Effect of an Imidazolineoxyl Nitric Oxide on Prostaglandin Synthesis in Experimental Shock: Possible Role of Nitrogen Dioxide in Prostacyclin Synthase Inactivation

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The effect of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a nitric oxide (NO) scavenger that yields nitrogen dioxide (NO$_2$) in a rat endotoxemia model was investigated. Endotoxin (lipopolysaccharide [LPS]) increased NO synthase (NOS) activity and inducible NOS expression measured in lung and plasma levels of nitrite/nitrate, 6-oxo-prostaglandin (PG) F$_{1\alpha}$, thromboxane B$_2$, and PGF$_{2\alpha}$. Infusion of cPTIO significantly reduced LPS-induced mean arterial blood pressure decline and mortality and selectively reduced LPS-induced 6-oxo-PGF$_{1\alpha}$ plasma levels and prostacyclin synthase (PGIS) activity measured in the lung and aorta. In vitro, PGIS activity in aorta rings was not modified by SNAP (NO donor), cPTIO slightly inhibited the enzyme but not in the presence of L-N$^\omega$-monomethyl arginine, and SNAP in combination with cPTIO significantly inhibited PGIS. Thus, cPTIO may be beneficial in endotoxic shock because of NO scavenging and PGIS inactivation, which could be mediated by NO$_2$.

We reported elsewhere [1] that endothelial cells probably possess only 2 enzymes involved in the biosynthesis of prostanoids: cyclooxygenase (COX), also termed prostaglandin H (PGH) synthase, and prostacyclin synthase (prostaglandin I synthase; PGIS). The former catalyzes the transformation of arachidonic acid (AA) to prostaglandin (PG) H$_2$, which has constricting properties and platelet-activating properties. The latter catalyzes the subsequent transformation of PGH$_2$ into PGI$_2$, a potent relaxing and platelet-activating agent. The ratio of COX and PGIS activities regulates the balance of relaxing/constricting prostanoids in endothelial cells. Exacerbated COX activity by overexpression of COX-2, together with partial inactivation of PGIS, leads to the diminution of PGI$_2$ formation and the release of excess PGH$_2$ [1], which exerts the same actions as thromboxane (Tx) A$_2$, because both share the same receptor [2, 3].

Prostanoids and nitric oxide (NO) play a central role in the pathogenesis of diverse inflammatory and infectious disorders [4–7]. A growing body of experimental evidence indicates an interaction of NO and its derivatives with the AA metabolism through the COX pathway [1, 8, 9]. It has been suggested that NO formation is the limiting step in the peroxynitrite production in endothelial cells and may be induced by the inflammatory cytokines [10, 11]. Homolytic fission of peroxynitrous acid, formed by protonation of peroxynitrite, yields nitrogen dioxide (NO$_2$) and hydroxyl radical [12, 13]. Peroxynitrite inactivates PGIS by nitration of an active site-related tyrosine [14].

We observed [1] that NO was involved in the partial inactivation of PGIS during the exposure of endothelial cells to interleukin (IL)–1$\beta$. The implication of NO on PGIS inhibition in human endothelial cells exposed to IL-1$\beta$ was based on the fact that L-N$^\omega$-monomethyl arginine (L-NMMA), an NO synthase (NOS) inhibitor, abrogated the inhibitory effect of IL-1$\beta$ [1]. Indeed, 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium, which releases both NO and superoxide anion and acts as a source of peroxyynitrite, inactivated PGIS in both resting and IL-1$\beta$–treated endothelial cells [1].

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) is a NO scavenger that reacts with NO to yield NO$_2$ plus 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (cPTI) [15]. It impairs the vasorelaxation induced by acetylcholine [16] and exhibits a potent therapeutic value in a rat model of endotoxic shock [17]. The fact that inactivation of PGIS caused by the treatment of endothelial cells with IL-1$\beta$ was significantly enhanced by the presence of cPTIO during exposure to IL-1$\beta$ argues against a direct effect of NO on PGIS activity. Nitration of tyrosyl residues by NO$_2$ is possible through the formation of tyrosyl radicals [18–20]. Collectively, our previous results with human endothelial cells in culture with L-NMMA and cPTIO were consistent with the concept that the inactivation of the PGIS caused by the incubation with IL-1$\beta$ was mediated by NO$_2$ [1].

