Flurbiprofen Enantiomers Inhibit Inducible Nitric Oxide Synthase Expression in RAW 264.7 Macrophages

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Purpose. Using RAW 264.7 macrophages, the present study investigates the influence of optically pure enantiomers of the nonsteroidal anti-inflammatory drug flurbiprofen on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression.

Methods. iNOS and cyclooxygenase-2 (COX-2) mRNA levels were measured by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). Concentrations of nitrite (index of cellular NO production) and prostaglandin E_2 (index of COX-2 activity) in cell culture supernatants were determined by Griess assay and enzyme immunoassay, respectively.

Results. R(−)- and S(+)-flurbiprofen decreased LPS-induced iNOS mRNA and nitrite levels in an equipotent and concentration-dependent manner. Suppression of iNOS mRNA expression by R(−)- and S(+)-flurbiprofen was gene-specific in that both substances failed to inhibit LPS-induced COX-2 mRNA expression. By contrast, flurbiprofen enantiomers suppressed LPS-induced prostaglandin E_2 formation enantioselectively with S(+)-flurbiprofen being considerably more potent than its R(−)-antipode.

Conclusions. Our results show that R(−)- and S(+)-flurbiprofen, albeit differing in their potency as inhibitors of COX-2 activity, equipotently suppress iNOS expression. Because sustained high NO levels are associated with pain and tissue injury under various pathological conditions, a suppression of the inducible NO pathway may contribute to the pharmacological action of both R(−)- and S(+)-flurbiprofen.

KEY WORDS: 2-arylpropionic acids; flurbiprofen enantiomers; inducible nitric oxide synthase; cyclooxygenase-2; stereoselectivity.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to relieve pain, fever, and inflammation. The pharmacological activity of the commonly marketed NSAIDs is attributed predominantly to an inhibitory effect on the activity of the cyclooxygenase (COX) enzyme, which catalyzes the first step in the conversion of arachidonic acid to prostaglandins and thromboxanes. In the early 1990s, COX was demonstrated to exist as two distinct isoforms (1). Whereas many of the side effects of NSAIDs (e.g., gastrointestinal ulceration and bleeding, platelet dysfunctions) are due to a suppression of COX-1-derived prostanoids, compelling evidence suggests that inhibition of prostanoids produced by COX-2 can be ascribed to the pharmacological activity of NSAIDs (for review see reference 2). However, this effect is not considered to explain the broad spectrum of anti-inflammatory and analgesic effects of NSAIDs sufficiently (for review see reference 3). Apart from inhibiting the synthesis of prostaglandins, NSAIDs exert a range of COX-independent actions, including suppression of the activity of neutrophils (4), alteration of protein kinase C activity (4), and inhibition of the activation of transcription factors (5–7).

The 2-arylpropionates represent the most important and widely used group of chiral NSAIDs. By virtue of a chiral carbon atom on the propionic acid side chain, they exist as enantiomeric pairs. 2-Arylpropionic acids are usually marketed as racemates containing an equimolar mixture of the respective S(+)- and R(−)-enantiomer. Whereas the S(+)-enantiomer represents an effective, but unselective COX inhibitor, the R(−)-enantiomer is much less active in this respect (8,9). However, experimental data from this and other laboratories suggest that certain pharmacological effects of 2-arylpropionic acids cannot be attributed exclusively to the S(+)-enantiomer (8,10–12). For flurbiprofen, behavioral studies indicate that both S(+)- and R(−)-flurbiprofen cause antinociceptive effects after systemic administration in rats (3,8,12), suggesting that at least the analgesic action of NSAIDs is poorly correlated with the inhibition of prostaglandin synthesis.

Nitric oxide (NO) formed by the inducible NO synthase (iNOS) has been implicated as a mediator of pain and tissue injury in various inflammatory and autoimmune diseases (13). Hence, interest in mechanisms underlying the regulation of iNOS, as well as pharmacological approaches that interfere with its expression, has substantially increased in recent years. To obtain further insights into the pharmacological effects of flurbiprofen, the present study investigated the influence of its optically pure enantiomers on lipopolysaccharide (LPS)-induced mRNA expression of iNOS in RAW 264.7 cells. The mouse macrophage-like cell line RAW 264.7 has been established as a model for studying molecular mechanisms and pathways involved in iNOS expression (14–16).

