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Cyclooxygenase-2 inhibition improves vascular endothelial dysfunction in a rat model of endotoxic shock: role of iNOS and oxidative stress

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Running Title: COX-2 and endothelial dysfunction

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Abbreviations: ACh, acetylcholine; BDK, bradykinin; COX, cyclooxygenase; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(5H)-furanone; EDTA, ethylenediamine tetraacetic acid; IL, interleukin; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; SMT, S-methylisothiourea; NA, noradrenaline; NO, nitric oxide; NOS, nitric oxide synthase; PSS, physiological salt solution; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SC-560, 5-(4-clorophenyl)-1-(4-metoxyphenyl)-3-trifluoromethylpirazole; SOD, superoxide dismutase; SQ-29548, 7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S(1alpha,2alpha(Z), 3alpha,4alpha)] -5-Heptenoic acid; TXA_2, thromboxane A_2; TNF-α, tumor necrosis factor-α. **Recommended section assignment:** Cardiovascular

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ABSTRACT

We investigated whether cyclooxygenase isoforms (COX-1, COX-2) and decreased NO availability contribute to endothelial dysfunction in endotoxemic rats. The involvement of ROS was also evaluated. Rats were injected with *Salmonella*-derived lipopolysaccharide or saline**.** After 6 hours, endothelial function of mesenteric resistance arteries was evaluated. In controls, ACh-induced relaxation was inhibited by the NOS inhibitor L-NMMA and unaffected by DFU (COX-2 inhibitor). In LPS-treated rats, the response to ACh was blunted compared with controls, less sensitive to L-NMMA and enhanced by DFU. COX-2 blockade improved also the inhibitory effect of L-NMMA on cholinergic relaxation. SC-560 (COX-1 inhibitor) did not modify the response to ACh in both groups. LPS-induced endothelial dysfunction was unaffected by the TxA_2 receptor antagonist SQ-29548. In vivo iNOS inhibition by S-methylisothiourea partly attenuated LPS-induced endothelial dysfunction. The antioxidants ascorbic acid and SOD normalized endothelium-dependent relaxation and restored the inhibitory action of L-NMMA on ACh. Responses to sodium nitroprusside were similar in both groups. In LPS-treated rats, RT-PCR showed a marked increase in mesenteric iNOS and COX-2 expressions, while eNOS and COX-1 were unchanged. LPS-induced COX-2 overexpression was reduced but not abrogated by S-methylisothiourea. LPS-induced COX-2 upregulation was also documented by immunohistochemistry. In conclusion, mesenteric resistance vessels from endotoxemic rats show impaired endothelial function due to reduced NO availability, a condition which is partly ascribable to an iNOS-dependent enhanced COX-2 expression, whereas TxA_2 does not seem to be involved. Oxidative stress is the main mechanism responsible for reduced NO availability and COX-2 might act as a source of ROS.

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Introduction

It is widely accepted that low-grade vascular inflammation plays a key role in the pathogenesis of atherosclerosis (Ross, 1999). Endothelial dysfunction, characterized by reduced nitric oxide (NO) availability and increased production of reactive oxygen species (ROS), is regarded as a crucial and early mechanism whereby vascular inflammation leads to atherosclerosis (Ross, 1999). A major involvement of endothelial dysfunction has been found also in severe forms of acute inflammation, such that occurring in endotoxemia elicited by Gram-negative bacterial lipopolysaccharide (LPS). Indeed, *in vitro* and *in vivo* evidence indicates an impairment of endothelium-dependent relaxation during endotoxemia by LPS (Szabo et al., 1997; Brandes et al., 1999; Piepot et al., 2000; Hernanz et al., 2004b). When considering the underlying mechanisms, increased levels of inflammatory cytokines have been reported to interfere with endothelial function during endotoxemia (Greenberg et al., 1993). Moreover, such cytokines promote the expression of several enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which in turn lead to an increased generation of NO as well as vasodilator and vasoconstrictor prostanoids (Moncada and Higgs, 1993; Camacho et al., 1998). This picture is further complicated by the evidence that COX-2 may act as a source of ROS (Katusic, 1996; O'Banion, 1999), which are responsible for NO breakdown (Cai and Harrison, 2000). However, the contribution of iNOS and COX-2, and particularly their putative synergistic or antagonistic roles in determining endothelial dysfunction, have not been clarified.

The present study was designed to examine endothelium-dependent relaxations of mesenteric small arteries in a rat model of endotoxic shock, to evaluate whether a reduction in NO availability and changes in COX-2 expression play a role in the pathogenesis of endothelial dysfunction

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secondary to endotoxemia. The participation of iNOS, thromboxane A_2 (TXA_2) and ROS in endotoxin-induced endothelial dysfunction was also investigated.

Methods

Animals

All experiments were carried out in accordance with the provisions of European Union Council Directive 86-609, recognized by the Italian Government. Male Sprague-Dawley rats, 250-300 g body weight, received physiological salt solution (PSS, control) or *Salmonella*-derived LPS (10 mg/Kg) intraperitoneally. Systolic blood pressure (SBP) and heart rate were measured by the tail-cuff method at baseline and 6 hours after injection of LPS or vehicle (BP recorder 58600, Ugo Basile, Italy). An average of 3 pressure readings was obtained. Six hours after treatment, rats were sacrificed under short anaesthesia with diethyl ether. This time was chosen on the basis of previous observations showing that COX-2 mRNA was markedly induced between 2 and 6 hours after LPS injection (Cao et al., 1995; Yamagata et al., 2001).

Preparation of Small Mesenteric Arteries for Reactivity Experiments

Superior mesenteric arteries were taken from the part of the mesenteric vascular bed that feeds the jejunum 8- to 10-cm distal to the pylorus and placed in cold PSS of the following composition (mmol/L): NaCl 120, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5, EDTA 0.026, glucose 5.5. A third-order branch of the mesenteric arterial tree (≈ 2 mm in length) was carefully dissected 1 mm from the intestine and cleaned of all adherent connective tissue under a dissecting microscope. The arterial segments were mounted in a pressure myograph chamber and slipped into two glass microcannulae. Both ends of the arterial segment were secured to the microcannulae with nylon ties. Intraluminal pressure was set to 45 mmHg with a servocontrolled pump. Vessels were then equilibrated for 1 hour with PSS that was bubbled with

95% air and 5% CO_2 to give a pH of 7.4, and heated to 37°C (Virdis et al., 2002). Lumen dimensions were determined by light microscopy and, via a video camera, displayed on a monitor and automatically measured by a software (Myoview, DMT, Aarhus, Denmark). Arterial segments were considered viable and used for experiments if they constricted by more than 50% of their resting lumen diameter in response to an extraluminal application of high-potassium (125 mM KCl) PSS containing 10 μM noradrenaline (NA).

Experimental Design

Endothelium-dependent and -independent relaxations were assessed by measuring the responses of mesenteric arteries retrieved from control or LPS-treated rats (n=8 each group) to cumulative concentrations of acetylcholine (ACh, 1 nM-100 μM) and sodium nitroprusside (0.01-100 μM), respectively. All experiments were performed in vessels precontracted with NA (10 μM). Since exposure of mesenteric vessels to LPS was previously shown to induce hyporeactivity to NA (Briones et al., 2000; Hernanz et al., 2004a), in preliminary experiments we performed a concentration-response analysis of NA effects (from 1 nM to 100 μM) to establish at which level this compound was able to elicit similar contractions in vessels from control and LPS-treated rats. After the titration study, the dose of 10 μM NA, which induced similar contracting responses in both groups, was selected.

Influence of COX-1, COX-2 Activity on NO Availability and Endothelial Function

The participation of COX-1 and COX-2 isoenzymes in modulation of endothelial function was assessed by construction of concentration-response curves to ACh following 30-min preincubation of mesenteric vessels with SC-560 (5-(4-clorophenyl)-1-(4-metoxyphenyl)-3-trifluoromethylpirazole 1 μM, selective COX-1 inhibitor) (Smith et al., 1998) or DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(5H)-furanone 1 μM, selective

COX-2 inhibitor). The latter drug was used because of its documented high selectivity for COX-2. For instance, in hamster ovarian cells expressing human COX-1 and COX-2, DFU was more selective for COX-2 (COX-1/COX-2 IC_{50} > 1000) as compared with NS-398 (COX-1/COX-2 IC_{50} ≈ 300). In addition, DFU was less potent than NS-398 in blocking COX-1 derived prostaglandin E_2 (PGE_2) production (Riendeau et al., 1997).

Concentrations of SC-560 and DFU were chosen according to preliminary experiments (n=4 mesenteric vessels from LPS-treated rats), where a concentration-ranging analysis was performed for SC-560 and DFU (0.1, 0.3, 1 and 10 μM) to identify their maximal effects on relaxant responses to ACh. Increasing concentrations of SC-560 failed to modify the relaxation to ACh. By contrast, DFU concentration-dependently increased the response to ACh, with a maximal effect observed at 1 μM (data not shown).