In the present investigation, we attempted to extend our previous in vitro observations, to test the hypothesis that effectively
there is an NO2-dependent inactivation of PGIS in vivo under inflammatory conditions that could be potentiated by cPTIO. The inhibitory action of cPTIO on PGIS could also contribute to the beneficial effect of cPTIO observed in endotoxic shock.

Methods

cPTIO and cPTI synthesis. cPTIO potassium salt was synthesized by modification of a method described elsewhere [15]. The starting compound, 2,3-hydroxylamino-2,3-dimethylbutane [21], was condensed at room temperature with 4-formilbenzoic acid in the presence of aqueous NaHCO3, leading to the intermediate compound 4-(1,3-dihydroxy-4,4,5,5-tetramethyltetrahydro-2-imidazolyl)benzoic acid. The latter compound was oxidized in dimethylformamide solution, using PbO2 as reagent and yielding cPTIO in the acid form as a deep-blue crystalline solid (melting point [m.p.], 188°C decomposition). The corresponding potassium salt was synthesized in high yield by dissolving this product in anhydrous dichloromethane and by neutralizing it with an alcohol solution of potassium tert-butoxide. cPTIO potassium salt was obtained as a high-purity blue precipitated solid (m.p., 215°C decomposition). No further purification steps were needed. cPTI was obtained from cPTIO by reaction with NO [15]. NO gas was bubbled though a phosphate-buffered (pH 7.4) cPTIO solution, and cPTI was purified by silica-gel column chromatography. Structures were confirmed by infrared, nuclear magnetic resonance, and electron spin resonance spectral information and by elemental analysis data. Purity was checked by thin-layer chromatography and by nonaqueous acidiometric titration.

Mean arterial blood pressure (MAP) measurement. The study was done with male Sprague-Dawley rats weighing 250–300 g. The animals received a standard diet and water ad libitum. All animals were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg); anesthesia was maintained by supplementary injections of anesthetic liquid. All samples were collected and placed in Eppendorf tubes containing 106 Soler et al. JID 2001;183 (1 January) mg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, and 100 μg/mL phenylmethylsulfonylfluoride, with a Potter-Elvehjem device placed in an ice-water bath. The homogenate was centrifuged at 8000 g for 30 min; the supernatant was collected, was treated with 200 μM aspirin in RPMI medium at 37°C for 20 min, and was centrifuged at 100,000 g for 90 min. The pellet was resuspended in 100 mM Tris-HCl (pH 8.5) containing 2 mM phenol. The protein content was measured by Bradford’s method, using the Bio-Rad protein assay, and was adjusted to 200 μg of protein/100 μL.

PGIS activity measurement. PGIS activity in aorta rings or lung homogenates was determined as described elsewhere [1], with small modifications. We incubated 100 U of COX-1 from ram seminal vesicles (Cayman Chemical) in 100 mM Tris-HCl (pH 8.5) containing 2 mmol/L phenol (200 μL, aorta rings; 100 μL, lung homogenates) with [14C]AA ([1-14C]AA; 55–58 mCi/mmol [American Isotope]; final concentration in incubation with tissues, 25 μM) at 30°C for 1 min. We added the aspirin-treated aorta ring or 100 μL of aspirin-treated lung homogenate to the COX preparation, and the mixture was incubated for 5 min at 37°C. The reaction was stopped by adding 350 μL of methanol:water:acetic acid (550:150:11). 14C-labeled 6-oxo-PGF1α was evaluated by high-performance liquid chromatography, as described elsewhere [22]. The quantity of COX-1 necessary to guarantee generation of a excess 14C-labeled endoperoxidase was determined in previous experiments by testing different amounts of isolated COX-1. 6-oxo-PGF1α was only observed when tissue was present in the preparation and the production of 6-oxo-PGF1α using 50 U COX-1 per incubate, was maximal (n = 5); 100 U of COX was chosen for the study.

