Hypertension alters role of iNOS, COX-2, and oxidative stress in bradykinin relaxation impairment after LPS in rat cerebral arteries

Raquel Hernanz, Ana M. Briones, María J. Alonso, Elisabet Vila, and Mercedes Salaices. Hypertension alters role of iNOS, COX-2, and oxidative stress in bradykinin relaxation impairment after LPS in rat cerebral arteries. Am J Physiol Heart Circ Physiol 287: H225–H234, 2004. First published March 4, 2004; 10.1152/ajpheart.00548.2003.—This study was performed to investigate the role of reactive oxygen species and inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) metabolites in the lipopolysaccharide effect on bradykinin-induced relaxation in middle cerebral arteries from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). LPS exposure (10 μg/ml for 1–5 h) reduced bradykinin relaxation; this effect appeared earlier and was greater in arteries from SHR than WKY rats. LPS also reduced the relaxation to the NO donor diethylamine (DEA)-NO; however, LPS modified neither the bradykinin relaxation after inhibiting NO synthesis with Nω-nitro-l-arginine (0.1 mM) nor endothelial NOS expression. In arteries from WKY rats, the respective iNOS and COX-2 inhibitors aminoguanidine (0.1 mM) and NS-398 (10 μM) and the superoxide anion scavenger SOD (100 U/ml) reduced the LPS effect on bradykinin relaxation; however, the thromboxane A2 (TxA2)/PGH2 receptor antagonist SQ-29548 (1 μM) and the H2O2 scavenger catalase (1,000 U/ml) did not modify the LPS effect. In arteries from SHR, all of these drugs reduced the LPS effect. LPS exposure (5 h) increased superoxide anion levels in arteries from both strains and TxA2 levels only in SHR. COX-2 expression rose to a greater extent after 5 h. In conclusion, in segments from WKY rats, LPS exposure (5 h) increased superoxide anion scavenger SOD (100 U/ml) reduced the LPS effect on bradykinin relaxation; however, the thromboxane A2 (TxA2)/PGH2 receptor antagonist SQ-29548 (1 μM) and the H2O2 scavenger catalase (1,000 U/ml) did not modify the LPS effect. In arteries from SHR, all of these drugs reduced the LPS effect. LPS exposure (5 h) increased superoxide anion levels in arteries from both strains and TxA2 levels only in SHR. COX-2 expression rose to a similar level in arteries from both strains after 1 and 5 h of LPS incubation, whereas expression of CuZn- and Mn-SOD only increased after 5 h. In conclusion, in segments from WKY rats, LPS reduced bradykinin-induced relaxation through increased production of NO (from iNOS) and superoxide anion. The greater LPS effect observed in arteries from SHR seems to be related to higher participation of reactive oxygen species and contractile prostanoids (probably TxA2).

lipopolysaccharide; inducible nitric oxide synthase; cyclooxygenase-2; rat; reactive oxygen species

ENDOTOXIC SHOCK INDUCED by bacterial products such as lipopolysaccharide (LPS) is associated with reduced responsiveness of vasoconstrictor stimuli and hypotension as well as decreased systemic vascular resistance (38). In addition, altered endothelium-dependent vasodilator responses have also been described, although the results are contradictory. A few authors have described unchanged (31) or increased (33) relaxation, whereas most report impairment after LPS treatment (3, 31). Alterations in endothelial nitric oxide (NO) release (31) or in the NO relaxation mechanism (3, 40) have been implicated in the impaired endothelium-dependent response after endotoxin exposure. Increased production of NO from the inducible isoform of NO synthase (iNOS) (19) and of reactive oxygen species (ROS) might play an important role in this effect of LPS. However, the role of other factors such as the inducible isoform of cyclooxygenase-2 (COX-2) metabolites, the production of which has also been described to mediate other vascular effects of LPS (4, 27), has not yet been analyzed.

Hypertension is considered to be a chronic inflammatory disease (42) with elevated proinflammatory cytokine blood levels (10, 43). Increased activity and protein expression by iNOS and COX-2 (10, 13, 16) as well as increased ROS production (24) have also been described in hypertension. The effect of LPS on vasoconstrictor response has been found to be greater in several vascular beds from hypertensive rather than normotensive rats (6, 7). In addition, differences in the participation of NO from iNOS, COX-2 metabolites, and ROS in these effects have also been described (6, 7, 10, 43). On the other hand, contradictory results of in vivo effects of LPS in hypertensive rats have been reported. Thus, higher resistance (1) or higher mortality (46) in spontaneously hypertensive rats (SHRs) after LPS exposure have both been described.

