Biochemical and Biophysical Research Communications **274,** 197–202 (2000)
doi:10.1006/bbrc.2000.3123, available online at http://www.idealibrary.com on IDEAL®

Salicylate Metabolites Inhibit Cyclooxygenase-2-Dependent Prostaglandin E_2 Synthesis in Murine Macrophages

Burkhard Hinz, Volker Kraus, Andreas Pahl, and Kay Brune
Department of Experimental and Clinical Pharmacology and Toxicology, Friedrich Alexander University Erlangen–Nürnberg, Fahrstrasse 17, D-91054 Erlangen, Germany

Received June 22, 2000

The poor cyclooxygenase (COX) inhibitor and major aspirin metabolite salicylic acid is known to exert analgesic and anti-inflammatory effects by still unidentified mechanisms. In RAW 264.7 macrophages, lipopolysaccharide (LPS)-induced COX-2-dependent synthesis of prostaglandin E_2 (PGE_2) was suppressed by aspirin (IC_{50} of 5.35 μM), whereas no significant inhibition was observed in the presence of sodium salicylate and the salicylate metabolite salicyluric acid at concentrations up to 100 μM. However, the salicylate metabolite gentisic acid (2,5-dihydroxybenzoic acid; 10–100 μM) and salicyl-coenzyme A (100 μM), the intermediate product in the formation of salicyluric acid from salicylic acid, significantly suppressed LPS-induced PGE_2 production. In contrast, γ-resorcylic acid (2,6-dihydroxybenzoic acid) as well as unconjugated coenzyme A failed to affect prostanoid synthesis, implying that the *para*-substitution of hydroxy groups and the activated coenzyme A thioester are important for COX-2 inhibition. Using real-time RT-PCR, none of the salicylate derivatives tested were found to interfere with COX-2 expression. Overall, our results suggest that certain metabolites of salicylic acid may contribute to the pharmacological action of its parent compound by inhibiting COX-2-dependent PGE_2 formation at sites of inflammation.

Key Words: **salicylic acid; non-steroidal anti-inflammatory drugs; cyclooxygenase-2; RAW 264.7 cells.**

Non-steroidal 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 (1, 2). Recently, it has been demonstrated that COX exists as two genetically distinct isoforms. COX-1 is constitutively expressed as a "housekeeping" enzyme in most tissues and mediates physiological responses such as regulation of platelet function and cytoprotection of the stomach. COX-2 encoded by an immediate-early gene can be up-regulated by various proinflammatory agents, including endotoxin, cytokines and mitogens (3–5) and has been shown to be the isoform primarily responsible for the synthesis of prostanoids involved in pathological processes such as acute and chronic inflammatory states (6, 7). Most of the NSAIDs inhibit both COX-1 and COX-2, although they vary in their relative potencies against the two isozymes (8). Whereas the mechanism-based side effect of NSAIDs (e.g., gastrointestinal toxicity, platelet dysfunction) are due to suppression of COX-1-derived prostanoids, the pharmacological action of NSAIDs has been ascribed to inhibition of COX-2 activity at sites of inflammation.

However, the theory that suppression of prostaglandin biosynthesis accounts for the pharmacological effects of NSAIDs has been questioned by comparing the actions of salicylate and aspirin (9). Salicylate does not, unlike its acetylated derivative aspirin, inhibit COX-1 and COX-2 activity *in vitro* (1, 10, 11). However, salicylate has been shown to exert a comparable analgesic and anti-inflammatory action as aspirin (12). Several suggestions have been made to describe how salicylates exert their pharmacological effects. From the data published by Kopp and Ghosh (13) it appears that inhibition of the transcription factor nuclear factor κB (NF-κB) could be a mechanism by which salicylates exert their anti-inflammatory action. However, relatively high concentrations of sodium salicylate (i.e., higher than that obtained after therapeutic dosing) were required to provide inhibition of NF-κB activa-

Abbreviations used: CoA, coenzyme A; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; NSAIDs, non-steroidal anti-inflammatory drugs; NF-κB, nuclear factor κB; PGE_2, prostaglandin E_2; salicyl-CoA, salicyl-coenzyme A.

0006-291X/00 $35.00

tion. Moreover, it has been proposed previously that salicylic acid mediates inhibition of prostaglandin synthesis via a metabolite (14, 15).