To verify the possibility that the effects of DFU were specific for cholinergic relaxations, vessels isolated from control and LPS-treated rats (n=4 each) were used to construct concentration-response curves to bradykinin (BDK, from 0.1 nM to 1 μM), another endothelial agonist acting through cholinergic-independent receptor pathways (Flavahan et al., 1991), either in the absence or in the presence of DFU. Moreover, to assess whether the effect of DFU was selective for endothelium-dependent relaxation, this COX-2 inhibitor was tested against concentration-response curves to sodium nitroprusside.

To evaluate NO availability, concentration-response curves to ACh were constructed after 30-min preincubation with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA, 100 μM). Then, to examine whether COX-2 could influence NO availability, ACh was tested in an additional group (n=6) of LPS-treated rats under simultaneous presence of L-NMMA and DFU.

Finally, to ascertain the possible contribution of COX-2-derived TXA_2 on endothelial dysfunction elicited by endotoxemia, mesenteric vessels were isolated from LPS-treated animals (n=4) and concentration-response curves to ACh were constructed after 30-min incubation with the TxA_2 receptor antagonist SQ-29548 (1 μM).

Interaction between iNOS and COX-2

To examine the possible role of iNOS in the modulation of COX-2 expression and function, both control and LPS-treated rats (n=6 each) were administered with the selective iNOS inhibitor S-methylisothiourea (SMT, 25 mg/Kg by intraperitoneal route, 30 min before LPS or PSS injection) (Muntane et al., 2000). Six hours after LPS or PSS treatment, mesenteric vessels were isolated and concentration-response curves to ACh were constructed either in the absence or in the presence of DFU.

Influence of ROS on Endothelial Function

In this further set of experiments (n=6 for each group), concentration-response curves to ACh were determined after 30 min preincubation with the antioxidant compounds ascorbic acid (10 mM) or superoxide dismutase (SOD, 100 U/ml). In addition, to evaluate whether impaired NO availability in endotoxemic rats was attributable to increased ROS production, ACh was infused during simultaneous incubation with L-NMMA and ascorbic acid or SOD. Subsequently, to investigate whether COX-2 could be implicated in the generation of ROS, additional mesenteric vessels from LPS-rats (n=6 for each group) were assayed with ACh after simultaneous incubation with DFU and ascorbic acid or SOD.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Expression of mRNA for COX and NOS isoforms was assessed by reverse transcription-polymerase chain reaction (RT-PCR). Specimens of mesenteric vessels were disrupted with cold glass pestles and total RNA was isolated by Trizol® (Life Technologies, Carlsbad, CA, USA) and chloroform. Total RNA (1 μg) served as template for cDNA synthesis in a reaction using 2 μl random hexamers (0.5 μg/μl) with 200 U of MMLV-reverse transcriptase in a buffer containing 500 μM deoxynucleotide triphosphate mixture (dNTP) and 10 mM dithiothreitol. cDNA samples were subjected to PCR in the presence of specific primers based on the nucleotide sequences of cloned rat COX and NOS isoforms (Ferraz et al., 1997; Tanaka et al., 2002). PCR, consisting of 5 μl of RT products, Taq polymerase 2.5 U, dNTP 100 μM and primers 0.5 μM, was carried out by a PCR-Express thermocycler (Hybaid, Ashford, Middlesex, U.K.). For COX isoforms, amplification conditions were: 1 min at 94°C, two min at 60°C, 1 min at 72°C for 35 cycles. For NOS isoforms, after 3 min at 94°C, the cycle condition was 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles, followed by 7 min at 72°C. Aliquots of RNA not subjected to RT were included in PCR reactions to verify the absence of genomic DNA. The efficiency of RNA extraction, RT and PCR was evaluated by primers for rat β-actin. PCR products were separated by 1.8% agarose gel electrophoresis in a Tris buffer 40 mM containing 2 mM EDTA, 20 mM acetic acid (pH 8), and stained with ethidium bromide. PCR products were then visualized by UV light and subjected to densitometric analysis by NIH image software (Scion Corporation, Frederick, MD, USA). The relative expression of target mRNA was normalized to that of β-actin.

Immunostaining of COX-2

After collection, mesenteric specimens were immediately fixed in cold 4% paraformaldehyde

diluted in phosphate buffered saline (PBS). Samples were dehydrated with ethanol, treated with xylene and embedded in paraffin at 56°C. Sections were sequentially exposed to the following solutions as previously described (Bernardini et al., 1999): 1% hydrogen peroxide in methanol for 5 min; proteinase K (0.05 mg/ml; Boehringer-Mannheim, Germany) in PBS for 10 min; normal swine serum (1:20, Sigma Chemicals, St. Louis, Missouri); primary antibody solutions, 1:100 diluted in 0.1% bovine serum albumin (BSA) and 0.1% sodium azide in PBS (overnight at 4°C). Goat anti-COX-2 polyclonal antibody (cod. no. sc-1746) (Santa Cruz Biotechnology, Santa Cruz, CA) was employed (1:100 diluted in 0.1% BSA and 0,1% sodium azide-PBS). Detection of immunoprecipitates was performed by an indirect streptavidine-peroxidase method, based on biotinylated immunoglobulins, peroxidase-labelled streptavidin complex, and finally to 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Dakopatts, Glostrup, Denmark), as previously reported (Bernardini et al., 1996). All reactions were performed at room temperature in a chamber humidified with PBS unless otherwise specified; washing between each step was performed with PBS. Negative controls were obtained by substituting the primary antibodies with a non-immune goat serum or with PBS plus 0.1% BSA. Endogenous peroxidases and avidin-binding activity were assayed by incubation of slides with DAB alone or with streptavidin-horse radish peroxidase complex/DAB, respectively.

Cytokine Assays

At the time of sacrifice, serum and plasma from trunk blood were collected for the determination of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) by means of enzyme-linked immunosorbent assay commercial kits (R&D Systems, Minneapolis, MN, USA).

Drugs and Solutions

Salmonella-derived LPS, SMT (S-methylisothiourea), SC-560 (5-(4-clorophenyl)-1-(4-metoxyphenyl)-3-trifluoromethylpirazole), L-NMMA (N^G-monomethyl-L-arginine), ACh (acetylcholine), BDK (bradykinin), NA (noradrenaline), sodium nitroprusside, ascorbic acid and SOD (superoxide dismutase) were purchased from Sigma Chemicals (St Louis, MO, USA), and SQ-29548 (7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S(1alpha,2alpha (Z),3alpha,4alpha)]-5-Heptenoic acid) from Cayman Chemical (Ann Arbor, MI, USA). DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(5H)-furanone) was kindly provided by Merck Research Laboratories (Rahway, NJ, USA). Drug solutions were made in distilled water except for SMT (dissolved in PSS) and SC-560, DFU and SQ-29548 (dimethylsulphoxide). Further dilutions were prepared with distilled water.

Data Analysis

Results are presented as mean±SEM and analyzed by repeated measures ANOVA, followed by a Student-Newman-Keuls test or by unpaired *t-test* where appropriate. A value of $P<0.05$ was considered statistically significant. Maximal NA-, ACh-, BDK- and sodium nitroprusside-induced responses (E_{max}) were calculated as maximal percentage decrements or increments of lumen diameter. “n” indicates the number of experiments.

Results

Physiological parameters, Cytokine Plasma Levels and Lumen Diameters of Mesenteric Resistance Arteries

Body weight was similar in control and LPS-treated animals. LPS but not PSS injection caused a decrease in SBP and an increase in heart rate 6 h after treatment. As expected, LPS-treated rats exhibited an increase in plasma levels of TNF-α, IL-1β and IL-6 compared with

controls. Lumen diameters of vessels from control and LPS-treated rats did not significantly differ (Table 1).

Contractile Reactivity to NA

NA concentration-dependently induced contractions in mesenteric vessels from both control and LPS-treated animals. Vessels from LPS-treated rats showed hyporeactivity to the first four concentrations of NA compared with controls, a difference which no longer occurred when higher concentrations were applied (Figure 1). Indeed, NA reached its maximum contracting effect at the concentration of 10 μM, with similar values in both groups (E_{max} 60.6±1.3 % and 55.6±2.6 % in controls and LPS-treated rats, respectively). No further significant increments were detected at the concentration of 100 μM (Figure 1). Based on these findings, 10 μM NA was used to pre-contract vessels in subsequent experiments.

Effects of SC-560, DFU and L-NMMA on Endothelium-Dependent Relaxations

In control rats, relaxation of resistance arteries evoked by ACh (E_{max}: 97.8±0.9%) was not modified by exposure to SC-560 (98.5±0.6%) or DFU (97.8±1.0%) (Figure 2A), whereas it was significantly blunted by L-NMMA (E_{max} : 64.1±1.6%; inhibition: -33.7±1.0%) (Figure 2A and 6).