Despite systemic hypotension, endotoxic shock is associated with an early decrease in cerebral blood flow and perfusion pressure as well as increased cerebrovascular resistance, all of which would be involved in the altered neurological function that is found in sepsis (12, 29). Our working hypothesis is that alteration of endothelium-dependent relaxations might contribute to these effects. The effects of LPS on cerebral arteriole diameter (4, 27) or on the responses induced by different vasoconstrictors in cerebral arteries (5, 41) have been studied. However, to our knowledge, the effect of endotoxin on endothelium-dependent relaxation in cerebral arteries has not yet been analyzed. The present study analyzes the effect of LPS on endothelium-dependent relaxation in isolated rat middle cerebral arteries (MCAs) and determines whether iNOS- and COX-2-derived products and ROS are involved in this effect. This study was performed in parallel on cerebral arteries from normotensive as well as hypertensive rats because of the known effects of hypertension on regulation of iNOS and COX-2 expression.
Materials and Methods

Six-month-old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid. Systolic arterial pressure was measured using an automatic sphygmomanometer with a tail-cuff method device, which was placed on the tail of pretrained rats that were placed for 1 h in a warm (37°C) chamber and restrained. Measurements of blood pressure were repeated at least three times, and the average systolic blood pressure readings were calculated as 220 ± 5 (SHR, n = 6) and 146 ± 4 mmHg (WKY, n = 6; P < 0.05). For the experiments, rats were decapitated, and the brain was removed and placed in cold (4°C) Krebs-Henseleit solution (KHS) bubbled with a 95% O2-5% CO2 mixture.

MCAs were dissected for reactivity experiments, superoxide anion measurement, and cytosolic Cu/Zn- and Mn-SOD protein expression analysis. All arteries of the circle of Willis were used for measurements of thromboxane A2 (TxA2) production and for endothelial NO synthase (eNOS) and COX-2 protein expression analysis to obtain a sufficient amount of protein.

All experiments complied with the current Spanish and European laws (RD 223/88 MAPA and 609/86).

Reactivity experiments. Ring segments of 2 mm in length [internal diameter: 234.1 ± 1.9 (n = 31) and 190.1 ± 1.9 μm (n = 34) for WKY rats and SHR, respectively; P < 0.05] were mounted in a small-vessel, dual-chamber myograph for measurement of isometric tension according to the method described (26).

Segment contractility was tested by an initial exposure to a high-K+ solution (120 mM K+–KHS). After precontraction with 10 μM serotonin [5-hydroxytryptamine; 1.2 ± 0.1 (n = 31) and 1.3 ± 0.1 mM/mm (n = 34), for WKY rats and SHR, respectively; P > 0.05], a first administration of 1 μM bradykinin (BK) was made. Afterward, five consecutive additions of serotonin and BK were performed at 1-h intervals in the absence or presence of 10 μg/ml LPS. In some experiments, concentration-response curves for BK (1 nM to 10 μM) in the absence and presence of LPS (5 h) were also performed.

To analyze the participation of iNOS-derived NO on the effect of LPS on the BK (1 μM) response, the inhibitor of this enzyme, aminoguanidine (0.1 mM), was used. The participation of COX-2-derived contractile prostanoids was analyzed with the specific COX-2 inhibitor NS-398 (10 μM) and the TXA2/PGH3 receptor antagonist SQ-29548 (1 μM). The involvement of ROS on the effect of LPS was studied using SOD (100 U/ml) and catalase (1,000 U/ml), the respective superoxide anion and H2O2 scavengers. All of these drugs were added 30 min before the corresponding BK administration.

To ascertain whether LPS alters the possible non-NO component of BK relaxation, the effect of a 5-h incubation with LPS on the concentration-response curves for BK was analyzed in the presence of the nonselective NOS inhibitor Nω-monomethyl-L-arginine (L- NMMA; 0.1 mM).

To analyze the effect of LPS on the vasodilator responses induced by the NO donor diethylenamine (DEA)-NO (0.1 nM to 10 μM) and papaverine (0.1 nM to 0.1 mM), concentration-response curves were drafted for segments precontracted with serotonin after 1 and/or 5 h of LPS incubation. Curves for parallel control segments were also created in the absence of LPS.