The aim of the present study was to investigate the influence of three salicylate metabolites on the COX-2-dependent formation of prostaglandin E_2 (PGE_2) in murine macrophages. The macrophage cell line (RAW 264.7) used in our experiments has been established as a suitable model to investigate compounds interfering with lipopolysaccharide (LPS)-inducible inflammatory cascades including the COX-2 pathway (16–18). Here we show that the salicylate metabolites gentisic acid and salicyl-coenzyme A (CoA) inhibit LPS-induced PGE_2 formation in RAW 264.7 cells. In conclusion, our results imply that certain metabolites of salicylic acid may contribute to the pharmacological action of its parent compound by inhibiting COX-2-derived prostanoid synthesis *in vivo* at sites of inflammation.

MATERIALS AND METHODS

Materials. Dulbecco's modified essential medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose was purchased from BioWhittaker (Verviers, Belgium). Fetal calf serum and penicillin-streptomycin were bought from Boehringer-Mannheim (Mannheim, Germany). LPS from *E. coli* (serotype 026:B6), aspirin, sodium salicylate, salicyluric acid, gentisic acid (2,5-dihydroxybenzoic acid), γ-resorcylic acid (2,6-dihydroxybenzoic acid), CoA, hydrocortisone and all other reagents were purchased from Sigma (Deisenhofen, Germany).

Preparation of salicyl-CoA. Salicyl-CoA was synthesized according to a modification of the protocol published by Mieyal *et al.* (19). Briefly, a suspension of salicylic acid in toluene was heated until all acid was dissolved. The equimolar concentration of thionyl chloride was dissolved in toluene and slowly added to the hot solution. Afterwards, the solution was refluxed for two hours, and toluene was removed under reduced pressure. The synthesized salicyl chloride was subsequently added dropwise to a rapidly stirred solution containing CoA in water adjusted to pH 8.0 with LiOH. The pH was kept at 7–8 by periodic additions of 2 M LiOH. After 1 h the suspension was adjusted to pH 3 with concentrated HCl, and the formed precipitate was removed by centrifugation. Aqueous and organic phases were evaporated to dryness under reduced pressure. The residue was extracted 10 times with 10 ml acetone:methanol (10:1, v/v) until chloride was undetectable in the extract. Thereafter, the residue was dissolved in phosphate buffer and monitored by fractionating HPLC. For qualitative analysis, salicyl-CoA was dissolved in D_2O and analyzed by ^1H NMR and UV spectroscopy. Quantification of salicyl-CoA was performed after hydrolysis with NaOH (2 M) by HPLC technique.

Cell culture. RAW 264.7 cells (ATCC TIB 71; American Type Culture Collection, Rockville, MD, USA) were maintained and subcultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

Incubation protocol. RAW 264.7 cells were seeded at 5×10^5 cells/well in 24-well plates. Cells were preincubated with the respective test compound for 30 min. Thereafter, LPS was added and the incubation was continued for an additional 24 h. Afterwards, supernatants were removed for determination of PGE_2 and cells were lysed for subsequent RNA isolation. Total RNA was isolated using the RNeasy total RNA Kit (Qiagen, Hilden, Germany).

Determination of PGE_2. PGE_2 concentrations were determined using a commercially available enzyme immunoassay kit (Cayman, Ann Arbor, MI). Basal PGE_2 levels were taken to represent the lower limit of stimulated mediator release (i.e., 0%), whereas the mean of PGE_2 levels determined in the LPS-treated group was used as the maximal possible mediator release (i.e., 100%). The percentage stimulation of PGE_2 release was calculated according to the following formula: % stimulation = {[PGE_2 level (LPS + test compound) − PGE_2 level (basal)]/[PGE_2 level (LPS) − PGE_2 level (basal)]} × 100%.

Quantitative RT-PCR analysis. β-Actin (internal standard) 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-carboxytetramethylrhodamine; TAMRA) at an internal thymidine. 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), the latter being defined as the cycle at which the 6FAM fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3 to 10. COX-2 mRNA levels were normalized to the housekeeping gene β-actin according to the following formula: C_T (COX-2) − C_T (β-actin) = ΔC_T. Subsequently, respective 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 of approximately 100%.