In LPS-injected rats, relaxation to ACh was attenuated (P<0.001) in comparison with controls (E_{max}: 69.9±3.6%) and significantly improved, but not normalized, by DFU (84.6±2.9%, P<0.05 vs control) (Figure 2B). Relaxation to ACh was unaffected by SC-560 (70.2±3.9%) (Figure 2B).

Similarly to results obtained with ACh, relaxation to BDK was significantly (P<0.001) blunted in vessels from LPS-treated rats (E_{max}: 73.3±2.1%) compared with controls (E_{max}: 95.2±1.9%). Moreover, DFU, while not modifying the response to BDK in controls (E_{max}:

94.7±2.1%), significantly (P<0.01) improved, but did not normalize, relaxation to BDK in vessels from LPS-treated rats (E_{max}: 82.1±1.3%, P<0.05 vs controls).

In vessels from LPS-treated rats, the inhibitory effect exerted by L-NMMA on relaxation evoked by ACh (62.4±2.5%; inhibition: -7.5±1.6%), was significantly lower compared with controls (Figure 6). Under the same conditions, DFU application not only improved the relaxant responses to ACh (ACh alone: 71.1±0.3%; ACh plus DFU: 84.2±1.1%), but also enhanced the inhibitory effect of L-NMMA on endothelial-mediated relaxation (ACh plus L-NMMA: 62.1±1.8%; inhibition: -9.0±1.4%; ACh plus L-NMMA and DFU: 61.4±1.9%; inhibition: -22.8±1.4%) (Figure 2C and 6). Upon incubation with DFU, the blunting effect of L-NMMA on maximal response to ACh, although improved, was still significantly lower compared to controls (Figure 6).

In vessels from LPS-treated rats, the relaxant response to ACh (E_{max}: 70.3±1.7%) was not affected by incubation with SQ-29548 (E_{max}: 71.2±2.6%).

Endothelium-independent relaxation by sodium nitroprusside was similar in control (E_{max}: 96.6±0.7%) and LPS-treated vessels (E_{max}: 96.5±0.8%) and was not modified by DFU (E_{max}: 96.2±1.1 and 96.9±1.5 in control and LPS-treated vessel, respectively).

Interaction Between iNOS and COX-2

In vessels from animals treated with SMT plus LPS, relaxation to ACh, although attenuated in comparison with controls, was significantly increased (E_{max} : 84.1±1.6%) when compared to LPS alone (Figure 3). Interestingly, following iNOS inhibition, the relaxant response to ACh was no longer enhanced by DFU (E_{max}: 86.1±1.9%) (Figure 3). In rats treated with SMT alone, the response to ACh (E_{max}: 96.9±2.8%) was similar to that recorded in controls, and was not affected by DFU (E_{max} : 96.5±2.7%).

Effect of Ascorbic Acid on Responses to Acetylcholine

In control rats, relaxation to ACh (98.1±0.7%) was not modified by ascorbic acid (98.3±0.7%). By contrast, the antioxidant drug significantly improved endothelium-dependent relaxation in endotoxemic rats (ACh, 71.0±0.6%; ACh with ascorbic acid, 96.4±0.9%; Figure 4A). Moreover, ascorbic acid restored the inhibitory effect of L-NMMA on agonist-induced relaxation (ACh plus L-NMMA, 62.8±0.6%; inhibition: -8.2±1.1%; ACh plus L-NMMA and ascorbic acid, 61.7±0.6%; inhibition: -34.7±1.2%; Figures 4A and 6). Under ascorbic acid, the relaxing effect of ACh on vessels from LPS-treated rats was no longer different from that exerted by ACh alone in control rats (Figure 6). Similarly, upon exposure to ascorbic acid, the blunting effect of L-NMMA on maximal relaxation elicited by ACh in endotoxemic rats (-34.7±1.2%) was similar to that measured in control animals (-32.9±1.0%).

When ascorbic acid and DFU were applied to vessels from LPS-treated rats, the COX-2 inhibitor did not further improve the enhancing action of ascorbic acid on the maximal relaxant response elicited by ACh (ACh, 70.5±0.6%; ACh plus DFU, 84.8±0.5%; ACh plus ascorbic acid, 96.6±0.3%; ACh plus ascorbic acid and DFU, 95.5±0.2%; Figure 4B).

Effect of SOD on Responses to Acetylcholine

As expected, in control rats relaxation to ACh (98.5±0.6%) was not modified by SOD (98.0±0.9%). By contrast, and similarly to that observed with ascorbic acid, in endotoxemic rats SOD administration normalized the vascular response to ACh and restored the inhibitory effect of L-NMMA on ACh-induced relaxation (ACh, 68.3±0.8%; ACh with SOD, 97.2±1.1%; ACh plus L-NMMA, 61.4±0.9%; inhibition: -6.9±0.9%; ACh plus L-NMMA and SOD, 64.7±1.1%; inhibition: -32.5±1.1%; Figure 5A and 6). When SOD and DFU were simultaneously applied to mesenteric vessels from LPS-treated rats, no further improvement of the maximal relaxant

response elicited by ACh was observed (ACh, 70.1±0.8%; ACh plus DFU, 82.4±0.7%; ACh plus SOD, 95.9±0.4%; ACh plus SOD and DFU, 96.2±0.3%; Figure 5B).

RT-PCR Analysis of COX and NOS Isoforms Expression

RT-PCR analysis showed a basal expression of mRNA encoding COX-1, COX-2 and eNOS, but not iNOS, in control rats. After treatment with LPS, a significant induction of iNOS and COX-2 was detected, whereas eNOS and COX-1 expressions were not affected (Figure 7). As expected, treatment with SMT alone failed to modify both COX-1 and COX-2 expressions. By contrast, while not modifying COX-1, the iNOS inhibitor significantly reduced, without completely abolishing, the LPS-induced COX-2 expression in mesenteric vessels (Figure 8).

Immunohistochemical Analysis of COX-2

In control mesenteric arteries, a weak COX-2 immunopositivity was detected (Figure 9A). By contrast, in vessels from LPS-injected rats, a marked enhancement in COX-2 immunostaining was observed, both in endothelial cells and within the muscle layer (Figure 9B).

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Discussion

In the present study we observed that endotoxic shock secondary to LPS injection is characterized by small mesenteric artery endothelial dysfunction, a finding which is in agreement and extends previous data obtained in different models of endotoxic shock, where a reduced endothelial function was also shown (Szabo et al., 1997; Brandes et al., 1999; Piepot et al., 2000).

With respect to the mechanisms responsible for endothelial dysfunction, to assess NO availability we employed the NOS inhibitor L-NMMA. In LPS-injected rats, the inhibition by L-NMMA on cholinergic relaxations was lower than in controls. Concomitantly, we detected an enhanced expression of iNOS in mesenteric vascular walls, whereas eNOS was unaffected. These findings, while extending to the mesenteric vascular district the concept that endotoxemia is associated with up-regulated iNOS expression (Hocherl et al., 2002), indicate that LPS-induced endothelial dysfunction is characterized by a significant reduction in stimulated NO availability. The different behavior of NOS isoforms in response to LPS (up-regulation of iNOS and unmodified eNOS expression), together with the reduced NO availability, is in line with a previous report on rabbit carotid arteries exposed to inflammatory cytokines (Kessler et al., 1997), showing that iNOS up-regulation was associated with an unmodified eNOS expression and an impaired endothelial function.

The major novel finding of our study concerns the involvement of COX-2 in endothelial dysfunction elicited by endotoxic shock. In mesenteric vessels from LPS-treated rats DFU, but not SC-560, improved endothelium-dependent relaxation, indicating that COX-2, but not COX-1, contributes to the pathogenesis of endothelial dysfunction associated with endotoxemia. The selectivity of the COX-2 inhibitor for endothelium-dependent relaxation was supported by the

observation that DFU improved the relaxant response of mesenteric vessels to different endothelial agonists, such as ACh and bradykinin, without effects on the response to sodium nitroprusside. Of note, DFU improved, without normalizing, endothelium-dependent relaxation, suggesting that COX-2-independent pathways are also responsible for LPS-induced vascular effects. In keeping with our functional results, an up-regulation of COX-2 expression in the mesenteric district, secondary to endotoxemia, was detected by RT-PCR. Furthermore, immunohistochemical analysis revealed a marked up-regulation of COX-2, but not COX-1, in the endothelial layer of mesenteric arteries from LPS-treated rats. These findings provide the first demonstration of COX-2 localization in endothelial cells of rat small resistance arteries, and strongly support our findings that endothelial COX-2 is involved in endotoxin-induced endothelial dysfunction. Interestingly, DFU restored partly the inhibitory effect of L-NMMA on cholinergic relaxation in LPS-injected rats, indicating that selective COX-2 blockade improves NO availability, and providing the first evidence for the existence of a close correlation between endothelial COX-2 expression and reduced NO-dependent relaxation in endotoxic shock.

