Origins of Prostaglandin E$_2$: Involvements of Cyclooxygenase (COX)-1 and COX-2 in Human and Rat Systems

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ABSTRACT

Prostaglandin (PG) E$_2$ is a major cyclooxygenase (COX) product at inflammatory sites where it contributes to local increases in blood flow, edema formation, and pain sensitization. Using rats in vivo and rat and human blood in vitro, we have examined the roles of COX-1 and COX-2 in the production of PGE$_2$. In anesthetized rats treated with bacterial lipopolysaccharide (LPS) to induce the expression of COX-2, the marked increase in PGE$_2$ production that followed bolus intravenous injection of arachidonic acid (3 mg kg$^{-1}$) was strongly inhibited by diclofenac but largely unaffected by the COX-2-selective inhibitor DFP (5,5-dimethyl-3-(2-propoxy)-4-methanesulfonylphenyl)-2(5H)-furanone. In rat blood in vitro, aspirin strongly inhibited the production of PGE$_2$ that followed either acute exposure to calcium ionophore, A$_2$3187 (calcimycin) (50 μM, 15 min), or incubation with LPS for 18 h. In contrast, human whole blood only produced significant levels of PGE$_2$ when incubated with LPS. Rat leukocytes expressed COX-2 and produced PGE$_2$ when exposed to LPS but not when acutely stimulated with A$_2$3187. Rat platelets, but not human platelets, also produced significant amounts of PGE$_2$ when acutely stimulated with A$_2$3187. These data show that when exposed to an inflammatory stimulus, rat whole blood produces increased levels of PGE$_2$ through induction of COX-2 in blood leukocytes. Rat blood, unlike human blood, may also produce copious amounts of PGE$_2$ via the actions of COX-1 enzyme constitutively present in platelets. These data may well explain why in rats COX-2-selective inhibitors have been reported not to produce the full anti-inflammatory effects associated with standard nonsteroid anti-inflammatory drugs.

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ABBREVIATIONS: PGE$_2$, prostaglandin E$_2$; COX, cyclooxygenase; NSAIDs, nonsteroid anti-inflammatory drugs; DFP, 5,5-dimethyl-3-(2-propoxy)-4-methanesulfonylphenyl)-2(5H)-furanone; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; A$_2$3187, calcimycin; PRP, platelet-rich plasma.
the British Pharmacological Society (Giuliano and Warner, 2001).

Materials and Methods

Materials

All compounds used were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. Antibodies for immunoblotting and DFP (Leblanc et al., 1999) were a gift from Merck Frosst (Quebec, Canada). For the radioimmunoassays, antiserum to PGE2 was obtained from Sigma-Aldrich; [3H]PGE2 was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, UK).

Surgical Procedure in Rats

Male Wistar rats were obtained from Tuck (Rayleigh, UK) and kept according to the guidelines set by the Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures (1989). Rats (220–250 g) were anesthetized with thiobutabarbital sodium (Inactin; 120 mg kg\(^{-1}\), i.p.). Body temperature was maintained at 37°C by means of a homeothermic blanket connected to a rectal probe. The trachea was cannulated (1.67 × 2.42-mm tubing) to facilitate ventilation. The right carotid artery was cannulated (0.58 × 0.96-mm tubing) and connected to a pressure transducer for the monitoring of systemic blood pressure, which was displayed on a computer linked to a digital data acquisition system (PowerLab 8/s, ADInstruments, Hastings, UK). The jugular vein was also cannulated (0.40 × 0.80-mm tubing) to allow injection of drugs and/or infusion of saline as necessary. At the end of each experiment, animals were killed by an overdose of anesthetic.

In Vivo Experimental Design

Upon completion of the surgical procedure, animals in the control and bacterial lipopolysaccharide (LPS) groups were injected (i.p.) or the selective COX-2 inhibitor DFP (10 mg kg\(^{-1}\), i.p.) as described below. The plasma obtained was stored (-40°C) until measurement of PGE2 by a radioimmunoassay.