NOS activity measurement. Rats were killed at the indicated times after administration of LPS, and NOS activity was quantified in lung homogenates by measuring the conversion of L-[3H]arginine to L-[3H]citrulline, as described elsewhere [23, 24]. In brief, lungs were homogenized as described above without aspirin pretreatment, the homogenates were centrifuged at 8000 g for 10 min at 4°C, and supernatants were used to measure NOS activity. The protein content was measured by Bradford’s method, and tissue homogenate containing 200 μg of protein was added to tubes containing a buffer consisting of 50 mM potassium phosphate (pH 7.2), 0.24 mM CaCl2, 1.2 mM MgCl2, 50 mM L-valine, 1 mM L-citrulline, 100 μM NADPH, 10 μM tetrahydrobiopterin, 20 μM levels of 6-oxo-PGF1α (stable metabolite of PGF1α), TXB2 (stable metabolite of TXA2) and PGE2, were evaluated by specific EIA (Cayman Chemical) after solid-phase extraction by following the manufacturer’s instructions.

Measurement of nitrite/nitrate plasma concentration. Plasma samples were ultrafiltered through a 10-kDa molecular cutoff filter (Millipore) and analyzed by a nitrate/nitrite fluorometric assay kit (Cayman Chemical), according to the manufacturer’s instructions.

Preparation of aorta rings. After removal, the aorta was dissected free of connective tissue and cut into ~5–mg rings. The aorta rings were then incubated in RPMI culture medium containing 200 μM acetylsalicylic acid (aspirin) at 37°C for 20 min. The medium containing aspirin was removed before the PGIS activity assay.

Lung homogenate preparation. After removal, lungs were frozen in liquid nitrogen and were stored at ~80°C until use. They were mechanically homogenized in 10 mL Hepes (pH 7.2) containing 320 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, and 100 μg/mL phenylmethylsulfonylfluoride, with a Potter-Elvehjem device placed in an ice-water bath. The homogenate was centrifuged at 8000 g for 30 min; the supernatant was collected, was treated with 200 μM aspirin in RPMI medium at 37°C for 20 min, and was centrifuged at 100,000 g for 90 min. The pellet was resuspended in 100 mM Tris-HCl (pH 8.5) containing 2 mM phenol. The protein content was measured by Bradford’s method, using the Bio-Rad protein assay, and was adjusted to 200 μg of protein/100 μL.

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L-arginine, and 10⁶ cpn L-[³H]arginine (63 Ci/~mol; Amersham). Incubations were done in duplicate at 37°C for 30 min. The reaction was terminated by removal of substrate and dilution by addition of 500 μL of H2O/Dowex AF 50W-X8 Na⁺-form (Sigma) 1:1 (vol: vol; pH 7.5), 890 μL of H2O was added to the incubation mix, and 1 mL of supernatant was removed, and the presence of [³H]citrulline was evaluated by liquid scintillation counting.

Specific inducible NOS (iNOS) mRNA analysis. Rats were killed at the indicated times after administration of LPS or vehicle, and iNOS mRNA was quantified in lung homogenates. Total RNA was isolated by the Ultraspec isolation system (Biotecx Laboratories). Lungs were mechanically homogenized in Ultraspec buffer. The specific mRNA levels were estimated by a quantitative reverse transcription–polymerase chain reaction (RT-PCR) protocol. RNA (5 μg) was reverse-transcribed by incubation with 75 U of murine leukemia virus reverse transcriptase in a reaction buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 0.4 μM antisense primer (described later), 30 U of RNAase inhibitor, and 1 mM dNTPs (Pharmacia Biotech) in a final volume of 30 μL (all from Perkin-Elmer unless indicated). The reaction mixture was incubated for 30 min at 42°C, stopped by heating for 5 min at 99°C, and cooled for 5 min at 5°C. The PCR was carried out in a DNA thermal cycler (model 480; Perkin-Elmer) with a reaction mixture (100 μL) containing 8 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μM sense/antisense primers, 2.5 U Taq polymerase, and 4 μCi [³H]dCTP (deoxy [¹²⁵]Icytidine 5'-triphosphate ammonium salt, 64 Ci/~mol; Amersham). Serial half dilutions of the cDNA were made to test linearity. The primers (Progenetic SL) used for iNOS were 5'-CACAAAGCCACATCG-GATTTC-3' (sense) and 5'-TGCATACCACTTCAACCCGAG-3' (antisense) [25]. The RNA for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was amplified and used as the internal control. The sense and antisense primers used for GAPDH were 5'-TCCCTCAAGATCTGTCAGCAA-3' and 5'-AGATCCACACCG-GATACATT-3', respectively [26]. Thirty cycles were done as follows: 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for all samples. The amplification products were separated by electrophoresis in a 1.5% wt/vol low-melting-point agarose gel containing ethidium bromide. Radioactivity associated with the specific band was evaluated as described elsewhere [27, 28]. The radioactivity was normalized with respect to GAPDH.