Another set of experiments analyzed the mediators involved in BK relaxation. For this purpose, the effects of 30 min of preincubation with L-NMMA (0.1 mM), the nonselective COX inhibitor indomethacin (10 μM), catalase (1,000 U/ml), and L-NMMA plus the inhibitor of the Ca2+-activated K+ channels tetraethylammonium (TEA; 5 mM) on the concentration-response curves of BK were all determined.

At the end of all experiments, segments were thoroughly washed and KHS was replaced by 120 mM K+–KHS; once the contraction was stable, 0.1 mM papaverine was added. The maximum response of the arteries [2.1 ± 0.1 (n = 31) and 1.7 ± 0.1 mM/mm (n = 34) for WKY rats and SHR, respectively; P < 0.05] was determined by the difference between the tone generated by the first exposure to 120 mM K+–KHS and that produced by 0.1 mM papaverine.

Western blot analysis. Cerebral arteries were incubated for 1 or 5 h in oxygenated KHS (37°C) with or without LPS. Afterward, the arteries were quick frozen in liquid nitrogen and kept at −70°C until analysis of protein expression.

Proteins from homogenized cerebral arteries (15 μg for eNOS and COX-2 and 5 μg for cytosolic Cu/Zn- and Mn-SOD) were separated by 7.5% (eNOS and COX-2) or 12% (Cu/Zn- and Mn-SOD) SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes overnight, and membranes were then incubated with mouse monoclonal antibody for eNOS (1:2,500 dilution; Transduction Laboratories; Lexington, UK) or rabbit polyclonal antibody for COX-2 (1:250 dilution; Cayman Chemical; Ann Arbor, MI), Cu/Zn-SOD (0.05 μg/ml; StressGen; Victoria, Canada), or Mn-SOD (0.02 μg/ml; StressGen) detection. Membranes were washed and incubated with a 1:2,000 dilution of anti-mouse or anti-rabbit IgG antibody (Transduction Laboratories) conjugated to horseradish peroxidase. The immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminescence system (ECL Plus, Amersham International; Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). The bands on the immunoblot were quantified using the NIH Image computer program (version 1.56). The same membrane was used to determine α-actin expression using a mouse monoclonal antibody (1:3,000,000 dilution; Boehringer Mannheim; Mannheim, Germany).

Homogenates from human endothelial cells, stimulated mouse macrophages, and rat brain were used as the positive controls for eNOS, COX-2, and Cu/Zn- and Mn-SOD, respectively.

Superoxide anion measurement. The oxidative fluorescent dye hydroethidine was used to evaluate the in situ production of superoxide anion as described previously (25). Hydroethidine permeates cells freely and, in the presence of superoxide anion, it is oxidized to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 488 nm and has an emission spectrum of 610 nm.

At the end of the functional studies, segments from both rat strains were immersed in the embedding medium Tissue-Tek OCT (Bayer Quimica Farmacéutica; Barcelona, Spain), frozen in liquid nitrogen, and kept at −70°C until superoxide anion measurement. Frozen ring segments were cut into 14-μm-thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer. Fresh buffer containing hydroethidine (2 μM) was topically 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 Leica TCS SP2 laser scanning confocal microscope equipped with a krypton-argon laser. Control and LPS-incubated tissues were processed and imaged in parallel. Laser settings were identical for image acquisition from all specimens. Fluorescence was detected with a 543-nm long-pass filter. Autofluorescence of the internal elastic lamina was detected with the 488-nm line of the microscope. In each experiment, some of the sequentially cut sections from LPS-treated arteries were incubated for 2 h with polyethylene glycol-conjugated SOD (PEG-SOD, 500 U/ml).

Measurements of TxA2 production. Arteries from WKY rats and SHR were incubated in oxygenated KHS in the absence or presence of LPS. At time 1 h, serotonin (10 μM) was added for 3 min, followed by the addition of BK (1 μM) for 10 min. At this time, the medium was collected for further analysis and replaced with a fresh medium. At time 5 h, we added serotonin for 5 min and, afterward, BK (10 μM). At this time, the medium was collected again. The measurements of TxB2, which is the metabolite of TxA2, were determined using a commercial enzyme immunoassay kit (R&D Systems Europe; Oxon, UK).
**RESULTS**

BK (1 nM to 10 μM) induced endothelium-dependent vasodilator responses that were similar in arteries from both strains of rats (Fig. 1). The NOS inhibitor l-NMMA (0.1 mM) reduced the concentration-response curve to BK in segments from both rat strains (Fig. 1). Incubation with TEA (5 mM) did not modify the effect of l-NMMA on BK relaxation (results not shown). Neither indomethacin (1 μM) nor catalase (1,000 U/ml) modified the relaxation to BK (results not shown).

