Lipopolysaccharide-Induced Increase of Prostaglandin E\(_2\) Is Mediated by Inducible Nitric Oxide Synthase Activation of the Constitutive Cyclooxygenase and Induction of Membrane-Associated Prostaglandin E Synthase

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Lipopolysaccharide-Induced Increase of Prostaglandin E$_2$ Is Mediated by Inducible Nitric Oxide Synthase Activation of the Constitutive Cyclooxygenase and Induction of Membrane-Associated Prostaglandin E Synthase

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NO produced by the inducible NO synthase (NOS2) and prostanooids generated by the cyclooxygenase (COX) isoforms and terminal prostanooid synthases are major components of the host innate immune and inflammatory response. Evidence exists that pharmacological manipulation of one pathway could result in cross-modulation of the other, but the sense, amplitude, and relevance of these interactions are controversial, especially in vivo. Administration of 6 mg/kg LPS to rats i.p. resulted 6 h later in induction of NOS2 and the membrane-associated PGE synthase (mPGES) expression, and decreased constitutive COX (COX-1) expression. Low level inducible COX (COX-2) mRNA with absent COX-2 protein expression was observed. The NOS2 inhibitor aminoguanidine (50 and 100 mg/kg i.p.) dose dependently decreased both NO and prostanooid production. The LPS-induced increase in PGE$_2$ concentration was mediated by NOS2-derived NO-dependent activation of COX-1 pathway and by induction of mPGES. Despite absent COX-2 protein, SC-236, a putative COX-2-specific inhibitor, decreased mPGES RNA expression and PGE$_2$ concentration. Ketoprofen, a nonspecific COX inhibitor, and SC-236 had no effect on the NOS2 pathway. Our results suggest that in a model of systemic inflammation characterized by the absence of COX-2 protein expression, NOS2-derived NO activates COX-1 pathway, and inhibitors of COX isoforms have no effect on NOS2 or NOS3 (endothelial NOS) pathways. These results could explain, at least in part, the deleterious effects of NOS2 inhibitors in some experimental and clinical settings, and could imply that there is a major conceptual limitation to the use of NOS2 inhibitors during systemic inflammation. The Journal of Immunology, 2001, 167: 3962–3971.

Nitric oxide produced by the inducible NO synthase (NOS2) is an essential component of the host innate immune and inflammatory response to a variety of pathogens, such as intracellular bacteria, viruses, fungi, and parasites (1, 2). Nevertheless, as for other components of the host inflammatory and immune response, excessive activation of NOS2 results in cardiovascular (3) and organ dysfunction (4) in clinical (5) or experimental situations of inflammatory disease of both septic (6) and nonseptic (7) etiology. Despite these well-documented deleterious effects on the host physiology, pharmacological inhibition of NOS2 enzymatic activity does not always attenuate organ dysfunction and can even in some situations worsen it. For instance, inhibition of NOS2 enzyme activity with N$^\gamma$-monomethyl-L-arginine (L-NMMA) in humans with septic shock attenuated arterial hypotension, but also decreased cardiac output and did not decrease mortality (5). In an experimental model, NOS2 inhibition with L-NMMA resulted in intrahepatic vascular thrombosis (4). The current paradigm used to explain the deleterious effects of pharmacological inhibition of NOS2 enzyme activity is that, due to their lack of specificity, NOS2 inhibitors such as L-NMMA also attenuate NO synthesis by the constitutive endothelial NOS isoform (NOS3). Other less frequently explored hypotheses are that NOS2 enzymatic inhibitors, by decreasing NO release: 1) result in an imbalance favoring the activated vasoconstricting systems, or 2) decrease the expression and/or activity of other NO-dependent vasodilatory systems. We focused on the latter possibility and hypothesized that inhibition of NOS2 activity could alter the vasodilatory and inflammatory pathway mediated by the cyclooxygenases (COX).

Prostanoids, including PGs, prostacyclins, and thromboxanes, are synthesized from three enzymatic pathways: arachidonic acid is released from membrane glycerophospholipids by phospholipase A$_2$ (PLA$_2$) (8) and is the substrate of the PGH synthase (PGH-S) that generates the common intermediate PGH$_2$ (9). PGH$_2$ is subsequently converted to various prostanoids (PGE$_2$, PGD$_2$, etc.).
PGF<sub>2</sub><sub>α</sub>, 6-keto-PGF<sub>1α</sub> (6-k-PGF<sub>1α</sub>), and thromboxane B<sub>2</sub> (TXB<sub>2</sub>) by terminal prostanoid syntheses (10). Different isoforms of PLA<sub>2</sub>, including a cytosolic PLA<sub>2</sub>, a secreted PLA<sub>2</sub>, and a calcium-independent cytosolic PLA<sub>2</sub>, are involved in supplying arachidonic acid to the PGH-S (8). PGH-S catalyzes the committed step in the synthesis of prostanooids and carries out two distinct activities, the COX and peroxidase activities (9). Two isoforms of COX have been identified: a constitutive isoform (COX-1) expressed in most tissues, responsible for the physiological production of PG, and an inducible isoform (COX-2) encoded by an immediate-early gene induced by cytokines, mitogens, and endotoxins in inflammatory cells, accounting for the elevated production of PG during inflammation (11). Terminal prostanoid syntheses catalyze the conversion of PGH<sub>1</sub> to biologically active prostanoids. Among these, two isoforms of PGE synthase (PGES) have been recently characterized: a cytosolic isoform involved in immediate PGE<sub>2</sub> biosynthesis (12) and a membrane-associated isoform (mPGES) induced by proinflammatory stimuli and involved in delayed PGE<sub>2</sub> biosynthesis (13).