Effect of an NO donor and NOS inhibitor in the presence or absence of cPTIO on PGIS activity in vitro. Under sterile conditions, aortas from untreated rats were cleared of periadventitial connective tissue and cut into ~3-mm rings. Rings were placed in sterile 24-well plates containing 1 mL of RPMI containing 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% fetal bovine serum. To this medium, we added the following conditions: none, 100 μM SNAP (NO donor), 100 μM SNAP plus 100 μM cPTIO, 100 μM cPTIO, 100 μM SNAP plus 100 μM cPTIO, 100 μM SNAP plus 100 μM cPTIO, 1 mM L-NMMA or 100 μM cPTIO plus 1 mM L-NMMA. Aorta rings were then incubated at 37°C for 4 h in a 5% CO₂ atmosphere. After medium was recovered for nitro/nitrate evaluation, aorta rings were incubated with [¹⁵N]-labeled endoperoxidase, to determine PGIS activity.

Statistics. We used SigmaStat software (SPSS Science) for statistical analysis. Statistical significance between groups was assessed by Student’s t test for 2 groups. One-way analysis of variance and the Student-Newman-Keuls tests were used to compare >2 groups. P < .05 was considered significant.

Results

MAP and survival in normal rats and in rats treated with LPS or with LPS plus cPTIO. Administration of cPTIO alone caused a slight increase in MAP, particularly during the first 2 h, without any significant effect on survival during the observation period (Figure 1). This increase was not due to volume loading during the infusion, because infusion of vehicle did not cause significant variation of MAP (not shown). Time-dependent hypotension was observed in LPS-treated rats, as shown by a double wave-shaped MAP curve (Figure 1A). The first phase of LPS-treated rats at ≤2 h after LPS infusion was similar to that of rats treated with LPS plus cPTIO. In a second phase,
a decrease in MAP was seen in the LPS-treated rats; rats treated with LPS and cPTIO had significantly higher MAP values. Infusion of cPTIO also resulted in a dramatic reduction of mortality caused by LPS administration (figure 1B). Infusion of cPTIO did not significantly modify the values of MAP and mortality, compared with the LPS group (n = 15; not shown).

Expression of iNOS and NOS activity in rats treated with LPS. As expected, administration of LPS produced a substantial time-dependent elevation of NOS activity in lung homogenates (figure 2). There was also a time-dependent induction of iNOS in terms of mRNA steady-state levels in lung homogenates. mRNA for iNOS increased until 3 h after LPS administration and then decreased. NOS activity increased throughout the observation period.

Nitrite/nitrate plasma levels. Consistent with the NOS activity results, LPS induced a time-dependent increase of plasma levels of nitrite/nitrate, with values similar to those found in rats treated simultaneously with LPS and cPTIO (figure 3).

Prostanoid plasma levels. Figure 4 depicts plasma levels of 6-oxo-PGF$_{1\alpha}$ (stable metabolite of PGI$_2$), TxB$_2$ (stable metabolite of TxA$_2$), and PGF$_{2\alpha}$. LPS administration caused an increase of the 3 prostanoids analyzed. Rats treated with LPS and cPTIO exhibited prostanoid plasma levels similar to those treated with LPS, with the exception of 6-oxo-PGF$_{1\alpha}$. After 3 h of LPS administration, levels of 6-oxo-PGF$_{1\alpha}$ were lower in the rats treated with LPS plus cPTIO, although differences in the LPS treatment group only reached statistical significance 4 h after LPS injection. These results suggested a selective inhibitory action of cPTIO on PGIS activity. To rule out the possibility that the effect of cPTIO on 6-oxo-PGF$_{1\alpha}$ was due to cPTIO formation, rats were infused with cPTIO equimolar dose of cPTI and treated with LPS. Plasma levels of 6-oxo-PGF$_{1\alpha}$ were then measured (n = 4): No differences were observed when compared with the LPS group (not shown).