Withdrawal of Blood

Rats. Male Wistar rats (250–280 g) were injected with a lethal dose of pentobarbitone sodium (Sagittai; 120 mg kg\(^{-1}\), i.p.). Upon occurrence of deep anesthesia, a laparotomy was performed, and blood from the abdominal aorta was collected into a tube containing 30 IU ml\(^{-1}\) heparin using a plastic syringe connected to an 18-gauge needle. Animals were then killed by thoracotomy.

Humans. Blood from healthy volunteers (25–55 years) who had not taken NSAIDs for at least 2 weeks was withdrawn from the antecubital vein using a 16-gauge butterfly needle and collected in a plastic tube containing heparin (20 IU ml\(^{-1}\), final).

Isolation of Rat Leukocytes

Rat leukocytes were isolated from heparinized whole blood by dextran sedimentation of erythrocytes. In detail, 0.8 parts of blood as that used to obtain platelet-rich plasma (PRP). In Protocol 2 and 3, leukocytes were isolated from rat blood as described above. The cells obtained were resuspended at a density of 8 × 10\(^6\) ml\(^{-1}\) in culture medium plus 10% fetal bovine serum and plated into 96-well plates (100 µl/well). These were then treated as described for whole blood under Protocol 1.

Protocol 2. Leukocytes were isolated from rat blood as described above. The cells obtained were resuspended at a density of 8 × 10\(^6\) ml\(^{-1}\) in culture medium plus 10% fetal bovine serum and plated into 96-well plates (100 µl/well). These were then treated as described for whole blood under Protocol 1.

Protocol 3. Rat platelet-rich plasma was obtained by centrifuging freshly collected heparinized (30 IU ml\(^{-1}\)) blood at 200g for 10 min. Blood in the platelet-rich plasma was aliquoted into 96-well plates (100 µl/well) and treated with either vehicle (0.1% DMSO in culture medium), aspirin (100 µM), or DFP (10 µM). Following incubation for 30 min (37°C, 5% CO\(_2\)/95% air), the Ca\(^{2+}\) ionophore A23187 (50 µM) was added, and rat platelet-rich plasma was incubated for further 15 min. The plates were then centrifuged (as above), and the supernatant was stored until measurement of PGE2 by a radioimmunoassay. The treatment just described was also carried out in parallel on whole blood and washed leukocytes originating from the same batch of blood as that used to obtain platelet-rich plasma (PRP).

Protocol 4. Freshly taken human and rat blood was aliquoted in plastic tubes (100 µl/tube) and then treated with the Ca\(^{2+}\) ionophore A23187 (50 µM). Fifteen minutes later the tubes were centrifuged (1500g, 4°C, 5 min), and the plasma obtained was stored (-40°C) until measurement of PGE2 by a radioimmunoassay.

Western Blot Analysis

Rat leukocytes from a single animal (5 × 10\(^5\)–7 × 10\(^7\) cells) were divided into three aliquots. One aliquot (basal) was immediately processed to be later used for immunoblotting. The remaining two aliquots were seeded onto individual 35-mm Petri dishes and incubated (37°C, 5% CO\(_2\)/95% air), one in the absence and one in the presence of LPS (100 µg ml\(^{-1}\)). Eighteen hours later cell extracts were prepared (lysis buffer: 50 mM tris-HCl, 10 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM peptatin A, and 0.2 mM leupeptin in ddH\(_2\)O) and later used for gel electrophoresis and Western blot analysis. The protein concentrations of cell lysates were determined by the Bradford colorimetric assay (Bradford, 1976). Equal amounts of protein (20 µg) were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis for 1 h at 100 V. The proteins were then electro-transferred to nitrocellulose (Hybond-C, Amersham Biosciences UK, Ltd.) at 80 V for 1 h. Following electro-transfer, the blots were incubated overnight at 4°C in blocking solution (5% w/v dry low-fat milk and 0.1% v/v Tween 20 in phosphate-buffered saline) on an orbital shaker. The blots were then washed (3 × 5 min) with washing buffer (TWEEN 20 0.1% v/v in phosphate-buffered saline) before being probed (1 h at room temperature) with anti-COX-1 (1:3000) or anti-COX-2 antibody (1:5000) diluted in blocking solution. Following incubation with the primary antibody, the blots were washed (3 × 5 min) with blocking solution before being probed (1 h room temperature) with the horseradish peroxidase-conjugate secondary antibody (supplied as part of the Phototope Kit, see below) diluted 1:2000 in blocking solution. The blots were then developed using Phototope-HRP Western blot detec-
tion kit (New England Biolabs, Hitchin, UK). Images were captured on Hyperfilm (New England Biolabs) and acquired by a Macintosh computer connected to a densitometer. Densitometric analyses were by Molecular Analyst (Bio-Rad, Hemel Hempstead, UK).