In addition to their role in inflammation, prostanoids have also been shown to modulate vasodilatation. Interestingly, COX-2 was shown to synergize with NO in mediating LPS-induced cerebral hyperemia (14), whereas the vasoconstrictor thromboxane antagonizes NO-mediated vasodilatation (15). Several in vitro reports have suggested that NO activates COX activity, but these observations are still controversial, and in vivo data are scarce (for a recent review, see Ref. 16). Moreover, it has been suggested in vitro that the COX products could modulate NOS2 activation through increased cAMP content in cells expressing NOS2 (reviewed in Ref. 17).

The aims of this in vivo study in rats stimulated with LPS were to investigate: 1) the effects of aminoguanidine (AG), a NOS2 enzymatic inhibitor on the COX pathway with a special interest on the time course of these effects; 2) a potential effect of prostanoid synthesis inhibition on NOS2 pathway activation.

**Materials and Methods**

**Animals**
Male Wistar Kyoto rats (250–350 g) were housed and treated in accordance with accepted practices for humane laboratory animal care.

**Preparation of the reagents**
All chemicals and reagents were purchased from Sigma (Saint Quentin Fallavier, France), unless specified otherwise. *Salmonella typhimurium* (Flavier, France), unless specified otherwise. *Salmonella typhimurium* (Flavier, France), unless specified otherwise. *Salmonella typhimurium* (Flavier, France), unless specified otherwise.

**Experimental protocols**
A low dose LPS-mediated activation of the NOS2 pathway (18, 19) was obtained by i.p. injection of LPS (6 mg/kg body weight (bw)) to all rats, except those of the control group (n = 4) that received 500 μl of 0.9% NaCl i.p.

**Protocol 1.** Studies with the NOS2 inhibitor, AG. Fifteen minutes before and 3 h after LPS injection, AG was injected i.p. at doses of 50 μg/kg bw (LPS + 50 μg/kg AG group, n = 6) or 100 μg/kg bw (LPS + 100 μg/kg AG group, n = 6). These rats were compared with LPS-treated rats that received AG vehicle (n = 6) and with the control group. The doses of AG were chosen in accordance with published data on dose vs efficacy vs toxicity reports (20, 21).

**Protocol 2.** Studies with the non specific COX inhibitor ketoprofen and the COX-2-specific inhibitor SC-236. Fifteen minutes before LPS injection, rats received either 5 mg/kg bw ketoprofen by gavage (LPS + ketoprofen group, n = 6), or 10 mg/kg bw SC-236 i.p. (LPS + SC-236 group, n = 6). As controls, additional LPS-treated rats were given either ketoprofen vehicle (n = 6) or SC-236 vehicle (n = 6). Fifteen minutes before LPS injection. Because ketoprofen and SC-236 vehicles had no significant effect on the parameters studied (data not shown), rats that received LPS and ketoprofen or SC-236 vehicles were pooled (LPS group, n = 12). LPS + ketoprofen and LPS + SC-236 groups were compared with LPS and control groups. The doses of ketoprofen (22) and SC-236 (23) were chosen from published reports.

Six hours after LPS administration, rats from protocols 1 and 2 were anesthetized with 100 mg sodium thiopental (Nesdonal; Rhône Poulenc Rorer) i.p., and the thorax and abdomen were dissected. Blood samples were recovered by cardiac puncture and centrifuged at 600 × g for 10 min, and plasma was stored at −70°C. Tissue samples from liver, spleen, kidney, lung, and heart were excised, rapidly rinsed in ice-cold saline, frozen, and stored at −70°C.

**Analysis of liver mRNA expression by semiquantitative RT-PCR**
Semiquantitative RT-PCR was performed to estimate mRNA expression of the inducible and endothelial isoforms of NOS, the constitutive and inducible isoforms of COX, and the membrane-associated isoform of PGES.

**Extraction of total RNA**
Total RNA was extracted from the different samples using Tri-Reagent (Amplinex, Soufflwaukee, France). The RNA concentration was measured in triplicate before and after dilution to ~1 μg/μl by spectrophotometric analysis at 260 nm. The RNA purity was determined by the ratio A<sub>260</sub>/A<sub>280</sub> (all samples between 1.6 and 2), and its integrity was confirmed by the existence of clear bands for 18S and 28S RNA after electrophoresis through a 0.8% agarose gel.

**Reverse transcription**
Five micrograms of total RNA in 10 μl of diethyl pyrocarbonate-treated water were added to each reaction mixture containing 10 mM Tris-Cl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 μM oligo(dT) 16 , 100 mM RNase inhibitor (GeneAmp RNA PCR kit; PE Applied Biosystems, Courtaboeuf, France), and 0.25 U/μl RNase A (Pharmacia Biotech, Orsay, France) to a final volume of 20 μl, and incubated for 30 min at 37°C, followed by 5 min at 75°C. After 5 min on ice, the RNA was mixed with 2.5 μl 100 μM murine leukemia virus reverse transcriptase (PE Applied Biosystems) and incubated for 30 min at 42°C, followed by 5 min at 90°C for denaturation of murine leukemia virus reverse transcriptase. The cDNA samples were stored at −20°C.

