Sodium Salicylate Inhibits Prostaglandin Formation without Affecting the Induction of Cyclooxygenase-2 by Bacterial Lipopolysaccharide in Vivo

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ABSTRACT

The mechanisms underlying the anti-inflammatory properties of salicylate are not well understood. In particular, while salicylate inhibits prostaglandin production in vivo it only weakly inhibits cyclooxygenase (COX)-1 or -2 activity in vitro. Thus, it has often been suggested that in vivo salicylate may inhibit the expression rather than the activity of COX, particularly COX-2. Using a model of acute COX-2 expression in the rat, we show that salicylate inhibits COX-2 activity in vivo without affecting COX-2 expression. In anesthetized rats LPS (6 mg kg⁻¹, i.p.) increased the expression of COX-2 as evidenced by increased circulating levels of 6-keto-prostaglandin F1α (6-keto-PGF1α, a stable breakdown product of PGl2), greatly exaggerated formation of 6-keto-PGF1α, following arachidonic acid (AA) challenge (3 mg kg⁻¹, i.v.), and increased expression of COX-2, but not COX-1, protein. Diclofenac (3 mg kg⁻¹, i.p.) or the COX-2 selective agent diisopropyl fluorophosphate (10 mg kg⁻¹, i.p.) decreased the LPS-induced increase in circulating 6-keto-PGF1α, and the exaggerated 6-keto-PGF1α production following AA challenge. Sodium salicylate (20 or 120 mg kg⁻¹, i.p.) (administered either 1 h prior, or once per day for 3 days prior, to LPS injection) reduced only the LPS-induced increase in circulating 6-keto-PGF1α, but not the exaggerated 6-keto-PGF1α production following AA challenge or the expression of COX-2. Thus, salicylate inhibits LPS-induced COX-2 activity in a manner that is overcome by provision of excess substrate and independent of effects on COX-2 expression. In conclusion, our results exclude mechanisms other than direct enzyme inhibition as responsible for the anti-COX effects of salicylate.

Inhibition of cyclooxygenase (COX), and therefore prostaglandin production, is the common mechanism of action of nonsteroid anti-inflammatory drugs (NSAIDs; Vane, 1971). As is now well appreciated, COX exists as two isoforms. In general terms, cyclooxygenase-1 (COX-1) is constitutive and present in, for example, the endothelium, stomach, and kidney, whereas cyclooxygenase-2 (COX-2) is induced by pro-inflammatory cytokines and endotoxin in cells in vitro and at inflammatory sites in vivo (Mitchell and Warner, 1999). Although revisited following the discovery of COX-1 and COX-2, Vane’s findings still explain the biochemical mechanism of action of NSAIDs. However, the mechanism of action of salicylate remains a debated issue.

In vivo salicylate is approximately equipotent to aspirin as an anti-inflammatory (Smith et al., 1975) and as an inhibitor of prostaglandin formation at inflammatory sites (Higgs et al., 1987). However, in vitro salicylate is a much weaker inhibitor of prostaglandin formation than aspirin (Vane, 1971). This simple observation has led many researchers to look for alternative mechanisms of action to explain the anti-inflammatory activity of salicylate. Of particular popularity has been the idea that salicylate and aspirin exert their anti-inflammatory effects by inhibiting the activation of NF-κB (Kopp and Ghosh, 1994). For example, salicylate has been shown to suppress a number of NF-κB-mediated responses, including chemokine and adhesion molecule gene expression (Gautam et al., 1995; Weber et al., 1995), cytokine-dependent transcription of inducible nitric-oxide synthase (iNOS) (Farivar et al., 1996), and cellular kinase activity (Frantz and O’Neill, 1995). Thus, despite some contrasting evidence (O’Sullivan et al., 1993; Barrios-Rodiles et al. 1996) by the end of 1996, salicylate was being reviewed as a transcription-modulating drug (Beauparlant and Hiscott, 1996; Cai et al., 1996; Goodnight, 1996).

