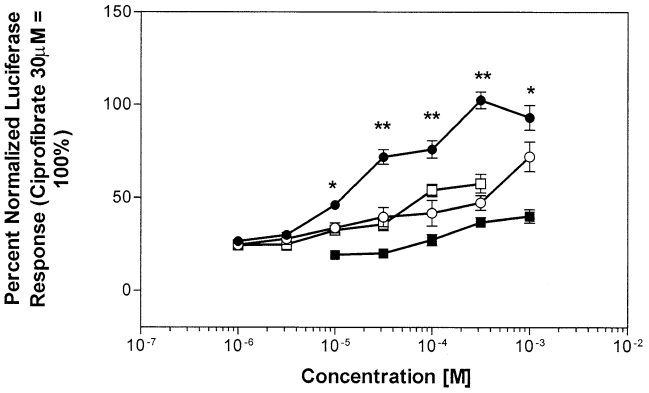

Fig. 2. Concentration–response curves of NSAIDs for activation of rat PPARα activity in H4IIEC3 cells transfected with PPREAB-luc plasmid. Key: (□) indomethacin, (■) *S*-naproxen, (●) *S*-ibuprofen, and (○) *R*-ibuprofen. Results are expressed as percent response of ciprofibrate (30 μM) run in the same experiment, with respect to luciferase activity assessed by light production. Each point on the curve is the mean ± SEM of 5–7 experiments. Each concentration in an experiment was done in duplicate. The presence of one (*) or two (**) asterisks indicates that the mean values of *S*- and *R*-ibuprofen isomers were significantly different at the 5 and 1% level, respectively.

endogenous PPARα [35], to assess the functional significance of PPAR activation in this cell line. The results show that ibuprofen isomers produced the same isomeric selectivity (*S*-ibuprofen > *R*-ibuprofen) for the stimulation of ACO activity and the activation of PPARα (Fig. 3) in H4IIEC3 cells. These findings indicate that these nuclear receptors were activated in a stereo-dependent fashion by the isomers of ibuprofen, and that the PPAR isozymes are pharmacologically distinct in susceptibility to activation by NSAIDs, with *S*-ibuprofen and *S*-naproxen being the most potent activators of the PPARα and PPARγ isoforms, re-

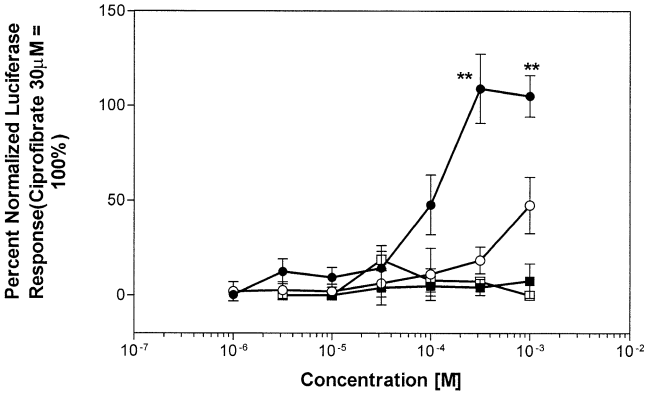

Fig. 3. Concentration–response curves for NSAID activation of rat PPARα activity in CV-1 cells transfected with rat PPARα and PPREAB-luc plasmid. Key: (□) indomethacin, (■) *S*-naproxen, (●) *S*-ibuprofen, and (○) *R*-ibuprofen. Results are expressed as a percent response of ciprofibrate (30 μM) run in the same experiment, with respect to luciferase activity as assessed by light production. Each point on the curve is the mean ± SEM of 5–7 experiments. Each drug concentration in an experiment was done in triplicate. Two asterisks (**) indicate that the mean values for *S*- and *R*-ibuprofen were significantly different at the 1% level.