MATERIALS AND METHODS

Materials

Dulbecco's modified essential medium (DMEM) with 4 mM L-glutamine and 4.5 g/l glucose was purchased from Bio Whittaker (Verviers, Belgium). Fetal calf serum and penicillin-streptomycin were bought from Boehringer Mannheim (Mannheim, Germany). Lipopolysaccharide (LPS) from *E. coli* (serotype 026:B6), dexamethasone, and all other reagents were purchased from Sigma (Deisenhofen, Germany). R(−)-flurbiprofen and S(+)-flurbiprofen were kindly provided by Knoll Pharmaceuticals (Nottingham, U.K.).

Cell Culture

RAW 264.7 cells (ATCC TIB 71; American Type Culture Collection, Rockville, MD) were maintained and subcultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomy-

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ABBREVIATIONS: COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs.

cin. The cells were grown in a humidified incubator at 37°C and 5% CO_2. All incubations were performed in serum-free medium.

Incubation Protocol

RAW 264.7 cells were seeded at 5×10^5 cells per well in 24-well plates. Following an adherence period, cells were preincubated with the respective test compound for 30 min. Thereafter, LPS was added to the cells and the incubation was continued for an additional 24 h. Supernatants were then removed for determination of nitrite and prostaglandin E_2 levels and for stereoselective HPLC analysis of the flurbiprofen enantiomers. Subsequently, cells were lysed for RNA isolation. Total RNA was isolated using the RNeasy total RNA Kit (Qiagen, Hilden, Germany).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

β-Actin (internal standard), iNOS, and COX-2 mRNA levels were determined by quantitative real-time RT-PCR. Briefly, this method uses the 5′,3′ exonuclease activity of r*Tth* DNA polymerase to cleave a probe during PCR. A probe consists of an oligonucleotide coupled with a reporter dye (6-carboxyfluorescein; 6FAM) at the 5′ end of the probe and a quencher dye (6-carboxy-tetramethylrhodamine; TAMRA) at an internal thymidin. Following the cleavage of the probe, reporter and quencher dye become separated, resulting in an increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye using the integrated thermocycler and fluorescence detector ABI PRISM™ 7700 Sequence Detector (Perkin-Elmer, Weiterstadt, Germany).

Quantification of mRNA was performed by determining the threshold cycle (C_T), which is defined as the cycle at which the 6FAM fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3–10. iNOS and COX-2 mRNA levels were normalized to the housekeeping gene β-actin according to the following formula: C_T (iNOS or COX-2) – C_T (β-actin) = ΔC_T. Subsequently, respective iNOS and COX-2 mRNA levels were calculated using the $\Delta\Delta C_T$ method, i.e., ΔC_T values representing mRNA from cells treated with LPS in combination with a test compound were set in relation to the ΔC_T value representing mRNA levels from cells treated with LPS alone according to the following formula: ΔC_T (LPS + test compound) – ΔC_T (LPS) = $\Delta\Delta C_T$ (LPS + test compound). The relative mRNA level for the respective test compound was calculated as $2^{-\Delta\Delta C_T} \times 100\%$ based on the results of control experiments with an efficiency of the PCR reaction of approximately 100%.

RT-PCR was performed in 25-μl reaction volumes containing 1× reaction buffer (50 mM bicine, 115 mM KOAc, 10 μM ethylenediaminetetraacetic acid [EDTA], 8% [w/v] glycerol, pH 8.2), $Mn(OAc)_2$ solution (4 mM for β-actin- and COX-2 mRNA; 5 mM for iNOS mRNA), 300 μM deoxynucleotides triphosphates (dATP, dCTP, dGTP, dTTP; GeneCraft, Münster, Germany), 0.2 μM primer, 0.1 μM probe and 1.25 U r*Tth* DNA Polymerase (GeneCraft). The following thermal profile was used: 2 min 50°C, 30 min 60°C, 5 min 95°C and 45 cycles of 95°C for 15 sec, 60°C for 1 min. RNA samples were amplified using commercially synthesized primers specific for murine β-actin, iNOS, and COX-2 (TIB MOLBIOL, Berlin, Germany). Sequences of the primers and probes were as follows:

β-actin sense primer 5′-TCACCCACACTGTGCCCATCTACGA

β-actin antisense primer 5′-GGATGCCACAGGATTCCATACCCA

β-actin probe 5′-(6FAM)TATGCTC(TAMRA)TCCCTCACGCCATCCTGCGT

iNOS sense primer 5′-TGCCCCTTCAATGGTTGGTA

iNOS antisense primer 5′-ACTGGAGGGACCAGCCAAAT

iNOS probe 5′-(6FAM)CGCTACAACA(TAMRA)TCCTGGAGGAAGTGG

COX-2 sense primer 5′-TTTGTTGAGTCATTCACCAGACAGAT

COX-2 antisense primer 5′-CAGTATTGAGGAGAACAGATGGGATT

COX-2 probe 5′-(6FAM)CTACCATGGTC(TAMRA)TCCCCAAAGATAGCATCA

Determination of Nitrite

Nitrite was determined by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl medium samples. The optical density at 550 nm was measured using a microtiter plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite prepared in medium. The percentage of stimulation of nitrite release was calculated according to the following formula: % stimulation = [nitrite level (LPS + test compound)] / [nitrite level (LPS)] × 100%.

Determination of Prostaglandin E_2

Prostaglandin E_2 concentrations were determined using a commercially available enzyme immunoassay kit (Cayman, Ann Arbor, MI). Basal prostaglandin E_2 levels were taken to represent the lower limit of stimulated mediator release (i.e., 0%), whereas the mean of prostaglandin E_2 levels determined in the LPS-treated group was used as the maximal possible mediator release (i.e., 100%). Using these limitations, the percentage of stimulation of prostaglandin E_2 release was calculated according to the following formula: % stimulation = [prostaglandin E_2 level (LPS + test compound) – prostaglandin E_2 level (basal)]/[prostaglandin E_2 level (LPS) – prostaglandin E_2 level (basal)] × 100%.

Stereoselective Determination of Flurbiprofen Enantiomers

The concentrations of the flurbiprofen enantiomers in cell culture media were determined by stereoselective HPLC analysis using a chiral α_1-acid glycoprotein column as described previously (17).

Cell Viability Assay

A cytotoxic effect of the test substances was excluded by a cell viability test based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.6-benzene disulfonate; Boehringer Mannheim,

Germany) by mitochondrial dehydrogenases in metabolically active cells.

Statistics

Comparisons between groups were performed with Student's two-tailed *t*-test using the absolute values of the respective experiment. $P < 0.05$ was considered to be statistically significant.

RESULTS

Incubation of RAW 264.7 macrophages with LPS for 24 h caused a marked up-regulation of iNOS mRNA and a substantial increase in nitrite formation to 30.48 ± 1.44 μM (mean ± SEM, n = 3). Under basal conditions iNOS mRNA and nitrite levels were not detectable. To evaluate the influence of flurbiprofen enantiomers on iNOS expression, cells were pretreated with the respective compound at increasing concentrations for 30 min before inducing iNOS with LPS for 24 h. According to Fig. 1, R(−)- and S(+)-flurbiprofen inhibited LPS-induced nitrite accumulation in cell culture supernatants in a concentration-dependent manner by up to 32% [R(−)-flurbiprofen at 100 μM] and 35% [S(+)-flurbiprofen at 100 μM]. Respective effects were registered at nontoxic concentrations of the enantiomers, as determined by measuring mitochondrial cell activity (data not shown). Moreover, both flurbiprofen enantiomers markedly decreased LPS-induced iNOS mRNA levels by up to 59% [R(−)-flurbiprofen at 100 μM] and 64% [S(+)-flurbiprofen at 100 μM)] (Fig. 2). Under the same experimental conditions, dexamethasone (1 μM) caused an 85% inhibition of LPS-induced iNOS expression accompanied by a 69% inhibition of nitrite accumulation in cell culture supernatants (Table I).