RT-PCR was performed in 25-μl reaction volumes containing 1× reaction buffer (50 mM Bicine, 115 mM KOAc, 10 μM EDTA, 8% (w/v) glycerol, pH 8.2), $Mn(OAc)_2$ solution (4 mM), 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, Münster, Germany). 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 s, 60°C for 1 min. RNA samples were amplified using commercially synthesized primers specific for murine β-actin 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. COX-2 sense primer, 5′-TTTGTTGAGTCATTCACCAGACAGAT; COX-2 antisense primer, 5′-CAGTATTGAGGAGAACAGATGGGATT; COX-2 probe, 5′-(6FAM) CTACCATGGTC (TAMRA)TCCCCAAAGATAGCATCA.

RESULTS

Incubation of RAW 264.7 macrophages with LPS for 24 h substantially increased PGE_2 levels in cell culture supernatants from 0.46 ± 0.17 ng/ml (mean ± SEM, $n = 7$) under basal conditions to 29.7 ± 5.3 ng/ml (mean ± SEM, $n = 12$). To evaluate the influence of different salicylic acid derivatives on prostanoid formation, RAW 264.7 cells were pretreated with the respective test compound for 30 min before inducing COX-2 expression with LPS for an additional 24 h. According to Fig. 1, aspirin inhibited LPS-induced PGE_2 forma-

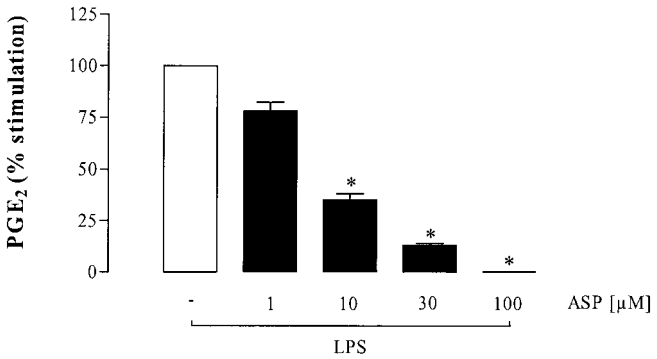

FIG. 1. Influence of aspirin (ASP; 1–100 μM) on LPS-induced PGE_2 synthesis in RAW 264.7 cells. Following a 30-min preincubation of cells with aspirin, LPS (10 μg/ml) was added to the cultures and the incubation was continued for an additional 24 h. PGE_2 levels were determined in cell culture supernatants. Values are means ± SEM of $n = 3$ observations. *$P < 0.05$, treatment vs control (open column), Student's t test.

tion with an IC_{50} of 5.35 μM, whereas sodium salicylate caused only a slight decrease of PGE_2 biosynthesis (17% inhibition at 100 μM) (Fig. 2). Incubation of cells with the oxymetabolite gentisic acid (2,5-dihydroxybenzoic acid) led to a significant attenuation of LPS-induced PGE_2 production at a concentration as low as 10 μM reaching a 62% inhibition at 100 μM (Fig. 3). In contrast, an isomer of gentisic acid, γ-resorcylic acid (2,6-dihydroxybenzoic acid), was virtually inactive in this respect (Fig. 3). Treatment of cells with the glycine conjugate and major salicylate metabolite, salicyluric acid, failed to suppress PGE_2 synthesis, whereas a significant inhibition was observed in the presence of salicyl-CoA, the intermediate product in the formation of salicyluric acid (Fig. 4). Under the same experimental conditions, unconjugated CoA had no influence on LPS-induced PGE_2 synthesis (Fig. 4). To assess the influence of the test compounds on COX-2 expression,

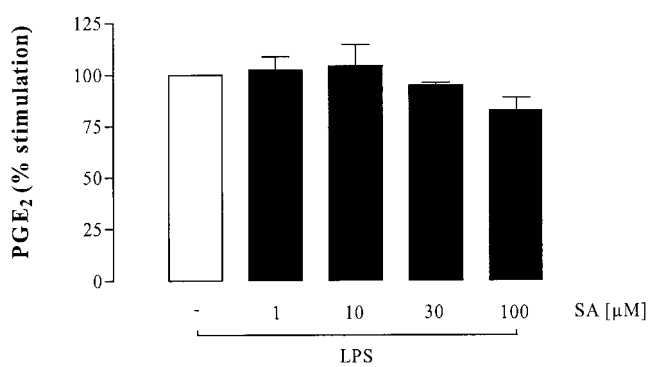

FIG. 2. Influence of sodium salicylate (SA; 1–100 μM) on LPS-induced PGE_2 synthesis in RAW 264.7 cells. Following a 30-min preincubation of cells with sodium salicylate, LPS (10 μg/ml) was added to the cultures and the incubation was continued for an additional 24 h. PGE_2 levels were determined in cell culture supernatants. Values are means ± SEM of $n = 3$ observations.