Data Analysis

Data are expressed as mean ± S.E.M. of separate determinations as specified in individual figure legends. Unless otherwise stated, statistical analyses were performed by applying a one-way analysis of the variance followed by Dunnett’s post-test. A P value smaller than 0.05 indicated a statistical difference.

For Figs. 3 and 5, PGE₂ formation was calculated as the difference between the PGE₂ measured after Ca²⁺ ionophore treatment and the PGE₂ accumulated in the same samples over 18 h. All graphs and analyses were by Prism 3.0 (GraphPad Software, San Diego, CA).

Terminology

In the writing of this article, we have used the word “accumulation” to signify the release of mediators in the absence of stimuli such as arachidonic acid or Ca²⁺ ionophore. Conversely, the expressions “stimulated release”, “formation”, or “acute formation” refer to the release of mediators following the application to the system of arachidonic acid or Ca²⁺ ionophore.

Results

In Vivo. Treatment of rats with LPS caused a marked increase in the levels of PGE₂ that followed injection of arachidonic acid (3 mg kg⁻¹) (control, 4.5 ± 1.4 ng ml⁻¹, n = 6; LPS-treated, 18.4 ± 3.7 ng ml⁻¹, n = 7). Diclofenac given to LPS-treated rats inhibited the release of PGE₂ back to approximately control levels (6.1 ± 1.5 ng ml⁻¹, n = 5, P < 0.05). The selective COX-2 inhibitor DFP produced a smaller insignificant reduction in PGE₂ formation in these LPS-treated rats (12.7 ± 1.6 ng ml⁻¹, n = 5, P > 0.05).

In Vitro: Accumulation and Acute Formation of PGE₂ in LPS-Treated Blood (Protocol 1). Basal accumulation of PGE₂ in rat blood under control conditions was 0.8 ± 0.1 ng ml⁻¹ over the 18-h incubation period. LPS caused concentration-dependent increases in the accumulation of PGE₂ (Fig. 1) that were inhibited by dexamethasone, aspirin, and DFP (P < 0.05; Fig. 1). Indeed, in blood incubated with 1 μg ml⁻¹ LPS, aspirin inhibited PGE₂ accumulation to below-detection levels.

Following incubation with A23187 (50 μM for 15 min) under control conditions, the concentration of PGE₂ in rat blood rose to 10.4 ± 0.9 ng ml⁻¹ (Fig. 2). This was approximately 10 times greater than the amount of PGE₂ that accumulated in the same samples over 18 h (Fig. 1). Interestingly, preincubation with LPS had no effect on the level of PGE₂ released by A23187 (Fig. 2).

The effects on PGE₂ formation of dexamethasone, aspirin, or DFP were similar at all concentration of LPS (P > 0.05, one-way analysis of the variance plus Bonferroni’s test; Fig. 2). On average, dexamethasone and DFP inhibited the stimulated release of PGE₂ by 21 ± 4 and 35 ± 7%, respectively. Aspirin inhibited the acute formation of PGE₂ by, on average, 78 ± 3%.

