

# Inhibition of Cyclooxygenase-2 Expression by 4-Trifluoromethyl Derivatives of Salicylate, Triflusal, and Its Deacetylated Metabolite, 2-Hydroxy-4-trifluoromethylbenzoic Acid

ALBERTO FERNÁNDEZ DE ARRIBA, FERNANDO CAVALCANTI, AGUSTÍ MIRALLES, YOLANDA BAYÓN, ANDRÉS ALONSO, MANUEL MERLOS, JULIÁN GARCÍA-RAFANELL, and JAVIER FORN

*Department of Pharmacology, Uriach Research Center, Barcelona, Spain (A.F.de A., F.C., A.M., M.M., J.G.-R., J.F.); and Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, Facultad de Medicina, Valladolid, Spain (Y.B., A.A.)*

Received November 2, 1998; accepted January 19, 1999

This paper is available online at <http://www.molpharm.org>

## ABSTRACT

The therapeutic potential of drugs that block the induction of cyclooxygenase-2 has been emphasized. When two 4-trifluoromethyl salicylate derivatives [2-acetoxy-4-trifluoromethylbenzoic acid (triflusal) and its deacetylated metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB)] were compared with aspirin and sodium salicylate as cyclooxygenase-2 (COX-2) inhibitors, we observed that in bacterial lipopolysaccharide-activated human blood, triflusal, aspirin, and HTB, but not sodium salicylate, inhibited COX-2-mediated prostaglandin  $E_2$  (PGE<sub>2</sub>) production (IC<sub>50</sub> = 0.16, 0.18, 0.39, and >10 mM, respectively). However, only triflusal and aspirin inhibited purified COX-2 enzyme. To test this apparent discrepancy, we realized that HTB and triflusal (but neither aspirin nor salicylate) produced a concentration-dependent inhibition of COX-2 protein expression in peripheral human mononuclear cells. This

observation was further confirmed in a rat air pouch model in vivo, in which both aspirin and triflusal inhibited PGE<sub>2</sub> production (ID<sub>50</sub> = 18.9 and 11.4 mg/kg p.o., respectively) but only triflusal-treated animals showed a decrease in COX-2 expression. This different behavior may be, at least in part, due to the ability of HTB and triflusal to block the activation of the transcription factor nuclear factor- $\kappa$ B to a higher extent than aspirin and sodium salicylate. Thus, in addition to inhibiting the COX-2 activity at therapeutic concentrations, triflusal is able to block through its metabolite HTB the expression of new enzyme, and hence the resumption of PGE<sub>2</sub> synthesis. Triflusal and HTB may exert beneficial effects in processes in which de novo COX-2 expression is involved and, in a broader sense, in pathological situations in which genes under nuclear factor- $\kappa$ B control are up-regulated.

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin (PG)H<sub>2</sub>, the precursor of a wide group of biologically active mediators such as PGE<sub>2</sub>, prostacyclin, and thromboxane A<sub>2</sub>. Two isoforms of this enzyme have been identified (Smith et al., 1996): COX-1, a constitutively expressed enzyme, and COX-2, an isoform induced in response to many proinflammatory agents, as well as mitogens and tumor promoters. COX-2 is expressed in activated macrophages, monocytes, and several other cell types (Mitchell et al., 1995) and has been identified in chronic inflammatory conditions in vivo (Vane et al., 1994). Moreover, recent discoveries have shown that COX-2 seems implicated in either physiological processes such as ovulation (Sirois and Dore, 1997) and delivery (Sawdy et al., 1997; Lim et al., 1997) or pathological states, such as colonic cancer, Alzheimer's disease, heart failure, and even hypertension (Levy, 1997; Oka and Takashima, 1997;

Hartner et al., 1998; Wong et al., 1998). In this scenario, there has been great interest in the role or roles of COX-2 and the usefulness of drugs that selectively block this isoenzyme (Frölich, 1997; see also Pennisi, 1998).

The first anti-inflammatory synthetic drug, which remains the most popular worldwide, is aspirin. Despite this, the mechanism by which aspirin and salicylate derivatives exert their therapeutic effects, far from being established, remains open to debate. In the early 1970s, Vane (1971) demonstrated that sodium salicylate, aspirin, and other commonly used NSAIDs inhibited cyclooxygenase. From then on, numerous studies have supported the hypothesis that this is how salicylates work (Mitchell et al., 1997). However, there are some doubt as to the molecular basis for the therapeutic properties of salicylate derivatives, and due to recent descriptions of the surprising effects of these drugs on some central cell regulatory events and gene regulation processes, the

**ABBREVIATIONS:** COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay; HTB, 2-hydroxy-4-trifluoromethylbenzoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HUVEC, human umbilical vein endothelial cell; LPS, bacterial lipopolysaccharide; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NSAID, nonsteroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; PG, prostaglandin.

mechanism of action of salicylates, in both the inflammatory response and the atherothrombotic processes, remains under discussion (Frölich, 1997).

Salicylates have been shown to inhibit the mitogen-activated protein kinase cascade (Schwenger et al., 1996) and the expression of several key enzymes as type IIA phospholipase A<sub>2</sub> (Vervoordeldonk et al., 1996) or the inducible isoform of NO synthase (Farivar and Brecher, 1996). A molecular mechanism by which salicylates could exert part of these effects was proposed by Kopp and Ghosh (1994) when they reported that salicylates inhibited the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation through the stabilization of I $\kappa$ B. Because NF- $\kappa$ B regulates the expression of many genes, this mechanism of action was rapidly implicated in the effects of salicylates on inflammation (Pierce et al., 1996), atherogenesis (Weber et al., 1995), or neuroprotection (Grilli et al., 1996).

COX-2 is an early gene expressed in response to many cytokines, and its transcriptional regulation is, at least in part, under the control of NF- $\kappa$ B (Newton et al., 1997). Thus, the inhibition of this transcription factor by salicylates could block COX-2 expression. However, several studies failed to demonstrate an inhibition of COX-2 expression by aspirin and other NSAIDs in various experimental models and species (O'Sullivan et al., 1993; Barrios-Rodiles et al., 1996). Furthermore, recent studies suggest that sodium salicylate exerts its anti-inflammatory action because it is a weak competitive inhibitor of arachidonic acid in the active site of the enzyme, rather than via its action on NF- $\kappa$ B activation (Loll et al., 1995; Mitchell et al., 1997). Another controversial aspect of the newly reported effects for aspirin and salicylate is the high concentration, higher than that obtained after therapeutic doses, needed to elicit significant effects (Kopp and Ghosh, 1994; Mitchell et al., 1997).

Nevertheless, the previous observations that salicylates inhibit the NF- $\kappa$ B-mediated gene expression prompted us to analyze the potential inhibition of the bacterial lipopolysaccharide (LPS)-induced COX-2 expression by triflusal and its deacetylated metabolite, HTB (Fig. 1). Triflusal is an antiplatelet drug that despite its structural analogy to aspirin,

exhibits different pharmacological and pharmacokinetic properties (McNeely and Goa, 1998). Here, we report that triflusal and HTB at therapeutic doses are potent inhibitors of NF- $\kappa$ B activation, de novo COX-2 synthesis, and PGE<sub>2</sub> production in both human PBMCs and a rat model of inflammation in vivo.