Inhibition of iNOS expression was a nonenantioselective process in that R(−)-flurbiprofen was not inverted into its S(−)-antipode during a 24.5-h incubation period as assessed by stereoselective HPLC analysis (Table II). Small amounts of the S(+)-enantiomer [<1% at 10–100 μM R(−)-flurbiprofen] quantified in cell culture media immediately after addition of the R(−)-enantiomer to the cells and following a 24.5-h incubation period are most likely due to an impurity rather than chiral inversion of R(−)-flurbiprofen. Conversely,

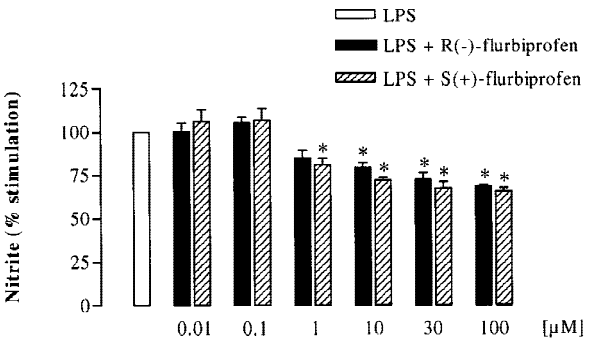

Fig. 1. Effect of R(−)- and S(+)-flurbiprofen (0.01–100 μM) on LPS-induced nitrite levels in cell culture supernatants of RAW 264.7 cells. Incubations were carried out as described in Materials and Methods. Values are means ± SEM of n = 3 observations. *P < 0.05, treatment vs. LPS control (open column), Student's *t*-test. Similar results were obtained from three individual experiments.

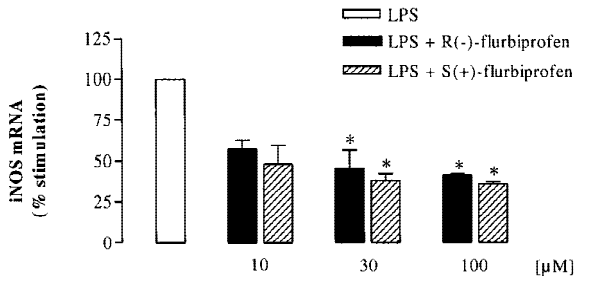

Fig. 2. Effect of R(−)- and S(+)-flurbiprofen (10–100 μM) on LPS-induced iNOS mRNA expression in RAW 264.7 cells. Incubations were carried out as described in Materials and Methods. Values are means ± SEM of n = 3 observations. *P < 0.05, treatment vs. LPS control (open column), Student's *t*-test. Similar results were obtained from three individual experiments.

a small amount of R(−)-flurbiprofen (<0.3%) was determined to be an impurity in the S(+)-enantiomer (data not shown).

Concurrently with the transcriptional induction of iNOS, a marked increase in COX-2 mRNA was observed in LPS-stimulated RAW 264.7 cells, whereas COX-2 mRNA was hardly detectable under basal conditions. As shown in Fig. 3, both flurbiprofen enantiomers failed to inhibit LPS-induced COX-2 mRNA expression. To assess the influence of the flurbiprofen enantiomers on COX-2 activity, prostaglandin E_2 levels were measured in cell culture supernatants. Incubation of RAW 264.7 macrophages with LPS for 24 h substantially increased PGE_2 levels from 1.56 ± 0.14 ng/ml (mean ± SEM, n = 3) under basal conditions to 16.0 ± 2.35 ng/ml (mean ± SEM, n = 3). S(+)-flurbiprofen (IC_{50} = 0.0061 μM) was 46-fold more potent than its R(−)-antipode (IC_{50} = 0.28 μM) in inhibiting LPS-induced prostaglandin E_2 formation (Fig. 4). When cells were incubated with dexamethasone, an almost complete suppression of COX-2 expression and concomitant formation of prostaglandin E_2 was observed (Table I).

DISCUSSION

The present study demonstrates an inhibitory effect of R(−)- and S(+)-flurbiprofen on the expression of iNOS in LPS-stimulated RAW 264.7 macrophages accompanied by marked decreases in nitrite levels in cell culture supernatants. These findings suggest that the pharmacological activity of flurbiprofen is not exclusively due to a suppression of COX activity, but may in part be associated with a suppression of iNOS activation. Respective inhibitions were registered at concentrations as low as 10 μM, which are within the range of plasma concentrations being achieved during oral therapy with the single enantiomers of flurbiprofen (18). Moreover, the concentration and uptake of flurbiprofen enantiomers can be enhanced at sites of inflammation through capillary damage leading to extravasation of protein-bound substances (19).