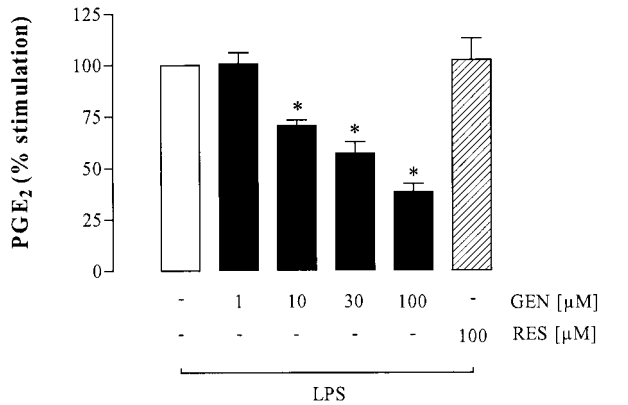

FIG. 3. Influence of gentisic acid (GEN; 1–100 μM) and γ-resorcylic acid (RES; 100 μM) on LPS-induced PGE_2 synthesis in RAW 264.7 cells. Following a 30-min preincubation of cells with the respective test compound, LPS (10 μg/ml) was added to the cultures and the incubation was continued for an additional 24 h. PGE_2 levels were determined in cell culture supernatants. Values are means ± SEM of $n = 3$ observations. *$P < 0.05$, treatment vs control (open column), Student's t test.

COX-2 mRNA levels were determined using quantitative real-time RT-PCR. Following a 24-h incubation of cells with LPS, a marked increase in COX-2 mRNA levels was observed. However, all salicylic acid derivatives tested failed to inhibit LPS-induced COX-2 mRNA expression (Fig. 5). In contrast, incubation of cells with hydrocortisone (1 μM) being used as a positive control led to a 92% inhibition of LPS-induced COX-2 expression (Fig. 5) accompanied by a 95% inhibition of PGE_2 formation in cell culture supernatants (data not shown).

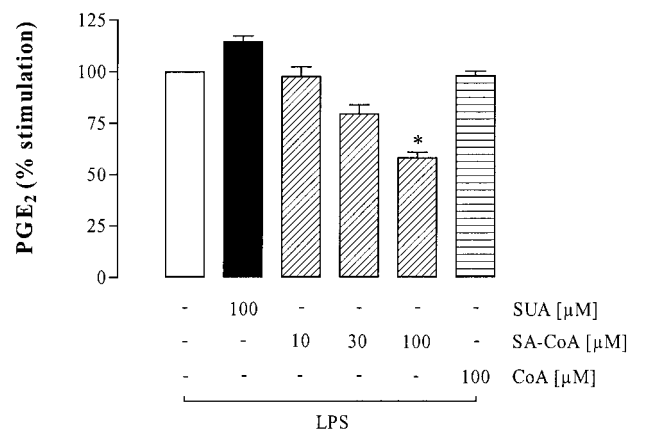

FIG. 4. Influence of salicyluric acid (SUA; 100 μM), salicyl-CoA (SA-CoA; 10–100 μM) and CoA (100 μM) on LPS-induced PGE_2 synthesis in RAW 264.7 cells. Following a 30-min preincubation of cells with the respective test compound, LPS (10 μg/ml) was added to the cultures and the incubation was continued for an additional 24 h. PGE_2 levels were determined in cell culture supernatants. Values are means ± SEM. of $n = 3$ observations. *$P < 0.05$, treatment vs control (open column), Student's t test.

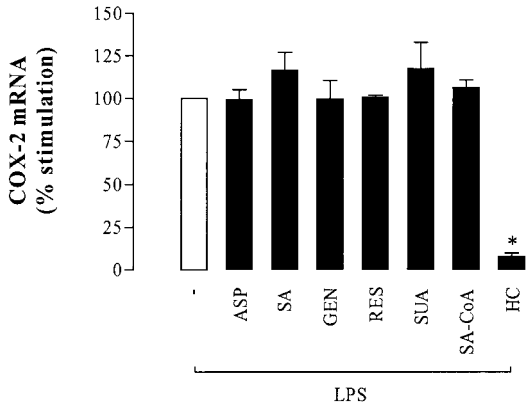

FIG. 5. Influence of aspirin (ASP; 100 μM), sodium salicylate (SA; 100 μM), gentisic acid (GEN; 100 μM), γ-resorcylic acid (RES; 100 μM), salicyluric acid (SUA; 100 μM), salicyl-CoA (SA-CoA; 100 μM), and hydrocortisone (HC; 1 μM) on LPS-induced COX-2 mRNA expression. Following a 30-min preincubation of cells with the respective test compound, LPS (10 μg/ml) was added to the cultures and the incubation was continued for an additional 24 h. COX-2 mRNA levels were determined as described under Materials and Methods. Values are means ± SEM of $n = 3$ observations. $^*P < 0.05$, treatment vs control (open column), Student's t test.