## Materials and Methods

**Reagents.** Tissue culture media and fetal bovine serum were purchased from GIBCO BRL Life Technologies S.A. (Madrid, Spain). Nonidet P-40 was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), and nitrocellulose membranes were purchased from Bio-Rad Laboratories S.A. (Barcelona, Spain). Bicinchoninic acid protein assay and molecular weight protein standards were obtained from Pierce Chemical (Rockford, IL). Enzyme-linked immunosorbent assay (ELISA) kits specific for PGE<sub>2</sub> and thromboxane B<sub>2</sub> were purchased from Amersham Ibérica S.A. (Madrid, Spain). Tris, toluene, Tween-20, and dimethyl sulfoxide were from Merck Química S.A. (Barcelona, Spain). 2-Acetoxy-4-trifluoromethylbenzoic acid and 2-hydroxy-4-trifluoromethylbenzoic acid were from URIACH Laboratories (Barcelona, Spain). Arachidonic acid (peroxide free) was purchased from Cayman Chemical Co. (Ann Arbor, MI) and stored at -20°C. Reagents for electrophoresis were obtained from Amersham Pharmacia Biotech GmbH (Madrid, Spain). LPS from *Escherichia coli* serotype 026:B6 (LPS) and all the other non-specified reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

**Whole Human Blood Assays.** Human whole blood (heparinized, 10 U/ml) was obtained at the Hospital de Sant Pau (Barcelona, Spain) from donors whom had no apparent inflammatory conditions and had not taken any NSAIDs during the previous 2 weeks. To determine COX-2 activity, the blood was aliquoted (1 ml) and incubated with compound ( $10^{-5}$  to  $5 \times 10^{-3}$  M) or vehicle (dimethyl sulfoxide, 0.5% final) in the absence or presence of LPS (10  $\mu$ g/ml) for 24 h at 37°C by gentle shaking (Patrignani et al., 1994). After the incubation, plasma was separated by centrifugation (10 min at 1000g, 4°C) and kept at -70°C until assayed for PGE<sub>2</sub> by specific ELISA.

**Enzyme Activity Determinations.** Cyclooxygenase activity (COX-2 from sheep placenta; Cayman Chemical Co.) was determined by measuring spectrophotometrically the turnover of the chromogen *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG<sub>2</sub> to PGH<sub>2</sub>. The reaction mixture (1 ml) contained 1  $\mu$ M hematin and 2 mM phenol as cofactors in Tris-HCl buffer (100 mM, pH 8.1). The enzyme (30–40 units) was preincubated at 37°C with 5  $\mu$ l of the vehicle alone (controls) or 5  $\mu$ l of the test compounds in dimethyl sulfoxide for 1 to 10 min at 37°C. The reaction was started by the addition of freshly prepared arachidonic acid (100 or 10  $\mu$ M) and TMPD (170  $\mu$ M) and measured by the change in absorbance at 611 nm for 30 s. The initial rate (linear for approximately 15 s) was measured, and the nonspecific rate of oxidation in the absence of enzyme was subtracted before calculation of the percentage of inhibition.

**Isolation and Culture of Human Mononuclear Cells.** Human peripheral blood mononuclear cells (PBMCs) were isolated from whole human blood by density gradient centrifugation in Ficoll solution ( $d = 1.077$  g/ml, Biochrom KG; Cultek S.L., Madrid, Spain). Then, 50-ml centrifuge tubes were filled with 15 ml of Ficoll. Heparinized (10 U/ml) human whole blood was diluted in equal parts with PBS, and 25 ml was carefully poured over the Ficoll solution. The tubes were then centrifuged for 20 min at 1200g, and the mononuclear cells (80–100% recovery) were collected from the whitish interphase between the plasma and the separating solution. Cells were washed twice with PBS (first cycle, 10 min at 300g; second cycle, 10 min at 200g) and then resuspended ( $3 \times 10^6$  cells/ml) with culture

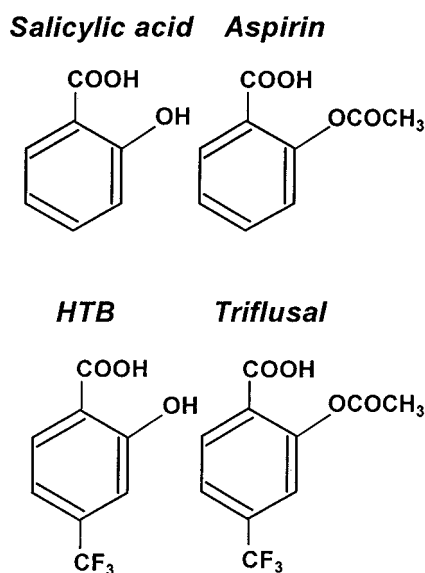


Fig. 1. Chemical structures of triflusal, HTB, aspirin and salicylic acid.

medium (RPMI-1640, supplemented with 10% heat-inactivated FCS).

Cells were placed in appropriate plastic culture plates, incubated for 1 to 2 h at 37°C in 5% CO<sub>2</sub>/air, and then treated with the test compounds for 24 h. Cell counts were performed with a Celltak  $\alpha$  MEK-6108-K (Radiometer S.A., Madrid, Spain), and standard Wright-Giemsa staining was performed to examine by light microscopy whether the cells displayed the morphological features of viable human mononuclear cells. Monocytes compose an average of 10% of PBMCs. The remaining 90% of cells were mostly lymphocytes that do not normally express COX (Spencer et al., 1998). Before the 24-h incubation, cell viability exceeded 95% as determined with trypan blue. Cell viability at the end of the incubation was determined by trypan blue exclusion and with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, as described later.

**Immunoblot Assays.** Human PBMCs, stimulated or activated with LPS and in the presence or absence of the drugs tested, were transferred to centrifuge tubes and pelleted by centrifugation for 5 min at 1200g. The supernatants were collected and stored at -70°C for later determination of PGE<sub>2</sub> by specific ELISA. Pelleted cells were washed twice with PBS, and an aliquot (0.5 ml) was used for the MTT assay. The remainder (2 ml;  $\sim 5 \times 10^6$  cells) was recentrifuged (600g, 10 min), the PBS was removed, and the cells were resuspended by gentle shaking in 50  $\mu$ l of lysis buffer (1% Nonidet-40, 1 mM EDTA in PBS) and incubated for 15 min in ice. The detergent-insoluble material was removed by centrifugation at 20,000g for 15 min at 4°C, and the protein concentration of the supernatants was determined by the bicinchoninic acid protein assay reagent. The cell extracts were then boiled (5 min) in a ratio of 1:1 with gel-loading buffer [50 mM Tris, 10% (w/v) SDS, 10% (v/v) glycerol, 50  $\mu$ l/ml 2-mercaptoethanol, and 2 mg/ml bromophenol blue]. Samples were centrifuged at 10,000g for 2 min, loaded (50  $\mu$ g/lane) onto a 4% stacking gel/7.5% separating, and subjected to electrophoresis (1 h at 200 V). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad; 2 h at 100 V) and overnight blocking at 4°C with PBS buffer containing 0.1% (w/v) Tween-20 and 20% dried lowfat milk. The membranes were washed with PBS containing 0.05% Tween-20 and incubated 1 h in a goat polyclonal antibody raised against purified human COX-1 or COX-2 protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blots were washed three times (PBS, 0.05% Tween-20) and incubated for 30 min with a horseradish peroxidase-conjugated secondary antibody raised in rabbit against goat (Pierce). Nitrocellulose membranes were washed twice, and bound antibody was visualized by enhanced chemiluminescence (ECL Kit; Amersham Ibérica S.A.).