**Amplification**
The PCR were made using amplifiers for NOS2, NOS3, COX-1, COX-2, mPGES, and the housekeeping gene β-actin. The following amplifiers were chosen in regions of low homology between constitutively expressed and inducible genes to avoid cross reactions: 5′-GATTTGAGGAAGGAGCCGGC-3′ (sense) and 5′-GCCCTTTTCTGCTTCAATAGG-3′ (antisense) for NOS2; 5′-TACGGGACGGACAAATCCAC-3′ (sense) and 5′-CAGGCTCTGACTTCCCTCTGATC-3′ (antisense) for NOS3; 5′-GATGACGGGTCTGTCTTGAT-3′ (sense) and 5′-TCTTCTAGGCGTGCTCCAG-3′ (antisense) for COX-1; 5′-TTACGAAAGTGTCTTCGGAAGT-3′ (sense) and 5′-GATCATGGTCATCAGCTTTTCTT-3′ (antisense) for COX-2; 5′-ATGACTTCCTCCATGTTGTTCGTCG-3′ (sense) and 5′-GTCTCCCAACTTGGG-3′ (antisense) for mPGES; 5′-GACTTCTGAGCTGAGGTGTT-3′ (sense) and 5′-GACCCTTGTTGGCCATAGG-3′ (antisense) for β-actin. Because of high expression level, NOS2, COX-1, and mPGES amplification was performed in duplex with β-actin, whereas NOS3, COX-2, and β-actin amplification was performed in different tubes. PCR was performed in a DNA thermal cycler (Bio-Rad Laboratories, Ivry-sur-Seine, France) using a 2-μl cDNA sample in a total reaction volume of 10 μl with 1 μM of each amplifier, 1X PCR buffer II, 1 mM dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.05 U/μl AmpliTaq DNA polymerase (Gene Amp RNA PCR kit; PE Applied Biosystems).
Amplification cycle numbers and annealing temperatures were optimized for each amplimer pair. A Gene Ruler 100-bp DNA Ladder Plus (Euromedex) was used to determine the size of the PCR products. Preliminary experiments were performed to document that the PCR was completed during the exponential phase of amplification and that the amplification was linear. The identity of the PCR products was confirmed by sequencing using ABI PRISM Dye Terminator Cycle sequencing Ready Reaction kit and ABI PRISM 310 PE Applied Biosystems.

**Densitometric analysis of PCR products**

The PCR products were separated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and viewed using UV light on a transilluminator. Densitometry of the resulting bands was performed with a Bio-Rad Gel Doc 1000 (Bio-Rad Laboratories). Results were expressed as a ratio of the OD of the band of the PCR product of interest to that of β-actin.

**Western blot analysis of NOS2, NOS3, COX-1, and COX-2 protein expression**

Tissue samples were homogenized with a Polytron PT 1200 (Kinematica, Littau, Switzerland) in 10 vol of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.2 mM sodium orthovanadate, 1% Triton X-100, (v/v), and 0.5% Nonidet P-40 (v/v)). De-tergents were purchased from ICN Biomedicals (Orsay, France). The homogenates were centrifuged at 12,000 × g for 15 min. Protein concentration in the supernatant was measured by the method of Lowry. A total of 150 μg of proteins from each tissue sample was denatured by boiling for 10 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) SDS, 0.36% (v/v) glycerol, 0.06% (v/v) 2-ME, 12% (w/v) bromphenol blue) separated by electrophoresis on a 7.5–4% SDS-PAGE (Mini Protein II; Bio-Rad), and transferred electrotheroretically overnight at 4°C (Trans Blot Electrotheroretic Cell; Bio-Rad) on polyvinylidene difluoride membranes (Sequiot-Blot PVDF Membrane; Bio-Rad) in 20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3. After blocking for 1 h with 3% BSA (fraction V; Euromedex) in TBS (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST solution)), membranes were incubated with 1/1000 diluted primary antibodies (anti-murine NOS2 or rabbit anti-human NOS3) followed by 1/40 to 1/200 (both from Oxford Biomedical Research), were used as positive controls for detection of NOS proteins. Native ovine protein from seminal vesicles and placenta, and LPS (both obtained from Transduction Laboratories, Lexington, KY) were used as positive controls for detection of COX proteins. Native ovine COX-1 and COX-2 polyclonal Abs (Cayman Chemical, Ann Arbor, MI) or rabbit anti-human COX-1 or COX-2 polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA, and Oxford Biomedical Research, Oxford, MD) for detection of NOS2, NOS3, COX-1, and COX-2 proteins, respectively. After washing, the membranes were incubated for 1 h with a 1/10,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad). Immunocomplexes were revealed using the nitroblue tetrazolium-5-bromo-4-cylo-3-indolyl phosphate (NBT-BCIP) or the ECL (ECL Western blotting; Amersham, Arlington Heights, IL) methods. A human endothelial cell line was derived from the aortic endothelial cell line and a NOS2 mouse macrophage lysate obtained from RAW 264.7 cells stimulated with IFN-γ and LPS (both obtained from Transduction Laboratories, Lexington, KY) were used as positive controls for detection of COX proteins. Native ovine COX-1 and COX-2 proteins purified from seminal vesicles and placenta, respectively (both from Oxford Biomedical Research), were used as positive controls for detection of COX proteins. High range prestained SDS-PAGE standards (Bio-Rad) were used for molecular mass determination. Densitometry of the resulting bands was performed using a Bio-Rad GS-690 Imaging Densitometer. As for RT-PCR technique, we previously verified that the intensity of the bands was proportional to the quantity of protein submitted to the immunodetection.

**Immunohistochemical analysis of COX-2 protein expression**

Control and experimental tissue sections (20 μm thick) were collected on the same gelatin-coated slides, and treated with the same immunohistochemical protocol. Sections were successively covered with different dilutions (1/40 to 1/200) of the polyclonal rabbit anti-human COX-2 serum (Santa Cruz Biotechnology) for 48 h at 4°C. After washing, the primary Ab was stained with a goat anti-rabbit IgG conjugated with FITC (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were counterstained with Evans blue before analysis with an epifluorescent microscope (Leica, Rueil Malmaison, France). The lack of immunostaining when sections were incubated with nonimmune rabbit serum or PBS to replace the primary Ab recognizing the COX-2 protein attested the specificity.

**Measurement of nitrate and nitrite in plasma**

The concentration of stable nitrate and nitrite, the end products of NO oxidation, was determined by the Griess reaction. A total of 100 μl of plasma was added to 50 μl of biosmoted water and submitted to nitrate reduction by 0.1 U/ml nitrate reductase (EC 1.6.6.2, from Aspergillus spe-cies) in the presence of 5 μM flav adenine dinucleotide and 30 μM NADPH. Incubation with l-lactate dehydrogenase (EC 1.1.1.27, type II, from rabbit muscle) and 0.3 mM sodium pyruvate allowed NADPH to oxidize. Samples were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H3PO4). After a 15-min incubation period at room temperature, the absorbance was read at 540 nm using a DU 640 B Beckman spectrophotometer (Beckman Instruments, Brea, CA). Nitrite concentration in samples was determined by extrapolation from a sodium nitrite standard curve (working range: 0.45–65 μM nitrate). All samples were tested in triplicate, and the background nitrite concentration of water was subtracted from the extrapolated nitrite concentration.

