Role of angiotensin II in endothelial dysfunction induced by lipopolysaccharide in mice

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Lund DD, Brooks RM, Faraci FM, Heistad DD. Role of angiotensin II in endothelial dysfunction induced by lipopolysaccharide in mice. Am J Physiol Heart Circ Physiol 293: H3726–H3731, 2007. First published October 26, 2007; doi:10.1152/ajpheart.01116.2007.—Endotoxin [or lipopolysaccharide (LPS)] increases levels of superoxide in blood vessels and impairs vasomotor function. Angiotensin II plays an important role in the generation of superoxide in several disease states, including hypertension and heart failure. The goal of this study was to determine whether the activation of the renin-angiotensin system contributes to oxidative stress and endothelial dysfunction after endotoxin. We examined the effects of enalapril (an angiotensin-converting enzyme inhibitor) or L-158809 (an angiotensin receptor blocker) on increases of superoxide and vasomotor dysfunction in mice treated with LPS. C57BL/6 mice were treated with either enalapril (60 mg·kg⁻¹·day⁻¹) or L-158809 (30 mg·kg⁻¹·day⁻¹) for 4 days. After the third day, LPS (10–20 mg/kg) or vehicle was injected intraperitoneally, and one day later, vasomotor function of the aorta was examined in vitro. After precontraction with FGF₂₅α, the maximal responses to sodium nitroprusside were similar in the aorta from normal and LPS-treated mice. In contrast, the relaxation to acetylcholine was impaired after LPS (54 ± 5% at 10⁻⁵, mean ± SE) compared with vessels treated with vehicle (82 ± 1%; P < 0.05). Enalapril improved (P < 0.05) relaxation in response to acetylcholine to 81 ± 6% after LPS. L-158809 also improved relaxation in response to acetylcholine to 77 ± 4% after LPS. Superoxide (measured with lucigenin and hydroethidine) was increased (P < 0.05) in aorta after LPS, and levels were reduced (P < 0.05) following enalapril and L-158809. Thus, after LPS, enalapril and L-158809 reduce superoxide levels and improve relaxation to acetylcholine in the aorta. The findings suggest that activation of the renin-angiotensin system contributes importantly to oxidative stress and endothelial dysfunction after endotoxin.

endotoxin; enalapril; L-158809; endothelial function

WE AND MANY OTHER INVESTIGATORS have demonstrated that bacterial endotoxin [or lipopolysaccharide (LPS)] impairs endothelial vasomotor function. Superoxide in blood vessels is thought to contribute to the impairment of endothelial function after LPS (12, 13, 20). Mechanisms that account for the generation of superoxide after LPS, however, are not entirely clear.

Angiotensin II appears to play a key role in the generation of superoxide in several disease states, including hypertension and heart failure (9, 10, 21, 30). The activation of NADPH oxidase appears to contribute importantly to increases in the generation of superoxide by angiotensin II (3, 11, 28, 36).

Recent evidence suggests that angiotensin II may contribute to vascular dysfunction after LPS (33). This hypothesis is supported by the finding that an angiotensin-converting enzyme (ACE) inhibitor attenuated endothelial dysfunction in the aortas of rabbits 5 days after LPS. The first goal of the present study was to determine whether an ACE inhibitor, enalapril, prevents endothelial dysfunction <1 day after LPS. Because LPS causes mice to reduce food and water intake, we sought to reduce the indirect effects on blood vessels by studying vasomotor responses much sooner after LPS than in a previous study (33).

Enalapril also inhibits bradykininase and thus increases levels of bradykinin (a vasodilator) as well as reducing levels of angiotensin II (23). Thus altered endothelial function after LPS and enalapril could be mediated by an increase in levels of bradykinin. The second goal of this study was to determine whether L-158809, an angiotensin type II (AT₂) receptor antagonist (6), which would not alter levels of bradykinin, also attenuates endothelial dysfunction after LPS.

Because angiotensin II generates superoxide (11), it seemed likely that enalapril and L-158809 may alter endothelial function after LPS by an effect on superoxide in blood vessels. The third goal of this study was to measure superoxide levels in blood vessels treated with enalapril or L-158809 after LPS.