DISCUSSION

Aspirin and salicylic acid are well-known NSAIDs for over a century now. Following oral administration, aspirin appears to be rapidly deacetylated to salicylic acid, the latter being the major mediator of the analgesic and anti-inflammatory properties of aspirin (20, 21). However, unlike aspirin and other NSAIDs, salicylic acid possesses virtually no inhibitory effect on the activity of the COX isozymes *in vitro* (1, 10, 11). On the other side, sodium salicylate has been demonstrated to be an effective inhibitor of prostaglandin formation *in vivo* at sites of inflammation (22) and to be equally effective against arthritis as aspirin (12). Thus, information regarding the influence of salicylate metabolites on COX-2-derived prostaglandin biosynthesis could lead to a better understanding of how salicylic acid exerts its pharmacological action.

In the present study we observed a substantial inhibition of COX-2-derived PGE_2 synthesis in RAW 264.7 macrophages in the presence of aspirin, whereas incubation of cells with salicylic acid, the immediate metabolite of aspirin *in vivo*, did not result in significant suppression of LPS-induced PGE_2 levels. On the other hand, a considerable attenuation of PGE_2 formation was observed in the presence of gentisic acid which is produced *in vivo* by hydroxylation of salicylate and accounts for less than 5% of the ingested salicylic acid (23). However, albeit the fraction metabolized to gentisic acid is rather small, the latter has been implicated as a metabolite with antioxidant properties that is predominantly formed under inflammatory conditions by polymorphonuclear leukocytes producing reactive oxygen radicals (24–27). In agreement with these studies, febrile patients undergoing salicylate treatment have previously been shown to excrete higher levels of gentisate than healthy subjects (28). In our study, gentisic acid significantly inhibited PGE_2 formation at a concentration as low as 10 μM which is within the range of plasma and synovial fluid concentrations being achieved in patients with rheumatoid arthritis receiving salicylate (24, 25). Moreover, the concentration and uptake of salicylate and, consequently, gentisic acid can be enhanced at sites of inflammation through capillary damage leading to extravasation of protein-bound substances (29). Interestingly, inhibition of PGE_2 synthesis was confined to gentisic acid (2,5-dihydroxybenzoic acid) and was not elicited by its isomer γ-resorcylic acid (2,6-dihydroxybenzoic acid) suggesting that the specific para-substitution of the hydroxy groups confers the capacity of gentisic acid to interfere with COX-2-derived prostanoid formation.

Like many other carboxylic acid xenobiotics, salicylic acid is conjugated with glycine before excretion. This conjugation involves a two step pathway including the activation of salicylic acid to a CoA thioester by the action of a CoA synthetase (30) and the subsequent transfer of the acyl moiety to the amino group of glycine by the action of a glycine *N*-acylase (31). In the present study the glycine conjugate and major salicylate metabolite salicyluric acid, which accounts for up to 65% of ingested salicylic acid and aspirin, respectively (23, 32), failed to inhibit LPS-induced PGE_2 synthesis. On the contrary to this finding, salicyl-CoA, the intermediate thioester formed during the biosynthesis of salicylurate in liver and kidney (30, 33), caused a significant inhibition of PGE_2 production in RAW 264.7 cells. The inhibition of COX-2-derived PGE_2 biosynthesis by *R*(−)-ibuprofenoyl-CoA (intermediate in the metabolic chiral inversion of the pharmacologically inactive *R*(−)-ibuprofen into its potent *S*(+)-antipode) demonstrated in a previous study from this lab (34) prompted us to analyze the action of salicyl-CoA on PGE_2 formation. According to our results, the introduction of a CoA moiety into the salicylate molecule confers comparable properties. In contrast to salicyl-CoA, unconjugated CoA failed to affect PGE_2 synthesis suggesting that inhibition of the inducible COX-2 isozyme is confined to the activated CoA thioester. However, no studies have been published so far focusing on the determination of salicyl-CoA following administration of clinically used doses of aspirin and salicylate, respectively.