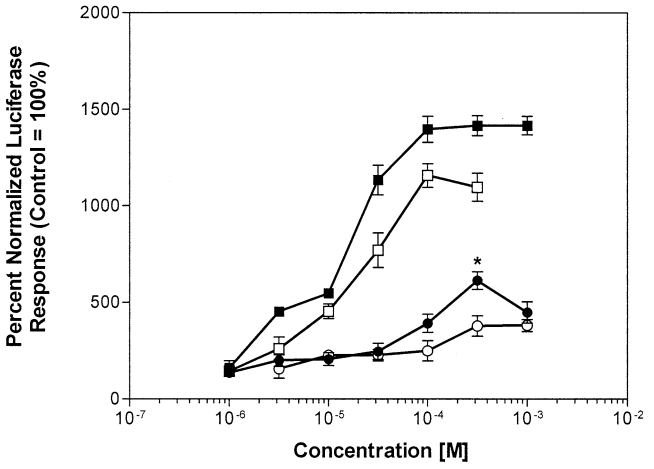

Fig. 4. Concentration–response curves for NSAID activation of rat PPARγ activity in CV-1 cells transfected with rat PPARγ and PPREaP2-luc plasmid. Key: (□) indomethacin, (■) *S*-naproxen, (●) *S*-ibuprofen, and (○) *R*-ibuprofen. Results are expressed as percent-fold induction over control (cell without treatment), with respect to luciferase activity assessed by light production. Each point on the curve is the mean ± SEM of 5–7 experiments, each in triplicate. The asterisk (*) indicates that the mean values for *S*- and *R*-ibuprofen were significantly different at the 5% level.

spectively. Based upon the different profiles of biological activity for PPAR activations, we suggest that both PPAR isoforms may be involved in the anti-inflammatory actions of NSAIDs, as has been suggested by others [20,21].

Initial measurements of COX activity established that the effective concentration-50 values for AA [EC_{50} values ~ 4 μM] were essentially the same for both PGHS enzymes. AA (10 μM) was used in all subsequent oxygenase assays to determine the inhibitory concentration-50 (IC_{50}) values of various NSAIDs; the use of this AA concentration concurs

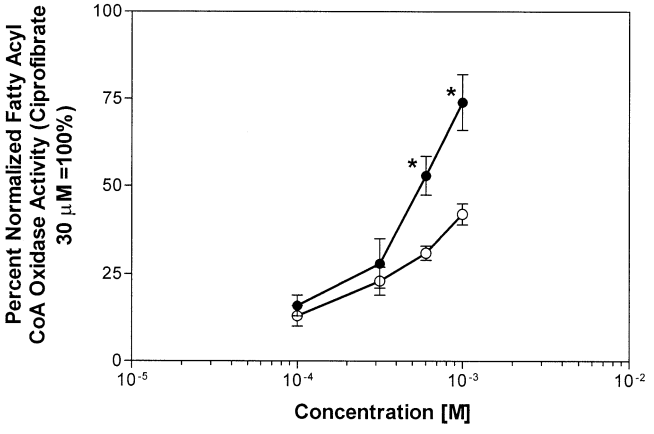

Fig. 5. Concentration-response curves of ibuprofen isomers for activation of peroxisomal ACO activity in H4IIEC3 cells. Key: (●) *S*-ibuprofen and (○) *R*-ibuprofen. Results are expressed as percent normalized to ciprofibrate 30 μM within the same experiment. Each point on the curve is the mean ± SEM of 3–4 experiments. Within each experiment, the individual drug concentrations were done as duplicate or triplicate determinations. An asterisk (*) indicates that the mean values for *S*- and *R*-ibuprofen were significantly different at the 5% level.

with that of Meade *et al.* [44]. As indicated in Table 1, the NSAIDs tested produced concentration-dependent inhibitions of oxygen consumption by the two PGHS isozymes. The propionic acid derivatives ibuprofen (including *RS*-, *S*-, and *R*-isomers) and *S*(+)-naproxen inhibited PGHS-1 and PGHS-2 activities with the same rank order of potency (Table 1). However, substantial differences were noted between the stereoisomers of ibuprofen on these two isozymes (Fig. 6, Table 1). Differences between the IC_{50} values toward PGHS-1 and PGHS-2 for this group of NSAIDs ranged from about a 27:4:1:5-fold preference for PGHS-1 with *S*-ibuprofen:*RS*-ibuprofen:*R*-ibuprofen:*S*-naproxen, respectively (Table 1). In our studies, the rank order inhibition of both PGHS isozymes by NSAIDs was *S*-naproxen = indomethacin > *S*-ibuprofen. These results are in agreement with the report of Mukherjee *et al.* [3] for the inhibitory effects of these NSAIDs on PGHS-1. Our findings with ibuprofen and its isomers show some differences on PGHS enzyme selectivity as compared with the work of others [16,44]. In the latter reports, there was little isozyme selectivity observed for ibuprofen. We have found about a 4- and 27-fold selectivity of *RS*- and *S*-ibuprofen, respectively, as inhibitors of PGHS-1 versus PGHS-2. We also observed that indomethacin and *S*-naproxen were 6- and 5-fold more potent as inhibitors of the PGHS-1 isozyme. These variations in relative inhibitions by NSAIDs may be explained, in part, by differences in the assay conditions and source of PGHS isozymes (human and murine vs ovine origins). Our studies used ovine PGHS isozymes, and we measured initial rates of oxygen consumption by the PGHS enzymes rather than the end-products of AA metabolism that were used by the other investigators [16,44].

