Disruption of COX-2 and eNOS does not confer protection from cardiovascular failure in lipopolysaccharide-treated conscious mice and isolated vascular rings

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Short title: eNOS and blood pressure in endotoxemia

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Abstract

It was hypothesized that a serial stimulation of vascular cyclooxygenase-2 with subsequent activation of endothelial nitric oxide synthase (eNOS) is responsible for decrease in blood pressure, cardiac performance and vascular reactivity in endotoxemia caused by lipopolysaccharide (LPS). The hypothesis was tested in catheterized, conscious, freely moving wild type mice and mice (C57BL/6J background) with targeted deletion of COX-2 and eNOS that were given an intravenous LPS bolus (2 mg/kg, 055:B5). In vitro studies were performed on murine aorta rings. LPS caused a concomitant decrease in mean arterial blood pressure (MAP) and heart rate (HR) that was significant after three hours and sustained through the experiment (eight hours). The LPS-induced changes in MAP and HR were not different from control in COX-2-/- and eNOS-/- mice. A prostacyclin receptor (IP) antagonist (BR5064), blocked the hypotensive effect of an IP agonist (beraprost), but did not attenuate the LPS-induced decrease in MAP and HR. LPS decreased eNOS and nNOS mRNA abundances in several organs while iNOS mRNA was enhanced. In aortic rings, LPS suppressed $\alpha_1$-adrenoceptor-mediated vascular tone. Inhibition of COX-2 activity (NS 398), disruption of COX-2, endothelium removal or eNOS deletion (eNOS-/-) did not improve vascular reactivity after LPS while the NO synthase blockers 1400W and L-NAME prevented loss of tone. COX-2 and eNOS activities are not necessary for LPS-induced decreases in blood pressure, heart rate and vascular reactivity Inducible NOS activity appears crucial. COX-2 and eNOS are not obvious therapeutic targets for cardiovascular rescue during gram-negative endotoxemic shock.

Key words: iNOS, prostacyclin, nitric oxide, endothelium.
Introduction

Gram-negative bacterial endotoxemia with sepsis is a systemic response to infection that occurs with an increasing incidence. The systemic inflammatory response may be complicated by cardiovascular collapse with fatal blood pressure decline and multiorgan failure (8). It remains a significant challenge to define pharmacologic targets for intervention that protect and improve cardiovascular performance. Cyclooxygenase type-2 (COX-2) expression and activity is stimulated in macrophages and endothelial cells by gram-negative bacterial wall-derived lipopolysaccharide (LPS) (1). The levels of the potent vasodilators prostaglandin E2 (PGE2) and prostacyclin (PGI2) are increased in patients with septic shock (6). The effect of COX inhibitors on survival and cardiovascular parameters in septic shock is, however, contradictory. In septic patients, ibuprofen reduced urinary levels of metabolites of prostacyclin and thromboxane A2 but not the incidence or duration of shock or the rate of survival (24) (7). Ibuprofen rapidly reversed hypotension in dogs exposed to endotoxin (2) and improved several vascular parameters in pigs (21). Indomethacin improved survival but had no significant effect on MAP in dogs exposed to endotoxin (22), whereas in rats, indomethacin did not reduce mortality rate (3). In anesthetized rats, the LPS-induced acute decrease in arterial blood pressure was attenuated significantly by a selective COX-2 inhibitor (rofecoxib) and a non-selective COX inhibitor (indomethacin) (25). Mice with targeted deletion of COX-2 (19) and mice treated with COX-2 inhibitors (34) exhibit improved survival when challenged with LPS, but the cardiovascular effects of LPS have not previously been examined in COX-2−/− mice. Nitric oxide (NO) produced by inducible NO synthase (iNOS) is of major importance for changes in blood pressure during sepsis (23). Apparently, the activity of endothelial NO synthase (eNOS) is required for the induction of iNOS and arterial blood pressure is protected in LPS-treated anesthetized eNOS−/− mice (17), (38), although lethality is not affected (35). PGE2 stimulates eNOS activity and PGE2-mediated vasodilatation depends on the presence of
endothelium and eNOS (27). A similar endothelium-dependency is seen also for PGI₂-induced vasodilatation (36). These findings prompted the hypothesis that serial activation of COX-2 and eNOS by LPS may account for the involvement of both enzymes in the circulatory response to LPS. This hypothesis predicts that functional deletion of COX-2 or eNOS protects cardiovascular status equally well against LPS-induced cardiovascular depression through attenuated vasodilatation of resistance vessels. The hypothesis was tested in vivo in mice with targeted disruption of COX-2 and eNOS that were challenged with Escherichia Coli-derived LPS (055:B5). Arterial blood pressure and heart rate were measured continuously in freely moving mice with chronic indwelling catheters mounted in a swivel system. To further address the involvement of the vasculature, in vitro myograph experiments were performed with isolated, LPS-treated, aortic rings prepared from wild type, COX-2⁻/⁻ or eNOS⁻/⁻ mice.
Materials & Methods