**Viability Determination (MTT Assay).** The viability of cells treated with any of the compounds tested was determined by assaying the ability of mitochondrial dehydrogenases to convert the soluble tetrazolium salt, MTT, into an insoluble purple formazan through cleavage of the tetrazolium ring. After 24-h incubation with vehicle or the drug tested, the cells were counted (Celltak  $\alpha$  MEK-6108-K) and washed two times with PBS. Then, the cells were resuspended (half the original volume) in a medium with MTT (Sigma) at a concentration of 5 mg/ml and without phenol red or FBS. Cells were incubated for 4 h at 37°C in 5% CO<sub>2</sub>. At the end of the incubation, the medium was removed, the cells were washed once, and the converted purple dye was solubilized with dimethyl sulfoxide at the same volume. Absorbance of converted dye was measured at 550 nm with background subtraction at 630 nm in a microplate reader (Ceres UV900 HD; Bio-tek Instruments Inc., Winoski, VT).

**Electrophoretic Mobility Shift Assay.** Human PBMCs were washed with ice-cold hypotonic lysis buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, and 0.6% Nonidet P-40). The cells were allowed to swell on ice for 10 min and vortexed vigorously for 10 s. Unbroken cells were eliminated by centrifugation (1000g, 10 min, 4°C), and the nuclei were collected

by centrifugation at 15,000g for 1 min in a microcentrifuge. The nuclear pellet was resuspended in high salt extraction buffer containing 25% glycerol and 0.5 M KCl, and the nuclear extract was obtained by pelleting for 30 min at 105,000g in a Optima TL ultracentrifuge (Beckman) using a TLA 100.2 rotor. Then, 22-mer double-stranded oligonucleotide probes containing NF- $\kappa$ B sequence were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase and separated from the unincorporated label by minicolumn chromatography. The kilobase sequence used was sense, 5'-AGTTCAGGG-GAATTTCCCAGGC-3', and the complement, 5'-GCCTGGGAAAT-TCCCCTGAACT-3'. Nuclear protein (10  $\mu$ g) was incubated for 20 min on ice with radiolabeled oligonucleotide probes ( $2-6 \times 10^4$  cpm) in a 25- $\mu$ l reaction buffer containing 2  $\mu$ g of poly(dI/dC), 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 8% Ficoll, and 4% glycerol. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4% nondenaturing polyacrylamide gel in Tris-borate/EDTA buffer at 175 V for 3 h at 4°C. The gel was dried and autoradiographed with an intensifying screen at -80°C for 2 to 12 h. The specificity of the DNA-protein complex was confirmed by competition with a 300-fold molar excess of unlabeled nucleotide containing the consensus sequences. Quantification of the DNA-protein complex containing the NF- $\kappa$ B sequence was carried out by densitometric scanning using software of the series Discovery 3.1 from PDI-Pharmacia.

**Air Pouch Model of Inflammation.** For determination of PGE<sub>2</sub> in exudate and stomach, male Lewis rats (175-200 g) were used. Air cavities were produced by a s.c. injection of 20 ml of sterile air into the intrascapular area. Every 2 days, 10 ml of air was injected again into the cavity to keep the space open. Seven days after the first injection, 2 ml of a 1% solution of  $\lambda$ -carrageenan (Sigma) in saline was injected into the air pouch to produce an inflammatory reaction. The animals were sacrificed 24 h later, and the volume of exudate was measured. The type and number of cells present in the exudate were determined with a Coulter Counter and standard Wright-Giemsa staining. Cells were pelleted by centrifugation at 1200g for 5 min at 4°C, and PGE<sub>2</sub> was determined in the supernatant by specific ELISA. Cells were resuspended in 2 ml of saline and submitted to a hypotonic shock with 6 ml of distilled water for 20 s to selectively eliminate red blood cells. The isotonicity of the solution was restored with 2 ml of 3.5% (w/v) NaCl, and the cells were collected by centrifugation (1000g, 10 min, 4°C), resuspended in lysis buffer at  $2 \times 10^8$  cell/ml, and used for immunoblotting (see above).

Immediately after the exudates were collected, the stomachs were excised and frozen at -70°C. The day of analysis, stomachs were homogenized in 70% ethanol. The homogenates were centrifuged (1000g, 10 min, 4°C), and the supernatants were dried under N<sub>2</sub> stream and resuspended in ELISA buffer for PGE<sub>2</sub> determination.

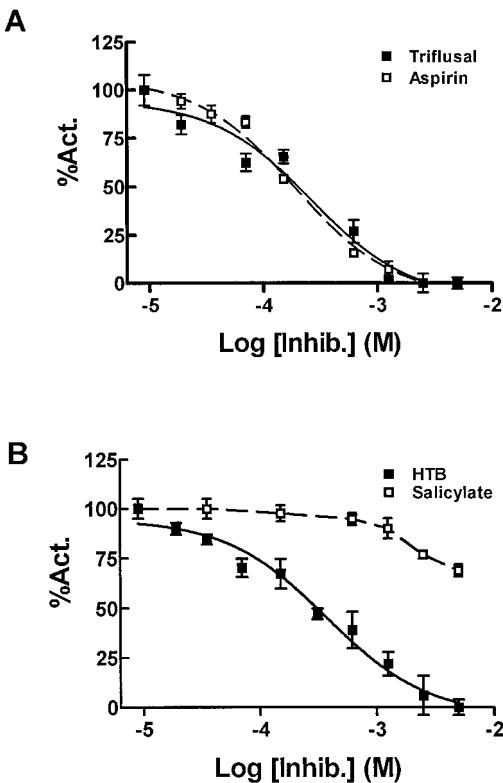
## Results

**Effects of Salicylates on Whole Human Blood Assays.** Heparinized (10 U/ml) whole human blood obtained from healthy donors was treated in vitro with sodium salicylate, HTB, triflusal, aspirin, and other NSAIDs. The COX-2 activity was measured as PGE<sub>2</sub> production in blood incubated with LPS (10  $\mu$ g/ml) for 24 h. Under unstimulated conditions, plasma PGE<sub>2</sub> averaged  $0.8 \pm 0.5$  ng/ml (mean  $\pm$  S.D.,  $n = 15$ ). When the blood was incubated with LPS for 24 h, an substantial increase in PGE<sub>2</sub> production was observed ( $11.5 \pm 2.3$  ng/ml,  $n = 15$ ). Sodium salicylate, HTB, triflusal, and aspirin inhibited LPS-induced PGE<sub>2</sub> increase in a dose-dependent manner (Fig. 2). HTB was much more potent than salicylate, with IC<sub>50</sub> values of  $0.39 \pm 0.09$  and  $>10$  mM, respectively. On the contrary, both aspirin and triflusal behaved as equipotent COX-2 inhibitors in this assay with similar IC<sub>50</sub> values (Table 1). The nonselective COX inhibitor



indomethacin and the selective COX-2 inhibitor NS-398 totally abrogated PGE<sub>2</sub> production with apparent IC<sub>50</sub> values far lower than 1 μM (Table 1), proving that the PGE<sub>2</sub> increase in blood incubated with LPS is produced mainly by a COX-2-dependent mechanism, as previously reported (Patrignani et al., 1994).