Different mechanisms have been proposed to explain the anti-inflammatory effects of salicylic acid suggesting that its pharmacological action may involve multiple steps with the formation of active metabolites being only one possible mode of action. Accordingly, both aspirin and sodium salicylate have been reported to inhibit neutrophil activation by interfering with signal

transduction pathways independently of inhibition of prostaglandin synthesis (35). Moreover, salicylates have been recently shown to inhibit COX-2 expression in human umbilical vein endothelial cells and foreskin fibroblasts (36). However, this observation is not in line with other studies performed in murine fibroblasts (37), rabbit alveolar macrophages (38), human macrophages (39) or murine NIH 3T3 cells (40). According to our results, none of the salicylate derivatives tested suppressed COX-2 mRNA levels. On the other hand and in agreement with previous findings (4, 5, 37, 38), COX-2 expression was inhibited by the glucocorticoid hydrocortisone which was used as a positive control. In several studies, sodium salicylate at millimolar concentrations has been shown to suppress NF-κB-mediated transcriptional activation by inhibiting degradation of the IκB molecule (13, 41, 42). As the expression of a number of inflammatory mediators requires the activation of the transcription factor NF-κB, this effect has been suggested as a further possible mechanism by which salicylate exerts its anti-inflammatory action. However, to inhibit NF-κB activation salicylate concentrations were required that are beyond the levels achieved *in vivo* even after administration of antirheumatic salicylate doses (12). As pointed out by Franz and O'Neill (43), salicylates may non-specifically inhibit kinase activities at supratherapeutic concentrations supporting the view that inhibition of NF-κB may simply be attributable to non-specific and toxic properties of high salicylate concentrations. It therefore appears questionable, whether an interference with the activation of NF-κB accounts for the pharmacological action of salicylic acid.

In summary, our data demonstrate that the salicylate metabolites gentisic acid and salicyl-CoA inhibit COX-2-derived PGE_2 synthesis suggesting that salicylic acid requires further bioactivating steps before acting as an inhibitor of the COX-2 isozyme *in vivo*. In conclusion, our results imply an additional mechanism for the action of salicylic acid, and may thus add a new aspect to a pharmacological enigma regarding the actions of aspirin and salicylate.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 353/A9). We thank Martin Gillich and Eva Hoier for expert technical assistance.