Mice

The investigation conforms with the “Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)” and the experimental protocol was approved by the Danish Animal Experiments Inspectorate (2009/561-1749) under the Danish Ministry of Justice. C57BL/6J wild type mice and C57BL/6J mice with targeted deletion of COX-2 and eNOS were studied. COX-2 -/- mice and their wild type littermates (COX-2 +/-) from heterozygos breeding were obtained from Jackson Laboratories on a predominant C57bl/6 background and further backcrossed for seven generations (C57Bl/6) before experiments. Genotyping was done with DNA from tail biopsies using the REDExtract-NAmp tissue PCR kit (Sigma). PCR used the following primers: (COX-2 -/-) 5'- GCC-CTG-AAT-GAA-CTG-CAG-GAC-G-3', 5'-CAC-GGG-TAG-CCA-ACG-CTA-TGT-C-3', (COX-2 +/-) 5'-CAC-CAT-AGA-ATC-CAG-TCCGG-3' and 5'-ACC-TCT-GCG-ATG-CTC-TTC-C-3'. In studies with eNOS -/- mice (Jackson Laboratories), C57BL/6J wild type mice were used as controls. All experiments in mice were performed in accordance with the Danish national guidelines for care and handling of animals. Mice were 8-10 weeks of age and were kept on a 12:12-hour light: dark cycle with free access to standard mice chow and tap water.

Blood pressure measurement

Mice were anaesthetized (ketamine (50 mg/kg) and xylazine (10 mg/kg, i.p.) and catheters were placed in the femoral artery and vein for blood pressure measurement and drug infusion, respectively (27). Mice were then placed in individual cages and catheters were attached to a swivel (Instech Laboratories) so they were able to move freely. Mice recovered for four days after
operation, and in that period the arterial catheters were kept open with infusion of 10 µl/h heparin solution (100 U in glucose). Before the experiment the artery line was connected to a pressure transducer and data were collected at 200 Hz. Systolic, diastolic and mean blood pressures together with heart rate were determined.

Experimental series: Blood pressure and heart rate was measured for half an hour before mice were given a bolus of LPS (2 mg/kg I.V., 055:B5 E. Coli, Sigma) or vehicle. Blood pressure and heart rate were then measured for eight hours. In one series, mice were followed for 20 hours after the LPS bolus. Experiments were performed in wild type, COX-2−/−, littermate COX-2+/+ and eNOS−/− mice (C57BL/6J). In one series blood pressure and heart rate was measured for ½ h in wild type (C57/Bl6) mice before they were given a bolus (0.1 mg/kg) of the IP2-receptor antagonist, BR5064 (a gift from Bayer Schering Pharma) followed by continuous infusion (0.005 g/kg/h) for one hour. This was followed by a bolus of LPS (2 mg/kg). The specificity of BR5064 was tested in other mice by first giving the mice a bolus of Beraprost (20 µg/kg, Cayman Chemicals) leading to a decrease in blood pressure within a few minutes. When blood pressure was stable again a bolus of BR5064 (0.1 mg/kg) was given followed by continuous infusion (0.005 mg/kg/h) for one hour. At the end of each experiment blood samples were drawn and the aorta, liver, heart, and kidneys were removed for further examination.

Measurements of eNOS, iNOS, nNOS and COX-2, α1a- and β1-adrenoceptor mRNA and eNOS protein

Q-PCR: total RNA from kidney, liver, aorta and heart from control and LPS-treated animals was isolated by TRIzol Reagent extraction according to the manufacturer’s instructions (Invitrogen) and used as template for Q-PCR analysis as described by Stubbe et al, (37). cDNA amplification was made using iQ SYBR Green Supermix (Bio-Rad), primer sequences are shown in table 1. A melting
curve analysis confirmed specificity post-run for each plate setup and a standard curve was constructed by plotting threshold cycle (Ct values) against serial dilutions. Purified PCR product was used to generate a standard curve.