**Measurement of prostanooid concentrations in plasma and liver**

The concentration of the end products of COX activity, PGE2, 6-k-PGF1α, and TXB2, was determined using ELISA kits (Neogen, Lexington, KY), following the manufacturer’s instructions. Ten-fold dilutions of plasma samples were used for the three assays. Liver samples were homogenized with a Polytron PT 1200 in 5 vol extraction buffer (15% (v/v) methanol and 1% (w/v) indomethacin in 0.1 M PBS, pH 7.5). The homogenates were centrifuged at 12,000 × g for 15 min. Supernatants were diluted 10-fold for PGE2 and 6-k-PGF1α assays and 50-fold for TXB2 assay. The enzymatic reaction was stopped by addition of 50 μl of 1 M HCl, and absorbance was read at 450 nm. Prostanoid concentrations were extrapolated from standard curves and were expressed in nanograms per milliliter for plasma and in picograms per microgram of protein for liver homogenates. Protein concentration was measured by the method of Lowry.

**Statistical analysis**

Statistical analysis was performed using the StatView IV software (Abacus Concepts, Berkeley, CA). Results are expressed as mean ± SEM. Comparisons among several groups were performed with nonparametric ANOVA (Kruskall-Wallis test). Comparisons between two groups were performed with the Mann-Whitney test. A p value <0.05 was considered statistically significant.

**Results**

**Effect of AG on NOS2 and NOS3 expression and activity**

As expected, rats from control group had no detectable NOS2 expression, and LPS administration (LPS group) induced NOS2 mRNA (Fig. 1) and protein (Fig. 2) expression in the liver and other organs (results not shown). Because NOS2 and NOS3 mRNA and protein expression were highest in the liver as compared with other organs, NOS-COX interactions were only studied in this organ. Expression of NOS3 mRNA (Fig. 1) and protein (Fig. 2) was similar in the liver from rats of both control and LPS groups. Control rats had low plasma nitrite and nitrate concentration; this concentration was significantly increased by LPS (Table I). Administration of 50 or 100 mg/kg AG did not modify liver NOS2 and NOS3 mRNA (Fig. 1) and protein (Fig. 2) expression compared with LPS group. In contrast, AG dose dependently decreased plasma nitrite and nitrate concentration. A dose of 100 mg/kg was necessary to completely inhibit the LPS-mediated increase in plasma nitrate and nitrite concentration (Table I). There was no decrease of plasma nitrate/nitrite concentration when AG was administered at 50 and 100 mg/kg to control rats (results not shown).

**Effect of AG on COX-1, COX-2 expression and PG concentration**

Control rats had high level COX-1 mRNA expression in the liver, significantly decreased by LPS challenge (Fig. 3). Whereas rats from control group had undetectable liver COX-2 mRNA expression, LPS challenge only moderately induced COX-2 mRNA expression in the liver, but this induction was too low to be quantified (Fig. 3). The COX-2 protein was undetectable in Western blot experiments for all groups of rats, even when using the very sensitive chemiluminescence detection method (results not shown). A barely detectable induction of the COX-2 protein was observed in...
liver sections from LPS-treated rats, but not control rats submitted to immunohistochemical detection (data not shown). To document our ability to detect COX-2 protein induction in a similar model, we performed additional Western blot experiments with tissue homogenates from rats injected with 15 mg/kg LPS and sacrificed 9 h later. Under these conditions, COX-2 protein expression was significantly induced in the liver and heart (data not shown). Taken together, these data demonstrate that 6 h after 6 mg/kg LPS injection, there is low level COX-2 mRNA expression and very low level or absent COX-2 protein expression.

Low, but reproducibly detectable plasma concentrations of 6-k-PGF$_1\alpha$, PGE$_2$, and TXB$_2$ were measured in rats from control group. LPS challenge significantly increased the concentrations of these three prostanoids (Table I).

Administration of AG did not modify liver COX-1 and COX-2 mRNA expression (Fig. 3), but dose dependently attenuated the LPS-mediated increase in 6-k-PGF$_1\alpha$ and TXB$_2$ concentrations compared with rats from LPS group (Table I). AG dose dependently decreased PGE$_2$ concentration, but not to the level measured in control rats. Thus, following AG administration, plasma and liver PGE$_2$ concentrations remained 10- and 2-fold higher, respectively, than in control rats (Table I).

Effect of ketoprofen and SC-236 on NOS2 and NOS3 expression and activity

In LPS-injected rats, neither ketoprofen nor SC-236 significantly modified liver NOS2 and NOS3 mRNA (Fig. 4), protein (Fig. 5) expression, or plasma nitrite and nitrate concentration (Table II). Administration of SC-236 decreased 6-k-PGF$_1\alpha$ and TXB$_2$ plasma concentrations in LPS-injected rats, but this decrease was not statistically significant. In contrast, the decrease of PGE$_2$ plasma and liver concentrations elicited by SC-236 in LPS-treated rats was statistically significant. The modest and statistically not significant effect of SC-236 on plasma and liver concentrations of 6-k-PGF$_1\alpha$ and TXB$_2$ in the presence of an important effect of ketoprofen is consistent with 1) a lack of effect of SC-236 on PGE$_2$, and 2) absent or low level COX-2 expression. Plasma concentrations of the three prostanoids studied were significantly lower in rats from LPS + ketoprofen group than in rats from LPS + SC-236 group (Table II).

**Time-dependent NOS-COX cross-talk**

Plasma concentrations of nitrite and nitrate, as well as plasma and liver concentrations of PGE$_2$ peaked at 6 h following LPS administration and slowly returned to control values at 48 h. Administration of AG completely abolished the increase in plasma nitrite and nitrate concentration, and only partly attenuated the increase in plasma and liver concentrations of PGE$_2$ (Table III and Fig. 7).