A “cross-talk” between iNOS and COX-2, with a strict dependence of COX-2 up-regulation on iNOS activation, has been previously demonstrated in the heart (Shinmura et al., 2002), whereas *in vitro* studies in other tissues gave conflicting results (Goodwin et al., 1999). Our experiments revealed that, after in vivo iNOS inhibition, the LPS-induced endothelial dysfunction was attenuated and that COX-2 blockade by DFU was no longer able to improve the response to ACh. Concomitantly, RT-PCR analysis showed that, under iNOS inhibition, LPS-induced COX-2 up-regulation was reduced, but not completely abrogated. Taken together, these findings indicate that the enhancement of COX-2 expression and activity occurs downstream of iNOS induction, thus demonstrating the existence of a hierarchical relationship between iNOS

and COX-2 in mesenteric vessels. Of importance, under iNOS inhibition, the endothelium-dependent relaxation, although improved, was still attenuated compared with controls, thus supporting the concept that COX-2-independent pathways contribute to LPS-induced effects.

In line with a recent study on patients with Crohn's disease, a clinical condition characterized by severe inflammation of gut wall and mesenteric district (Tabernero et al., 2003), LPS up-regulated COX-2 expression also in the mesenteric muscle layer. However, at least in our experimental conditions, the increased muscular expression of COX-2 does not appear to interfere with relaxant responses mediated by NO pathways, as demonstrated by the unmodified relaxations to sodium nitroprusside in vessels from LPS-treated rats. Rather, it is conceivable that up-regulated muscular COX-2 interacts with other endogenous mediators involved in the control of vascular contractility. This hypothesis is in keeping with previous data (Tabernero et al., 2003), where COX-2 inhibition attenuated NA-induced vasoconstriction, suggesting an involvement of COX-2 products in the regulation of vascular responses to vasoconstrictor mediators.

The increased iNOS expression, together with the decreased NO availability secondary to endotoxemia, raises the possibility that NO might be inactivated by an enhanced production of ROS, as supported by previous evidence (Brandes et al., 1999; Macarthur et al., 2000; Hernanz et al., 2004b). To address this issue, we first used ascorbic acid, an antioxidant compound capable of scavenging superoxide anions at high concentrations (Jackson et al., 1998). In LPS-vessels, the blunted endothelium-dependent relaxation was completely reversed by exposure to ascorbic acid. Furthermore, in the presence of ascorbic acid, L-NMMA increased its inhibiting effect on endothelium-dependent relaxations to values not different from those observed in control vessels. These findings suggest a major involvement of ROS in reducing NO availability

in endotoxemia. However, the beneficial effect of ascorbic acid might depend on mechanisms other than scavenging superoxide, such as stimulation of NO production, reduced lipid peroxidation or increased production of tetrahydrobiopterin (Tomasian et al., 2000). For these reasons, we tested also SOD, a specific superoxide anion scavenger. The results obtained with this scavenger were similar to those achieved with ascorbic acid. Taken in conjunction, such findings indicate that, in the presence of antioxidant compounds, the activity of NO pathway is restored, thus demonstrating that ROS represent the main mechanism accounting for NO breakdown during endotoxemia in resistance arteries. In line with this proposal, SOD abolished the LPS-induced endothelial dysfunction in isolated cerebral arteries (Hernanz et al., 2004b). Moreover, previous reports demonstrated that the enhanced NO production, secondary to iNOS up-regulation (Stoclet et al., 1999; Piepot et al., 2000), succeeded in reacting with superoxide anions to generate peroxynitrite, a substance responsible for endothelial cytotoxic effects (Katusic, 1996). Overall, it is conceivable that enhanced ROS production could play a crucial role in reducing endothelial NO availability in rats with endotoxic shock. However, tissue ROS production and nitrite/nitrate concentrations were not measured in our study, and therefore the above hypothesis could not be substantiated by direct mechanicistic evidence. Furthermore, it is worth mentioning that different durations of endotoxemia might lead to the recruitment of other pathophysiological mechanisms, with induction of different vascular responses. For instance, SOD did not restore, but further deteriorated, ACh-induced relaxation in aorta rings from animals exposed to LPS for 12-30 hours (Brandes et al., 1999).

As far as the pathogenesis of oxidative injury is concerned, there is evidence that COX-2 may be a source of oxygen radicals (Katusic, 1996; O'Banion, 1999). Indeed, the catalytic activity of cyclooxygenase consists of a series of radical reactions which use molecular oxygen and

generate intermediate ROS (Marnett, 2000). Thus, it is conceivable that an increase in COX-2 activity during endotoxemia might promote an overproduction of ROS. In our experiments the reduced endothelium-dependent relaxation was partly restored by COX-2 blockade, while being completely normalized by ascorbic acid or SOD. Moreover, when mesenteric vessels were simultaneously exposed to DFU plus ascorbic acid or SOD, no further potentiation of the relaxing responses to ACh was observed. Taken together, these data suggest that ROS production, occurring in the presence of endotoxic shock, can be partly ascribed to COX-2-dependent mechanisms, leading to endothelial dysfunction. Although the molecular mechanisms whereby COX-2 promotes ROS production, and ROS can then impair the endothelial function during endotoxemia, were not examined in the present study, some pathways putatively involved in these processes have been schematically outlined in Figure 10. It is also noteworthy that COX-2 seems to account for a great part, but not for the whole production of ROS, suggesting that COX-2-independent sources of ROS might contribute to endothelial dysfunction. Although elucidation of this aspect deserves further investigation, inflammatory cytokines, with particular regard for TNF-α-induced activation of NADPH oxidase, are likely candidates (Frey et al., 2002).

Besides enhancing ROS generation, COX-2 overexpression might also produce vasoconstrictor prostanoids (Cipollone et al., 2001). To address this issue we used the TxA_2 receptor antagonist SQ-29548 and found that this drug failed to modify the endothelium-dependent relaxation of mesenteric vessels from LPS-treated rats, a finding which is in keeping with previous data obtained in cerebral arteries isolated from WKY rats (Hernanz et al., 2004b). Overall, these data, together with our results showing that the response to sodium nitroprusside

was similar in control and LPS-treated rats, exclude the participation of vasoconstrictor prostanoids acting on TxA_2 receptor in COX-2 mediated endothelial dysfunction.

In conclusion, the present study indicates that small mesenteric vessels from rats with endotoxic shock are characterized by impaired endothelial function due to reduced NO availability, and that such an alteration is partly dependent on iNOS-dependent COX-2 over-expression. It also suggests that ROS represent the main mechanism responsible for reduced NO availability and that COX-2 could contribute to their generation.

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Footnotes

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Legends for Figures

Figure 1. Contracting response elicited by increasing concentrations of NA in mesenteric resistance arteries from controls and LPS-injected rats. Each point represents the mean of four experiments±SEM (vertical bars). *P<0.05.

Figure 2. Endothelium-dependent relaxations elicited by ACh in mesenteric resistance arteries from control (panel A) or LPS-injected rats (panel B), in the absence (saline) or in the presence of L-NMMA, DFU or SC-560, and from LPS-injected rats (panel C) in the absence (saline) or in the presence of L-NMMA, DFU, or both. Each point represents the mean of six to eight experiments±SEM (vertical bars). *P<0.05; **P<0.01; ***P<0.001.

Figure 3. Endothelium-dependent relaxations to ACh in mesenteric resistance arteries from control, LPS and SMT + LPS-injected rats, under basal conditions (saline) or in the presence of DFU. Each point represents the mean of six to eight experiments±SEM (vertical bars). **P<0.01.

Figure 4. Endothelium-dependent relaxations to ACh in mesenteric resistance arteries from LPS-injected rats. Panel A: relaxant responses in the absence (saline) or in the presence of L-NMMA, ascorbic acid (Asc. Ac.), or both. Panel B: relaxant responses in the absence (saline) or in the presence of Asc. Ac., DFU, or both. Each point represents the mean of six experiments±SEM (vertical bars). *P<0.05; **P<0.01; ***P<0.001.

Figure 5. Endothelium-dependent relaxations to ACh in mesenteric resistance arteries from LPS-injected rats. Panel A: relaxant responses in the absence (saline) or in the presence of L-NMMA, SOD, or both. Panel B: relaxant responses in the absence (saline) or in the presence of SOD, DFU, or both. Each point represents the mean of six experiments±SEM (vertical bars). **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure 6. Inhibition exerted by L-NMMA on maximal relaxing responses to ACh in mesenteric resistance arteries from control and LPS-injected rats, either in the absence (saline) or in the presence of DFU or ascorbic acid (Asc. Ac.) and SOD. Each column represents the mean of six to eight experiments±SEM. ****P*<0.001 vs other groups; ***P*<0.01 vs controls; ‡*P*<0.01 vs LPS+DFU.