*Western blotting:* Liver tissue was homogenized using a sucrose/imidazole buffer modified from Jensen et al. (29). 30 µg of protein was separated on a 4-15% Tris-HCl gel (Biorad) and transferred to a Immobilon-P PVDF membrane (Millipore). Membranes were blocked with 5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) for one hour followed by incubation overnight at 4°C with primary antibody diluted 1:1000 in 5% non-fat dry milk in TBST. Primary antibody was anti-eNOS (abcam, ab5589). The antigen-antibody complex was visualized by horseradish peroxidise-conjugated secondary antibody (P0448, diluted 1:2000, Dako) using the ECL plus Western Blotting Detection System (GE Healthcare; Hillerod, Denmark). Antibody against β actin (1:2000, abcam) was used for loading control.

**Isometric force measurements in aorta rings:**

Isometric force measurements in aorta rings were obtained in a Halpern-Mulvany myograph (Model 610 M, Danish Myo Technology A/S, Aarhus, Denmark). The mice were killed by a blow to the head followed by decapitation. The thoracic aorta was rapidly excised and placed in ice-cold physiological salt solution (PSS) as described (27). This was followed by dissection and cutting the aorta into rings (1.5-2 mm length) before incubation at 37°C with LPS (50 µg/mL, E. Coli, 055:B5, Sigma) or vehicle for 18 hours. Incubation-media contained D-MEM (L-Glutamine and Hepes, Gibco) with 0.5% BSA (7.5% Gibco), penicillin (100 u/mL, Sigma) and streptomycin (100 µg/mL, Sigma). The rings were then suspended in the myograph at 37 °C in PSS with LPS, equilibrated with 5% CO₂ in air (pH of 7.4). The rings were normalised at resting tension and the viability of the vascular smooth muscle and endothelial cells was tested by adding phenylephrine [PE, at three
increasing concentrations ([EC_{50}, EC_{80}, and EC_{100}]) followed by acetylcholine (10^{-6} M). After
normalisation the rings were contracted with PE (EC_{50}) for one hour and the experiment was ended
by adding acetylcholine (10^{-6}M).

Experimental series: 
**Series 1:** Rings from wild type mice (C57BL/6J) incubated with LPS (50 µg/ml) or vehicle. 
**Series 2:** Rings from COX-2⁻/⁻ mice were incubated with LPS or vehicle and rings from C57BL/6J were incubated with LPS and the selective COX-2 inhibitor NS398 (10 µmol/L) or LPS alone. 
**Series 3:** Rings from eNOS⁻/⁻ mice incubated with LPS or vehicle and rings from C57BL/6J incubated with LPS and the NOS inhibitor L-NAME (10^{-4}mol/L) and LPS or L-NAME alone. 
**Series 4:** The endothelium was removed in rings from C57BL/6J by 1% Triton X-100 followed by incubation with LPS and L-NAME, LPS alone or vehicle. 
**Series 5:** Rings from wild type mice (C57BL/6J) incubated with LPS (50 µg/ml) and the iNOS inhibitor 1400W (1 µmol/L, Cayman Chemical) or LPS alone. LPS, L-NAME and 1400W were dissolved in PSS.

**Statistical evaluation**

Blood pressure data were integrated over ½ hour before LPS and thereafter for each hour. Changes in mean values of blood pressure with time were compared by one way ANOVA. If ANOVA was significant, blood pressure values after LPS were compared with blood pressure before LPS (control) by unpaired t-test with Bonferroni correction. P<0.05 was considered significant. Between groups, e.g. COX-2 genotypes, blood pressure decrease after LPS at 8 hours (delta blood pressure) was compared by unpaired students t-test. Data testing vascular reactivity and expression levels of specific mRNA and protein molecules were evaluated by unpaired students t-test. For each series separate controls were always run in parallel. P<0.05 was considered significant.
Results

Effect of lipopolysaccharide (LPS) on arterial blood pressure and heart rate in wild-type mice

Blood pressure was followed for eight hours after LPS or vehicle injection. Baseline mean arterial blood pressure in catheterized freely moving C57BL/6 mice was 106.5±2.2 mmHg and heart rate was 613.1±11.7 bpm. Injection of a vehicle bolus (isotonic NaCl) had no acute effect on MAP or HR. After LPS injection (2 mg/kg I.V.), blood pressure decreased significantly after two and a half hours and stabilized at a level around 75 mmHg after six hours which was an average decline of 32.2±3.8 mmHg (Figure 1A-B, n=11). There was an almost synchronous decrease in heart rate after LPS at three hours which stabilized at a rate averaging 50% of control (around 300 bpm) at six hours (Figure 1C-D).