**Effects of Salicylates on Purified COX-2 Enzyme.** To determine whether COX-2 enzyme activity was modified by sodium salicylate, aspirin, triflusal, or HTB, the effect of these compounds on the oxidation of arachidonic acid (100 and 10 μM) by the purified sheep COX-2 enzyme was examined. The results obtained with these compounds at a substrate concentration of 100 μM, as well as those obtained with indomethacin and NS-398, are compared in Table 1. Aspirin and triflusal showed a time-dependent profile of inhibition with a maximal effect after 10 min of preincubation with the enzyme. Neither sodium salicylate nor HTB at concentrations up to 10 mM significantly affected COX-2 activity



**Fig. 2.** Effects of salicylates on whole human blood assay. COX-2 activity was measured as PGE<sub>2</sub> production in heparinized (10 U/ml) human blood incubated with LPS (10 μg/ml) for 24 h in the presence of triflusal or aspirin (A) and HTB or salicylic acid (B) at the indicated concentrations. Values are expressed as a percentage of the PGE<sub>2</sub> measured in vehicle-treated control and reflect the mean ± S.E.M. (n = 3–5).

TABLE 1

IC<sub>50</sub> Inhibition of COX-2 activity by NSAIDs

Heparinized whole human blood was incubated at 37°C with compound or vehicle in presence of LPS (10 μg/ml) for 24 h. Activity was determined measuring the PGE<sub>2</sub> released in the medium. Inhibition of purified COX-2 from sheep placenta (Cayman Chemical Co.) was determined spectrophotometrically. The enzyme (30–40 U) was preincubated at 37°C for 7 min. with triflusal, aspirin, HTB, and salicylate or for 2 min with indomethacin and NS-398 before activity determination. Results are mean ± S.D. (n = 3).

	Triflusal	Aspirin	HTB	Salicylate	Indomethacin	NS-398
	μM					
Human blood	160 ± 10	180 ± 50	390 ± 90	>5000	0.78 ± 0.03	0.12 ± 0.04
Purified COX-2	280 ± 40	420 ± 80	>10000	>10000	0.98 ± 0.06	0.042 ± 0.003

independently of the time of preincubation (Table 1). When the lowest concentration (10 μM) of substrate was used, the profiles of aspirin, triflusal, HTB, and sodium salicylate were not modified (data not shown). As expected, both indomethacin and NS-398 completely blocked COX-2 activity, with apparent IC<sub>50</sub> values of 0.98 and 0.042 μM, respectively (Table 1).

**Induction of COX-2 Expression by LPS in Human PBMCs.** COX-2 was not detected by immunoblotting in untreated human PBMCs. Overnight incubation of these cells also failed to induce COX-2 expression. However, LPS (10 μg/ml) produced an up-regulation of COX-2 protein. To optimize conditions for LPS-induced COX-2 expression in cultured PBMCs, a dose- and time-response study was performed. As shown in Fig. 3A, cells exposed overnight to LPS (0.1–50 μg/ml) showed a dose-dependent increase in COX-2 levels. On the other hand, COX-2 expression is detected as early as 2 h after stimulation with 10 μg/ml LPS and reached the maximum expression after 19 h of incubation (Fig. 3A). PGE<sub>2</sub> generation almost paralleled COX-2 protein expression (Fig. 3B). Modification of COX-1 expression was not detected in any of the conditions tested (data not shown).

**Effects of Exposure to Salicylates on PGE<sub>2</sub> Release and COX-2 Expression in Human PBMCs Stimulated by LPS.** Human PBMCs, either untreated or LPS activated, were assayed for PGE<sub>2</sub> released into the medium. As shown in Fig. 3B, untreated PBMCs released low levels of PGE<sub>2</sub> after 19-h incubation (3 ± 0.5 ng/ml), but exposure to 10 μg/ml LPS led to a dose-dependent increase in PGE<sub>2</sub> production with a ~5-fold increase (15 ± 3 ng/ml). The COX-2-selective inhibitor NS-398 totally prevented the LPS-induced PGE<sub>2</sub> production (data not shown), confirming that COX-2, and not COX-1, is involved in this process.

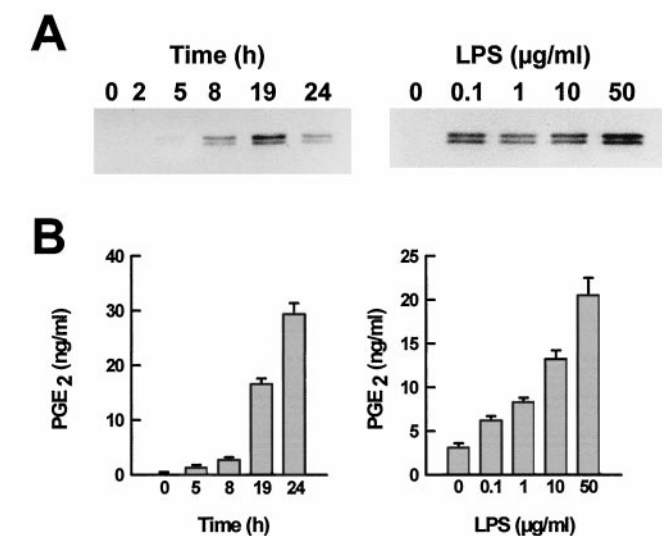
When incubated together with LPS, sodium salicylate or aspirin (at concentrations up to 5 mM) had no effect on the LPS-induced COX-2 expression (Figs. 4A and 5A). On the other hand, aspirin, but not sodium salicylate, completely inhibited PGE<sub>2</sub> release (Figs. 4B and 5B). Similarly, the COX-2-selective inhibitor NS-398 totally inhibited PGE<sub>2</sub> release but failed to prevent COX-2 expression at concentrations up to 50 μM (data not shown). However, at the same concentration range tested for the other salicylates, HTB and triflusal caused an inhibition of PGE<sub>2</sub> release and COX-2 expression in a concentration-dependent manner (Figs. 4 and 5). Cell viability measured by trypan blue exclusion and with the MTT assay was more than 90% of the control regardless of the treatment used.

**Inhibition of NF-κB Activation by Salicylates in Human PBMCs Stimulated by LPS.** To determine the possible implication of the transcription factor NF-κB on the inhibition of COX-2 expression in PBMCs, we compared the effect of

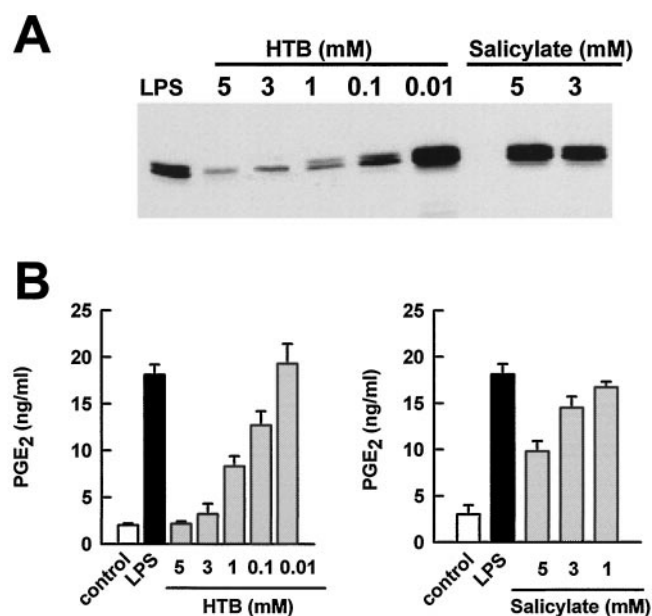
different concentrations of aspirin, salicylate, triflusal, and HTB on the activation of NF- $\kappa$ B in LPS-activated PBMCs. As shown in Fig. 6A, the incubation of PBMCs with LPS induced a rapid and prominent activation of NF- $\kappa$ B as judged from the