The abundance of NOS2 mRNA in the liver after LPS administration was increased significantly during the next 24 h, peaking at 6 h. After an initial decrease 6 h post-LPS injection, COX-1 mRNA abundance surprisingly increased until 24 h after LPS injection. No effect of AG was observed on NOS2 and COX-1 mRNA expression during the course of the experiment (Table III).

Expression of NOS3 mRNA remained stable, while COX-2 mRNA expression was induced at 6 and 12 h following LPS injection, but was too low to be quantified (data not shown).
Table 1. Effect of AG on plasma nitrite/nitrate concentration and plasma and liver prostanoids concentrations measured 6 h after LPS injection

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + 50 mg/kg AG</th>
<th>LPS + 100 mg/kg AG</th>
</tr>
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<tbody>
<tr>
<td>Nitrite/nitrate (Plasma (µM))</td>
<td>2.50 ± 0.14</td>
<td>4.61 ± 0.25*</td>
<td>3.46 ± 0.26†</td>
<td>2.44 ± 0.45‡</td>
</tr>
<tr>
<td>6-k-PGF&lt;sub&gt;1α&lt;/sub&gt; (Plasma (ng/ml))</td>
<td>1.22 ± 0.22</td>
<td>2.41 ± 0.29*</td>
<td>1.93 ± 0.12*</td>
<td>1.62 ± 0.08‡</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; (Liver (pg/µg protein))</td>
<td>0.06 ± 0.01</td>
<td>0.63 ± 0.14*</td>
<td>0.43 ± 0.06*</td>
<td>0.26 ± 0.06†</td>
</tr>
<tr>
<td>TXB&lt;sub&gt;2&lt;/sub&gt; (Plasma (ng/ml))</td>
<td>2.17 ± 1.48</td>
<td>45.17 ± 6.38*</td>
<td>32.80 ± 8.69*</td>
<td>24.79 ± 3.87*</td>
</tr>
<tr>
<td>TXB&lt;sub&gt;2&lt;/sub&gt; (Liver (pg/µg protein))</td>
<td>0.28 ± 0.02</td>
<td>0.81 ± 0.12*</td>
<td>0.59 ± 0.06*</td>
<td>0.54 ± 0.08‡</td>
</tr>
<tr>
<td>Liver (pg/g protein)</td>
<td>0.10 ± 0.02</td>
<td>0.26 ± 0.04*</td>
<td>0.20 ± 0.05</td>
<td>0.14 ± 0.02†</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± SEM; n = 4 for control group; n = 6 for LPS, LPS + 50 mg/kg AG, and LPS + 100 mg/kg AG groups. *, †, and ‡: p < 0.05 as compared to control, LPS, and LPS + 50 mg/kg AG groups, respectively.

Effect of LPS, AG, ketoprofen, and SC-236 on mPGES mRNA expression

Expression of mPGES mRNA, undetectable in the liver of rats from control group, was significantly induced following LPS administration. Although AG and ketoprofen did not modify mPGES mRNA abundance, SC-236 injection resulted in a significant and reproducible decrease of mPGES mRNA expression (Fig. 8).

Discussion

The main findings of this study are as follows: 1) inhibition of NOS2 enzyme activity with the relatively specific NOS2 inhibitor AG decreases the liver and plasma concentrations of COX products 6-k-PGF<sub>1α</sub>, PGE<sub>2</sub>, and TXB<sub>2</sub>; 2) because of the low level/absent COX-2 protein expression and lack of effect of SC-236 on plasma and liver concentrations of 6-k-PGF<sub>1α</sub> and TXB<sub>2</sub>, the observed effects of AG suggest that NOS2-derived NO activates the COX-1 pathway; 3) in this model of systemic inflammation, in addition to the NOS2-mediated COX-1 activation, the increased plasma and liver concentrations of PGE<sub>2</sub> are the result of LPS-mediated induction of mPGES mRNA expression; 4) in the absence of COX-2 protein expression, the putative COX-2 inhibitor SC-236 decreases the liver and plasma concentrations of PGE<sub>2</sub>, and also decreased mPGES mRNA expression in the liver, an observation that to the best of our knowledge is reported for the first time; 5) in contrast to previous in vitro reports, administration of COX inhibitors did not change the LPS-induced NOS2 pathway activation in vivo.

Methodological discussion

Ketoprofen is a potent nonsteroidal anti-inflammatory drug whose therapeutic efficacy has been demonstrated in numerous diseases over 20 years (24). It inhibits both COX-1 and COX-2 activities. In a model of carrageenan-induced paw edema in rats, a dose of 5 mg/kg exhibited maximal inhibition of carrageenan-induced inflammation (22). A dose of 0.5 mg/kg ketoprofen given orally

![FIGURE 3](image3.png)

**FIGURE 3.** Effect of AG on mRNA expression of COX isoforms in the liver. A, Representative RT-PCR profile of rat liver COX-1, COX-2, and β-actin mRNA expression. The PCR products were detected as 598-, 304-, and 232-bp bands for COX-1, COX-2, and β-actin, respectively. B, Densitometric analysis. Results are expressed as mean ± SEM of relative COX/β-actin mRNA abundance. COX-2 mRNA expression was too low to be quantified; n = 4 for control group; n = 6 for LPS, LPS + 50 mg/kg AG, and LPS + 100 mg/kg AG groups. *, p < 0.05 as compared with control group.