In separate mice, circulatory parameters were followed for 20 hours after LPS injection (n=6). These mice survived and blood pressure did not decrease further between six and twenty hours. None of the tested mice died during the experiments (Figure 1 B and D, inserts at right). Analysis of tracks with higher time resolution (average each 15 min) confirmed the above: blood pressure declined significantly at 150 min with no significant change in heart rate until 180 min which then also declined.

Effect of LPS on arterial blood pressure and heart rate in COX-2 / mice and COX-2 /+

The resting mean arterial blood pressure in C57BL/6J mice with targeted deletion of COX-2 (COX-2 / ) averaged 112.3±3.3 mmHg and in C57BL/6J wild type littermates (COX-2 /+) 106.0±3.3 mmHg, which was not significantly different (Figure 2 A). There was no blood pressure difference between genders in COX-2 / mice. Bolus injection of LPS resulted in a blood pressure decrease that was significant after two hours in COX-2 / and then stabilized around 80 mmHg (Figure 2A-B).
The delta BP value after LPS was 26.2±3.6 mmHg for COX-2+/+ and 31.7±4.9 mmHg for COX-2−/− and not significantly different between the two strains. Heart rate was 622.2±20.6 bpm in resting COX-2−/− mice and an LPS bolus decreased it to 421.5±45.9 bpm (Figure 2 C-D). The decrease in heart rate caused by LPS was not significantly different between wild type littermates and COX-2−/− (ΔHR 244.1±55 and 202.7±56 bpm, respectively).

Effect of a prostacyclin receptor (IP) antagonist on the LPS-induced cardiovascular functional decline

Efficient blockade of IP was controlled by bolus injection of the stable prostacyclin receptor agonist beraprost (20 µg/kg). In wild type mice infused with vehicle, beraprost injection yielded an acute, reversible decrease in blood pressure (Figure 3A). BR5064 (0.1 mg/kg bolus followed by 0.005 mg/kg/h continuous infusion) abolished the effect of IP agonist infusion (Figure 3B). Administration of BR5064 had no significant effect on resting blood pressure or heart rate (Figure 3C, E). BR5064 infusion to mice did not prevent a significant decrease in blood pressure in response to an LPS bolus (Figure 3C-D). Thus, after four hours, blood pressure had decreased significantly in LPS treated, BR5064-infused mice and then stabilized at this level for the duration of the experiment. The delta BP (ΔBP 19.7±3.8) was not significantly different from that observed in vehicle-infused, LPS-treated mice. Heart rate decreased significantly in response to LPS in BR5064-infused mice in a way not significantly different from that in vehicle infused mice (Figure 3 E-F).

Effect of LPS on arterial blood pressure and heart rate in eNOS−/− mice

C57BL/6J mice with targeted deletion of eNOS displayed a significantly elevated resting blood pressure compared to wild type mice: MAP was 130.5±3.1 mmHg compared to 106.5±2.2 mmHg in
control mice (Figure 4A). Heart rate was 657.4±13bpm in eNOS<sup>−/−</sup> mice (n=6) compared to 613.1±12 bpm in wild type mice, which was not significantly different. In eNOS<sup>−/−</sup> mice, LPS treatment yielded a significant decrease in arterial blood pressure that was apparent three hours after the bolus injection (Figure 4A-B). Blood pressure then stabilized at a level around 90 mmHg. The delta MAP decrease at six hours was 43.2±5 mmHg with no significant difference between wild type control mice and eNOS<sup>−/−</sup> mice. In response to LPS, heart rate displayed a significant decrease after three hours and stabilized around 300 bpm (Figure 4C-D). The decrease in heart rate after six hours was 350.3±19 bpm in wild type mice and was not significantly different between wild type and eNOS<sup>−/−</sup> mice.