The relaxation induced by the first single administration of 1 μM BK was also similar in segments from WKY rats (79.7 ± 3.0%, n = 31) and SHR (81.5 ± 2.1%, n = 34; P > 0.05). Relaxations induced by the subsequent administrations of 1 μM BK were impaired in a time-dependent manner in arteries from both strains of rats (P < 0.05); however, the decrease was greater in arteries from SHR than WKY rats (Fig. 2). The COX-2 inhibitor NS-398 (10 μM) increased the BK relaxation in segments from SHR (Fig. 2) but not WKY rats (data not shown). Similar results were observed when segments were treated with the TXA2/PGH2 receptor antagonist SQ-29548 (1 μM; data not shown).

The NO donor DEA-NO (0.1 nM to 10 μM) induced concentration-dependent vasodilator responses in MCAs from both strains of rats. The sensitivity [pD2, 7.3 ± 0.1 (n = 11) and 6.7 ± 0.2 (n = 14)] for WKY and SHR, respectively; P < 0.05] but not the maximum response (Emax, 121.2 ± 13.3 and 118.5 ± 6.7% for WKY and SHR, respectively; P > 0.05) was greater in segments from WKY rats than from SHR.

*Effect of LPS on vasodilator responses.* Incubation with LPS (10 μg/ml for 1–5 h) induced a time-dependent (P < 0.05)
reduction in the relaxation to a single concentration of BK (1 μM) in arteries from both strains of rats. The effect of LPS appeared earlier (1 vs. 4 h) and was greater in arteries from SHR than WKY rats as measured by percentage of LPS reduction [at 1 h: 0.7 ± 3.1 vs. 22.9 ± 4.1 for WKY and SHR, respectively; P < 0.05 (n = 25); and at 5 h, 33.8 ± 6.1 vs. 63.6 ± 4.6 for WKY and SHR, respectively; P < 0.05 (n = 21)].

LPS incubation at 5 h also induced a rightward shift in the concentration-response curve to BK (1 nM to 10 μM) in both rat strains with a reduction of maximum response only in arteries from SHR (Fig. 3A).

Because NO is an important mediator of the BK-induced vasodilator response, changes in eNOS expression and the ability of MCAs to respond to exogenous NO after LPS exposure were analyzed. Western blot analysis revealed that the expression of eNOS protein was similar in control arteries from WKY rats and SHRs; after both 1 and 5 h of LPS incubation, the expression of this isoform was unmodified (Fig. 3B; P > 0.05). However, incubation with LPS for 5 h reduced the relaxation elicited by the NO donor DEA-NO in WKY rats and SHR (Fig. 3C): the comparison of dAUC values indicates that the effect of LPS was similar in both strains [WKY, 35.4 ± 7.4 (n = 9) and SHR, 32.7 ± 6.1% (n = 9); P > 0.05]. LPS reduced the relaxation in response to DEA-NO similarly at 1 and 5 h (data not shown). To ascertain whether LPS could alter the non-NO component of BK relaxation, the effect of LPS was analyzed in the presence of L-NMMA. Figure 3A shows no additional inhibitory effect of LPS (5 h) on the concentration-response curve to BK in arteries treated with L-NMMA.

LPS incubation for 5 h did not modify the concentration-response curve to papaverine (0.1 nM to 0.1 mM) in segments from either strain (results not shown).

![Fig. 3. A: effects of L-NMMA (0.1 mM), lipopolysaccharide (LPS, 10 μg/ml, 5-h incubation), and LPS plus L-NMMA on the corresponding concentration-response curves to BK in segments of MCAs from WKY rats (n = 4, 9) and SHR (n = 4, 6). B: representative blots for endothelial nitric oxide synthase (eNOS) expression in cerebral arteries incubated for 1 and 5 h in the absence or presence of LPS (n = 10, 12). C: effect of LPS (5-h incubation) on the concentration-response curve for diethylamine (DEA)-NO in segments of MCAs from WKY rats (n = 8, 9) and SHR (n = 8, 9). Results are expressed as a percentage of the previous tone. *P < 0.05 vs. control.](http://ajpheart.physiology.org/)

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Participation of NO from iNOS on effect of LPS on BK relaxation. In the absence of LPS, aminoguanidine (0.1 mM) did not modify the relaxation to BK in arteries from WKY rats and SHR (data not shown). However, this inhibitor abolished the LPS effect in MCAs from WKY rats and significantly improved the impaired BK relaxation in arteries from SHR from the first hour of incubation; Fig. 4 only shows the effects at 1 and 5 h.