The *R*-isomer of ibuprofen was found to be 32-fold less active than *S*-ibuprofen as an inhibitor of PGHS-1, whereas the isomers were weak and equally effective as inhibitors of PGHS-2 activity (Table 1). Our findings are consistent with the view that the *R*-isomers of these chiral NSAIDs possess low inhibitory activities against prostaglandin synthesis, and are generally ineffective as anti-inflammatory agents, *in vitro* and *in vivo* [2,3]. However, the work of Brune and coworkers [45] with the isomers of flurbiprofen has demonstrated that both isomers exhibited potent *in vivo* analgesic properties, which differed from their reported selectivity for inhibition of prostaglandin synthesis. Our finding of a comparable effect of the ibuprofen isomers at high concentrations on PGHS-2 (1 mM concentration range) as well as pharmacokinetic parameters [3] may provide explanations for the observed differences in the *in vivo* and *in vitro* activities observed with NSAID isomers.

Experiments were also conducted with these drugs to determine their corresponding potencies for inhibiting AA (300–500 μM)-induced aggregation and serotonin release responses (Fig. 7, Table 2). A progressive increase in the time lag phase of the aggregation response and a decrease in the maximal aggregation responses to AA were observed in the presence of increasing concentrations of each compound

Table 1
Concentration-dependent inhibitory effects of NSAIDs on AA-induced oxygen consumption by PGHS-1 and PGHS-2 synthase enzymes

Compound	PGHS-1 activity		PGHS-2 activity	
	pIC_{50}[a]	IC_{50} (μM)	pIC_{50}[a]	IC_{50} (μM)
S(+)-Ibuprofen	4.43 ± 0.17*	37.2	3.00 ± 0.04	1000
R(−)-Ibuprofen	2.93 ± 0.02	1175	3.01 ± 0.12	997
RS-Ibuprofen	3.87 ± 0.10*	135	3.24 ± 0.10	575
Indomethacin	6.17 ± 0.14**	0.68	5.38 ± 0.10**	4.2
S(+)-Naproxen	6.11 ± 0.07**	0.78	5.38 ± 0.08**	4.2

[a] Values are the means ± SEM of N = 4–8 experiments. pIC_{50} is equal to the negative logarithm of the drug concentration that produces a 50% inhibition of oxygen consumption induced by AA.

* Mean values of *S*- and *RS*-ibuprofen were significantly different ($P < 0.05$) from that of *R*-ibuprofen at $P < 0.05$.

** Mean values of indomethacin and *S*(+)-naproxen were significantly dffferent ($P < 0.05$) from the mean values of *S*-, *R*- and *RS*-ibuprofen. In addition, the mean values of indomethacin and *S*(+)-naproxen were not significantly different ($P > 0.05$).

(Fig. 7). Each drug inhibited these responses in a concentration-dependent manner with a rank order of inhibitory potency of indomethacin > *S*-ibuprofen > *RS*-ibuprofen > *S*-naproxen > *R*-ibuprofen, and the corresponding inhibitory potencies (IC_{50} values, μM) for these drugs against AA-induced aggregation and secretion were indomethacin (0.49, 0.43) > *S*-ibuprofen (1.6, 1.6) > *RS*-ibuprofen (5.8, 5.1) > *S*-naproxen (9.3, 11.8) > *R*-ibuprofen [66,155], respectively (Table 2).