appearance of  $\kappa$ B-binding activity in the nuclear extracts. The mean increase of the binding activity elicited by LPS was  $3.5 \pm 0.6$ -fold ( $n = 4$ , Fig. 6B) and was more prominent than that produced by TNF- $\alpha$ , an agonist that has been used in several studies dedicated to assessment of the effect of salicylates on NF- $\kappa$ B activation. Figure 6C shows that incubation for 15 min with either sodium salicylate or HTB before the addition of 10  $\mu$ g/ml LPS produced a significant reduction in  $\kappa$ B-binding activity in nuclear extracts of these cells. Attempts to compare the potency of these compounds were carried out by densitometric scanning of the protein/oligonucleotide complexes in three independent experiments. Figure 6D shows that 3 mM HTB produced an inhibition of  $85 \pm 7\%$  of  $\kappa$ B-binding complexes, whereas the inhibition observed for the same concentration of sodium salicylate was  $35 \pm 12\%$ . Similar experiments were carried out with aspirin and triflusal (electrophoretic mobility shift assay; not shown), and in keeping with previous findings in human umbilical vein endothelial cells (Y. Bayón, A. Alonso, and M. Sanchez-Crespo, unpublished observations), triflusal was also more potent than aspirin in the inhibition of  $\kappa$ B-binding activity in PBMCs ( $87 \pm 3\%$  and  $48 \pm 5\%$  of inhibition at 3 mM, respectively;  $n = 3$ ).

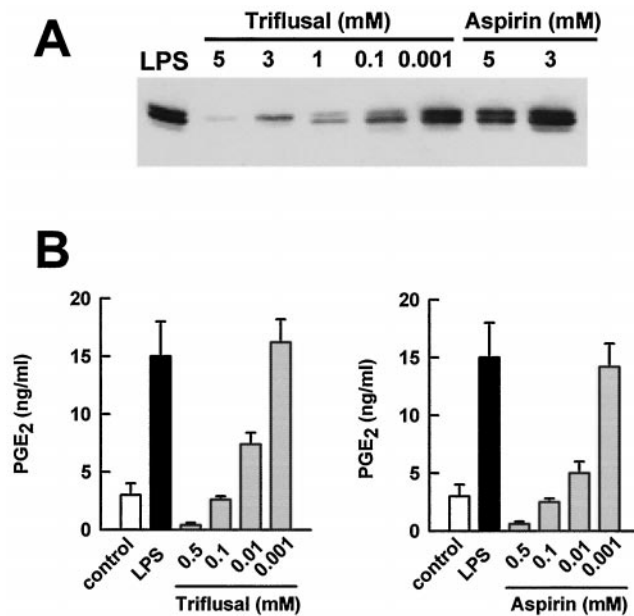
**Effects of Triflusal and Aspirin on COX-2 Expression and Activity in Infiltrating Cells in the Rat Air Pouch.** Carrageenan induced an outstanding prostaglandin release into the exudate fluid when it was administered into the intrascapular area of the control rats ( $72.3 \pm 14.7$  ng/ml). Vasodilation, edema, and significant increase in cellular influx were also observed after carrageenan injection. Polymorphonuclear leukocytes, monocyte/macrophages, and lympho-



**Fig. 3.** Time and dose dependence of LPS-induced COX-2 expression (A) and PGE<sub>2</sub> generation (B) by PBMCs. PBMCs ( $3 \times 10^6$  cells/ml) isolated from healthy donors were exposed to a fixed LPS concentration (10  $\mu$ g/ml) for 2 to 24 h or to increasing LPS concentrations (0.1–50  $\mu$ g/ml) for a fixed time of 19 h. COX-2 protein expression (A) and PGE<sub>2</sub> concentration in the medium (B) were determined by immunoblotting and a specific ELISA, respectively, as described in *Materials and Methods*. Immunoblots represent a typical experiment of two with comparable results. PGE<sub>2</sub> values are the mean  $\pm$  S.E.M. of determinations from two separate experiments.



**Fig. 4.** Effects of sodium salicylate and HTB on PGE<sub>2</sub> release and COX-2 expression in human PBMCs stimulated by *E. coli* LPS. PBMCs ( $3 \times 10^6$  cells/ml) were stimulated with LPS (10  $\mu$ g/ml) for a fixed time of 19 h in the presence or absence of HTB (0.01–5 mM) or sodium salicylate (1–5 mM). A, COX-2 protein expression determined by immunoblotting of cells incubated with LPS and vehicle (indicated as LPS) or with LPS and HTB or sodium salicylate; data shown are from a representative study performed four times with comparable results. B, PGE<sub>2</sub> concentration in the culture medium of cells incubated without LPS (Control), with LPS and vehicle (LPS), and with LPS and increasing concentrations of HTB or sodium salicylate (Salicylate); values reflect the mean  $\pm$  S.E.M. of duplicate determinations from three separate experiments.



**Fig. 5.** Effects of aspirin and triflusal on PGE<sub>2</sub> release and COX-2 expression in human PBMCs stimulated by *E. coli* LPS. PBMCs ( $3 \times 10^6$  cells/ml) were stimulated with LPS (10  $\mu$ g/ml) for a fixed time of 19 h in the presence or absence of triflusal (0.001–5 mM) or aspirin (0.001–5 mM). A, COX-2 protein expression determined by immunoblotting of cells incubated with LPS and vehicle (indicated as LPS) or with LPS and triflusal or aspirin; data shown are from a representative study performed four times with comparable results. B, PGE<sub>2</sub> concentration in the culture medium of cells incubated without LPS (Control), with LPS and vehicle (LPS), and with LPS and increasing concentrations of triflusal or aspirin; values are the mean  $\pm$  S.E.M. of determinations from three separate experiments.

cytes accounted for 86%, 12%, and 2% of the infiltrating cells, respectively.

The administration of triflusal (3–30 mg/kg p.o.) to rats (Fig. 7) inhibited in a dose-dependent manner COX-2 protein expression in the cells present in the exudate and caused a decrease in PGE<sub>2</sub> levels with an ID<sub>50</sub> value of 11.4 mg/kg. Aspirin at the same doses did not exert any detectable effect on COX-2 expression, although it inhibited PGE<sub>2</sub> release to the same extent than triflusal (ID<sub>50</sub> = 18.9 mg/kg). In contrast, the inhibitory effect of triflusal on PGE<sub>2</sub> production in stomachs was 6-fold lower than that of aspirin (ID<sub>50</sub> = 13.9 and 2.3 mg/kg, respectively), as shown in Fig. 8.