**PGIS activity ex vivo.** After 4 h of LPS administration, PGIS activity was evaluated in lung homogenates and aorta rings (see Methods). As shown in figure 5, the loss of PGIS activity was not identical in these tissues. PGIS activity in lung homogenates of LPS-treated rats was lower than in controls, but this difference was not significant when evaluated in the aorta rings. In the animals treated with cPTIO, the PGIS activity was significantly lower than in control rats, although this was more pronounced in lung homogenates than in aorta rings. The lowest PGIS activity was found in samples from rats treated with both cPTIO and LPS. As shown in figure 5, there were no significant differences in PGIS activity ex vivo between LPS-treated rats and LPS-treated rats infused with cPTIO.

**Effect of NO donor and NOS inhibitor in the presence or absence of cPTIO on PGIS activity in vitro.** The above results suggested that NO$_2$, generated by the reaction of cPTIO with NO, inhibited PGIS. Thus, we incubated aorta rings from untreated rats for 4 h in a culture incubator with culture medium or medium containing 100 μM of SNAP (NO donor), 100 μM cPTIO, or both. Results are shown in figure 6A. SNAP did not exert any effect on PGIS activity, whereas cPTIO significantly inhibited the enzyme. When aorta rings were incubated with both cPTIO and SNAP, PGIS activity was significantly more inhibited than when incubated with cPTIO alone. L-NMMA alone did not affect the rats treated with LPS plus cPTIO.
PGIS activity. Nevertheless, when it was present in the incubation medium with cPTIO, L-NMMA abolished the inhibitory effect of cPTIO on PGIS activity. We found no significant effect of cPTI alone or in combination with SNAP on PGIS activity in vitro. As shown in figure 6B, the presence of SNAP increased nitrite/nitrate levels in the medium, indicating a higher formation of NO. Nitrite/nitrate levels in the medium were significantly lower in the presence of L-NMMA than in controls.

Discussion

This study was undertaken to observe the action of cPTIO on PGIS activity, extending our previous in vitro research that suggested an inhibitory action of NO on PGIS endothelial cells in vitro [1]. Yoshida et al. [17] found that cPTIO had a therapeutic value in experimental endotoxic shock. By using the most effective dose used by Yoshida et al., we observed a clear improvement in the MAP fall and a significant reduction in mortality caused by the administration of LPS in the rats given cPTIO in addition to LPS. Even though our administration protocol of cPTIO differed from that of Yoshida et al. [17], our results confirm their findings. Our results are also consistent with other reports suggesting that inhibition of excess of NO could be beneficial in shock (reviewed in [4, 5, 29, 30]).

We evaluated iNOS and NOS activity in the lung, which is one of the most affected organs in systemic inflammation syndromes. Our results with lung homogenates were consistent with those reported by Liu et al. [31], who analyzed heart, lung, and aortic and pulmonary arteries; iNOS expression and NOS activity were increased by the administration of LPS, which resulted in enhanced circulating levels of nitrite/nitrate. Nevertheless, NOS activity was significantly increased after 3 h of LPS administration, whereas the increase in nitrite/nitrate levels was observed just 1 h after LPS injection, indicating that, during the first phase of the syndrome, the MAP decline was due to NO coming from constitutive NOS, rather than from iNOS. This concept is supported by data reported by others (reviewed in [29]). As expected, no effect of cPTIO on nitrite/nitrate levels was observed because NO can easily be transformed into nitrite [32].