More recently, the idea that salicylate’s anti-inflammatory activity is exerted through inhibition of COX expression
rather than by inhibition of COX activity has gained new support (Wu, 1998; Xu et al., 1999). However, Mitchell et al. (1997) showed in vitro that the ability of salicylate to inhibit COX is influenced by experimental conditions, particularly by the supply of arachidonic acid, the substrate for COX substrate. Indeed, a moment’s reflection on enzyme/inhibitor interactions reminds us that an enzyme inhibitor that acts by competing with the substrate will appear increasingly less active in the presence of increasingly high levels of substrate. Thus, when the excess amounts of arachidonic acid often used in in vitro assays are reduced, salicylate readily inhibits COX activity at concentrations far below those required to inhibit NF-κB activation (Mitchell et al., 1997).

Despite the large volume of literature regarding salicylate, particularly the popular theory that salicylate inhibits the induction of COX-2 expression, no one has actually explored the effects of salicylate upon the expression and activity of COX-2 in vivo. Using a model of LPS-dependent COX-2 induction in the rat, we investigated whether there is any evidence for the transcription-modulating properties of salicylate characterized in vitro underlying the inhibition of prostaglandin production observed in vivo. Some of these results have been presented to the British Pharmacological Society (Giuliano and Warner, 2000).

**Experimental Procedures**

### Materials

All compounds used were obtained from Sigma (Poole, UK) unless otherwise stated. DFP (Leblanc et al., 1999) was a gift from Merck Frosst, Kirkland, Quebec, Canada. For the radioimmunoassays, antisera to 6-keto-PGF$_{1α}$ was obtained from Sigma, and [$^3$H]6-keto-PGF$_{1α}$ was purchased from Amersham Pharmacia Biotech UK, Ltd., (Little Chalfont, Buckinghamshire, UK).

### Surgical Procedure

Male Wistar rats (220–250 g; Tuck, Rayleigh, UK) were anesthetized by injection of thiobutabarbitual sodium (120 mg kg$^{-1}$, i.p.). Body temperature was maintained at 37°C by means of a homeothermic blanket (Harvard Apparatus, Edenbridge, UK) connected to a rectal probe. The trachea was cannulated to facilitate ventilation. The right carotid artery was cannulated and connected to a pressure transducer (PDCR 75, Druck Ltd., Leicester, UK) for the monitoring of systemic blood pressure through a digital recording device (PowelLab 85a, ADInstruments, Hastings, UK). The jugular vein was also cannulated to allow infusion of saline (4 ml kg$^{-1}$ h$^{-1}$) and injection of arachidonic acid (see below). Following surgery, animals were left for 30 min to stabilize.

### Experimental Design

**Single Administration of Drugs.** Upon completion of the surgical procedure, animals in the control and LPS groups were injected ($t = 0$ h) with 2 ml kg$^{-1}$ saline i.p or *Escherichia coli* LPS (serotype 0127: B8; 6 mg kg$^{-1}$, i.p.), respectively. Rats in the LPS group were treated either at time $t = -1$ h or $t = 4$ h with vehicle (10% dimethyl sulfoxide in saline) or 20 or 120 mg kg$^{-1}$ sodium salicylate. To avoid any direct effect on the induction of COX-2 protein diclofenac (3 mg kg$^{-1}$) or the selective COX-2 inhibitor DFP (10 mg kg$^{-1}$) were only administered at $t = 4$ h. Six hours after LPS was injected, animals were challenged with arachidonic acid (3 mg kg$^{-1}$), which was given as a bolus via the jugular vein. Blood samples (300 μl) were taken via the carotid artery cannula at $t = 0, 2, 4$, and $6$ h, and 1 min after administration of the AA bolus ($t = 6$ AA). The samples were centrifuged at 12,000g for 5 min (4°C), and the plasma was removed, supplemented with heparin (15 U ml$^{-1}$), National Veterinary Sup-

### Measurement of Plasma Prostaglandin 6-Keto-PGF$_{1α}$

Treatment of anesthetized rats with LPS induces the expression of COX-2 (Salvemini et al., 1995), which is correlated to elevations in the plasma levels of 6-keto-PGF$_{1α}$ (Hamilton et al., 1999). Therefore, as an index of COX-2 activity, we measured 6-keto-PGF$_{1α}$ levels in the plasma samples by radioimmunoassay, as previously described (Hamilton et al., 1999).