Figure 7. Upper panels: representative agarose gel showing RT-PCR products for (A) COX-1, COX-2, β-actin, and (B) eNOS, iNOS, β-actin in mesenteric vessels from control (CON) and LPS-treated rats. Bottom panels: column graphs referring to densitometric analysis of COX isoform (A) and NOS isoform (B) cDNA bands normalized to the expression of β-actin. M=size markers. Each column represents the mean of four-five experiments±SEM (vertical bars). **P*<0.05 vs respective controls.

Figure 8. Upper panels: representative agarose gel showing RT-PCR products for COX-1, COX-2 and β-actin in mesenteric vessels from rats treated with SMT alone (SMT) and LPS plus SMT. Bottom panels: column graphs referring to densitometric analysis of COX isoform cDNA

bands normalized to the expression of β-actin. M=size markers. Each column represents the mean of four-five experiments±SEM (vertical bars). *P<0.05 vs SMT alone.

Figure 9. Immunostaining for COX-2 in small mesenteric arteries from control (panel A; scale bar: 28 μm) and LPS-treated rats (panel B; scale bar: 40 μm). Sections of arterial segments show the endothelial cell layer inwards. Positive immunoreaction is observed as a brown precipitate (arrows).

Figure 10. Schematic diagram showing the hypothetic involvement of COX-2-derived reactive oxygen species (ROS) in LPS-induced endothelial dysfunction. Lipopolysaccharide (LPS) can directly or indirectly (via inflammatory cytokines and iNOS upregulation) induce COX-2 expression. The hyperactivity of COX-2 catalysis may be a source of ROS, including superoxide anions (O_2^-). Other sources of ROS may include TNF-α-induced activation of NADPH oxidase etc. Superoxide anions are able to react with nitric oxide (NO), leading to a reduced nitric oxide availability and concomitant hyperproduction of peroxynitrites ($ONOO^-$). These reactive species could react with various biologically active proteins involved in endothelium-mediated vascular relaxation, such as endothelial nitric oxide synthase (eNOS), thus inhibiting its activity. ROS could also oxidize lipoproteins (LDLs), wich in turn could interfere with endothelial function. Note that peroxynitrites could also exert direct endothelial cells damage. IL: interleukin; TNF-α: tumor necrosis factor α; oxLDLs: oxidized lipoproteins. **(+)** indicates the stimulation of enzyme expression. (For references see: (Cox and Cohen, 1996; Katusic, 1996; Pasquet et al., 1996; Szabo, 1996; Frey et al., 2002)

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Table 1. Physiological Parameters, Cytokine Plasma Levels and Lumen Diameters of Vessels from Control and LPS-Treated Rats

Parameter	Controls	LPS
Body weight (g)	322.0±15.5	331.7±17.4
SBP (mmHg)	124±6.5	92±4.6*
Heart rate (beats/min)	346±25	527±31*
Plasma TNF-α (pg/ml)	10.7±0.1	318.6±26.7**
Plasma IL-1ß (pg/ml)	30.0±0.1	935.4±199.5**
Plasma IL-6 (pg/ml)	69.4±8.1	1112.9±296.4**
Lumen diameter (μm)	249.7±8.1	257.3±10.1

n=8 for each group. SBP: systolic blood pressure; *P<0.05; **P<0.01

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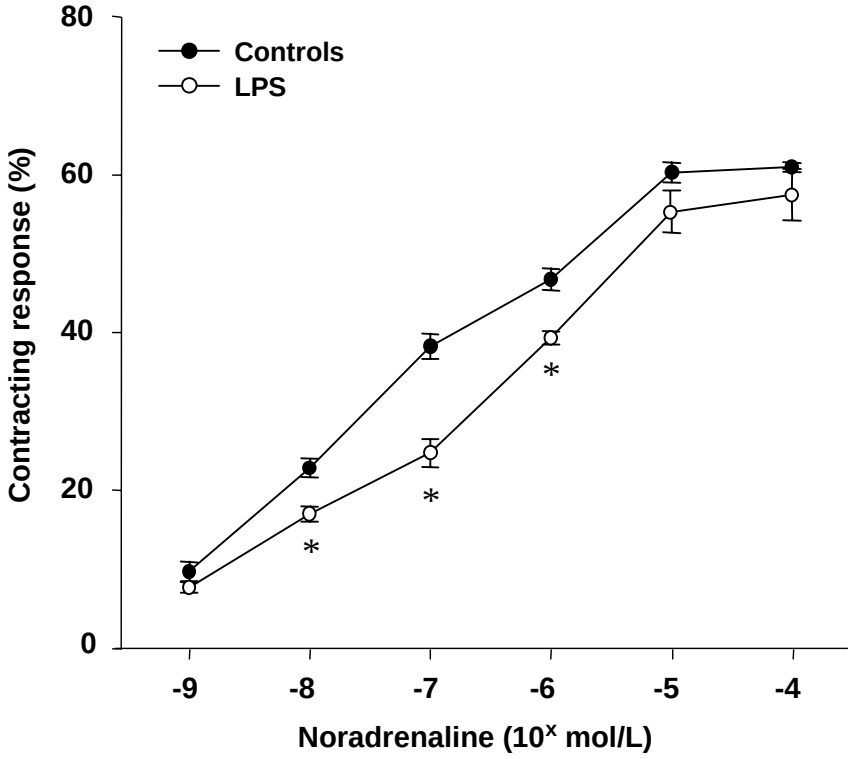

Figure 1

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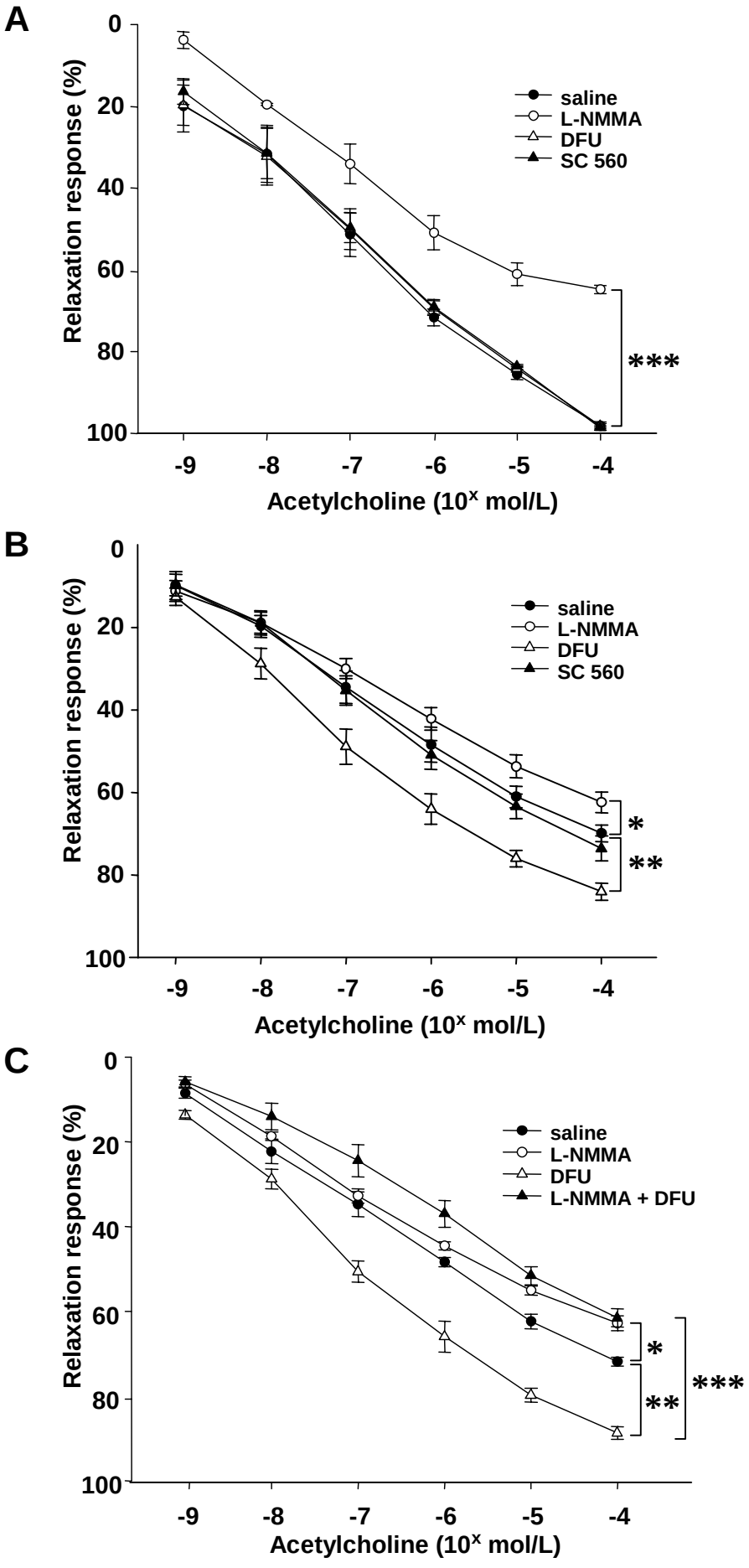