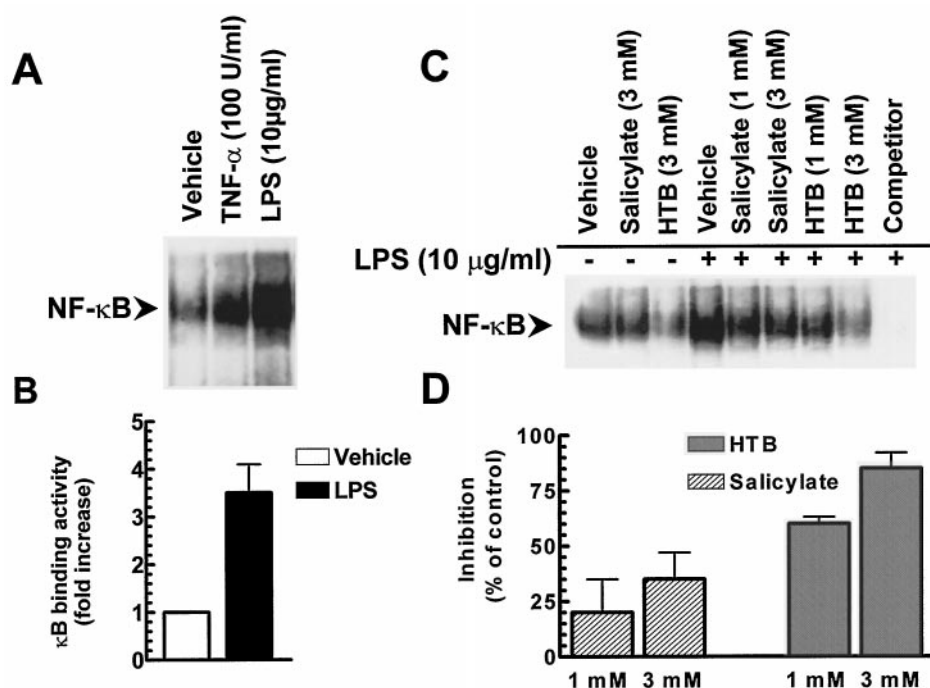
## Discussion

The search for selective COX-2 inhibitors started after the identification of this cytokine-inducible isoform of cyclooxygenase (Fu et al., 1990). So far, more than a dozen of these compounds have been described (Frölich, 1997), all of them behaving as competitive inhibitors of the enzyme. An alternative approach chosen by some research groups was to develop irreversible COX-2-selective inhibitors (Kalgutkar et al., 1998) that could prevent the resume of prostaglandin production once the drug plasma levels fall, in the same way as aspirin does with COX-1 and, to a lesser extent, with COX-2. Moreover, because of the fast response of COX-2 to induction by proinflammatory stimuli, another interesting approach could be to identify drugs that selectively block the expression of the enzyme (Pennisi, 1998).

In this context, the initial aim of the present study was to test the ability of two salicylate derivatives, triflusal and its deacetylated metabolite HTB, to inhibit COX-2-mediated PGE<sub>2</sub> synthesis in several experimental models. As a result of this study, the mechanism underlying PGE<sub>2</sub> synthesis inhibition by triflusal and HTB was elucidated, and it turned out to be clearly different than those of their counterparts aspirin and salicylate.

In a whole human blood assay *in vitro*, plasma PGE<sub>2</sub> levels increased 15-fold after 24-h incubation with 10 µg/ml LPS. Triflusal, aspirin, and HTB blocked this increase with apparent IC<sub>50</sub> values (Table 1) well within plasma concentrations found in the therapeutic use of triflusal or HTB, which can approach 1 mM (McNeely and Goa, 1998). Sodium salicylate in the range of doses tested (up to 5 mM) failed to exert any effect on this LPS-mediated PGE<sub>2</sub> synthesis. On the other hand, only aspirin and triflusal inhibited the purified COX-2 isoenzyme activity in a time-dependent fashion, whereas HTB and sodium salicylate were ineffective. Lack of inhibition of COX-2 activity by sodium salicylate has been reported by others (Frölich, 1997). It has also been reported that some salicylates are competitive inhibitors of arachidonic acid for the active site of the COX-2 (Frölich, 1997; Mitchell et al., 1997), and thus the substrate concentration becomes a rate-limiting factor. To evaluate the influence of this factor, two different concentrations of arachidonate (10 and 100 µM) were used. Neither HTB nor salicylate had any inhibitory effect, even at the lowest level of arachidonic acid tested.

Thus, the inhibition of LPS-induced PGE<sub>2</sub> release in human whole blood by HTB cannot be explained in terms of enzyme inhibition, and other explanations, such as a decrease in enzyme expression, had to be considered. Indeed, before the full establishment of the COX-1/COX-2 concept, Wu et al. (1991) showed that aspirin (and sodium salicylate) at concentrations as low as 60 nM partially inhibited the induction of COX in human umbilical vein endothelial cells (HUVECs) stimulated by interleukin-1. Moreover, the inhibition of COX expression was almost complete at an aspirin concentration of 2.7 mM (0.5 mg/ml). However, these striking results could not be reproduced by others using murine fibroblasts activated with phorbol ester (Kujubu and Herschman, 1992), rabbit alveolar macrophages stimulated with LPS (O'Sullivan et al., 1993), human macrophages stimulated with LPS (Barrios-Rodiles et al., 1996), the human pulmo-



**Fig. 6.** Effect of salicylate and HTB on the activation of NF-κB elicited by LPS. PBMCs were incubated with both TNF-α and LPS for 10 min to assess the effect of these stimuli on NF-κB activation (A). The increase of κB-binding activity produced by LPS in four independent experiments is shown in B. In other experiments, PBMCs were preincubated with salicylate and HTB before the incubation with LPS for an additional period of 10 min, and at the end of this periods the nuclear extract was collected and used for the assay of κB-binding activity. The lane marked "competitor" indicates that the nuclear extract was incubated with the <sup>32</sup>P-labeled probe in the presence of a 100-fold excess of unlabeled probe (C). The graph shows the inhibitory effect observed on κB-binding activity in LPS-treated cells expressed as mean ± S.E.M. of three blots quantified by densitometric scanning using software of the series Discovery 3.1 from PDI-Pharmacia (D).



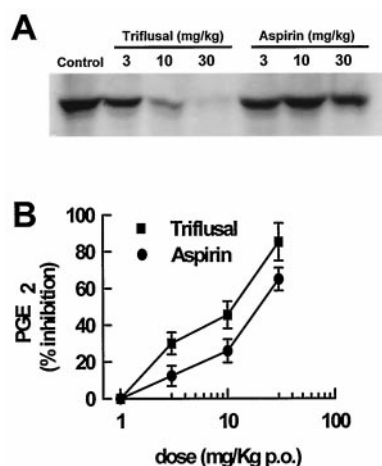
nary epithelial line A549 activated with interleukin-1 $\beta$  (Mitchell et al., 1997), or quiescent murine NIH 3T3 cells stimulated by the addition of 10% FCS (Spencer et al., 1998) or in an experimental system in which COX-2 expression in endothelial cells (HUVECs) was induced by coculture with tumor cells (Tsujii et al., 1998), despite the fact that much higher aspirin and/or sodium salicylate concentrations were tested. This disagreement could be due to a cell-specific regulation by salicylates of COX-2 expression, or it could reflect a stimulus-dependent response, as has been reported for the inhibition of tumor necrosis factor but not epidermal growth factor-induced mitogen-activated protein kinase activation by sodium salicylate (Schwenger et al., 1996). Nevertheless, because the human blood method we used is clearly dependent on LPS-induced de novo expression of COX-2 (Patrignani et al., 1994), we hypothesized that HTB and triflusal

could exert their effect on PGE<sub>2</sub> synthesis by suppressing COX-2 expression. This hypothesis was confirmed using human PBMCs, in which PGE<sub>2</sub> release and COX-2 expression were dose and time dependently activated by LPS (Fig. 3). HTB produced a concentration-dependent inhibition of both the PGE<sub>2</sub> release and the COX-2 expression. The inhibitory effect on LPS-induced COX-2 expression was evident at 0.1 mM and was complete at 3 mM. Unlike HTB, sodium salicylate, at concentrations up to 5 mM, had no effect on COX-2 expression. In addition, triflusal, but not aspirin, inhibited COX-2 expression to the same extent as HTB.