### Measurement of Nitrate/Nitrite Plasma Levels

LPS treatment of anesthetized rats induces an increase in NO production following inducible NO synthase (iNOS) expression (Thiemermann, 1997). In the circulation, NO is largely converted to nitrate, so nitrite/nitrate measurement can be used as an index of NO synthesis. The nitrate present in plasma samples from control and LPS-treated rats was enzymatically converted to nitrite following the procedure described by Schmidt et al. (1992). The total nitrite concentration was then determined spectrophotometrically by the Griess reaction.

### Western Blot Analysis

Rat lung or aortae were homogenized in 6 volumes of ice-cold protein extraction buffer (0.5 mM EDTA, 0.5 mM EGTA, 0.2 mM leupeptin, 0.07 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged at 17,500g (rotor Beckman 70.1 Ti) for 15 min. The resulting pellets were homogenized in extraction buffer containing 0.1% Triton X-100 and then centrifuged at 17,500g for 30 min. The supernatant was then collected and centrifuged at 100,000g for 1 h. Pellets obtained were taken to be the membrane fraction and resuspended in extracting buffer containing 1% Triton X-100. Protein concentration was determined by the Bradford colorimetric assay (Bradford, 1976). Protein extracts were then diluted in a 1:1 ratio with sample buffer and boiled for 5 min (sample buffer: 50 mM Tris-HCl pH 6.8, 10% w/v SDS, 10% w/v glycerol, 10% v/v 2-mercaptoethanol, and 0.02% w/v bromphenol blue). Equal amounts of protein (75 μg) were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis for 1 h at 100 V. The separated proteins were then electrotransferred to nitrocellulose (Hybond-C super, Amersham Pharmacia Biotech UK, Ltd.) at 80 V for 1 h. Following electrotransfer, the blots were incubated overnight at 4°C in blocking solution (5% w/v dried low-fat milk and 0.1% v/v Tween 20 in phosphate-buffered saline) on an orbital shaker. The blots were then washed (three times, 5 min each) with washing buffer (TWEEN 20 0.1% v/v in phosphate-buffered saline) before being probed (1 h at room temperature) with anti-COX-2 or anti-COX-1 antibody (rabbit anti-mouse; SPI Bio, Massy Cedex, France) diluted 1:1000 in blocking solution. Following incubation with the primary antibody, the blots were washed (three times, 5 min) with blocking solution before being probed (1 h at room temperature) with alkaline phosphatase-conjugate secondary antibody (anti-rabbit IgG; New England Biolabs, Ltd., Hitchin, UK) diluted 1:2000 in blocking solution. The blots were then developed using Phototope-horseradish peroxidase West-
ern blot detection kit (New England BioLabs, Ltd.). Images were captured on Hyperfilm (Amer sham Pharmacia Biotech UK, Ltd.) and acquired by a Macintosh computer connected to a densitometer (GS-700, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Densitometric analyses were by Molecular Analyst (Bio-Rad Laboratories Ltd.).

Data Analysis

Data are reported as mean ± S.E.M. Statistical analyses as described in individual legends were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Results

Effect of LPS on 6-Keto-PGF$_{1α}$ Formation. The circulating levels of 6-keto-PGF$_{1α}$ in naive rats were significantly increased following treatment with LPS (Fig. 1). Similarly, the acute production of PGF$_2$ following AA (3 mg kg$^{-1}$, i.v.) challenge (determined by the plasma levels of 6-keto-PGF$_{1α}$; Fig. 1, t = 6AA) was greatly exaggerated in animals exposed to LPS.

Effects of Salicylate, Diclofenac, and DFP on LPS-Associated 6-Keto-PGF$_{1α}$ Formation. In naive rats 20 or 120 mg kg$^{-1}$ salicylate (administered 1 h before LPS) significantly inhibited the elevation in circulating 6-keto-PGF$_{1α}$ caused by LPS treatment (Fig. 2). However, neither dose of salicylate affected the exaggerated response to AA injection seen in LPS-treated animals (Fig. 2). Even when administered to LPS-treated rats just 2 h before AA challenge (i.e., at t = 4 h after LPS) 20 or 120 mg kg$^{-1}$ salicylate still had no effect upon the exaggerated AA-induced 6-keto-PGF$_{1α}$ production (Fig. 3). Unlike salicylate, both diclofenac (3 mg kg$^{-1}$) and the selective COX-2 inhibitor DFP (10 mg kg$^{-1}$) reduced by more than 90% the exaggerated increase in 6-keto-PGF$_{1α}$ formation following AA challenge in LPS-treated rats (Fig. 3).