Also consistent with previously reported data [7], increased circulating levels of prostanoids were observed in LPS-treated rats. LPS increased plasma levels of 6-oxo-PGF₁α, TxB₂, and

![Figure 4. Effect of lipopolysaccharide (LPS; vehicle) on prostanoid plasma concentration as function of time. Bars are mean ± SD (n = 5). *P < .05. LPS-challenged rats infused with vehicle and LPS-challenged rats prophylactically treated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; LPS and cPTIO) vs. time 0. #P < .05, LPS and cPTIO group vs. LPS group. 6-oxo-PGF₁α, 6-oxo-prostaglandin F₁α; PGF₂α, prostaglandin F₂α; TxB₂, stable metabolite of TxA₂.](image)

![Figure 5. Effect of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) on prostacyclin synthase (PGIS) activity ex vivo in aorta rings (A) and lung homogenates (B). Results shown as pmol of 6-oxo-PGF₁α formed in 5 min per mg of tissue (A) or mg of protein (B). *P < .05, lipopolysaccharide (LPS; vehicle)-challenged rats prophylactically treated with cPTIO (LPS and cPTIO) or cPTI (LPS and cPTI). LPS-challenged rats infused with vehicle, or LPS-uninfused rats infused with cPTIO vs. untreated rats infused with vehicle (controls); #P < .05, compared with LPS group. Bars are mean ± SD (LPS and cPTI, n = 4; all others, n = 7).](image)
Figure 6. Effect of nitric oxide (NO) donor and nitric oxide synthase (NOS) inhibitor in presence or absence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) on prostacyclin synthase (PGIS) activity (A) and nitrite/nitrate levels (B) in vitro. Rings were incubated in medium with none (control), 100 \( \mu \text{M} \) NO donor (SNAP), 100 \( \mu \text{M} \) cPTIO, 100 \( \mu \text{M} \) SNAP + 100 \( \mu \text{M} \) cPTIO, 100 \( \mu \text{M} \) 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (cPTI), 100 \( \mu \text{M} \) SNAP + 100 \( \mu \text{M} \) cPTI, 1 mM L-NG-monomethyl arginine (L-NMMA), or 100 \( \mu \text{M} \) cPTIO + 1 mM L-NMMA at 37°C in 5% CO\(_2\) atmosphere for 4 h. Nitrite/nitrate levels were determined in incubation medium and PGIS activity in aorta rings. Bars are mean ± SD (\( n = 10 \), controls; \( n = 5 \), all others).

PGF\(_{2\alpha}\) by 1 h after LPS injection. In rats exposed to LPS, cPTIO did not significantly modify prostanoid levels, except for 6-oxo-PGF\(_{1\alpha}\) levels, which were inhibited after 3 h of LPS injection, suggesting a specific progressive action of cPTIO on PGI\(_1\) synthesis.

Results of the PGIS activity analysis 4 h after LPS injection indicated that LPS partially inactivated PGIS in lung homogenate samples. This was not so evident in the aorta rings. These results are consistent with findings from in vitro experiments in which exposure of endothelial and mesangial cells to a potent proinflammatory stimulus (e.g., IL-1\( \beta \)) partially inactivated PGIS [1, 11]. Administration of cPTIO significantly reduced PGIS activity in both aorta rings and lung homogenates: The effect was more relevant in the latter. Administration of both LPS and cPTIO reduced PGIS activity to a greater extent than LPS alone. The effect of cPTIO on 6-oxo-PGF\(_{1\alpha}\) plasma levels and on PGIS activity was not likely coincident, because many organs and cell types contributed to plasma levels and because the effect of LPS and cPTIO probably was not uniform in different tissues, as indicated by tissue analysis results. Although the inhibitory effect of cPTIO on 6-oxo-PGF\(_{1\alpha}\) plasma levels and on PGIS activity are not coincident, they are consistent with one another.