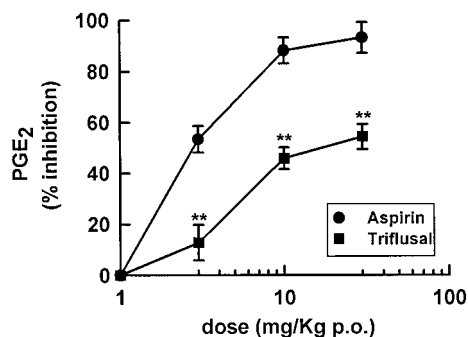
Provided that human COX-2 promoter has two putative NF- $\kappa$ B motifs and that NF- $\kappa$ B seems to be involved in the induction of COX-2 expression (Hwang et al., 1997; Newton et al., 1997), we wondered to what extent the effect of triflusal and HTB on COX-2 expression was mediated by the suppression of the LPS-inducible NF- $\kappa$ B activation. Our data show that the inhibitory effect of triflusal and HTB in NF- $\kappa$ B activation and COX-2 expression paralleled each other. Accordingly, NF- $\kappa$ B is clearly involved in the regulation of COX-2 expression in LPS-stimulated macrophages (D'Acquisto et al., 1997). However, the significant effect observed in NF- $\kappa$ B activation for aspirin and salicylate (Kopp and Ghosh, 1994; Weber et al., 1995) does not correspond with an observed similar effect on COX-2 expression in either this study or others (as mentioned). It agrees with a more complex regulation of COX-2 expression in which other transcription factors, as activator protein-1, NF-interleukin-6, or cAMP response element-binding protein, may be involved (Inoue et al., 1995; Miller et al., 1998).

In any case, HTB and triflusal are clearly more potent inhibitors of NF- $\kappa$ B than aspirin and sodium salicylate, showing a complete inhibition of  $\kappa$ B binding to the nuclear fraction at concentrations of 3 mM. Recently, the effect of both aspirin and salicylate on NF- $\kappa$ B activation has been mechanistically related to the selective inhibition of I $\kappa$ B kinase- $\beta$  (Yin et al., 1998) by interfering with ATP binding to the enzyme. It seems likely that a similar or related mechanism could explain the effect of both triflusal and HTB.

Next, we investigated whether the inhibition of COX-2 expression observed in the human PBMCs treated with triflusal or HTB in vitro can also be seen in a model of inflammation in vivo after treatment with triflusal at therapeutic doses. The rat s.c. air pouch model was used because it is characterized by the release of prostaglandins due to a rapid induction of COX-2 mRNA and protein in exudate cells (Masferrer et al., 1994). The administration of triflusal (3–30 mg/kg p.o.) blocked COX-2 expression in the cells present in the exudate and produced a dose-dependent decrease in PGE<sub>2</sub> synthesis. Aspirin at similar doses did not exert any detectable effect on COX-2 expression, although it inhibited PGE<sub>2</sub> to the same extent as triflusal. Thus, in vivo aspirin effect could be mainly attributed to enzyme inhibition, whereas COX-2 expression blockade better accounts for the inhibition of PGE<sub>2</sub> release by triflusal. Moreover, triflusal showed 6-fold less inhibitory effect than aspirin on PGE<sub>2</sub> production in the stomach, which is mainly due to COX-1. Thus, triflusal is 10 times more selective for COX-2 over COX-1 than aspirin (COX-1/COX-2 ID<sub>50</sub> ratio of 1.2 for triflusal and 0.12 for aspirin) in this model, and provided that COX-1 has cytoprotective effects in the gastric mucosa (Masferrer et al., 1994), this may explain the better gastric toler-



**Fig. 7.** Effect of aspirin and triflusal on COX-2 expression and PGE<sub>2</sub> synthesis at the site of inflammation in the carrageenan air pouch model in rats. Aspirin and triflusal were administered by gavage 30 min before the carrageenan injection. The animals were sacrificed 24 h later, and the exudate in the air pouch was collected. Cells of exudates from vehicle-treated (control) or triflusal- or aspirin-treated animals were pelleted by centrifugation, and COX-2 expression was determined by immunoblotting (A). The values are from a representative study (six animals per group) performed two times with comparable results. PGE<sub>2</sub> was determined in the exudate supernatants by specific ELISA (B). Values are expressed as a percentage of the PGE<sub>2</sub> measured in the exudate of vehicle-treated animals and reflect the mean  $\pm$  S.E.M. ( $n = 6$ ).



**Fig. 8.** Effect of aspirin and triflusal on PGE<sub>2</sub> synthesis in the stomachs of carrageenan-treated rats. Aspirin and triflusal were administered by gavage 30 min before the carrageenan injection. The animals were sacrificed 24 h later, and PGE<sub>2</sub> of the stomachs extracted as described in *Materials and Methods*. Values are expressed as a percentage of the PGE<sub>2</sub> measured in vehicle-treated animals and reflect the mean  $\pm$  S.E.M. ( $n = 6$ ). \*\* $p < .01$ , triflusal-treated versus aspirin-treated animals (Student's  $t$  test).

ance and lower incidence of bleeding events with triflusal compared with aspirin in the clinical use of both drugs (McNeely and Goa, 1998).

In conclusion, the data presented here show that triflusal and its main metabolite HTB are able to inhibit NF- $\kappa$ B activation and COX-2 expression in both human mononuclear cells in vitro and rat inflammatory cells in vivo. The concentrations required to elicit these effects are in keeping with the plasma concentrations achieved in therapeutic use (McNeely and Goa, 1998). Neither aspirin nor sodium salicylate shows significant effects on COX-2 expression in any of the experimental models tested. Thus, the introduction of the trifluoromethyl group in the 4-position of salicylates confers new properties to the molecule. To our knowledge, this is the first demonstration of an inhibition of COX-2 expression by salicylates at therapeutic concentration ranges. Moreover, because HTB does not affect COX-1 activity (De la Cruz et al., 1992), it becomes the first NSAID that selectively blocks the expression of the inducible isoform of COX, which makes it a possible and promising tool for knocking out this enzyme. These findings have several implications. First, triflusal and HTB may become useful tools in the investigation of the mechanism of action of salicylates in the diverse processes in which this family of drugs has been implicated. Second, because COX-2 expression and/or NF- $\kappa$ B activations play a role in a broad variety of pathological conditions, including inflammation, colorectal cancer, Alzheimer's disease, and stroke (Pennisi, 1998; Rustgi, 1998), the ability of triflusal and HTB to inhibit NF- $\kappa$ B activation/COX-2 expression at therapeutic concentrations may be useful in these diseases.

#### Acknowledgments

We thank Dr. Mariano Sanchez-Crespo from CSIC (Faculty de Medicina, Valladolid) for his helpful discussion and review of the manuscript and Assumpta Oliveras for her extremely appreciated technical assistance.