Pretreatment of rats for 3 days with either saline or salicylate caused no significant change in the basal circulating plasma levels of 6-keto-PGF$_{1α}$. In these pretreated animals exposure to LPS caused no elevations in the circulating levels of 6-keto-PGF$_{1α}$. In rats pretreated with saline, as in naive animals, LPS produced a greatly exaggerated response to bolus injection of AA (Figs. 1 and 4). This exaggerated response following exposure to LPS was not different in rats pretreated for 3 days with salicylate (Fig. 4).

Effects of Salicylate on LPS-Dependent Nitrate/Nitrite Accumulation. Because activation of NF-κB has been associated with the induction of iNOS activity, we measured the time-dependent increase in the circulating concentrations of nitrate/nitrite that followed LPS injection as a fur-
Salicylate Does Not Inhibit Cyclooxygenase-2 Induction

Discussion

Our study shows clearly in vivo, for the first time, that salicylate inhibits the activity of COX-2, i.e., formation of PGL₂, independent of an effect upon the expression of COX-2. Indeed, salicylate acts as we would expect a weak competitive inhibitor to act; it fails to reduce PGL₂ formation when excess substrate is present. It does not act as an inhibitor of COX-2 expression; no reduction in COX-2 expression was detected by Western blot analysis. Furthermore, if salicylate did act to reduce the amount of COX-2 enzyme this would not be so simply reversed as by the addition of excess substrate. Therefore, salicylate can be seen simply to act as an inhibitor of the enzymatic activity of COX-2, as we also show for the traditional NSAID diclofenac and the selective COX-2-inhibitor DFP. Therefore, there is no need for us to raise the possibility that salicylate acts in some way to influence the expression of COX-2, e.g., by inhibiting NF-κB.

Our previous studies have established a clear correlation between LPS administration, COX-2 induction and 6-keto-PGF₁α accumulation in anesthetized rats (Hamilton and Warner, 1998). This was confirmed when we found that LPS administration induced the cep-regulation of COX-2 protein (but not COX-1) and an increase in the plasma levels of 6-keto-PGF₁α. COX-2 expression was principally determined in lung tissue, because previous studies (Hamilton and Warner, 1998) have demonstrated that LPS administration induces the expression of COX-2 in the vascular endothelium, and the lung contains approximately half the body’s endothelial cells. For confirmation we also examined the expression of COX-2 in some aortic samples because in earlier studies applying immunohistochemical analysis to this model we have found induction of COX-2 expression in the endothelium of the aorta, heart, spleen, and kidney, as well as the lung (L. C. Hamilton and T. D. Warner, unpublished observations). The increased circulating levels of 6-keto-PGF₁α, that followed exposure to LPS were reduced by salicylate, diclofenac, and the COX-2-selective inhibitor DFP.

Subsequent challenge with arachidonic acid (3 mg kg⁻¹; the highest dose that could routinely be administered without immediate death of the experimental animals) revealed that COX-2 induction was associated with a greatly exaggerated increase in 6-keto-PGF₁α levels; i.e., that the synthetic capacity for 6-keto-PGF₁α was much more greatly enhanced than might appear from the simple measurement of circulating 6-keto-PGF₁α levels. However, unlike its effect on the 6-h circulating accumulation 6-keto-PGF₁α levels, and in contrast to diclofenac and DFP, salicylate was without effect on the exaggerated formation of 6-keto-PGF₁α, that followed bolus administration of AA. This was true even if rats were pretreated with salicylate for the preceding 3 days at a dose as high as 120 mg kg⁻¹; this pretreatment regimen was employed because salicylate’s serum half-life increases with dose (i.e., high, repeated doses allow the highest salicylate levels in tissues to be reached; Needs and Brooks, 1985).