**Effect of LPS on contraction of aortic rings in vitro**

Segments of thoracic aorta from C57BL/6J mice pre-incubated with LPS and were subsequently suspended in a myograph. The α1-adrenoceptor agonist phenylephrine was used at EC<sub>50</sub> (2x10<sup>−7</sup> mol/L) for 60 minutes to examine the ability of the rings to maintain tone. After incubation with LPS (50 µg/mL), the preparations exhibited a reproducible and significant reduction of contractility in response to phenylephrine (n=6) compared to control rings (n=5) (Figure 5A). At the end of each experiment, acetylcholine was added to test endothelial function. Acetylcholine relaxed the control rings whereas acetylcholine induced a significant contraction in LPS-treated rings (Figure 5C). LPS-treated rings from COX<sup>−/−</sup> mice (n=5) displayed a suppression of phenylephrine-induced tone that was indistinguishable from that observed in aorta harvested from wild type mice (Figure 5B). The COX-2 antagonist NS-398 had no significant effect on the vascular reactivity of LPS-exposed rings (Figure 5B, n=6). Aortic segments from eNOS<sup>−/−</sup> mice incubated with LPS also showed a reduced contractility (n=5) to phenylephrine stimulation not significantly different from that of arteries from wild type mice (Figure 5B, n=6). Inhibition of NO synthase activity by addition of L-
NAME or the iNOS inhibitor 1400w improved phenylephrine-induced contractility in LPS-exposed rings (each series n=6) to a level not significantly different from control rings or from preparations incubated with L-NAME only (Figure 5B). Removal of the endothelium abolished acetylcholine-mediated relaxations but did not affect contractility determined as the response to EC$_{50}$ of phenylephrine (16.1±2.2 mN, n=5, Figure 5B). Incubation of rings without endothelium with LPS reduced contractility (4.1±1.0 mN, n=6) in response to phenylephrine to a similar extent as that observed in preparations with endothelium (Figure 5B). Incubation of LPS-treated rings without endothelium with L-NAME resulted in a significantly improved contraction to phenylephrine (12.43±1.6 mN, n=6) compared to LPS-treated aorta without endothelium not exposed to the NOS inhibitor; there was no significant difference in contractility compared to control rings without LPS and endothelium.

Effect of LPS on eNOS, iNOS, nNOS, COX-2 and adrenoceptor expression in mouse organs

Total RNA from control and LPS-treated wild type and COX-2-- mouse liver, aorta, kidney and heart was subjected to qRT-PCR analysis. In liver and kidney, systemic LPS treatment yielded a significantly elevated level of iNOS mRNA in wild type and COX-2-- (Figure 6A). COX-2 mRNA was increased in liver (Figure 6D). In aorta and left cardiac ventricle there were no significant change in iNOS and COX-2 mRNA level (Figure 6A-D). The level of eNOS mRNA in liver, aorta and heart was significantly reduced in response to LPS infusion in wild type mice but not in COX-2--, whereas no significant change was observed in the kidney (Figure 6B). nNOS mRNA was significantly down-regulated in kidney and liver in LPS-treated wild type and COX-2-- mice, with the same tendency in aorta and heart (Figure 6C). Total eNOS protein abundance in liver tissue was not changed by LPS treatment in wild type and in COX-2-- mice as determined by western blotting analysis (Figure 6E). In contrast, iNOS protein level was significantly increased by LPS in wild
type and COX-2-/− mice compared to control (Figure 6F). The abundance of β₁-adrenoceptor mRNA in left ventricle was significantly decreased in wild type and COX-2−/− in response to LPS (Figure 6G). In aorta, there was no significant change in mRNA abundance of the α₁a-adrenoceptor (Figure 6H).
Discussion

The present study shows that LPS-induced suppression of heart rate and blood pressure in conscious, freely moving mice is not attenuated by targeted deletion of COX-2 or eNOS, or by pharmacologic inhibition of prostacyclin receptor. Results from isolated aortic rings exposed to LPS corroborated the interpretation that COX-2 and the endothelium/eNOS has no major role in the depression of vascular reactivity caused by lipopolysaccharide. By contrast, selective iNOS inhibition rescued vascular contractility. LPS induced marked and opposite changes in the expression of iNOS (up), nNOS (down) and eNOS (down) mRNAs in vivo which supports a crucial role of iNOS activity for the decline in cardiovascular function, in line with previous findings. Taken in conjunction, the present data show that activity of COX-2 or eNOS and therefore a serial coupling between them is less likely as cause for and therapeutic target of cardiovascular collapse in LPS-mediated endotoxemia.