In Vitro: Accumulation and Acute Formation of PGE₂ in LPS-Treated Leukocytes (Protocol 2). Incubation of rat washed leukocytes with LPS (1, 10, and 100 μg ml⁻¹) caused a concentration-dependent accumulation of PGE₂ over the 18-h incubation period (0.7 ± 0.3, 2.9 ± 0.7, and 6.3 ± 2.0 ng ml⁻¹, respectively; n = 8 for all). In control samples and in blood treated with LPS and test drugs, however, PGE₂ was below detection levels.

Similar to what was observed in whole blood (Fig. 2), rat washed leukocytes produced similar amounts of PGE₂ when exposed to A23187 (50 μM) irrespective of their previous incubation with LPS (Fig. 3; P > 0.05). The amounts of PGE₂ produced, however, were approximately one-third of the levels measured in treatment-matched whole-blood samples (Fig. 3). The acute formation of PGE₂ by LPS-treated rat washed leukocytes was effectively blocked by aspirin and,
conversely to what was observed in LPS-treated whole blood, by dexamethasone. DFP caused a partial inhibition of the acute formation of PGE2 that failed to reach statistical significance (Fig. 3).


The production of PGE2 by freshly isolated rat washed leukocytes following exposure to A23187 was below detection levels. Conversely, following A23187 treatment, PGE2 levels rose considerably and to similar extents (P < 0.05, t test) in rat whole blood and rat PRP (Fig. 4). This A23187-stimulated formation was strongly inhibited by aspirin (P < 0.05) but was unaffected by DFP (P > 0.05, vehicle versus drugs).

In Vitro: Stimulated Release of PGE2 in Rat and Human Whole Blood (Protocol 4).

Under control conditions, the amount of PGE2 produced by rat whole blood in response to the addition of A23187 (50 μM for 15 min) was approximately 20 times greater than that produced by human blood (rat, 24 ± 1.9 ng ml⁻¹; human, 1.2 ± 0.16 ng ml⁻¹; n = 9 for both).

In Vitro: Expression of COX-1 and COX-2 in Rat Leukocytes.

Western blot analysis of cell extracts demonstrated that freshly isolated leukocytes expressed low levels of COX-1 (Fig. 5) but contained no detectable COX-2 (Fig. 6). Maintenance of the cells in culture conditions for 18 h resulted in a 3-fold increase in COX-1 expression that was not affected by the presence of LPS (Fig. 5). Conversely, COX-2 expression in isolated leukocytes was not affected by exposure to culture conditions for 18 h but was greatly increased by incubation with LPS (Fig. 6).

Discussion

In this study, we show that the increase in the arachidonic acid-stimulated production of PGE2 seen in LPS-treated rats is coupled to both COX-1 and COX-2. A similar relationship was also found in rat whole blood in vitro, although not in human whole blood in vitro (Warner et al., 1999). Given the important role played by PGE2 in inflammation, these observations are consistent with the concept that COX-1 products contribute to inflammatory responses in rats and possibly other species, as reported by Wallace et al. (1998).

We have previously shown that LPS administered to the anesthetized rat induces both the up-regulation of COX-2 protein and an increase in the plasma levels of 6-keto-PGF1α (Hamilton et al., 1999; Giuliano et al., 2001). In these previous experiments, challenge with arachidonic acid revealed that COX-2 induction was associated with a much greater increase in the synthetic capacity for 6-keto-PGF1α than sug-
DFP strongly suggested that the accumulation of PGE_2 observed in isolated leukocytes exposed to LPS may well account for the accumulation of PGE_2 measured in LPS-treated whole blood from rats.