These observations lead to a clear conclusion about the...
salicylates, and in particular the instability of aspirin within such systems cannot account for the pharmacokinetics of supratherapeutic concentrations (0.0010.001 in cell culture systems and employed drugs at concentrations. For example, those studies that show transcription-
licylate are demonstrated are not relevant to in vivo conditions under which transcription-modifying properties of salicylate are demonstrated.

The rats at high doses for the preceding 3 days. Similar observations have been made in vitro, where high concentrations of salicylate have been reported to cause a complete return of synthetic capacity. Second, our observations give no support to the notion that salicylate inhibits the induction of COX-2. First, if salicylate had influenced by the supply of substrate (Mitchell et al., 1997). More importantly, although referring to therapeutic plasma concentrations (Insel, 1996) the majority of

**TABLE 1**

Effect of salicylate on NO\textsubscript{2}/NO\textsubscript{3} (\textmu M) accumulation in plasma

<table>
<thead>
<tr>
<th>Time (h) Blood Sample Taken</th>
<th>n</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no LPS)\textsuperscript{a}</td>
<td>4</td>
<td>21 ± 3</td>
<td>27 ± 2</td>
<td>32 ± 5</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>LPS + vehicle\textsuperscript{b}</td>
<td>5</td>
<td>24 ± 2</td>
<td>35 ± 2</td>
<td>111 ± 13\textsuperscript{**}</td>
<td>226 ± 33\textsuperscript{***}</td>
</tr>
<tr>
<td>LPS + salicylate 20\textsuperscript{b}</td>
<td>7</td>
<td>14 ± 5</td>
<td>22 ± 6</td>
<td>119 ± 13</td>
<td>275 ± 28</td>
</tr>
<tr>
<td>LPS + salicylate 120\textsuperscript{b}</td>
<td>5</td>
<td>17 ± 4</td>
<td>29 ± 4</td>
<td>152 ± 9</td>
<td>306 ± 26</td>
</tr>
<tr>
<td>LPS + diclofenac\textsuperscript{c}</td>
<td>4</td>
<td>27 ± 9</td>
<td>34 ± 5</td>
<td>131 ± 24</td>
<td>287 ± 53</td>
</tr>
<tr>
<td>LPS + DFP\textsuperscript{d}</td>
<td>5</td>
<td>14 ± 2</td>
<td>19 ± 3</td>
<td>109 ± 13</td>
<td>283 ± 32</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Control vs. vehicle, \textsuperscript{b}p < 0.05, \textsuperscript{**}p < 0.01, \textsuperscript{***}p < 0.001.

Our observations give no support to the notion that salicylate inhibits the induction of COX-2. First, if salicylate had reduced the expression of COX-2, increasing the supply of substrate (i.e., bolus administration of AA) should not simply cause a complete return of synthetic capacity. Second, our Western blot analysis showed that neither high nor low doses of salicylate had any effect on the induction of COX-2 in the lung or in the aortic endothelium. Consistent with this observation and in contrast to results from in vitro studies in which high concentrations of salicylate have been reported to reduce iNOS expression (Amin et al., 1995; Rege et al., 1996; Sakitani et al., 1997; Kim et al., 1998), we found salicylate to have no effect upon the increases in plasma nitrate induced by LPS, even when salicylate was given to the rats at high doses for the preceding 3 days.

Many authors have concluded that the anti-inflammatory activity of salicylate and aspirin could be explained by its ability to affect the activation of NF-\kappa B (Yin et al., 1998) or mitogen-activated protein kinases (Alpert et al., 1999), or by nonspecific effects on cellular kinases (Frant and O’Neill, 1995) rather than, or in addition to, the inhibition of COX. However, if salicylate acts as a transcription-modifying drug with effects on the NF-\kappa B pathway, why did we find no evidence of any such effect in vivo, despite reductions in the formation of PGI\textsubscript{2}? Most probably because the in vitro conditions under which transcription-modifying properties of salicylate are demonstrated are not relevant to in vivo conditions. For example, those studies that show transcription-modifying effects of salicylate or aspirin have all been conducted in cell culture systems and employed drugs at supratherapeutic concentrations (0.001–0.02 M). Obviously, such systems cannot account for the pharmacokinetics of salicylates, and in particular the instability of aspirin within the circulation (Needs and Brooks, 1985; Giuliano and Warner, 1999). More importantly, although referring to therapeutic plasma concentrations (Insel, 1996) the majority of