Participation of COX-2 mediators on effect of LPS on BK relaxation. In LPS-incubated segments from WKY rats and SHR, NS-398 (10 μM) abolished the inhibitory effect of LPS on BK relaxation at 3, 4, and 5 h of incubation but not at 1 and 2 h. SQ-29548 (1 μM) did not modify the effect of LPS on BK relaxation in segments from WKY rats but reversed it at all incubation times studied in MCAs from SHR. Figure 5A only shows the effects at 1 and 5 h. In the absence of LPS, both NS-398 and SQ-29548 also improved BK responses in SHR (see above); however, the effect was greater in the presence than in the absence of 5-h LPS incubation when expressed as the percentage of increase in BK relaxation (NS-398, 22.7 ± 6.2 vs. 125.9 ± 26.5 in the absence and presence of LPS, respectively; P < 0.05; SQ-29548, 50.4 ± 11.6 vs. 185.1 ± 33.8 in the absence and presence of LPS, respectively; P < 0.05).

COX-2 was only slightly expressed in arteries from SHR and WKY rats after 1 h of incubation in KHS; 5 h of incubation produced an increase in COX-2 expression that was greater in SHR than in WKY rats (Fig. 5B). When arteries were incubated for 1 or 5 h with LPS, the expression of COX-2 was further increased, abolishing the difference in COX-2 expression between the two rat strains (Fig. 5B).

In cerebral arteries from SHR, TxB 2 levels were higher after 5 h (5.2 ± 0.5 pg·ml⁻¹·μg protein⁻¹) than after 1 h [3.3 ± 0.5 pg·ml⁻¹·μg protein⁻¹ (n = 9); P < 0.05] of incubation in KHS. After 5 h of incubation with LPS, an increase in TxB 2 levels was observed in cerebral arteries from SHR [7.8 ± 0.7 pg·ml⁻¹·μg protein⁻¹ (n = 9); P < 0.05] but not in those from WKY rats [4.0 ± 0.9 vs. 5.0 ± 0.7 pg·ml⁻¹·μg protein⁻¹ in the absence and presence of LPS, respectively (n = 6); P < 0.05].

Participation of ROS on effect of LPS on BK relaxation. The superoxide anion scavenger SOD (100 U/ml) abolished the inhibitory effect of LPS on BK relaxation in arteries from both strains. In LPS-treated arteries from WKY rats, the H 2 O 2 scavenger catalase (1,000 U/ml) did not modify the response to BK. However, in arteries from SHR incubated with LPS, catalase abolished the effect of LPS on the relaxation to BK at all of the incubation times studied. Figure 6 only shows the effects at 1 and 5 h. Neither SOD nor catalase modified the BK response in the absence of LPS (data not shown).
Results are expressed as a percentage of relaxation elicited by BK at 0 h in segments of MCAs from WKY (n = 10, 11; A) and SHR (n = 7, 6; B) rats. Results are expressed as a percentage of relaxation elicited by BK at 0 h in each case. *P < 0.05 vs. control; #P < 0.05 vs. LPS.

An increase in ethidium bromide fluorescence was observed in LPS-treated arteries from both rat strains, which reflects an increase in superoxide anion generation (Fig. 7). Ethidium fluorescence was prominent in all three layers of the cerebral arterial segments. Incubation with PEG-SOD abolished the ethidium fluorescence, thereby confirming the specificity of the fluorescent signal for superoxide anion (results not shown). Cu/Zn-SOD and Mn-SOD protein expressions were detected in arteries from WKY rats and SHR after 1 and 5 h of incubation in KHS. In WKY rats but not in SHR, Mn-SOD expression was greater after 5 h of incubation than after 1 h. Incubation with LPS for 5 h but not for 1 h increased the expression of both Cu/Zn-SOD and Mn-SOD to similar levels in both strains of rats (Fig. 8).