METHODS

Animals. Adult male C57BL/6 mice (20–25 g) were studied. Enalapril (400 mg/kg of diet), an ACE inhibitor, was added to the diet of one group of mice. L-158809 (200 mg/kg), an AT₁ receptor antagonist, was added to the diet of the other mice. We have used enalapril and L-158809 in a previous study in rats (7) and adjusted the amount of drug based on food consumption of mice. Based on daily consumption of diet by mice (1.5 g/10 g body wt) the amount of enalapril received was 60 mg·kg⁻¹·day⁻¹ and L-158809 was 30 mg·kg⁻¹·day⁻¹. Control mice received rodent chow without enalapril or L-158809.

After 4 days of treatment with enalapril or L-158809, mice were treated with either vehicle or LPS (Escherichia coli stereotype 0128; B12, 10–20 mg/kg ip). Twenty-four hours later, mice were euthanized by an injection of pentobarbital sodium (150 mg/kg ip) followed by exsanguination.

The aorta was quickly removed and placed in cold (4°C) oxygenated Krebs solution containing (in mM) 133 NaCl, 4.7 KCl, 1.35 NaH₂PO₄, 16.3 NaHCO₃, 0.61 MgSO₄, 7.8 glucose, and 2.52 CaCl₂. Aortas were cut into rings (5–7 mm in length). All procedures and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Detection of superoxide. Hydroethidine, an oxidative fluorescent dye, was used to evaluate levels of superoxide in the aorta in situ, as described previously (20, 21). Cells are permeable to hydroethidine.
and, in the presence of superoxide, hydroethidine is oxidized to fluorescent ethidium bromide and trapped by intercalation with DNA. Unfixed frozen aortic rings were cut into 30-μm sections and placed on glass slides. Hydroethidine (2 × 10⁻⁶ mol/l) was applied to each tissue section and coverslipped. Slides were incubated in a light-protected, humidified chamber at 37°C for 30 min. Images were obtained with a laser-scanning, confocal microscope equipped with a krypton/argon laser. Aortas from normal and LPS-treated mice were processed and imaged in parallel.

Basal levels (without an addition of NADPH) of superoxide were also measured by lucigenin-enhanced chemiluminescence as described previously (20). Vessel segments were placed in 0.5 ml PBS and 5 μM lucigenin, and relative light units were measured for 5 min. Background counts were determined and subtracted, and values were normalized to surface area.

Vasomotor responses. Aortic rings were mounted on stainless steel hooks at optimal resting tension (0.5 g) in individual organ baths in Krebs bicarbonate solution at 37°C and aerated with 95% O₂-5% CO₂. Tension was adjusted periodically to the desired level during a 45-min equilibration period. Vascular rings were then precontracted with 10⁻⁵ mol/l PGF₂α and initially tested with 3 × 10⁻⁵ acetylcholine and washed.

Vascular rings were then precontracted to 50% to 70% of the contraction with 10⁻⁵ mol/l PGF₂α. Responses to acetylcholine (10⁻⁹ to 10⁻⁵ mol/l), to the endothelium-independent dilator sodium nitroprusside (10⁻⁹ to 10⁻⁵ mol/l), and to papaverine (10⁻⁸ to 10⁻⁴ mol/l) were examined after preconstriction of vessels. Responses to PGF₂α (10⁻⁹ to 10⁻⁵ mol/l) were also examined. Contractile responses are expressed as grams of tension, and relaxation is expressed as percent relaxation during precontraction produced by PGF₂α (10⁻⁵ mol/l).

Adhesion of leukocytes. Rings of aorta were pinned out flat, fixed in formalin, and stained with Wrights stain. Vessel segments were examined en face with a dissecting light microscope. In randomly selected fields, the number of leukocytes in a test grid was counted. Six cross-sectional fields were counted in each mouse, and the average value was calculated and reported as the number of leukocytes per squared millimeters of aorta.