The present report provides cardiovascular data obtained at the systemic level in conscious freely moving COX-2−/− mice, which constitute an attractive model to study the role of the enzyme independently of non-selective actions of COX inhibitors and the variable pharmacokinetic conditions that prevail during sepsis (18). The present model of LPS-induced endotoxemic shock resulted in hypotension and reduced heart rate with onset after two-three hours that lasted for at least twenty hours, while mice survived. The “mild” LPS dosing regimen (2 mg/kg I.V.) is different from a study designed to examine survival, where COX-2−/− mice were significantly protected from death compared to COX-2+/+ in response to LPS (3 daily injections of 40 mg/kg each by intraperitoneal injection or 100 mg/kg once) (19). The present observations suggest that the better survival in COX-2−/− mice and in mice given COX-2-selective blockers (34) (4) is less likely to be
caused by an improved cardiovascular function. Of note, two selective COX-2 inhibitors and the glucocorticoid-receptor agonist dexamethasone showed equal ability to suppress the excretion of prostacyclin metabolite 6-keto-PGF$_{1\alpha}$ in rats after LPS, but only dexamethasone improved arterial pressure and attenuated the rise in plasma levels of nitrite and nitrate caused by LPS (30). This finding supports a modest role of COX-2 activity for blood pressure decline compared to nitric oxide synthase activity. Efficient *in vivo* blockade of prostacyclin IP receptors was obtained but the IP antagonist did not attenuate the LPS-mediated suppression of MAP and heart rate. COX-2 being the dominant source for systemic prostacyclin (31), the present finding is in accord with the lack of effect of COX-2 on systemic hemodynamics (13). In rats subjected to endotoxemic shock, both a COX-2 inhibitor, rofecoxib, and a prostacyclin antagonist, CAY-10441, attenuated the LPS-mediated decrease in blood pressure (25) (26). These experiments were performed during isoflurane anaesthesia and used 5 times the present dose of LPS (10 mg/kg) given intravenously. Baseline blood pressure was 80-90 mmHg and during LPS, at or below 50 mmHg. These different experimental conditions may explain the discrepancies with the present findings obtained in conscious mice, although species differences may also be involved. Rats display a hyper dynamic circulatory response to LPS, with increased heart rate and cardiac output (almost doubled), while blood pressure drops and systemic vascular resistance is markedly lowered (26). This suggests a sequence where the primary event is either vasodilatation with a compensatory increase in heart rate or a directly mediated inflammatory tachycardia inducing a reflex decrease in peripheral resistance. In mice, however, the basal heart rate approximated 600 bpm and in no case was an increase in response to LPS recorded. A decline in blood pressure was noted first and within 30 minutes this was followed by a decline in heart rate with no compensatory tachycardia. The almost synchronous drop in blood pressure and heart rate suggest inadequate responsiveness in or effect of the sympathetic nervous system and/or suppressed inotropic state of the myocardium. Cytokines down-
regulate α-adrenoceptors in various organs during endotoxemia in rats (11), whereas the β3-adrenoceptor is upregulated in human myocardium from septic patients (32). Our results show a decrease of β1-adrenoceptor expression to 10% of control level in left ventricular myocardium while aortic α1a-adrenoceptor expression showed no alteration. If present at the level of functional receptors, this myocardial down regulation could cause a lower sensitivity towards sympathetic stimulation.