DISCUSSION

The results of the present study show that LPS inhibits the endothelium-dependent vasodilator responses induced by BK, and this inhibition is greater in segments of MCAs from hypertensive than from normotensive rats. Different types of participation by contractile prostanoids from COX-2, NO from iNOS, and the ROS could explain the differences found between the rat strains. To our knowledge, this is the first study in which the participation of all of these mediators in the LPS effect on endothelium-dependent relaxation has been studied in large cerebral arteries from normotensive and hypertensive rats.

BK induced similar endothelium-dependent relaxations in MCAs from normotensive and hypertensive rats as previously reported (5). However, sensitivity to the NO donor DEA-NO was reduced in segments from SHR, which is similar to what had previously been described for these arteries using sodium nitroprusside (5). In arteries from both rat strains, the vasodilator responses induced by a single administration of BK were reduced by successive administrations; this reduction was greater in MCAs from the hypertensive animals and led to lower BK responses in arteries from SHR than from WKY rats from the third administration. Other authors have suggested the possible implication of inducible enzymes in the impairment of Ach-induced relaxation during the experiment (44). In our case, we found that the specific COX-2 inhibitor NS-398 and the TxA2/PGH2 receptor antagonist SQ-29548 but not the iNOS inhibitor aminoguanidine prevented the reduction of the relaxation but only in arteries from SHR. Furthermore, a time-dependent generation of TxA2 by cerebral arteries from SHR was observed. These findings indicate the participation of COX-2-derived contractile prostanoids, probably TxA2, in the reduction of the relaxation observed in MCAs from SHRs and could help to explain the greater time-dependent reduction in BK relaxation observed in this strain. Additionally, COX-2 expression was greater in cerebral arteries from SHR than from WKY rats after 5 h of incubation in KHS. Elevations of COX-2 activity and protein expression have been found in several models of hypertension (6, 13, 16). This increase seems to be a consequence of circulating proinflammatory cytokines, which are known to be elevated in hypertension (10, 43).

**Effect of LPS on BK relaxation.** Endotoxic shock is characterized by hypotension and decreased systemic vascular resistance associated with reduced responsiveness to vasoconstrictor and vasodilator stimuli (38). However, an early decrease in cerebral blood flow and perfusion pressure and an increase in cerebrovascular resistance have also been described (12, 29). In the present study, the effect of LPS on BK relaxation was time dependent, and the resulting decrease in relaxation could well be involved in the deleterious cerebrovascular effects observed in sepsis. In peripheral arteries, other authors have also described an impairment of endothelium-dependent vasodilator responses after LPS exposure (3, 31, 32, 44). However, unaltered (31) or increased (33) relaxations have also been described.

In MCAs, BK relaxation is mainly mediated by endothelial NO as previously reported (5, 21). Alterations in the synthesis of this mediator could explain the depressor effect induced by LPS in BK relaxation. Thus LPS downregulates arterial eNOS protein expression (9). In contrast, an increase of eNOS expression in cerebral arteries (34) or no alteration in eNOS mRNA levels in aorta (2) have also been described after LPS. The present study finds no change of eNOS protein expression in MCAs after 1 or 5 h of LPS incubation and thereby excludes this mechanism as being possibly responsible for the impairment of BK relaxation, although a reduction in the activity of this isoform cannot be discarded. Another hypothesis to explain the LPS effect on BK relaxation could be an alteration in the mechanism of NO relaxation. In agreement, LPS incubation (1 and 5 h) reduced the relaxation induced by the NO donor DEA-NO in MCAs from WKY rats. The specificity of the LPS effect on NO relaxation was confirmed by the lack of effect of LPS on papaverine relaxation and on BK-induced relaxation when the NO component was inhibited with L-NMMA. However, it is difficult to explain why the effect of LPS on BK relaxation appeared later than on DEA-NO. A similar depression in responses to sodium nitroprusside has been described in peripheral arteries (3, 40). However, no effect of LPS on nitrovasodilator responses has been described despite the impairment in endothelium-dependent relaxation.
A reduction in soluble guanylate cyclase expression (37) and activity (40), described after LPS exposure, could be involved in the depressor responses to both nitrovasodilators and endothelium-dependent vasodilators.

Role of iNOS, COX-2, and ROS.