REFERENCES

1. Vane, J. R. (1971) *Nature New Biol.* **231,** 232–235.
2. Smith, W. L., and Marnett, L. J. (1991) *Biochim. Biophys. Acta* **1083,** 1–17.
3. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2692–2696.
4. Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., and Hwang, D. (1992) *J. Biol. Chem.* **267,** 25934–25938.
5. Mitchell, J. A., Belvisi, M. G., Akarasereenont, P., Robbins, R. A., Kwon, O. J., Croxtall, J., Barnes, P. J., and Vane, J. R. (1994) *Br. J. Pharmacol.* **113,** 1008–1014.
6. Herschman, H. R. (1996) *Biochim. Biophys. Acta* **1299,** 125–140.
7. Brune, K., Zeilhofer, H. U., and Hinz, B. (1999) *in* Fast Facts—Rheumatology Highlights 1998–99 (Emery, P., Ed.), pp. 18–24, Health Press, Oxford.
8. Patrignani, P., Panara, M. R., Sciulli, M. G., Santini, G., Renda, G., and Patrono, C. (1997) *J. Physiol. Pharmacol.* **48,** 623–631.
9. Weissmann, G. (1993) *J. Lipid Mediat.* **6,** 275–286.
10. Vargaftig, B. B. (1978) *J. Pharm. Pharmacol.* **30,** 101–104.
11. Cromlish, W. A., and Kennedy, B. P. (1996) *Biochem. Pharmacol.* **52,** 1777–1785.
12. Preston, S. J., Arnold, M. H., Beller, E. M., Brooks, P. M., and Buchanan, W. W. (1989) *Br. J. Clin. Pharmacol.* **27,** 607–611.
13. Kopp, E., and Ghosh, S. (1994) *Science* **265,** 956–959.
14. Vane, J. R., and Botting, R. M. (1995) *Inflamm. Res.* **44,** 1–10.
15. Blackwell, G. J., Flower, R. J., and Vane, J. R. (1975) *Biochim. Biophys. Acta* **398,** 178–190.
16. Suh, N., Honda, T., Finlay, H. J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q. W., Nathan, C., Gribble, G. W., and Sporn, M. B. (1998) *Cancer Res.* **58,** 717–723.
17. Hinz, B., Brune, K., and Pahl, A. (2000) *Biochem. Biophys. Res. Commun.* **271,** 353–357.
18. Hinz, B., Brune, K., and Pahl, A. (2000) *Biochem. Biophys. Res. Commun.* **272,** 744–748.
19. Mieyal, J. J., Webster, L. T., Jr., and Siddiqui, U. A. (1974) *J. Biol. Chem.* **249,** 2633–2640.
20. Levy, G. (1979) *Drug Metab. Rev.* **9,** 3–19.
21. Ali, M., McDonald, J. W., Thiessen, J. J., and Coates, P. E. (1980) *Stroke* **11,** 9–13.
22. Whittle, B. J., Higgs, G. A., Eakins, K. E., Moncada, S., and Vane, J. R. (1980) *Nature* **284,** 271–273.
23. Shen, J., Wanwimolruk, S., Purves, R. D., McQueen, E. G., and Roberts, M. S. (1991) *J. Pharmacokinet. Biopharm.* **19,** 575–595.
24. Cleland, L. G., Whitehouse, M. W., and Betts, W. H. (1985) *Drugs Exp. Clin. Res.* **11,** 463–467.
25. Grootveld, M., and Halliwell, B. (1986) *Biochem. J.* **237,** 499–504.
26. Sagone, A. L., Jr., and Husney, R. M. (1987) *J. Immunol.* **138,** 2177–2183.
27. Haynes, D. R., Wright, P. F. A., Gadd, S. J., Whitehouse, M. W., and Vernon-Roberts, B. (1993) *Agents Actions* **39,** 49–58.
28. Kapp, E. M., and Coburn, A. F. (1942) *J. Biol. Chem.* **145,** 549–565.
29. Brune, K., and Lanz, R. (1985) *in* Handbook of Inflammation (Bonta, I. L., Bray, M. A., and Parnham, M. J., Eds.), Vol. 5, pp. 413–449, Elsevier, Amsterdam.
30. Killenberg, P. G., Davidson, E. D., and Webster, L. T., Jr. (1971) *Mol. Pharmacol.* **7,** 260–268.
31. Schachter, D., and Taggart, J. V. (1953) *J. Biol. Chem.* **203,** 925–934.
32. Needs, C. J., and Brooks, P. M. (1985) *Clin. Pharmacokinet.* **10,** 164–177.
33. Tishler, S. L., and Goldman, P. (1970) *Biochem. Pharmacol.* **19,** 143–150.
34. Neupert, W., Brugger, R., Euchenhofer, C., Brune, K., and Geisslinger, G. (1997) *Br. J. Pharmacol.* **122,** 487–492.

35. Abramson, S. B., Leszczynska-Piziak, J., Clancy, R. M., Philips, M., and Weissmann, G. (1994) *Biochem. Pharmacol.* **47,** 563–572.
36. Xu, X.-M., Sansores-Garcia, L., Chen, X.-M., Matijevic-Aleksic, N., Du, M., and Wu, K. K. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 5292–5297.
37. Kujubu, D. A., and Herschman, H. R. (1992) *J. Biol. Chem.* **267,** 7991–7994.
38. O'Sullivan, M. G., Huggins, E. M., Jr., and McCall, C. E. (1993) *Biochem. Biophys. Res. Commun.* **191,** 1294–1300.
39. Barrios-Rodiles, M., Keller, K., Belley, A., and Chadee, K. (1996) *Biochem. Biophys. Res. Commun.* **225,** 896–900.
40. Spencer, A. G., Woods, J. W., Arakawa, T., Singer, I. I., and Smith, W. L. (1998) *J. Biol. Chem.* **273,** 9886–9893.
41. Gautam, S. C., Pindolia, K. R., Noth, C. J., Janakiraman, N., Xu, Y. X., and Chapman, R. A. (1995) *Blood* **86,** 2541–2550.
42. Vervoordeldonk, M. J., Pineda Torra, I. M., Aarsman, A. J., and van den Bosch, H. (1996) *FEBS Lett.* **397,** 108–112.
43. Franz, B., and O'Neill, E. A. (1995) *Science* **270,** 2017–2019.