#### References

- Barrios-Rodiles M, Keller K, Belley A and Chadee K (1996) Nonsteroidal anti-inflammatory drugs inhibit cyclooxygenase-2 enzyme activity but not mRNA expression in human macrophages. *Biochem Biophys Res Commun* **225**:896–900.
- D'Acquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M and Carnuccio R (1997) Involvement of NF- $\kappa$ B in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett* **418**:175–178.
- De la Cruz JP, Mata JM and Sanchez De la Cuesta F (1992) Triflusal versus aspirin on the inhibition of human platelet and vascular cyclooxygenase. *Gen Pharmacol* **23**:297–300.
- Farivar RS and Brecher P (1996) Salicylate is a transcriptional inhibitor of the inducible nitric oxide synthase in cultured cardiac fibroblasts. *J Biol Chem* **271**:31585–31592.
- Frölich JC (1997) A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzyme. *Trends Pharmacol Sci* **18**:30–34.
- Fu JY, Masferrer JL, Seibert K, Raz A and Needleman P (1990) The induction and suppression of prostaglandin H<sub>2</sub> synthase (cyclooxygenase) in human monocytes. *J Biol Chem* **265**:16737–16740.
- Grilli M, Pizzi M, Memo M and Spano P (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF- $\kappa$ B activation. *Science (Wash DC)* **274**:1383–1385.
- Hartner A, Goppelt-Strube M and Hilgers KF (1998) Coordinate expression of cyclooxygenase-2 and renin in the rat kidney in renovascular hypertension. *Hypertension* **31** (Suppl 2):201–205.
- Hwang D, Jang BC, Yu G and Boudreau M (1997) Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: Mediation through both mitogen activated protein kinase and NF- $\kappa$ B signaling pathways in macrophages. *Biochem Pharmacol* **54**:87–96.
- Inoue H, Yokoyama C, Hara S, Tone Y and Tanabe T (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. *J Biol Chem* **270**:24965–24971.
- Kalgtutkar AS, Crews BC, Rowlinson SW, Garner C, Seibert K and Marnett LJ (1998) Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science (Wash DC)* **280**:1268–1270.
- Kopp E and Ghosh S (1994) Inhibition of NF- $\kappa$ B binding by sodium salicylate and aspirin. *Science (Wash DC)* **265**:956–959.
- Kujubu DA and Herschman HR (1992) Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene. *J Biol Chem* **267**:7991–7994.
- Lim H, Paria BC, Das SK, Dinchuck JE, Langenbach R, Trazskos JM and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91**:197–208.
- Levy GN (1997) Prostaglandin H synthases, non-steroidal antiinflammatory drugs and colon cancer. *FASEB J* **11**:234–247.
- Loll PJ, Picot D and Garavito RM (1995) The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H<sub>2</sub> synthase. *Nat Struct Biol* **2**:637–643.
- Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC and Seibert K (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* **91**:3228–3232.
- McNeely W and Goa KL (1998) Triflusal. *Drugs* **55**:823–835.
- Miller C, Zhang M, He Y, Zhao J, Pelletier JP, Martel-Pelletier J and Di Battista JA (1998) Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphate activity in human chondrocytes: Co-stimulation of AP-1 and CRE nuclear binding proteins. *J Cell Biochem* **69**:392–413.
- Mitchell JA, Larkin S and Williams TJ (1995) Cyclooxygenase-2: Regulation and relevance in inflammation. *Biochem Pharmacol* **50**:1535–1542.
- Mitchell JA, Saunders M, Barnes PJ, Newton R and Belvisi MG (1997) Sodium salicylate inhibits cyclooxygenase-2 activity independently of transcription factor (nuclear factor- $\kappa$ B) activation: Role of arachidonic acid. *Mol Pharmacol* **51**:907–912.
- Newton R, Kuitert LM, Bergmann M, Adcock IM and Barnes PJ (1997) Evidence for involvement of NF- $\kappa$ B in the transcriptional control of COX-2 gene expression by IL-1 $\beta$ . *Biochem Biophys Res Commun* **237**:28–32.
- Oka A and Takashima S (1997) Induction of cyclooxygenase 2 in brain of patients with Down's syndrome and dementia of Alzheimer type: Specific localization in affected neurones and axons. *Neuroreport* **8**:1161–1164.
- O'Sullivan MG, Huggins Jr EM and McCall CE (1993) LPS-induced expression of prostaglandin H synthase-2 in alveolar macrophages is inhibited by dexamethasone but not by aspirin. *Biochem Biophys Res Commun* **191**:1294–1300.
- Patrignani P, Panara MR, Greco A, Fusco O, Natoli C, Iacobelli S, Cipollone F, Ganci A, Crémion C, Maclof J and Patrono C (1994) Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. *J Pharmacol Exp Ther* **271**:1705–1712.
- Pennisi E (1998) Building a better aspirin (Research News). *Science (Wash DC)* **280**:1191–1192.
- Pierce JW, Read MA, Ding H, Lusinskas FW and Collins T (1996) Salicylates inhibit I $\kappa$ B- $\alpha$  phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. *J Immunol* **156**:3961–3969.
- Rustgi AK, (1998) Cyclooxygenase-2: The future is now (News and Views). *Nat Med* **4**:773–774.
- Sawdy R, Slater D, Fisk N, Edmonds DK and Bennett P (1997) Use of a cyclooxygenase type-2-selective non-steroidal anti-inflammatory agent to prevent preterm delivery. *Lancet* **350**:265–266.
- Schwenger P, Skolnik EY and Vilcek J (1996) Inhibition of tumor necrosis factor-induced p42/p44 mitogen-activated protein kinase activation by sodium salicylate. *J Biol Chem* **271**:8089–8094.
- Sirois J and Dore M (1997) The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as determinant of the ovulatory process. *Endocrinology* **138**:4427–4434.
- Smith WL, Garavito RM and DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* **271**:33157–33160.
- Spencer AG, Woods JW, Arakawa T, Singer II and Smith WL (1998) Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J Biol Chem* **273**:9886–9893.
- Tsuji M, Kawano S, Tsujii S, Sawaka H, Hori M and DuBois RN (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93**:705–716.
- Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature (London)* **231**:232–235.
- Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J and Willoughby DA (1994) Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci USA* **91**:2046–2050.
- Vervordeltonk MJ, Pineda TI, Aarsman AJ and van den Bosch H (1996) Aspirin inhibits expression of the interleukin-1 $\beta$ -inducible group II phospholipase A<sub>2</sub>. *FEBS Lett* **397**:108–112.
- Weber C, Erl W, Pietsch A and Weber PC (1995) Aspirin inhibits nuclear factor- $\kappa$ B mobilization and monocyte adhesion in stimulated human endothelial cells. *Circulation* **91**:1914–1917.
- Wong SCY, Fukuchi M, Melnyk P, Rodger I and Giada A (1998) Induction of cyclooxygenase-2 and activation of nuclear factor- $\kappa$ B in myocardium of patients with congestive heart failure. *Circulation* **98**:100–103.
- Wu KK, Sanduja R, Tsai AL, Ferhanoglu B and Loose-Mitchell DS (1991) Aspirin inhibits expression of the interleukin-1 $\beta$ -induced-prostaglandin H synthase expression in cultured endothelial cells. *Proc Natl Acad Sci USA* **88**:238–2387.
- Yin M-J, Yamamoto Y and Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I $\kappa$ B kinase- $\beta$ . *Nature (London)* **396**:77–80.

Send reprint requests to: Dr. Javier Forn, Department of Pharmacology, Uriach Research Center, Degá Bahí, 59-67, 08026 Barcelona, Spain. E-mail rd@uriach.com