Surprisingly, rat washed leukocytes appeared to be only partially capable of contributing the levels of PGE_2 measured in whole blood following Ca^{2+} ionophore treatment. In particular, the amounts of PGE_2 produced by control and LPS-treated leukocytes challenged with A23187 were about one-third of those observed in similarly treated whole blood. Most importantly and in contrast to what we observed in whole blood, dexamethasone inhibited the acute formation of PGE_2 in LPS-treated washed leukocytes. Although the effect of dexamethasone was not further investigated, one might speculate that in LPS-treated leukocytes dexamethasone may have curtailed PGE_2 production by down-regulating not only COX-2 but also COX-1 or, alternatively, by reducing the activity of phospholipase A_2. Notably, as shown by Western blot analyses, the expression of COX-1 protein was increased in leukocytes maintained in culture conditions for 18 h. Moreover, COX-1 expression has previously been reported to be sensitive to dexamethasone (Hamasaki et al., 1993; Jun et al., 1999).

Regardless of the mechanism involved, dexamethasone did not produce as great an inhibition of PGE_2 production in whole blood as it did in LPS-stimulated leukocytes. This indicated that a source other than the leukocytes was responsible for the stimulated formation of PGE_2 observed in whole blood. Indeed, a comparative analysis of rat platelet-rich plasma and washed leukocytes indicated that, in whole blood, platelets represent the main source of COX-1-derived PGE_2.

In summary, the experiments described here show that when exposed to LPS, rat whole blood produces increased levels of PGE_2 through induction of COX-2 in blood leukocytes. Although this model is not strictly one of inflammation, the acute application of LPS does induce the rapid expression of COX-2 and therefore provides an in vivo experimental system in which to study the interplays between COX-1 and COX-2. In addition, because in vitro assays employing LPS-treated whole blood as a source of COX-2 have been widely used to characterize the pharmacological activities of NSAIDs (Warner et al., 1999; Fitzgerald and Patrono, 2001), LPS treatment in vivo seems a further logical proving ground. Another caveat to keep in mind is that the rate of prostanoid formation following arachidonic acid challenge in vivo most likely is greatly in excess of that seen in inflammation. The application of excess substrate, however, does provide us once again with a system more aligned with those used to test NSAID activities in vitro (e.g., strong stimuli are applied to activate platelets and therefore drive COX-1 activity) (Warner et al., 1999; Fitzgerald and Patrono, 2001). At the same time, we must remember that in COX-2 overexpressing cells, for instance, prostanoid production may not be limited by arachidonic acid availability, and so our application of excess substrate could be biased toward COX-1 products. Taken together, of course, these considerations remind us that in this study we have measured COX-1 and COX-2 products in an acute in vivo system that does not directly model inflammatory disease.

Clearly, our data demonstrate that rat blood has the ability...
to produce copious amounts of PGE\(_2\) via the actions of COX-1 enzyme constitutively present in platelets. This observation begs a simple question. Is platelet production of PGE\(_2\) of any particular relevance? In comparison with the characterization of their function in blood clotting, the role of platelets as mediator and effector cells in inflammation has only recently been recognized (Klinger, 1997). Platelets participate in inflammatory events by releasing a considerable number of mediators and by interacting with leukocytes and endothelial cells. Furthermore, the contribution of platelets to inflammatory processes may not be solely restricted to interactions within the vasculature. In fact, several studies have shown that platelets can be found together with leukocytes in the exudates of numerous inflammatory diseases (Bazzoni et al., 1991). Indeed, of particular relevance to this discussion is the observation that platelets accumulate in the exudate provoked by the subdermal implantation of sponges in rats (Smith et al., 1976). Moreover, platelets also appear to accumulate in the carrageenan-induced paw edema in rats (Vincent et al., 1978). In these circumstances, one might expect, consistent with the results presented here, that platelet activation in vivo could result in a significant production of PGE\(_2\) from COX-1. Indeed, the PGE\(_2\) produced by platelets may be of extreme importance in the early stages of the inflammatory process.