Figure 2

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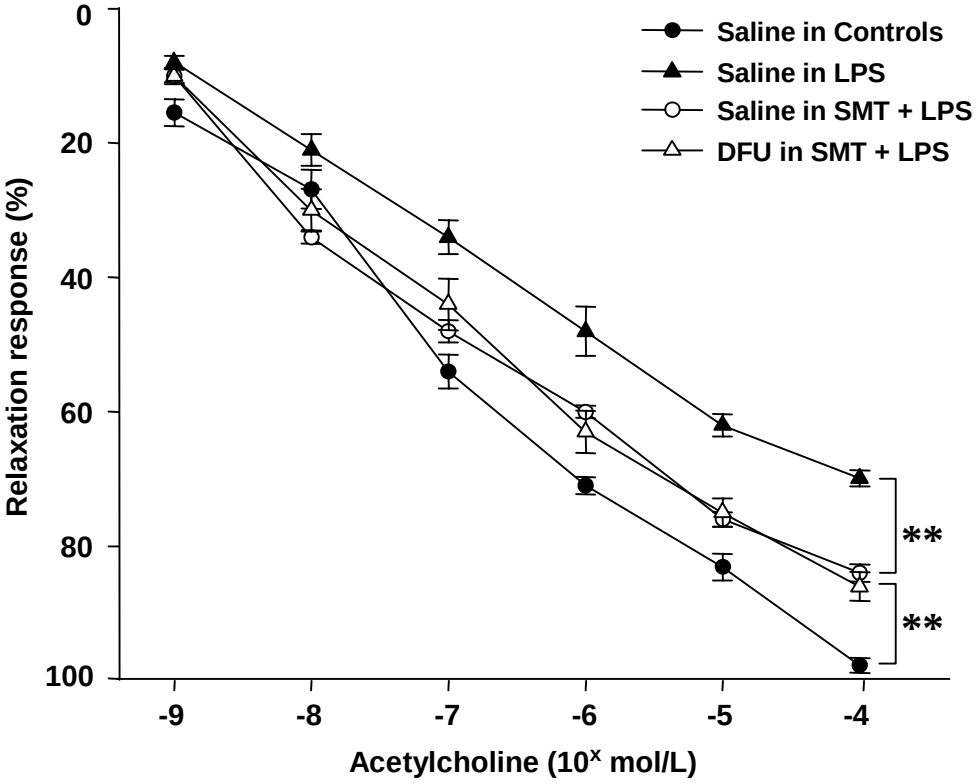

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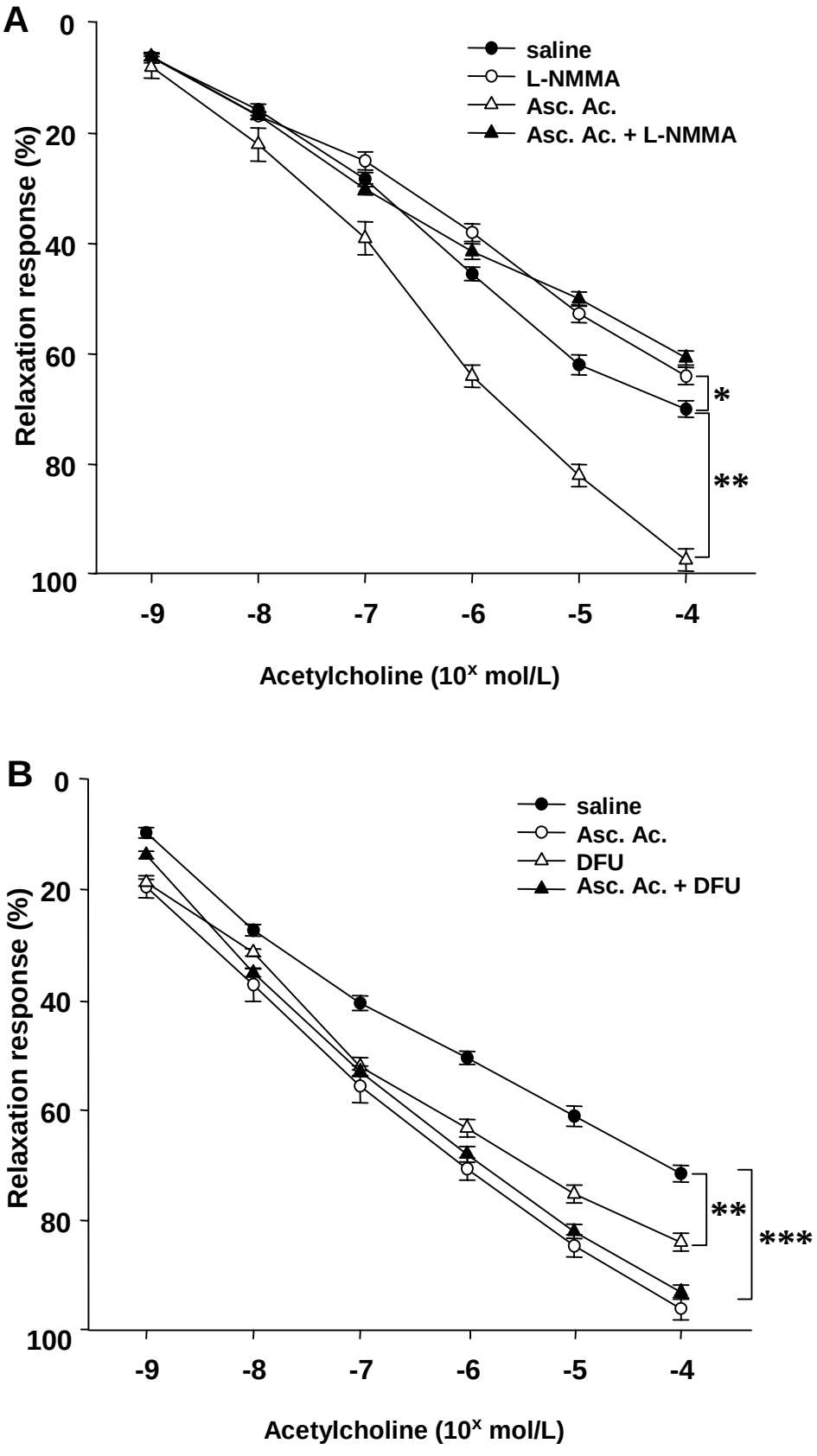

Figure 4

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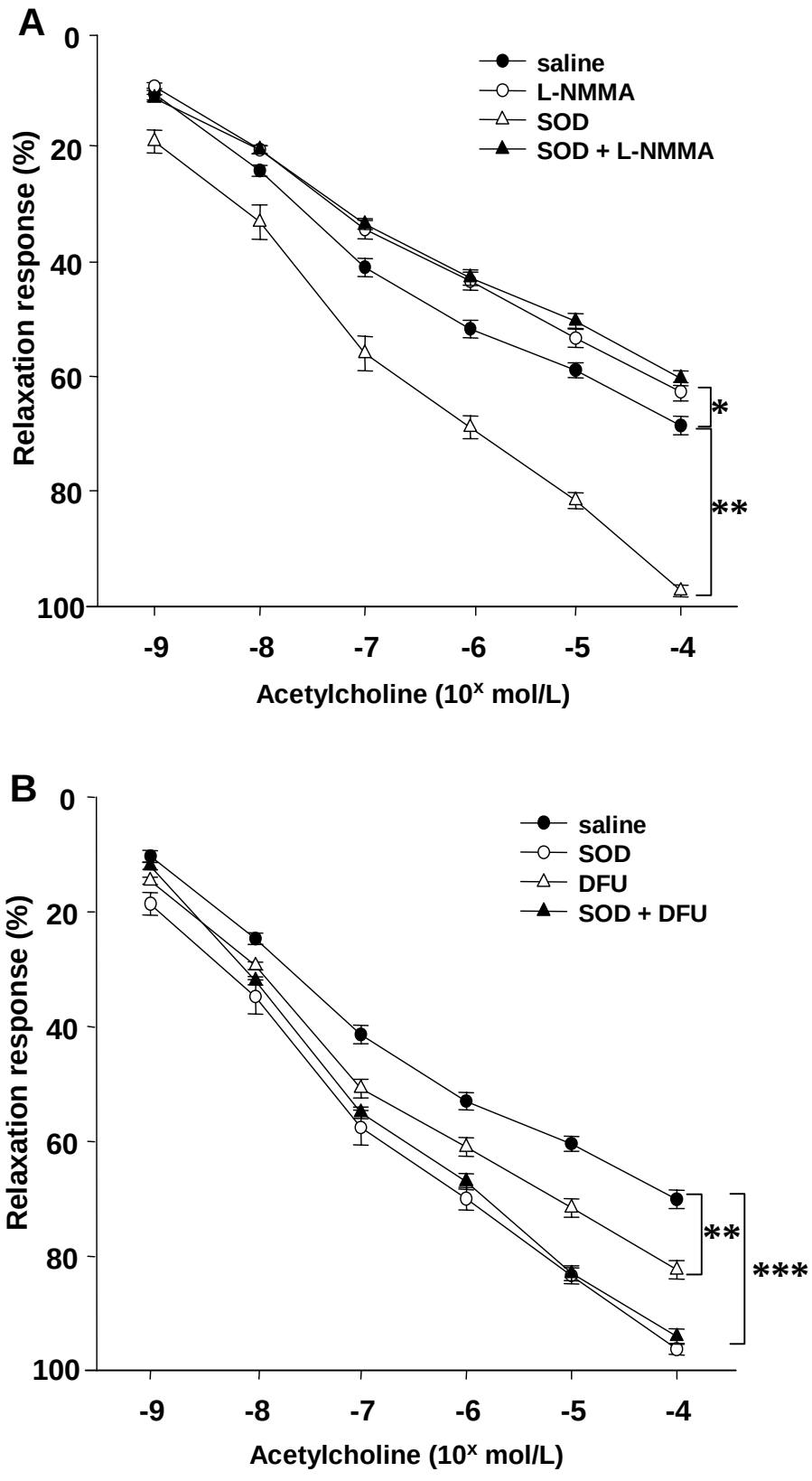