Drug. LPS, acetylcholine chloride, PGF₂α, sodium nitroprusside, and lucigenin (Sigma) were dissolved in normal saline. Enalapril and L-158809 were provided as a gift from Merck, and hydroethidine was obtained from Molecular Probes and suspended in dimethyl sulfoxide at a concentration of 10⁻² mol/l.

Statistical analysis. All data are expressed as means ± SE. Intergroup comparisons were performed using analysis of variance (one-way ANOVA) to test for differences among treatment groups, followed by Bonferroni’s corrected t-test. Differences were considered to be significant when P < 0.05.

RESULTS

Vasomotor responses. In aortic rings from mice given a control diet, maximal relaxation to acetylcholine was significantly less after LPS (54 ± 5%) than in vehicle-treated mice (88 ± 1%; Fig. 1A). The ACE inhibitor, enalapril, improved relaxation to acetylcholine after LPS. Following LPS, relaxation to acetylcholine was impaired less in vessels from mice given enalapril (81 ± 6%; Fig. 1A).

In separate groups of mice, in aortic rings of mice treated with LPS on the control diet, maximal relaxation to acetylcholine was significantly less (58 ± 6%) than in vehicle-treated mice given a control diet (88 ± 2%; Fig. 1B). Following LPS, relaxation to acetylcholine was impaired less in vessels from mice given the AT₁ receptor blocker, L-158809 (77 ± 4%; Fig. 1B), than in mice on a control diet.

Submaximal, but not maximal, responses to nitroprusside tended to be attenuated after LPS (Fig. 2, A and B). Treatment with enalapril or L-158809 in mice given LPS tended to improve responses to nitroprusside (Fig. 2, A and B). Responses to papaverine, a non-nitric oxide (NO)-mediated vasodilator, were not altered following LPS (Fig. 3).

After LPS, the constrictor responses to PGF₂α were similar after enalapril or control diets (Fig. 4A). Following LPS, constrictor responses to PGF₂α also were similar after L-158809 or control diets (Fig. 4B). Thus vasodilator responses after LPS were not altered by enalapril or L-158809.

Superoxide levels. Levels of superoxide (hydroethidium fluorescence) were higher in aortas from mice treated with LPS than in vehicle-treated mice on a control diet (Fig. 5, B vs. A). The increase in superoxide was observed primarily in endothelium and adventitia. After LPS, hydroethidine fluorescence was less in aortas from mice treated with enalapril (Fig. 5C) or L-158809 (Fig. 5D) than in mice on a control diet (Fig. 5B). In vehicle-treated mice, fluorescence in aortas was similar after enalapril, L-158809, or control diets (data not shown).

Superoxide levels (lucigenin) were higher in aortas from mice given LPS than in aortas from mice given vehicle (Fig. 6A). After LPS, superoxide levels were significantly less in aortas from mice given enalapril than in mice on control diets (Fig. 6B).
Superoxide in aorta from vehicle-treated mice was similar after enalapril or regular chow. Following LPS, superoxide levels were significantly lower in aortas from mice given L-158809 than those given regular chow (Fig. 6).

Leukocyte adhesion. The number of leukocytes bound to the aorta was greater in mice given LPS (1.35 ± 0.34, n = 8, P < 0.05) than in normal aortas (0.30 ± 0.08, n = 8). Adhesion of leukocytes in mice given LPS and pretreated with enalapril (0.59 ± 0.27, n = 4) or L-158809 (0.97 ± 0.30, n = 4) tended to be lower than with LPS but did not reach statistical significance.

**DISCUSSION**

The major new findings of this study are: 1) the impairment of relaxation to acetylcholine in aorta from mice within 1 day after treatment with LPS is prevented by enalapril; 2) the impairment of relaxation to acetylcholine is also prevented by L-158809, which suggests that protection against the impairment of responses after LPS by enalapril are not due to increases in bradykinin; and 3) the levels of superoxide, which are elevated in the aorta of mice following LPS, are reduced toward normal by both enalapril and L-158809. These findings suggest that the activation of the renin-angiotensin system after LPS plays a key role in oxidative stress and endothelial dysfunction.