AA is a platelet activator that mediates aggregation and serotonin secretion by subsequent metabolic conversion by COX to pro-aggregatory cyclic endoperoxides and thromboxane A_2 [46]. Our findings suggest that these NSAIDs inhibit AA-induced platelet aggregation and secretion through inhibition of PGHS-1. The IC_{50} values for *S*- and *R*-ibuprofen on human platelet aggregation, serotonin secretion, and on PGHS-1 and PGHS-2 activities were 1.6 μM/66.1 μM, 1.6 μM/155 μM, 37.2 μM/1175 μM, and 1000 μM/997 μM, respectively. The *S*-isomer was 32-, 41- and 96-times more potent than the *R*-isomer as an inhibitor of PGHS-1 activity, AA-induced platelet aggregation, and serotonin secretion. In contrast, activities of the ibuprofen isomers against PGHS-2 were nearly the same (see Table 1). In these studies, the relationships between the isomers of ibuprofen differed in PGHS-1 versus PGHS-2 inhibition; however, the ibuprofen isomers exhibited the same stereoselectivity for ovine PGHS-1 and inhibition of AA-induced human platelet aggregation and secretion, which is mediated by the PGHS-1 isozyme. We propose that the observed stereoselectivity (*S*-ibuprofen > *R*-ibuprofen) is related to their interactions directly with the PGHS isozymes in the cell and cell-free systems used. It is of interest that the *R*-enantiomer of ibuprofen was nearly ineffective until high concentrations were used. Previously, Caldwell *et al.* [47] reported that *R*-ibuprofen (and other related arylpropionic acids) undergoes conversion to the more active *S*-isomer, *in vitro* and *in vivo*. The results of these experiments demonstrate that the stereoselectivity of the ibuprofen isomers remains the same in human platelets and the ovine PGHS-1 isozyme, and suggest that little interconversion of these isomers has occurred in human platelets.

In these experiments, we demonstrated that the isomers of ibuprofen show a differential inhibitory pattern for the PGHS-1 and PGHS-2 enzymes, and PPAR isoforms. As indicated by Brune *et al.* [45] and Mukherjee *et al.* [3], the inhibition of PGHS-1 may not be the site of action of commonly used NSAIDs as higher concentrations than those required for inhibition of PGHS are necessary for *in vivo* effectiveness. Our findings present other explanations, namely, that the PGHS-2 and PPAR isoforms are other cellular targets for NSAID action. For example, our results show that NSAIDs required higher concentrations to block PGHS-2 than PGHS-1 activity, and that similar concentration ranges for these compounds were required for the activation of PPAR isoforms, *in vitro*. Thus, our results of higher concentrations required for PGHS-2 inhibition may, in part, explain the basis for the pharmacological actions of these NSAIDs, if indeed PGHS-2 is the major cellular target for these compounds. Moreover, our studies do not show a high rank order correlation between PPAR activation and PGHS-2 inhibition, an interaction that has been suggested by Gilroy and Colville-Nash [20]. Any attempt to extrapolate the results with our studies *in vitro* to the *in vivo* situation is speculative, since the pharmacological actions *in vivo* are dependent upon pharmacokinetic and pharmacodynamic properties of the individual NSAIDs [3]. Nevertheless, based upon our results, the activation of PPARα and PPARγ isoforms may be involved in the anti-inflammatory action of NSAIDs. In this regard, a new generation of drugs (celecoxib, rofecoxib, nabumetone) has been developed that exhibits higher selectivity for the inhibition of PGHS-2 activity, and these new modalities may have some benefit for the treatment of anti-inflammatory agents in arthritis with minimal adverse effects on the stomach and intestine [17]. Based upon our studies, we believe that chirality can further be used to identify additional drugs or to guide the synthesis of new compounds to produce greater anti-inflammatory activity with greater inhibition of the inducible PGHS-2 enzyme and activation of PPAR isoforms.