**Fig. 6.** A, Western blot analysis of COX-2 protein in rat lung homogenate. Lanes: 1, COX-1 standard (25 ng); 2, COX-2 standard (25 ng); 3, lung homogenate from control rat; 4–6, lung homogenates from LPS-treated rats injected at t = −1 h with vehicle (lane 4), salicylate 20 mg kg\textsuperscript{−1} (lane 5) or salicylate 120 mg kg\textsuperscript{−1} (lane 6). LPS treatment up-regulated COX-2 expression, and this was not affected by salicylate 20 or 120 mg kg\textsuperscript{−1}. Gel typical of three experiments. B, densitometric analysis of displayed gel and two additional experiments confirming up-regulation of COX-2 (\textsuperscript{***}, p < 0.001, one-way ANOVA plus Bonferroni’s test) and lack of effect of salicylate (p > 0.05).
the researchers investigating salicylate’s pharmacology have failed to appreciate that in the normal therapeutic concentration range salicylate is strongly bound by plasma proteins (80–90%) and that it is the unbound fraction that accounts for pharmacological effects in vivo, i.e., the total plasma concentrations of NSAIDs represent a scarcely meaningful clinical index (Brouwers and de Smet, 1994). It follows that in vitro testing of salicylate at these therapeutic plasma concentrations is cast serious doubts on the conclusions drawn in vivo. Indeed, in the LPS-treated rat, we cannot clearly see that sodium salicylate, even at very high sustained doses, has no effect on the expression of COX-2 protein, although it does inhibit COX-2-dependent formation of PGL2. The ability of salicylate to inhibit COX-2 activity in a weak substrate-dependent manner correlates well with its ability to act as an anti-inflammatory only at high doses; i.e., at inflammatory sites where phospholipase activity is increased and arachidonic acid more abundant (Nevalainen et al., 2000), salicylate is a weak COX inhibitor.

Despite the relative abundance of publications claiming that salicylate may exert its anti-COX effect through mechanisms other than inhibition of COX activity, we have found no such evidence in vivo. Indeed, in the LPS-treated rat, we could not demonstrate in vivo. Indeed, in all the studies cited above (Amin et al., 1995; Kepka-Lenhart et al., 1996; Sakitani et al., 1997; Kim et al., 1998), supratherapeutic concentrations of salicylate were employed in systems with low protein binding.

The physiological relevance of the findings of salicylates influencing COX expression has never been fully investigated, although an interesting study has been recently published (Xu et al., 1999). This study showed that pretreatment of mice with aspirin caused a dose-dependent reduction of COX-2 mRNA in peritoneal macrophages following administration of LPS. Surprisingly, however, these same authors did not explore the functional relevance of the reduction in COX-2 transcription. In particular, due to the instability of mRNA for COX-2 it is unlikely that it is linearly related to COX-2 protein expression. Furthermore, as is made clear here and in earlier studies (Hamilton et al., 1999), the formation of COX-2 products is highly regulated by the supply of substrate. Thus, despite a partial suppression of COX-2 mRNA, changes in COX-2 protein expression could well be insignificant and the total production of COX-2-derived prostanoids unaffected.

References


Fig. 7. A, Western blot analysis of COX-2 protein in rat aortic homogenates. Lanes: 1, COX-2 standard (25 ng); 2, COX-1 standard (25 ng); 3, aortic homogenate from control rat; 4 and 5, aortic homogenates from LPS-treated rats injected at t = −1 with vehicle (lane 4) or salicylate 120 mg kg−1 (lane 5). LPS treatment up-regulated COX-2 expression, and this was not affected by salicylate 120 mg kg−1. Gel typical of three experiments. B, densitometric analysis of displayed gel and two additional experiments confirming up-regulation of COX-2 (***, p < 0.001, one-way ANOVA plus Bonferroni’s test) and lack of effect of salicylate (p > 0.05).


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