As with other NSAIDs, the principle mode of action of flurbiprofen relies on the inhibition of the activity of COX, the rate-limiting enzyme of the arachidonic acid pathway. In previous studies, S(+)-flurbiprofen has been shown to be a considerably more potent inhibitor of prostaglandin and thromboxane production in various tissues than its R(−)-antipode (9). In agreement with these studies, we observed a remarkable enantioselectivity in our cellular system in that

Table I. Regulation of iNOS and COX-2 mRNA Expression by Dexamethasone (1 μM) in LPS-Stimulated RAW 264.7 Cells and Concomitant Changes in Nitrite and Prostaglandin E_2 (PGE_2) Levels in Cell Culture Supernatants[a]

	iNOS mRNA (% stimulation)	Nitrite (% stimulation)	COX-2 mRNA (% stimulation)	PGE_2 (% stimulation)
LPS + dexamethasone	15.14 ± 1.02*	31.22 ± 1.88*	0.67 ± 0.10*	5.69 ± 2.25*

[a]Incubations were carried out as described in Materials and Methods. Values are means ± SEM of n = 3 observations. *P < 0.05, treatment vs. LPS control, Student's *t*-test. Similar results were obtained from three individual experiments.

S(+)-flurbiprofen was 46-fold more potent in inhibiting LPS-induced prostaglandin E_2 formation than the R(−)-enantiomer. However, we, as well as others, have previously shown that both enantiomers are effective as analgesics (8,10,12), supporting the notion that at least the analgesic action of NSAIDs is poorly correlated with inhibition of prostaglandin synthesis (3). Recently, it has been suggested that a reduction of inflammation-induced intraspinal release of substance P may contribute to the observed antinociceptive effects of the enantiomers (20). Another mediator that has been shown to enhance central and peripheral nociception is NO (21). NO produced by macrophages or stimulated neurons has been reported to increase the sensitivity of peripheral nociceptors (22). On the basis of these findings it is also reasonable to assume that inhibition of iNOS expression is another possible mechanism by which R(−)- and S(+)-flurbiprofen exert an antinociceptive action. However, which one of the possible targets actually participates in flurbiprofen-mediated antinociception should be resolved by future studies.

A characteristic phenomenon with 2-arylpropionic acids is the unidirectional metabolic chiral inversion of the R(−)-enantiomer to its S(+)-form (23). Previous studies indicate that in contrast to R(−)-ibuprofen, chiral inversion of therapeutic doses of R(−)-flurbiprofen does not occur in humans and rats (17,18). Likewise, no inversion of R(−)-flurbiprofen to the S(+) form could be determined in our cellular system, indicating that inhibition of iNOS expression by the flurbiprofen enantiomers occurred in a nonenantioselective manner.

Ambiguous findings have been published on the influence of NSAIDs on iNOS expression. Data published by Amin et al. (24) showed that aspirin, salicylate, and indomethacin did not significantly inhibit iNOS mRNA expression at pharmacological concentrations. Likewise, Sakitani et al. (25) reported that sodium salicylate prevented the induction of interleukin-1β-induced NO formation in rat hepatocytes at a post-transcriptional step without affecting iNOS mRNA levels. In the case of flurbiprofen, Mariotto et al. (26) reported a failure of the substance to inhibit LPS-induced iNOS induction in rat neutrophils. On the other hand, flurbiprofen was found to decrease LPS-induced iNOS mRNA levels in rat stomach, suggesting that its transcriptional effect is cell-type-dependent (27). In the present study, inhibitions of iNOS expression caused by R(−)- and S(+)-flurbiprofen were more pronounced in comparison to the suppressions of nitrite levels. However, because both parameters were measured at the same time point, the earlier onset of action of flurbiprofen at the transcriptional level may account for the differential degrees of inhibition. The observed decreases in iNOS mRNA and nitrite levels are very likely not related to inhibition of COX-2 activity, because they were weaker and occurred at concentrations above those needed to suppress LPS-induced prostaglandin E_2 synthesis. It is therefore conceivable that both flurbiprofen enantiomers display a direct effect on regulatory elements (e.g., transcription factors) that control iNOS expression. In transcription factors responsive to LPS, the promoter of the murine macrophage iNOS gene contains consensus sequences for the binding of activator protein 1 (AP-1), nuclear factor-κB (NF-κB), and nuclear factor for interleukin-6 expression (NF-IL-6) (14). Moreover, interactions with signal transduction pathways involved in iNOS expression (i.e., protein kinase C, tyrosine kinase) (15) may contribute to the action of R(−)- and S(+)-flurbiprofen.