It is well established that LPS administration promotes NO overproduction through iNOS activation (39). In MCAs from WKY rats, LPS incubation induces iNOS expression (17). Three points of evidence suggest that iNOS induction plays a central role in the impairment of endothelium-dependent relaxation: 1) in peripheral arteries, iNOS inhibitors improved the depressor effect of endotoxin (19), 2) iNOS-deficient mice did not present hyporesponsiveness to vasodilator stimuli after LPS treatment (9), and 3) NO-dependent relaxations were impaired in cerebral arteries after iNOS gene transfer (15). In addition, the iNOS inhibitor aminoguanidine restored the relaxation induced by BK after LPS incubation in MCAs from WKY rats, which supports the participation of iNOS in the depressor effect of the endotoxin. Some hypotheses have attempted to explain the mechanisms involved in this participation. Thus the nitration of protein tyrosine residues by peroxynitrite, formed by the simultaneous generation of NO from iNOS and superoxide anion, has been shown to inhibit different enzymes involved in endothelium-dependent relaxation such as eNOS (30). Decreased eNOS activity has been described in response to high concentrations of NO such as would be produced after iNOS induction (8). In addition, a downregulation of soluble guanylate cyclase activity in vascular smooth muscle in response to iNOS-derived NO has been reported (28, 40). Alternatively, the impairment of BK relaxation might be related to generation of superoxide anion by iNOS rather than NO itself (3); also, the rapid reaction between superoxide anion and NO will decrease NO bioavailability.

In addition to NO from iNOS, other metabolites including those from COX-2 (4, 6, 11, 27) or from ROS-generating enzymes such as xanthine oxidase and NAD(P)H oxidase (3) have also been suggested to be involved in the effect of LPS on vasoconstrictor responses. However, little is known about their participation in the effect of LPS on endothelium-dependent relaxation. In MCAs from WKY rats, LPS increased COX-2 protein expression after 1 and 5 h of incubation. In addition, NS-398 abolished the depressor effect of LPS on the BK relaxation, which suggests the participation of COX-2-derived mediators in this effect. Nevertheless, the TxA2/PGH2 receptor antagonist SQ-29548 failed to modify the effect of the endotoxin on BK relaxation, and the TxA2 levels were not modified.
after LPS treatment. These results disprove the participation of vasoconstrictor prostanoids acting on the TxA2/PGH2 receptor in MCAs from WKY rats.

COX-2 could also generate superoxide anion (20, 45), which would participate in the LPS effect. Thus the impairment of endothelium-dependent relaxation after LPS administration has been associated with increased generation of superoxide anion (3, 32). Our results in MCAs showed that the superoxide anion scavenger SOD abolished the LPS effect on the vasodilator response to BK. In addition, an increase of superoxide anion levels was observed in cerebral arteries after LPS incubation. These results confirm the involvement of superoxide anion, which could be generated in part from COX-2, in the depressed BK response after LPS exposure. Superoxide anion can scavenge NO and thereby effectively reduce the bioavailability of endothelium-derived NO. In a previous report, we showed that superoxide anion and peroxynitrite induce vasoconstrictor responses in cerebral arteries (17). Furthermore, peroxynitrite formed by simultaneous generation of superoxide anion and NO from iNOS would also contribute to the impairment of endothelium-dependent relaxation (3). The increased expression of the Mn- and Cu/Zn-SOD isoforms observed after 5 h of LPS incubation would compensate for the increased superoxide anion production. Other authors have also reported induction of Mn-SOD after LPS exposure (3, 22). However, reduction by LPS of the Cu/Zn-SOD isoform has been reported in kidney (22). When superoxide anion is dismutated by SOD, H2O2 is generated, and this could also participate in the LPS effect (3). Nevertheless, the H2O2 scavenger catalase did not alter BK response in the presence of LPS in MCAs from WKY rats, which disproves this participation.

Influence of hypertension on LPS effect. LPS produced an inhibition of BK relaxation that appeared earlier in arteries from hypertensive rats. Furthermore, when a single concentration of BK was used, the LPS effect was clearly greater in SHR. However, when concentration-response curves were formed, the effect of LPS was only greater in arteries from SHR at the highest BK concentration used; differences in the two experimental protocols could explain this discrepancy. Similar to what happened with normotensive rats, LPS did not modify eNOS expression but reduced the relaxation induced by DEA-NO, which indicates the existence of alterations in the NO relaxation mechanism.