Figure 5

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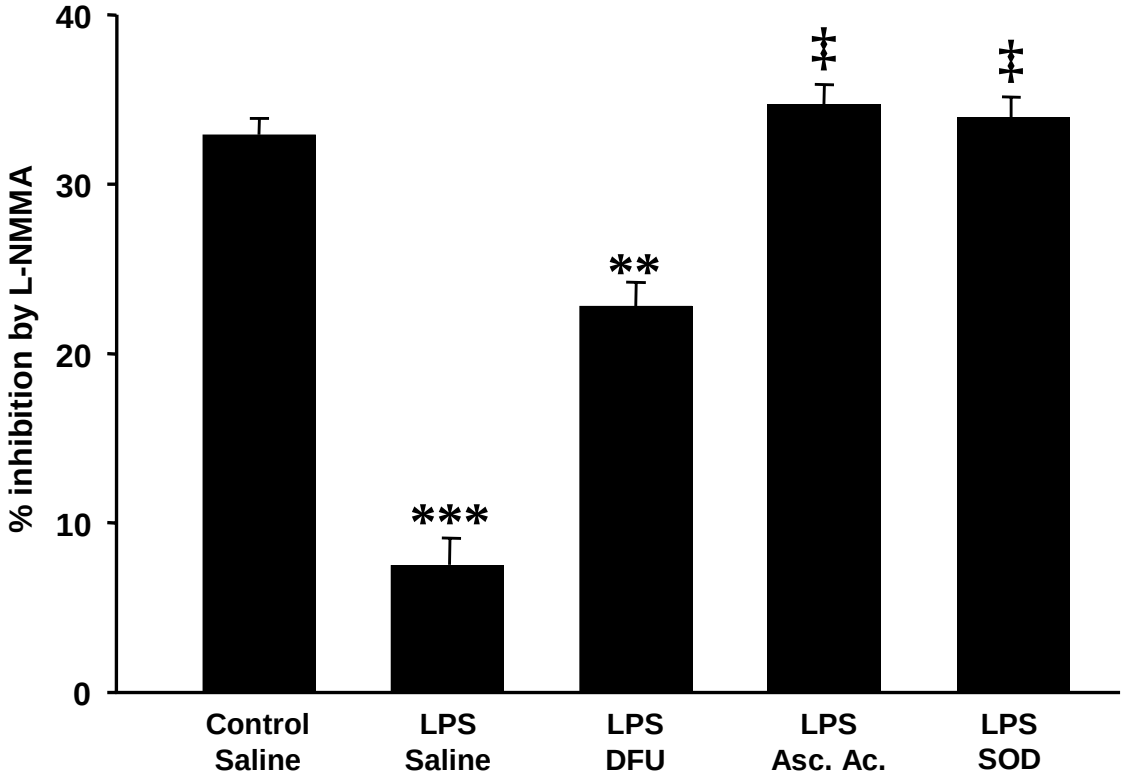

Figure 6

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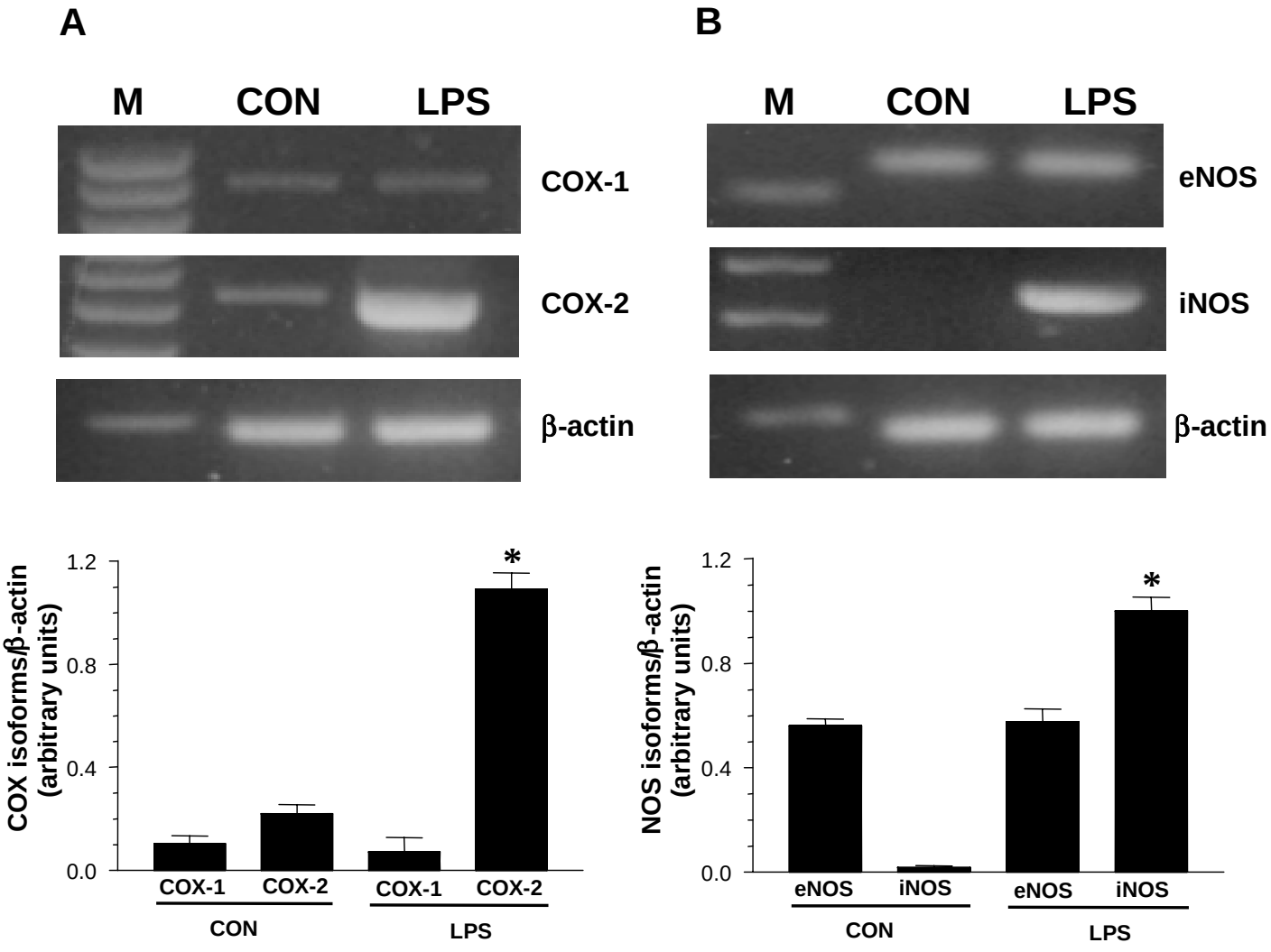