The effects of R(−)- and S(+)-flurbiprofen on iNOS expression were gene-specific in that neither enantiomer altered LPS-induced COX-2 mRNA levels. These data are in agreement with studies reporting a failure of NSAIDs to suppress COX-2 expression in rabbit alveolar macrophages (28) and THP-1 and U937 human macrophages (29). Moreover, and in

Table II. Optical Purity of R(−)-Flurbiprofen[a]

Added R(−)-flurbiprofen concentration	S(+)-flurbiprofen (%) in cell culture medium relative to the added R(−)-flurbiprofen concentration	
	t = 0	t = 24.5 h
10 μM	0.92 ± 0.15	0.84 ± 0.23
30 μM	0.87 ± 0.12	0.75 ± 0.09
100 μM	0.78 ± 0.02	0.72 ± 0.04

[a]Using stereoselective HPLC analysis, flurbiprofen enantiomers were determined in cell culture medium immediately before adding R(−)-flurbiprofen-containing medium to the cells (t = 0) and following a 24.5-h incubation of cells with R(−)-flurbiprofen in the presence of LPS (10 μg/ml; added 30 min after R(−)-flurbiprofen). Values are means ± SEM of n = 3 observations.

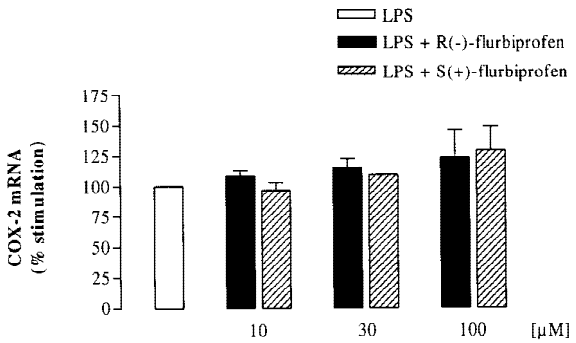

Fig. 3. Effect of R(−)- and S(+)-flurbiprofen (10–100 μM) on LPS-induced COX-2 mRNA expression in RAW 264.7 cells. Incubations were carried out as described in Materials and methods. Values are means ± SEM of n = 3 observations. Similar results were obtained from three individual experiments.

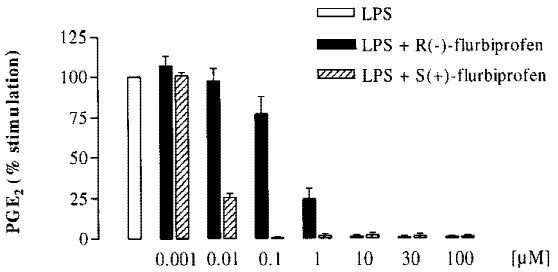

Fig. 4. Effect of R(−)- and S(+)-flurbiprofen (0.001–100 μM) on LPS-induced prostaglandin E_2 (PGE_2) levels in cell culture supernatants of RAW 264.7 cells. Incubations were carried out as described in Materials and Methods. Values are means ± SEM of n = 3 observations. *P < 0.05, treatment vs. LPS control (open column), Student's *t*-test: R(−)-flurbiprofen (1–100 μM), S(+)-flurbiprofen (0.01–100 μM). Similar results were obtained from three individual experiments.

support of the data presented here, we recently showed that in RAW 264.7 cells, aspirin and other salicylates, albeit suppressing COX-2-dependent prostaglandin E_2 production, do not interfere with COX-2 expression (30). By contrast, and in agreement with previous findings (1,13,28,30), both iNOS and COX-2 expression were inhibited by dexamethasone, which was used as a positive control in the present study.

Our results demonstrate a specific inhibition of iNOS expression by both enantiomers of the 2-arylpropionic acid flurbiprofen at therapeutically relevant concentrations. We conclude that a partial suppression of the inducible L-arginine-NO pathway might play a role in the pharmacological action of both flurbiprofen enantiomers. Moreover, this is the first study to show that the weak COX inhibitor R(−)-flurbiprofen, so far neglected as the less-potent entity of the racemate, is an equipotent inhibitor of iNOS mRNA expression in comparison with S(+)-flurbiprofen.

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