Although our experiments suggest that COX-1 is a major source of “inflammatory” PGE\(_2\) in the rat, they also indicate that this is not so in humans (Warner et al., 1999). Notably, as shown above, human blood can only produce a small fraction (1/20) of the levels of COX-1-derived PGE\(_2\) produced by rat blood. Indeed, we and others have taken the production of PGE\(_2\) in LPS-treated human as a signal of COX-2 activity and used it to characterize the activities of a range of NSAIDs (Warner et al., 1999; FitzGerald and Patrono, 2001), as has been done here in rat whole blood. At the same time, it is interesting to note that rats are often used in the characterization of NSAIDs and novel COX-2 inhibitors in inflammatory models (Chan et al., 1995; Riendeau et al., 1997). The data gathered in the present study support the idea that in these inflammatory models the contribution of COX-1-derived PGE\(_2\), both as a confounding factor and as a mediator, may have been overlooked. This is in agreement with the experiments of Wallace et al. (1998, 1999) who reported that COX-2 inhibitors, such as SC-58125, DFP-697, or NS-398, need to be administered at nonselective COX-1-inhibiting doses to obtain a significant anti-inflammatory effect in the carrageenan air pouch or in the carrageenan paw edema in rats. From this, Wallace and his colleagues proposed that COX-1 may be a major contributor to the inflammatory process in the animal models used. Importantly, the results shown here, although looking at systemic levels of prostanooids and not local production at an inflammatory site, are consistent with this hypothesis in that the production of COX-1-derived PGE\(_2\) by platelets may well underlie the involvement of COX-1 in inflammation. Therefore, it may not be surprising that in rats COX-2-selective inhibitors fail to produce the full anti-inflammatory effects observed with standard NSAIDs (Wallace et al., 1998, 1999). Most importantly, however, the differences found between human (this study and Warner et al., 1999) and rat blood suggest that extrapolations to humans of results from NSAID testing in the rat should only be made very cautiously. Indeed, the role of COX-1 in the production of “inflammatory” PGE\(_2\) in the rat was not reflected in human samples, supporting the use of COX-2-selective inhibitors as human anti-inflammatories.

References


Giuliano F, Mitchell JA, and Warner TD (2001) COX-1-derived PGE\(_2\): a comparison between PGE\(_2\) in the rat, they also indicate that this is not so in humans (Warner et al., 1999). Notably, as shown above, human blood can only produce a small fraction (1/20) of the levels of COX-1-derived PGE\(_2\) produced by rat blood. Indeed, we and others have taken the production of PGE\(_2\) in LPS-treated human as a signal of COX-2 activity and used it to characterize the activities of a range of NSAIDs (Warner et al., 1999; FitzGerald and Patrono, 2001), as has been done here in rat whole blood. At the same time, it is interesting to note that rats are often used in the characterization of NSAIDs and novel COX-2 inhibitors in inflammatory models (Chan et al., 1995; Riendeau et al., 1997). The data gathered in the present study support the idea that in these inflammatory models the contribution of COX-1-derived PGE\(_2\), both as a confounding factor and as a mediator, may have been overlooked. This is in agreement with the experiments of Wallace et al. (1998, 1999) who reported that COX-2 inhibitors, such as SC-58125, DFP-697, or NS-398, need to be administered at nonselective COX-1-inhibiting doses to obtain a significant anti-inflammatory effect in the carrageenan air pouch or in the carrageenan paw edema in rats. From this, Wallace and his colleagues proposed that COX-1 may be a major contributor to the inflammatory process in the animal models used. Importantly, the results shown here, although looking at systemic levels of prostanooids and not local production at an inflammatory site, are consistent with this hypothesis in that the production of COX-1-derived PGE\(_2\) by platelets may well underlie the involvement of COX-1 in inflammation. Therefore, it may not be surprising that in rats COX-2-selective inhibitors fail to produce the full anti-inflammatory effects observed with standard NSAIDs (Wallace et al., 1998, 1999). Most importantly, however, the differences found between human (this study and Warner et al., 1999) and rat blood suggest that extrapolations to humans of results from NSAID testing in the rat should only be made very cautiously. Indeed, the role of COX-1 in the production of “inflammatory” PGE\(_2\) in the rat was not reflected in human samples, supporting the use of COX-2-selective inhibitors as human anti-inflammatories.