**Vasorelaxation.** In the present study we observed significant impairment of endothelium-dependent relaxation of aorta to acetylcholine following LPS, which is consistent with data from previous studies (12, 13, 20). Evidence that angiotensin II contributes to endothelial dysfunction after LPS was provided in a study in which an ACE inhibitor, perindopril, was given to rabbits with endotoxic shock 5 days after LPS (33). We chose to study responses much sooner after LPS than in the previous study to reduce the indirect effects of prolonged endotoxin shock on blood vessels, including changes in blood volume and...
decreased food intake. To extend the findings of the previous study (33), we also measured superoxide and examined the effects of an angiotensin receptor blocker as well as an ACE inhibitor.

Effects of enalapril are related to the inhibition of conversion of angiotensin I to angiotensin II in plasma and tissue. Enalapril also blocks bradykininase, however, and prevents cleavage of endogenous bradykinin (23). Thus the effects of enalapril after LPS treatment could be produced by an increase in availability of bradykinin (a vasodilator), as well as a reduction of angiotensin II (a vasoconstrictor). We found, however, that L-158809, an angiotensin receptor blocker (25), which does not affect levels or responses to bradykinin, also improves endothelial function after LPS. These finding therefore support the conclusion that the renin-angiotensin system contributes importantly to endothelial dysfunction after LPS.

Relaxation to nitroprusside is only modestly impaired after LPS and responses to papaverine are virtually normal after LPS, which suggests that impaired responses to acetylcholine were not due to damage to aortic smooth muscle produced by LPS. In a previous study (8, 20), we found that extracellular superoxide dismutase (SOD) improved responses to acetylcholine after LPS, which provides additional evidence that altered vasomotor responses were not due to nonselective or irreversible damage to blood vessels.

Vasoconstriction. Although vasoconstriction in response to PGF$_{2\alpha}$ tended to be attenuated after LPS, the effect was small and did not reach statistical significance. These findings are consistent with a previous study (14), although we (20) and others (1, 6, 30, 32) have observed an impairment of vasoconstriction after LPS in other species. Although we did not observe significant effects of LPS on vasoconstrictor responses, the dose of LPS used was sufficient to produce a pronounced impairment of relaxation to acetylcholine.

Superoxide levels. Impairment of endothelial vasomotor function after LPS appears to involve increases in superoxide in blood vessels (1, 12, 13, 20). LPS activates several cytokines, which may contribute to the generation of superoxide after LPS (34). Tumor necrosis factor-$\alpha$ and interleukin-1$\beta$ (IL-1$\beta$) are elevated after LPS (22, 24). Both cytokines may contribute to the generation of superoxide in the vessels and lead to endothelial dysfunction.

In rats, increases in IL-1$\beta$ in the liver after injection of LPS were prevented by pretreatment with either an ACE inhibitor or an AT$_1$ receptor antagonist (22). The findings suggest that angiotensin II and its AT$_1$ receptor may modulate production of proinflammatory cytokines after LPS.

In addition to endothelial dysfunction following LPS, we also observed an elevation of superoxide in arteries after LPS. The data are compatible with the conclusion that superoxide is an important mediator of impaired endothelium-dependent relaxation after LPS (6, 16). Bioavailability of NO is modulated by the local generation of superoxide (4). We found that elevated levels of superoxide after LPS are reduced toward normal by enalapril or L-158809. Thus, following LPS, the generation of superoxide through the activation of the renin-angiotensin system contributes to the reduction of bioavailability of NO, generated by endothelium, and to endothelial dysfunction.

The effects of LPS on superoxide levels and the impairment of endothelial function are due to excessive production of superoxide beyond the rate of dismutation. Thus it is possible...
that increases in superoxide are due, at least in part, to altered rates of superoxide dismutation, in addition to increases in generation of superoxide. In a previous study, we demonstrated that gene transfer of extracellular SOD (ECSOD) attenuates the impairment of endothelium-dependent vascular relaxation produced by LPS (20). This finding does not allow a conclusion, however, about the effects of LPS on endogenous levels of the SODs. SOD activity is upregulated by preexposure to chronic hypoxia (35); preexposure of rats to 4 wk of chronic hypoxia increased CuZnSOD and MnSOD and reduced levels of reactive oxygen species after LPS. Other studies suggest that ECSOD mRNA is decreased in the renal cortex by an infusion of angiotensin II (36). In aortic smooth muscle cells, LPS increased the binding of angiotensin II and increased the number of receptors (2). Thus AT1 receptors may be upregulated by an exposure to LPS.