LPS is described to cause more hypotension and hyporeactivity to vasoconstrictors in SHR than in WKY rats; these effects are associated with elevated iNOS expression and activity as well as with increased nitrate and cGMP levels (5, 7, 43). In cerebral arteries from WKY rats (17) and SHR (unpublished results), no iNOS expression was detected after 1 h of LPS incubation; after 5 h, LPS increased iNOS expression to a similar level in arteries from both rat strains (5). In MCAs from SHR, aminoguanidine reduced but did not abolish the LPS effect on BK relaxation from the third hour of incubation; this suggests that iNOS induction is involved in the depressor effect at this incubation time, similar to what happens in WKY rats. However, mechanisms other than NO from iNOS could be responsible for the differences in the LPS effect observed between the two strains of rats.

COX-2 expression after LPS incubation was similar in cerebral arteries from SHR and WKY rats. In arteries from SHR, both NS-398 and SQ-29548 improved the BK relaxation more in the presence than in the absence of LPS, which suggests that vasoconstrictor prostanoids participate in the effect of the endotoxin in this strain. In agreement, TxA2 levels produced by cerebral vessels were greater after LPS treatment. Although the participation of COX-2-derived metabolites in the LPS effect on endothelium-dependent vasodilator responses has not been studied, contractile prostanoids by TxA2- PGH2 receptor activation have been implicated in the endothelial dysfunction observed in hypertension (23). The fact that in MCAs from SHR the effect of SQ-29548 but not NS-398 was abolished the LPS effect in arteries from SHR (unpublished results). Because SOD abolished the LPS effect in arteries from SHR from the third hour of incubation; this suggests that iNOS induction is involved in the depressor effect at this incubation time, similar to what happens in WKY rats. However, mechanisms other than NO from iNOS could be responsible for the differences in the LPS effect observed between the two strains of rats.

In MCAs from hypertensive rats, 5 h of LPS incubation also increased superoxide anion levels. Superoxide anion can scavenge NO and thereby effectively reduce the bioavailability of NO. In addition, superoxide anion is also a vasoconstrictor agent in MCAs from SHR (unpublished results). Because SOD abolished the LPS effect in arteries from SHR from the first hour of LPS incubation, a greater participation of ROS in the effect of endotoxin on arteries from SHR than on arteries from WKY rats could be suggested. The increase in superoxide anion production could be compensated by an upregulation of

Fig. 8. Representative Western blot and quantitative analysis for Cu/Zn-SOD (A) and Mn-SOD (B) protein expression in MCAs from rats incubated for 1 and 5 h in the absence (WKY rats, n = 5; SHR, n = 7) or presence (WKY rats, n = 5; SHR, n = 7) of 10 μg/ml LPS. Results are expressed as the ratio between signal for the Cu/Zn-SOD and Mn-SOD protein and signal for α-actin. *P < 0.05 vs. in absence; #P < 0.05 vs. 1 h.
Cu/Zn- and Mn-SOD expression after LPS exposure. SOD dismutates superoxide anion to H2O2, which could also participate in the LPS effect. Because catalase abolished the LPS effect in SHR but not in WKY rats confirms the involvement of H2O2 in the former. Greater participation of ROS in vascular responses has also been suggested in mesenteric resistance arteries from SHR (6). In this sense, increased sensitivity to contractile prostanoids and ROS has already been described in vessels from hypertensive animals (14, 18, 35).

In conclusion, the results of the present study suggest that LPS induces inhibition of the endothelium-dependent vasodilator response in MCAs from normotensive rats. This effect would contribute to explanation of the decrease in cerebral blood flow and perfusion pressure and the increase in cerebrovascular resistance observed in the earlier stages of endotoxic shock (12, 29), although experiments to evaluate cerebral blood flow are necessary to confirm this hypothesis. The LPS effect is mediated by the release of superoxide anion, which could be generated in part from COX-2, and of NO from iNOS. The higher participation of ROS and contractile prostanoids, among them TxA2, in arteries from hypertensive rats could be related to the greater depressor effect of endotoxin on the endothelium-dependent relaxation in this strain.

ACKNOWLEDGMENTS

The authors thank Dr. M. Carmen Fernández-Criado for the care of animals and C. F. Warren for linguistic assistance.

GRANTS

This study was supported by Grants BXX2000-0153 and SAF2003-00633 from Dirección General de Investigación and Grant C03-01 from Fondo de Investigación Sanitaria de la Seguridad Social.
A. M. Briones and E. Vila are members of the EC VASCAN 2000 Consortium (QLG1-CT-1999-00084).

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