![FIGURE 4](image4.png)

**FIGURE 4.** Effect of COX inhibitors on mRNA expression of NOS isoforms in the liver. A, Representative RT-PCR profile of rat liver NOS2, NOS3, and β-actin mRNA expression. The PCR products were detected as 578-, 819-, and 232-bp bands for NOS2, NOS3, and β-actin, respectively. B, Densitometric analysis. Results are expressed as mean ± SEM of relative NOS/β-actin mRNA abundance; n = 4 for control group; n = 12 for the LPS group; and n = 6 for LPS + ketoprofen and LPS + SC-236 groups. *, p < 0.05 as compared with control group.
inhibited by 50% the urinary PGE₂ secretion in rats; 5 mg/kg ketoprofen administered rectally produced a 87% inhibition of PGE₂ synthesis (24). Toxicity of ketoprofen arises from its inhibition of COX-1 activity, as for all nonsteroidal anti-inflammatory drugs. Nevertheless, repeated high doses are necessary to elicit deleterious side effects (24). Thus, we can assume that the single dose of 5 mg/kg used in our experiments was sufficient to block COX activity without inducing toxic effects. This is illustrated in Table II: ketoprofen completely inhibited the LPS-induced increase of plasma and liver 6-k-PGF₁α and TXB₂ and liver PGE₂ concentration.

The molecule SC-236 is a member of the family of the potent and selective inhibitors of COX-2 activity (23). Its selectivity toward COX-2 has been uniformly proven: it displays IC₅₀ values of 17 and 0.005 μM against human COX-1 and COX-2, respectively. Therefore, the ratio of selectivity IC₅₀ COX-1/IC₅₀ COX-2 is of 3400 (25). The specificity of this compound has also been tested in vivo using the rat air pouch-induced inflammation model: SC-236 inhibits COX-2-dependent PGE₂ production with an ED₅₀ of 0.3 mg/kg when given by gavage, and a dose of 2 mg/kg almost completely (99%) inhibited the COX-2-dependent PGE₂ production, whereas COX-1-dependent PGE₂ production was not affected at doses up to 10 mg/kg (26, 27). Moreover, an i.p. injection of 0.5 mg/kg SC-236 was sufficient to entirely reverse the curtailment of the edema evoked by allergen in infected rats (28). No toxicity was elicited by administration of up to 200 mg/kg SC-236 in rats (27). The long plasma half-life of SC-236 (117 h in rats (23)), its high specificity, lack of toxicity, and the low dose (2 mg/kg) necessary to inhibit COX-2 activity are strong arguments that the dose of 10 mg/kg used in our experiments: 1) would have been sufficient to completely and selectively block a potential COX-2 activity, and 2) had no effect on COX-1 activity.

AG belongs to the family of relatively specific inhibitors of NOS2 activity (21). Its selectivity toward NOS2 was recognized early (29, 30) and subsequently confirmed (21, 31). Interestingly, AG has beneficial effects in various experimental models of inflammation and shock (reviewed in Ref. 21). Moreover, Tracey et al. (20) reported an ED₅₀ value for AG of 19 mg/kg in rats injected with 5 mg/kg LPS, an 85% inhibition of the LPS-induced increase

### Table II. Effect of COX inhibitors on plasma nitrite/nitrate concentration and plasma and liver prostanoids concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + Ketoprofen</th>
<th>LPS + SC-236</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite/nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (μM)</td>
<td>2.50 ± 0.14</td>
<td>4.37 ± 0.32*</td>
<td>4.22 ± 0.68*</td>
<td>3.88 ± 0.67*</td>
</tr>
<tr>
<td>6-k-PGF₁α</td>
<td>1.22 ± 0.22</td>
<td>2.21 ± 0.13*</td>
<td>1.50 ± 0.10‡</td>
<td>1.80 ± 0.10‡</td>
</tr>
<tr>
<td>Liver (ng/ml)</td>
<td>0.06 ± 0.01</td>
<td>0.46 ± 0.07*</td>
<td>0.02 ± 0.04*</td>
<td>0.29 ± 0.08*</td>
</tr>
<tr>
<td>PGE₂</td>
<td>2.17 ± 1.48</td>
<td>4.08 ± 4.21*</td>
<td>15.56 ± 3.55‡</td>
<td>26.52 ± 2.34‡</td>
</tr>
<tr>
<td>Liver (pg/μg protein)</td>
<td>0.28 ± 0.02</td>
<td>0.82 ± 0.13*</td>
<td>0.32 ± 0.05†</td>
<td>0.53 ± 0.08‡</td>
</tr>
<tr>
<td>TXB₂</td>
<td>0.68 ± 0.08</td>
<td>1.29 ± 0.14*</td>
<td>0.64 ± 0.11*</td>
<td>1.07 ± 0.15‡</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± SEM; n = 4 for control group; n = 12 for LPS group, n = 6 for LPS + ketoprofen and LPS + SC-236 groups. †, ‡: p < 0.05 as compared to control, LPS, and LPS + ketoprofen groups, respectively.
in plasma nitrates/nitrites with 300 mg/kg AG, and a 10% mortality in rats receiving 100–300 mg/kg AG. Thus, its reported selectivity toward NOS2 and low toxicity are arguments that AG, at the doses used in our experiments (50 or 100 mg/kg twice), was a relatively specific NOS2 enzyme inhibitor. This was confirmed by the results presented in Table I: 100 mg/kg AG entirely inhibited the LPS-induced increase of plasma nitrite/nitrate concentration without decreasing this concentration below that observed in control rats.

**Characterization of NOS and COX isoform expression and end-product concentration after low dose LPS challenge in vivo**

Our results are consistent with previous reports (reviewed in Ref. 1) and demonstrate that 6 h after 6 mg/kg LPS injection in rats, NOS2 expression and plasma nitrite and nitrate concentration are increased, while NOS3 mRNA and protein expression remain unchanged. During the same time course, there was decreased COX-1 mRNA expression, slightly increased COX-2 mRNA, absent COX-2 protein expression, and highly increased plasma and liver concentration of the COX products. Decreased COX-1 expression by LPS has previously been reported (32, 33), and our results are consistent with these observations. In contrast, there was low level induction of COX-2 mRNA expression with undetectable COX-2 protein. The absence of major COX-2 induction following LPS challenge contrasts with previous reports showing COX-2 induction in many distinct cell types (for review, see Ref. 34). Additional Western blot experiments demonstrating induction of COX-2 protein in liver and heart of rats stimulated with 15 mg/kg LPS and sacrificed 9 h later further argued for absent COX-2 protein induction 6 h after injection of 6 mg/kg LPS in rats (Y. Devaux, C. Seguin, and D. Longrois, unpublished observations). Taken together, our results are consistent with previous reports that demonstrated that low dose LPS injection is a more efficient inducer of NOS2 as compared with COX-2 (35).