The lack of contribution of COX-2-derived prostanoids in the blood pressure response to LPS is in accord with modest and restricted up-regulation of COX-2 mRNA in the liver. The data agree with previous data from mice where COX-2 changes transiently in vivo (20). In accord with earlier reports (28) (35), MAP was increased by approximately 30 mmHg in eNOS−/− mice. A significant role has been attributed to eNOS in endotoxemic (17) (38) and anaphylactic (14) blood pressure decrease and the COX products PGE2 and prostacyclin can activate eNOS (22,27) (27,33). The present data indicate that both in conscious mice and in isolated vascular rings, eNOS is down-regulated and does not contribute to cardiovascular suppression. eNOS protein abundance is also decreased in endothelial cells in a model of cecal ligation and puncture in rats (39). Thus LPS modulates the expression of the three NOS isoforms differently since both eNOS and nNOS were suppressed and iNOS stimulated. Neuronal nitric oxide synthase (nNOS) is expressed in vascular smooth muscle cells (10) and endothelium (5). It contributes to endothelium-dependent relaxation in mouse aorta (12), and in accordance with our findings, nNOS is also down-regulated in cardiac myocytes from rats in a model of endotoxic shock (16). The suppression of vascular eNOS abundance is in accord with disappearance of the relaxation to acetylcholine. Actually, and surprisingly, in isolated preparations incubated with LPS, acetylcholine caused an increase in tension rather than a relaxation. This probably reflects the induction of endothelium-dependent contractions, as has been observed with another cytokine in the aorta of the rat (9).
The present data rather support that iNOS is the major source of NO in response to LPS, since iNOS mRNA was enhanced several times in liver and kidney while \textit{in vitro}, L-NAME as well as selective iNOS inhibition normalized LPS-suppressed vessel reactivity. This is in accord with observations in LPS-challenged awake mice, where systemic NO production depended solely on iNOS and where eNOS-derived NO was reduced (23). This finding has been reproduced also \textit{in vitro}, where iNOS activity suppressed eNOS expression and abolished acetylcholine-mediated dilatation in aortic rings of the mouse (15). The present in vitro observations suggest that LPS-stimulated iNOS activity in the arterial media layer is sufficient to suppress vascular reactivity. Although endothelial cells also may respond to LPS by upregulation of COX-2 (1) (and iNOS) this appears not to be necessary for suppression of vascular reactivity. The role of the endothelium in sepsis is although not completely clear.

\textbf{Perspective and significance}

In summary, the present study shows that a non-lethal, acute inflammatory challenge by infusion of Gram-negative bacterial wall-derived LPS to conscious unrestrained mice reduces simultaneously heart rate and mean arterial blood pressure \textit{in vivo} and vascular reactivity \textit{in vitro} independently of COX-2, prostacyclin receptors and endothelium/eNOS activity. Thus, at least in the mouse, a serial coupling between COX-2 and eNOS activities is unlikely to account for acute cardiovascular depression during endotoxemia while iNOS appears to be of major significance.

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Conflict of interest

None declared
Legends to figures

Figure 1.
Effect of an intravenous LPS bolus (2 mg/kg, n=11) or vehicle bolus (n=7) on arterial blood pressure (MAP) and heart rate (HR) measured by chronic indwelling catheters in conscious, freely moving wild-type mice.
A, Time course of mean arterial pressure in wild type mice after an LPS or vehicle (control) bolus. B, Bar graph depicts hourly averages of mean arterial blood pressure in wt mice after LPS bolus and statistical evaluation. Insert at right depict average blood pressure results from separate mice followed for 20 hours. C, Time course of heart rate changes in wt mice after an LPS or vehicle bolus. D, Bar graph depicts hourly averages of heart rate for 8 hours in wt mice after LPS bolus, and for separate mice followed for 20 hours (insert at right). Columns show means ± SEM. *: statistically significant difference between basal values of MAP and HR before bolus injection (0.5 hour) and after LPS bolus at p<0.05.

Figure 2.
Mean arterial blood pressure (MAP) and heart rate (HR) measured by indwelling catheters in conscious COX-2-/- mice (n=7) and their wild type littermates (COX-2 +/-, n=7) after LPS (2 mg/kg) or vehicle (control).
A, Time course of mean arterial pressure in COX-2 +/- and +/- mice after LPS or vehicle bolus. B, Bar graph depicts hourly averages of mean arterial blood pressure in COX-2 +/- mice after an LPS bolus.
bolus. C, Time course of changes in heart rate in COX-2**/− and **/+ mice after LPS or vehicle bolus. D, Bar graph depicts hourly mean heart rate in COX-2**/− mice after LPS bolus. Columns show means ± SEM.

*statistically significant difference between basal MAP and HR before (0.5 hour) and after LPS bolus (p<0.05).

**Figure 3.**

A, Blood pressure trace shows the effect of the prostacyclin receptor agonist beraprost (20μg/kg) on mean arterial blood pressure in the absence (A, left panel) and presence (B, right panel) of the prostacyclin receptor antagonist BR5064 (0,1μg/kg bolus and infusion 0,005 g/kg/h for one hour) in a wild type mouse.