Since aspirin and other NSAIDs are either nonselective against PGHS-2, or are preferential inhibitors of PGHS-1 activity [44,48], as also indicated in our studies (Tables 1),

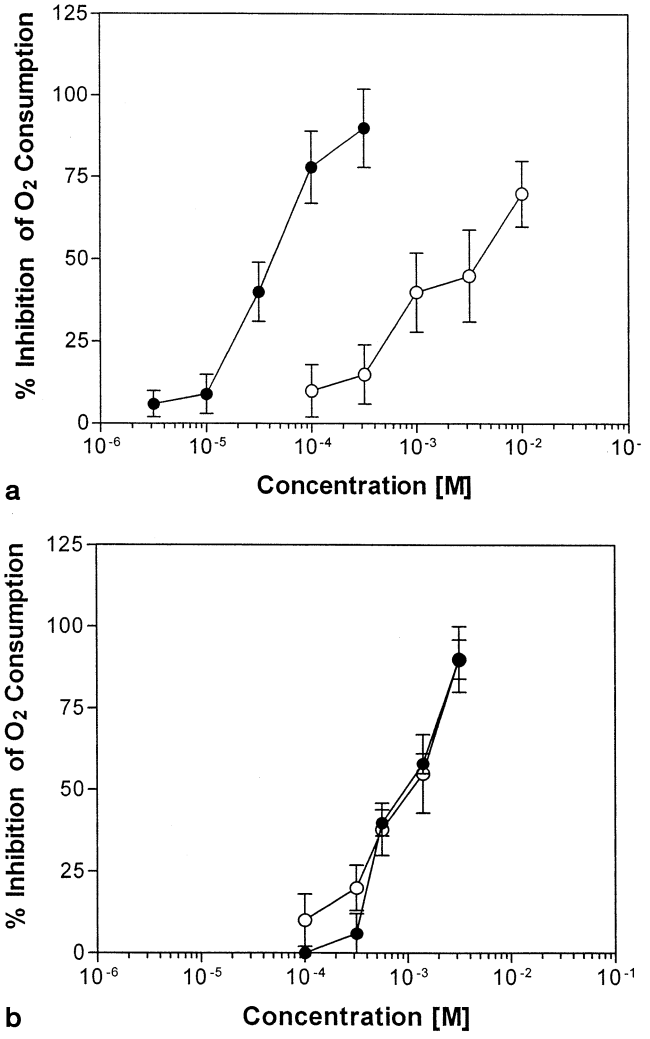

Fig. 6. Comparative concentration-dependent effects of ibuprofen isomers as inhibitors of oxygen consumption induced by arachidonic acid on PGHS-1 (upper panel) and PGHS-2 (lower panel). Key: (●) *S*-ibuprofen and (○) *R*-ibuprofen. The results (means ± SEM) are representative of at least three experiments.