The dramatic rise in plasma and liver prostanooid concentrations despite low level/absent COX-2 protein 6 h after LPS challenge is consistent with COX-1 being the main source of plasma and liver prostanooids in this experimental model, although participation of COX-2 in organs not studied cannot be excluded. The relative contribution of COX-1 and COX-2 in the inflammatory response is controversial. Some studies suggested that COX-1 is the major source of PG during inflammation (36, 37), whereas others suggested that the PGs that contribute to inflammatory responses are derived exclusively from COX-2 (11, 38). Other investigators have dissected the inflammatory response in two phases: an early phase (4 h) mediated by COX-1-generated PG, and a later phase (after 12 h) mediated by COX-2-derived PG (39). Thus, both COX-1 and COX-2 synthesize PG during the inflammatory response, but the relative contribution of one or the other isosform depends on the

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**FIGURE 7.** Time-dependent NOS-COX interaction. Results are expressed as mean ± SEM; *n = 4 for each group. *p < 0.05 as compared with control group.

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**FIGURE 8.** Effect of AG, ketoprofen, and SC-236 on mPGES mRNA expression in the liver. A, Representative RT-PCR profile of rat liver mPGES and β-actin mRNA expression. The PCR products were detected as 501- and 232-bp bands for mPGES and β-actin, respectively. B, Denitometric analysis. Results are expressed as mean ± SEM of relative mPGES/β-actin mRNA abundance; *n = 4 for control group; *n = 6 for LPS, LPS + 50 mg/kg AG, LPS + 100 mg/kg AG, LPS + ketoprofen, and LPS + SC-236 groups. *p < 0.05 as compared with control and groups that did not receive SC-236, respectively.
type of proinflammatory stimulus and the experimental time course after its administration. Our results are consistent with the observation that in a model of nonlethal systemic inflammation (6 mg/kg LPS in rats in vivo) with absent COX-2 protein, COX-1 is the major source of PG in the liver and plasma in the early phase.

The effects of AG are consistent with activation of the COX-1 pathway by NOS2-derived NO

Inhibition of NOS2 enzyme activity with AG dose dependently decreased not only plasma nitrite and nitrate concentrations, but also plasma and liver 6-k-PGF$_{1\alpha}$, PGE$_2$, and TXB$_2$ concentrations (Fig. 9). These effects of AG on plasma and liver prostanoid concentrations were not due to changes of COX-1 or COX-2 mRNA or protein expression. A direct effect of AG on COX activity is unlikely. Zingarelli et al. (33) have shown that AG inhibited COX-1, but not COX-2-derived 6-k-PGF$_{1\alpha}$ production. Nevertheless, a high dose of 3 mM AG was necessary to inhibit only 25% of COX-1 activity. In addition, we performed experiments in which AG was administered to rats that had no NOS2 expression. Under these conditions, AG did not decrease plasma nitrites/nitrates nor PG concentrations (data not shown). Taken together, these observations argue against a nonspecific direct effect of AG on COX activity in these in vivo experiments. Our results are consistent with previous studies that demonstrated that NO enhances COX activity (40–45). In contrast, several studies have shown that NO inhibits COX activity (46–50) or has no effect (51, 52). Specifically, studies performed in rats in vivo have shown that NOS2-produced NO either activates PG synthesis (42) or has no effect (52). The results of these conflicting reports have been recently reviewed by Goodwin et al. (16). The effects of NO on COX activity depend not only on experimental conditions, cell types, inhibitors, and inducers of NO synthesis used, but also on the isoforms of NOS and COX taken into account. A recent study with COX-1- and COX-2-deficient mice has shown that NO activates COX-1-derived PGE$_2$ production with no modification of COX-1 expression, and that NO inhibits COX-2-derived PGE$_2$ production with decreased COX-2 expression (53). In contrast, Sautetbin et al. (54) have shown that endogenous NO activates both COX-1 in normal rats and COX-2 in LPS-stimulated rats. Our results are consistent with the observation that in vivo, in the absence of COX-2 protein, NOS2-derived NO enhances COX-1-derived PG synthesis.

Time course of COX activation by NO

To further investigate the NOS-COX cross-talk in this experimental model, additional rats treated with 6 mg/kg LPS and 100 mg/kg AG or vehicle were sacrificed 12, 24, or 48 h after LPS injection (protocol 3). Our results show that the activation of COX pathway by NO is significant during 12 h after LPS injection and is correlated with induction of NOS2 expression and activity. The increase of COX products is paralleled by the increased nitrite and nitrate concentration (Table III). This result is not consistent with a long lasting effect of NOS2-derived NO on PG synthesis, nor with a hit-and-go effect. The decrease in COX-1 mRNA expression 6 h following LPS challenge is followed by a high increase in COX-1 mRNA expression between 12 and 24 h following LPS challenge (Table III). To the best of our knowledge, such a time course of COX-1 mRNA expression after LPS challenge in vivo has not been published yet. The biological significance of this delayed increase in COX-1 mRNA expression is currently under investigation.