C, Time course of mean arterial pressure in wt mice given an LPS bolus (n=10) and an LPS bolus with the IP receptor antagonist BR5064 (n=11). BR5064 was given at 0.5 h and at 1.5h, LPS was added. D, Bar graphs depict hourly mean arterial blood pressure in BR5064-treated wt mice after LPS bolus. E, Time course of average heart rate in wt mice after IP-antagonist BR5064 with and without LPS bolus. F, Bar graph shows hourly mean heart rate in IP antagonist-treated mice after LPS bolus. Columns show means ± SEM.* statistically significant difference between basal MAP and HR and after LPS bolus at P<0.05.

**Figure 4.**

Mean arterial blood pressure (MAP) and heart rate (HR) measured by indwelling catheters in conscious eNOS**/− mice (n=6) and eNOS**/+ (wt) mice (n=11) after an LPS bolus.

A, Time course of mean arterial pressure in eNOS**/− and wt mice after LPS (control trace is same as in Figure 1A). B, Bar graphs depict hourly mean arterial blood pressure in eNOS**/− mice before
after LPS bolus. C, Time course of mean heart rate in eNOS−/− and wt mice after LPS bolus. D, Bar graphs depict hourly mean heart rate decrease in eNOS−/− mice after LPS bolus. eNOS−/− mice were compared with wt control mice also shown in figure 1. Columns show means ± SEM * statistically significant difference between basal MAP and HR before (0.5 hour) and after LPS bolus at P<0.05.

Figure 5

A, Effect of the alpha-adrenoceptor agonist phenylephrine (PE) on contractile force of mouse aortic rings pre-incubated with and without LPS (50 µg/ml). At termination of the experiment, acetylcholine (Ach 1 µM) was added to elucidate endothelial function. Original recordings of two experiments performed in parallel.

B, Bar graphs illustrate mean contractile force in response to PE in aortic rings from: Wild type mice incubated with LPS (n = 6) and without LPS (control, n = 5); COX-2−/− mice with LPS (n = 5); wild type mice with the COX-2 inhibitor NS398 and LPS (n = 6); eNOS−/− mice with LPS (n = 5); wild type mice with the NOS inhibitor L-NAME (n = 6); wild type mice with L-NAME and LPS (n = 6); wild type mice with the iNOS inhibitor 1400W and LPS (N = 6); endothelium-denuded rings from wild type mice (n = 6); endothelium-denuded rings from wild type mice with LPS (n = 6); endothelium-denuded rings from wild type mice with L-NAME and LPS (n = 6). Columns show means ± SEM. *: statistically significant difference between control and LPS, #: significant difference between LPS alone and LPS+inhibitor. C, The panel shows mean contractile force in mouse aortic rings pre-constricted with phenylephrine (PE) before and after addition of acetylcholine (Ach). In control rings, Ach produced a significant relaxation whereas a significant contraction was seen in the LPS-preincubated rings. Columns show means ± SEM. *: statistically significant difference between control and acetylcholine.
Figure 6

Messenger RNA levels for iNOS (A), eNOS (B), nNOS (C) and COX-2 (D) in kidney, liver, aorta and heart as measured by qRT-PCR in wild type mouse tissues (wt) and tissue from COX-2 -/- treated with LPS (+) and controls (-). Tissues were harvested from mice 8 hours after the LPS bolus. Messenger RNA levels are shown relative to GAPDH (liver and aorta), TBP (kidney) and Tbc (heart) and expressed as fold change compared to control. Columns show mean values ± SEM. * Statistically significant difference between (-) and (+) (p< 0.05; unpaired t test). Protein level of total eNOS (E) and iNOS (F) in liver tissue from vehicle-treated and LPS-treated wild type mice and LPS-treated COX-2 -/- mice (n=4 for each condition).

Messenger RNA levels for β1-adrenoceptor in heart (G) and α1-adrenoceptor in aorta (H) measured by qRT-PCR in wild type mouse tissues (wt) and tissue from COX-2 -/- treated with LPS (+) and controls (-). Tissues were harvested from mice 8 hours after the LPS bolus. Messenger RNA levels are shown relative to GAPDH (aorta) and Tbc (heart) and expressed as fold change compared to control. Columns show mean values ± SEM. * Statistically significant difference between (-) and (+) (p< 0.05; unpaired t test).
References


30. Leach M, Hamilton LC, Olbrich A, Wray GM, and Thiemermann C. Effects of inhibitors of the activity of cyclo-oxygenase-2 on the hypotension and multiple organ dysfunction


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