Figure 7

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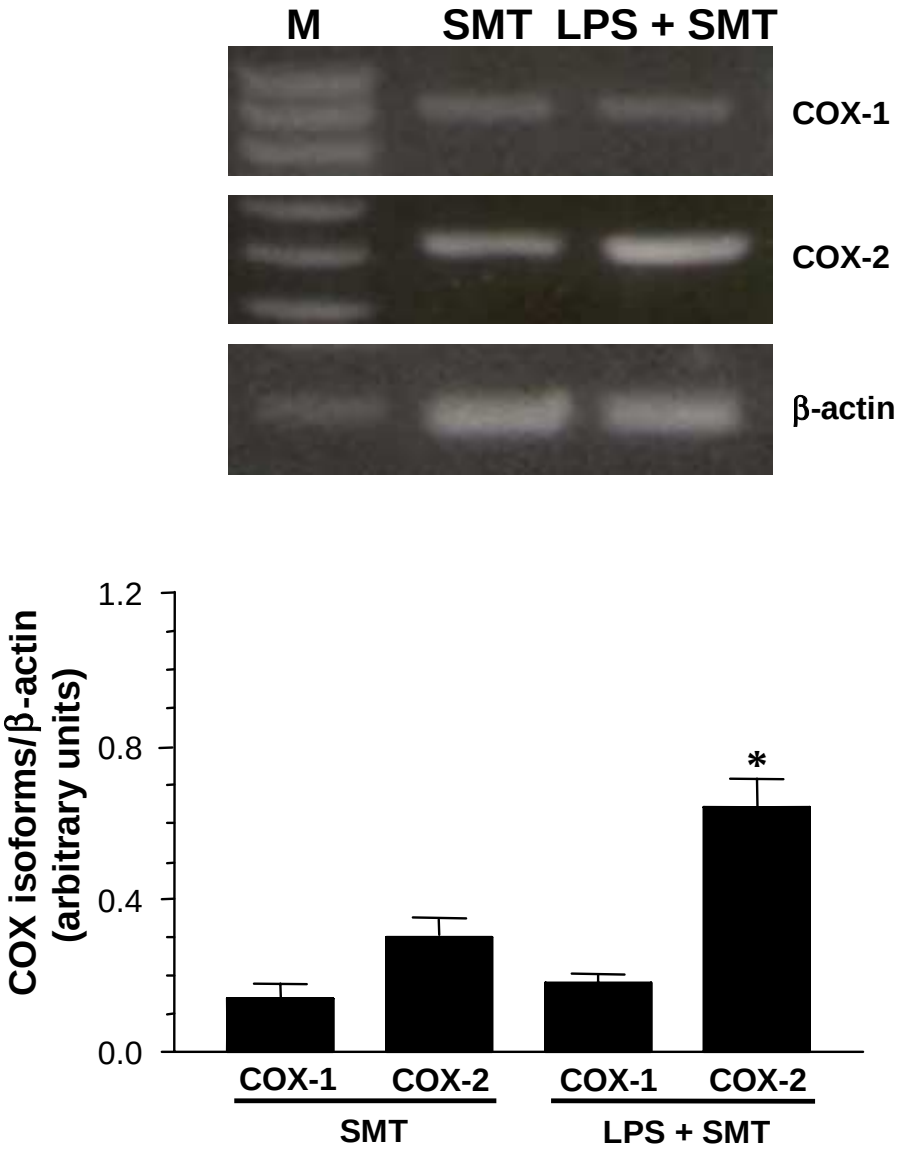

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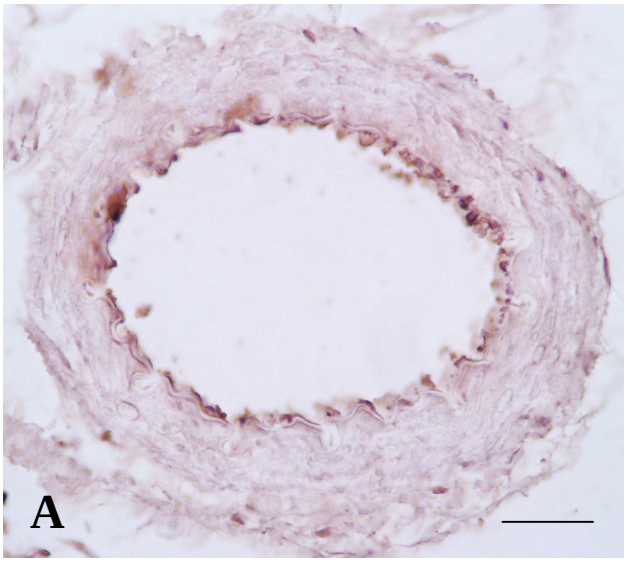

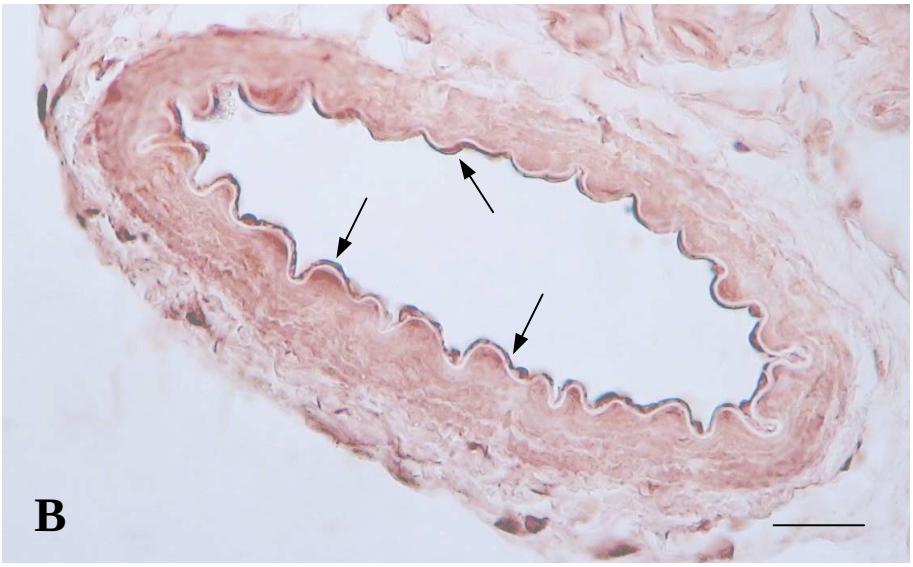

Figure 9

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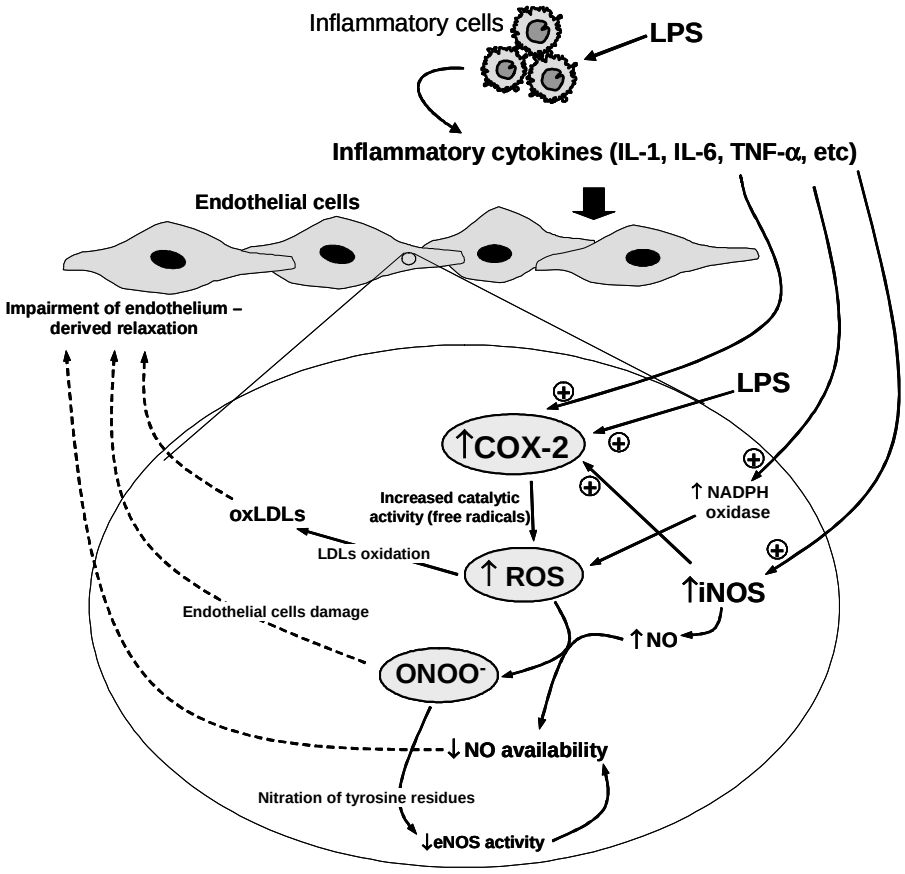

Figure 10