The final vascular constriction or relaxation is the result of the balanced action of constricting and relaxing agents. Definitive evaluation of the exact importance of PGI\(_2\) and, hence, the PGIS activity in the decline of blood pressure and mortality in endotoxemia should be obtained using specific PGIS inhibitors or PGI\(_2\) antagonists without altering other vasoactive agents. Nevertheless, as far we know, this is not yet possible. By using COX-2 selective inhibitors at doses that inhibited PGI\(_1\) formation, Leach et al. [33] observed an increase in the hypotension induced by LPS injection. We obtained similar results when we administered indomethacin or COX-2 selective inhibitors at doses that inhibited 6-oxo-PGF\(_{1\alpha}\), TxB\(_2\), and PGF\(_{2\alpha}\) by >80% (unpublished data). Leach et al. concluded that the increase in prostanoid formation does not contribute to the circulatory failure caused by LPS; however, they only determined plasma levels of 6-oxo-PGF\(_{1\alpha}\) and the other prostanoids that have vasoconstricting activity were probably inhibited by the COX-2 inhibitors. Inhibition of COX leads to the inhibition of both relaxing and constricting prostanoids and may alter production of other vasoactive agents that are regulated by prostaglandins, such as the renin-angiotensin system. In contrast, we observed that cPTIO selectively inhibited PGIS without modifying synthesis of other prostanoids in the LPS-induced shock.

The results of the in vitro experiments showed that NO alone did not exert any effect on PGIS since SNAP significantly increased nitrite/nitrate in the culture medium without appreciable modification of PGIS activity. In contrast, cPTIO decreased PGIS activity, an effect that was even more pronounced when both SNAP and cPTIO were present in the culture me-
Nevertheless, nitration of tyrosyl residues by NO$_2$ is also possible by means of the formation of tyrosyl radicals [18±20]. Of note, we were unable to demonstrate PGIS nitration because commercial antibodies against PGIS did not run well in our rat samples.

Cigarette smoke is one of the greatest exogenous sources of human exposure to NO [38, 39]. NO is converted to NO$_2$ within minutes and nitrogen oxides in the gas-phase of the cigarette smoke nitrate tyrosine [40]. Hong et al. [41] reported that AA metabolite(s) of the COX pathway is involved in the late phase of cigarette smoke-induced bronchoconstriction but did not identify the COX-derived compound involved in the bronchoconstriction. Our results allow us to speculate that an increased amount of PGH$_2$ released by vascular endothelium caused by NO$_2$-dependent partial inactivation of PGIS could account for the observations of Hong et al. Also, cigarette smoke impairs endothelium-dependent relaxation [42], and selective nitration of PGIS in atherosclerotic bovine coronary arteries has been reported elsewhere [43]. Ex vivo, hypoxia-reoxygenation generates peroxynitrite that inactivates PGIS, causing PGH$_2$-mediated coronary vasospasm in isolated bovine coronary arteries that depend on PGH$_2$ accumulation [44]. In vivo and ex vivo data reported by Zou and coworkers [43, 44] show that inhibition of PGIS is relevant in vascular response. In vivo data clearly indicate that, in atherosclerotic lesions in which nitrated PGIS was detected, formation of PGF$_2$ was reduced, and impaired vasorelaxation was observed [43]. Impaired vasorelaxation was corrected in the presence of a TxA$_2$/PGH$_2$ antagonist, whereas inhibition of TxA synthase was ineffective. Laboratory data strongly suggest that, when vascular PGIS is inhibited, the vasoconstrictor PGH$_2$ is released [43, 44].

In summary, our results show that cPTIO selectively reduces PGIS activity in vivo and in vitro when NO is supplied and cPTI does not exert any action on PGIS activity. Although our results are not conclusive with respect to the exact mechanism by which cPTIO plus NO inactivate PGIS, they are consistent with the concept that NO$_2$ (or a related compound) derived from NO could be involved in the PGIS inactivation caused by cPTIO. Chemical studies that include detection of nitrated PGIS will be performed to confirm such a mechanism. Our results are consistent with the hypothesis that selective inhibition of PGIS could contribute, in addition to NO scavenging, to the beneficial effect of cPTIO in experimental shock by reducing the formation of the potent vasodilator PGI$_2$. More research is needed to clarify the physiologic sources of NO$_2$ and the exact relevance of PGI$_2$ inhibition in physiopathologic situations in which NO formation is increased, such as chronic inflammation, systemic inflammatory syndromes, or atherosclerosis.

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References


13. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990; 87:1620–4.