Another potential source of angiotensin II in the vessel wall after LPS is leukocytes. The number of leukocytes bound to the aorta was greater in mice given LPS than in normal aortas. Adhesion of leukocytes in mice given LPS and pretreated with enalapril or L-158809 tended to be less than with LPS alone. Our previous studies with LPS in rats demonstrated an impairment of vasomotor function and an increase in adhesion of leukocytes to the aortic endothelium (20). The data did not allow us to determine whether the mechanism by which ECSOD improved vascular function after LPS depended more on a decrease in superoxide from the vessel wall or decrease in adhesion of leukocytes.

Leukocytes produce proinflammatory cytokines that activate oxidases (15, 18). These oxidases generate reactive oxygen species, including superoxide, and thus may contribute to elevated levels of oxidants in endothelium during inflammation (17, 31). Because angiotensin II is present in both macrophages (26) and monocytes (19), these cells are a possible source of angiotensin II and oxidative stress in the vessel wall. In contrast, arteries that were incubated with LPS in vitro, with no potential for leukocyte recruitment, also had impaired endothelium-dependent relaxation (14). Whether the impairment of endothelial function after LPS is produced by superoxide that is generated by leukocytes or vascular cells, our data suggest that treatment with enalapril or L-158809 is effective in the protection of endothelial function.

In summary, these studies suggest that the generation of angiotensin II during the initial stages (20 h) of inflammation induced by LPS may be important in the development of endothelial dysfunction. Angiotensin II increases the levels of superoxide in blood vessels, and superoxide presumably reacts with locally released NO to produce endothelial vasomotor dysfunction. Increased levels of vascular superoxide and endothelial dysfunction are prevented by a pretreatment with enalapril and L-158809. Data in this study demonstrate the protection of endothelium-dependent relaxation in arteries after LPS by the inhibition of generation of angiotensin II or by the inhibition of AT1 receptors. We speculate that angiotensin II contributes importantly to endothelial dysfunction associated with inflammation produced by LPS.

Although previous studies (38, 39) have suggested that the mouse aorta has low levels of AT1 receptors under normal conditions, others have shown that AT1A receptors play a predominant role in angiotensin II-induced superoxide production in the mouse aorta (27). Other studies have shown that LPS upregulates AT1 receptors (2, 37) in endothelial cells in vitro. LPS increased the mRNA and protein levels of AT1 receptors in rat pulmonary microvascular endothelial cells (37). In aortic smooth muscle cells, LPS increased the binding of angiotensin II and increased the number of receptors (2). Thus AT1 receptors may be upregulated by an exposure to LPS.

After LPS, although the upregulation of NAD(P)H oxidase may contribute to oxidative stress and endothelial dysfunction, there are other possible sources of superoxide, including xanthine oxidase and other enzymes. NAD(P)H and xanthine oxidase are upregulated in the rat aorta 30 h after LPS (1). Chemiluminescence from these vessels was reduced after treatment with oxypurinol, which suggests that xanthine oxidase may also contribute to oxidative stress after LPS.

Leukocytes are another potential source of angiotensin II in the vessel wall after LPS. The number of leukocytes bound to the aorta was greater in mice given LPS than in normal aortas. Adhesion of leukocytes in mice given LPS and pretreated with enalapril or L-158809 tended to be less than with LPS alone. Our previous studies with LPS in rats demonstrated an impairment of vasomotor function and an increase in adhesion of leukocytes to the aortic endothelium (20). The data did not allow us to determine whether the mechanism by which ECSOD improved vascular function after LPS depended more on a decrease in superoxide from the vessel wall or decrease in adhesion of leukocytes.

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