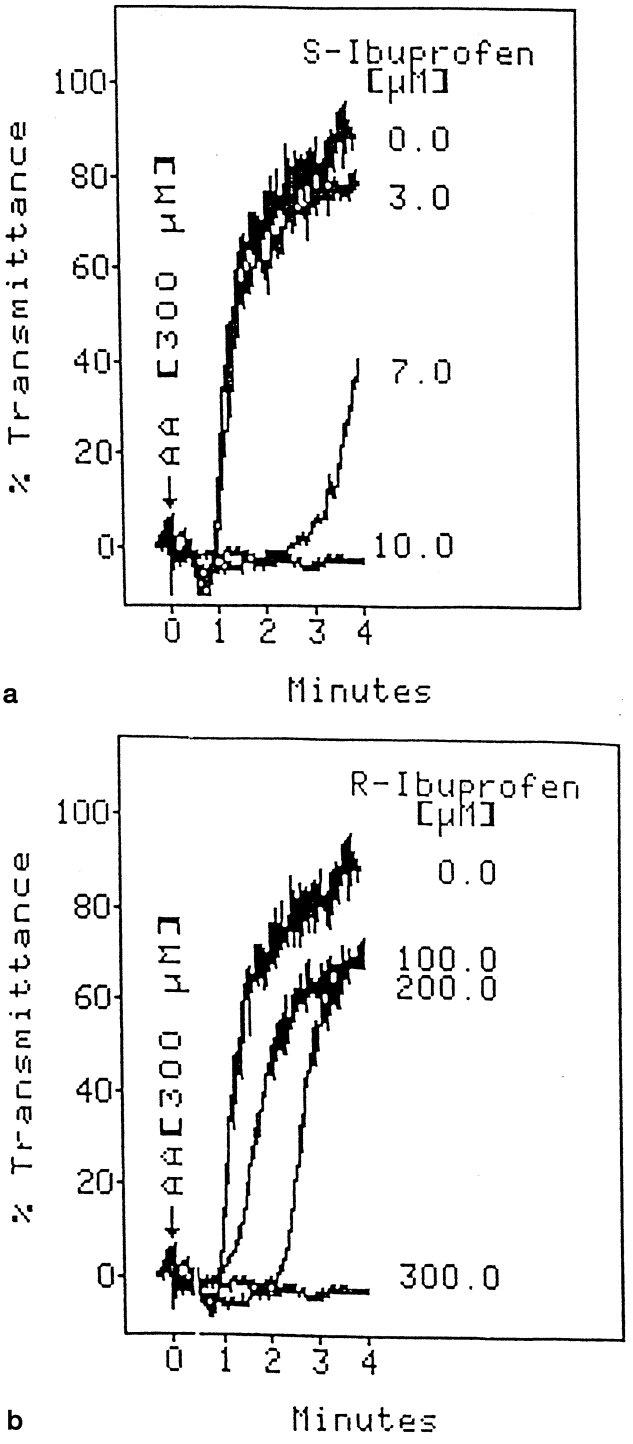

Fig. 7. Comparative concentration-dependent effects of ibuprofen isomers as inhibitors of arachidonic acid (AA)-induced aggregation in human PRP. Upper panel, *S*-ibuprofen; lower panel, *R*-ibuprofen. Results are representative of at least three experiments.

other cellular sites of action may exist, and include anti-inflammatory actions mediated through the activation of PPAR isoforms [20,21,49]. Furthermore, these comparisons of inhibitor IC_{50} values for the selected NSAIDs on PGHS-2 (ibuprofen isomers and *S*-naproxen) and PPAR isoforms are in the same concentration range. Thus, it is possible, due to the recognized involvement of PPARα and PPARγ on cell proliferation and inflammation, particularly the PPARγ isoform, that this nuclear receptor represents another cellular target for the action of NSAIDs.

Our studies have demonstrated that commonly used NSAIDs are concentration-dependent, selective activators of PPARα and PPARγ isoforms. The fact that NSAIDs activate PPARs might add to our understanding of their toxicity, and of the recently reported angiostatic and anti-cancer effects of these nuclear receptors [20,50–52]. Cyclopenetenone metabolites of AA generated by PGHS-2 have been proposed to activate PPARs, and a further investigation of this interrelationship is required to increase our understanding of the mechanism of anti-inflammatory action for NSAIDs [20]. Our future aims will be to test selective PGHS-2 inhibitors as regulators of PPAR iso-

Table 2
Concentration-dependent inhibitory effects of NSAIDs on AA-induced human platelet aggregation and serotonin secretion

Compound	Aggregation		Serotonin secretion	
	pIC_{50}[a]	IC_{50} (μM)	pIC_{50}[a]	IC_{50} (μM)
S(+)-Ibuprofen	5.79 ± 0.14*	1.61	5.79 ± 0.13*	1.61
R(−)-Ibuprofen	4.18 ± 0.17	66.1	3.81 ± 0.32	155
RS-Ibuprofen	5.24 ± 0.19*	5.79	5.29 ± 0.12**	5.1
Indomethacin	6.32 ± 0.20**	0.49	6.37 ± 0.26**	0.43
S(+)-Naproxen	5.03 ± 0.13	9.29	4.93 ± 0.06	11.8

[a] Values are the means ± SEM of N = 4–8 experiments. pIC_{50} is equal to the negative logarithm of the drug concentration that produces a 50% inhibition of AA-induced aggregation or serotonin release.

* Mean values of *S*- and *RS*-ibuprofen were significantly different ($P < 0.05$) from that of *R*-ibuprofen at $P < 0.05$.

** Mean values of indomethacin was significantly different ($P < 0.05$) from that of *S*(+)-naproxen.

forms, and to assess the pharmacological effects of commonly used NSAIDs as potential agents for the treatment of atherosclerosis, malignant diseases, and glucose homeostasis [24,49,53].

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