Mechanical explanation for NOS-COX cross-talk

Several mechanisms that could explain the activation of COX by NO have recently been reviewed by Goodwin et al. (16); the most likely to explain our results is the one described by Landino and coworkers (45). In addition to increasing the synthesis of NO, LPS also causes the synthesis of reactive oxygen species such as superoxide anions (O$_2^·$) that react spontaneously with NO to form the potent and versatile oxidant peroxynitrite (ONOO$^-$). Because ONOO$^-$ is an inorganic hydroperoxide, it is conceivable that it acts as an activator of the COX activity and as a substrate for the hydroperoxidase activity of PGH-S (45). In contrast, inhibition of COX activity through tyrosine nitration by ONOO$^-$ has been demonstrated, and COX-2 has been shown to be more sensitive to this inhibition than COX-1 (53, 55).

Induction of mPGES mRNA expression contributes to the increased plasma and liver concentrations of PGE$_2$ after LPS challenge in vivo

The nonspecific COX inhibitor ketoprofen completely abolished the LPS-mediated increase in plasma and liver prostanoid concentrations and attenuated the increase of plasma PGE$_2$ concentration. Administration of the COX-2-specific inhibitor SC-236 to LPS-stimulated rats did not decrease plasma and liver concentrations of TXB$_2$ and 6-k-PGF$_{1\alpha}$. This is consistent with low level/absent COX-2 expression in this experimental model. Nevertheless, SC-236 administration to LPS-injected rats significantly decreased PGE$_2$ concentration by ~40% (Table II). This result could be surprising because we have shown that the COX-2 protein was barely or not induced under these experimental conditions. To explain this paradigm, we investigated a potential effect of SC-236 on another enzyme responsible for prostanoid synthesis. PGE$_2$ is described as the main modulator of immune and inflammatory reactions (16), and was the most abundant PG produced following LPS injection in our experiments. Because AG totally abolished the
LPS-mediated increase in 6-k-PGF$_{1α}$ and TXB$_2$, but had partial effects on PGE$_2$ concentration, we focused on the enzyme specifically responsible for the synthesis of PGE$_2$. This enzyme is the terminal synthase metabolizing PGH$_2$ to PGE$_2$, i.e., the recently characterized PGES. Two isoforms of PGES have been characterized, one of which is the mPGES inducible by proinflammatory stimuli such as LPS (13). Our results show that, while mPGES mRNA expression was undetectable in the liver of control rats, LPS administration triggered induction of mPGES mRNA expression in the liver. This induction most probably accounted for the increased PGE$_2$ concentration measured in the plasma and liver of LPS-treated rats compared with control rats. Surprisingly, SC-236 administration significantly inhibited the LPS-mediated induction of mPGES mRNA expression (Fig. 8), suggesting for the first time an effect of SC-236 on mPGES mRNA transcription and/or stability. Whether this effect is direct or indirect remains to be established. Inhibition of mPGES activity by another COX-2-specific inhibitor, NS-398, has recently been reported and supposed to be related to similar structural properties in the active sites of COX-2 and mPGES (56). We can thus assume that the putative COX-2-specific inhibitor SC-236 inhibited mPGES rather than COX-2 activity in our experiments. Taken together, these observations are consistent with a documented inhibitory effect of SC-236 on mPGES mRNA expression and/or a putative inhibitory effect on activity that could account for a decreased PGE$_2$ production.

AG did not change the LPS-induced mPGES mRNA expression

Neither AG nor ketoprofen altered the LPS-mediated induction of mPGES mRNA expression in the liver (Fig. 8), suggesting that NOS2-derived NO and prostanoids had no effect on mPGES mRNA transcription and/or stability. In contrast, NO has been shown to induce PGES activity in vitro (57). Because the isomerization of PGH$_2$ to PGE$_2$ by PGES is a nonoxidative rearrangement that requires glutathion as the unique cofactor (58), it seems unlikely that NO activates PGE$_2$ through ONOO$^-$ as it has been suggested for activation of COX by NO. Nevertheless, PGES contains a tyrosine residue necessary for its catalytic activity (12) that could be a potential target for NO-derived tyrosine nitration. The mechanisms of a potential modulation of PGES expression and/or activity by NO need further investigation.

Prostanoids do not modify NOS expression and activity in LPS-challenged rats in vivo

Neither ketoprofen nor SC-236 significantly modified NOS2 and NOS3 mRNA (Fig. 4) and protein (Fig. 5) expression or nitrite and nitrate plasma concentrations (Table II) compared with rats from LPS group. Several experiments, all performed in vitro, have revealed activatory (59), inhibitory (60, 61), or absent (62) effects of PGE$_2$ on NO production. Our results are against any significant effect of prostanoids on NOS expression and activity in vivo.

Beneficial and deleterious effects of NOS2 inhibitors

One can speculate that potent or high dose inflammatory stimuli induce high level expression of NOS2 and COX-2, increased ONOO$^-$ concentrations, tyrosine nitration of COX-2, with decreased PG production. Under these circumstances, NOS2 inhibitors might have beneficial effects on host survival (typically high LPS doses in rodent models; Ref. 18). Other types of stimuli, such as the low dose LPS used in the present study, result in NOS2 induction and low level/absent COX-2 induction with NO or ONOO$^-$, resulting in increased COX-1-derived end products. In that case, NOS2 inhibitors could have deleterious effects because they result in the inhibition of two vasodilatory systems. This could be typically the case in human septic shock (5). A differential effect of AG on the different products of the terminal prostanoïd syntheses could contribute to explain the beneficial/deleterious effects of AG administration. Interestingly, in this study, TXB$_2$ (a vasoconstrictor prostanoïd) synthesis was inhibited at lower doses of AG compared with PGE$_2$ and 6-k-PGF$_{1α}$ (vasodilatory prostanoïds). These results could explain the differences observed between models in which high or low dose NOS2 inhibitors were administered (4).

In conclusion, these results demonstrate that, in a model of low dose LPS-mediated NOS2 activation and absent COX-2 protein expression, inhibition of NOS2 activity by AG dose dependently decreases the plasma concentration of COX-1-generated PGS. If these results are confirmed in other species and with proinflammatory stimuli other than LPS, they could imply that there is a major conceptual limitation to the use of NOS2 inhibitors in models of systemic inflammation.

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References


