Endothelial Dysfunction in Rat Mesenteric Resistance Artery after Transient Middle Cerebral Artery Occlusion

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ABSTRACT

Stroke triggers a local and systemic inflammatory response leading to the production of cytokines that can influence blood vessel reactivity. In this study, we aimed to assess whether cerebral ischemia/reperfusion could affect vasoconstriction and vasodilatation on mesenteric resistance arteries (MRA) from Wistar Kyoto rats. The right middle cerebral artery was occluded (90 min) and reperfused (24 h). Sham-operated animals were used as controls. Plasma levels of interleukin (IL)-6 and IL-1β were measured at 24 h. Vasoconstrictor and vasodilator responses were recorded in a wire myograph. Protein expression was determined by Western blot and immunofluorescence, and superoxide anion (O₂⁻) production was evaluated by ethidium fluorescence. In MRA, ischemia/reperfusion increased plasma levels of IL-6, O₂⁻ production, protein expression of cyclooxygenase-2, and protein tyrosine nitrosylation, but it impaired acetylcholine (ACh) vasodilatation without modifying the vasodilataions to sodium nitroprusside or the contractions to phenylephrine and KCl. Superoxide dismutase (SOD) and indomethacin reversed the impairment of ACh relaxation induced by ischemia/reperfusion. However, N⁰-nitro-L-arginine methyl ester affected similarly ACh-induced vasodilatations in MRA of ischemic and sham-operated rats. Protein expression of endothelial and inducible nitric-oxide synthase, copper/zinc SOD, manganese SOD, and extracellular SOD was similar in both groups of rats. Our results show MRA endothelial dysfunction 24 h after brain ischemia/reperfusion. Excessive production of O₂⁻ in MRA mediates endothelial dysfunction, and the increase in plasma cytokine levels after brain ischemia/reperfusion might be involved in this effect.

Stroke is an inflammatory process associated with cytokine release and with generation of free oxygen radicals (Mergenthaler et al., 2004; Hossmann, 2006). Cytokines and free oxygen radicals are associated with the endothelial dysfunction observed in several cardiovascular pathologies (Huang et al., 2006; Xu and Touyz, 2006; Dandona et al., 2007; Pennathur and Heinecke, 2007; Wang et al., 2007). Incubation with cytokines can affect vasoconstrictor and vasodilator responses in mesenteric resistance arteries (MRA; Briones et al., 2005b; Jiménez-Altayó et al., 2006) and in other resistance arteries (Vila and Salaices, 2005).

After stroke, there is an abrupt increase of blood pressure that can persist for several days (Varon, 2007), and around one third of the patients still remain hypertensive after 10 days (Varon and Marik, 2000). The mechanisms underlying hypertension after a cerebral accident have not been fully elucidated, but activation of sympathetic nervous system may be involved. Thus, it has been reported that contractile responses to noradrenaline are diminished in rat vas deferens (Boselli et al., 2002), an organ that is innervated by the sympathetic nervous system.

Resistance arteries, roughly having an internal diameter <300 μm, are important regulators of vasomotor tone and...
hence blood pressure. Disturbances of peripheral vascular resistance are basic contributors to different cardiovascular pathologies (Vila and Salacces, 2005; Wang et al., 2007). Therefore, alterations of the vascular tone of resistance arteries may play an important role in the pathogenesis and progression of cardiovascular diseases. The tone of resistance arteries depends on a complex interplay between endothelial and smooth muscle cells. Mesenteric resistance arteries are innervated by the sympathetic nervous system, and the contraction is mediated by noradrenaline acting on α1-adrenoceptors present in the smooth muscle cells. Endothelial cells contribute to the regulation of vascular tone, releasing several vasoactive factors (Vanhoutte, 2003; Villar et al., 2006), and the association between endothelial dysfunction and vascular disease is also well established (Trigg et al., 2003; Huang and Vita, 2006).

The aim of our study was to evaluate whether focal cerebral ischemia could influence the vascular responses on a remote site as in MRA that have an important role in blood pressure regulation.

Materials and Methods

Animals. Thirteen- to 15-week-old male Wistar Kyoto (n = 30) rats (Janvier, Le Genest Saint Isle, France) were used. Rats were housed under a 12-h day/night cycle, and they had free access to food and water before and after surgery. The investigation conforms to the Institute of Laboratory Animal Resources (1996).

Surgical Procedures and Measurement of Infarct Volume. Focal brain ischemia was produced by 90-min intraluminal occlusion of the right middle cerebral artery (MCA) with reperfusion (24 h) as reported previously (Jiménez-Altayó et al., 2007). Sham-operated animals were used as controls. Blood samples were obtained at 24 h after surgery. The investigation conforms to the instructions of the manufacturers.

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Plasma Levels of IL-6 and IL-1β. IL-6 and IL-1β content (picograms per milliliter) was evaluated in plasma with rat enzyme-linked immunosorbent assay kits (IL-6: Pierce Endogen, Rockford, IL; and IL-1β: BioSource, Invitrogen, Barcelona, Spain), following the instructions of the manufacturers.

Tissue Preparation. Segments of third-order branches [vasculature reactivity, supraoxide anion (O2−) production, and immunofluorescence studies] and second- and third-order branches (Western blot studies) of the mesenteric artery were dissected free of fat and connective tissue, and they were maintained in KHS. Vessels to be used for O2− production were placed in KHS containing 30% sucrose overnight. Next, they were transferred to a cryomold (Bayer Química, Heidelberg, Germany), using the same imaging settings in each case. Parallel sections were incubated with polyethylene glycol SOD (PEG-SOD; 500 U/ml). Quantitative analyses of O2− production was performed with MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA). Four areas per ring were sampled for each experimental condition. The integrated optical densities in the target region were calculated. All measurements were conducted blind.

Western Blot. Proteins from homogenized MRA [40 μg for eNOS, iNOS, and COX-2 and 15 μg for copper/zinc SOD (Cu/Zn SOD), manganese SOD (Mn SOD), and extracellular (EC) SOD] were electrophoretically separated by 7% (eNOS, iNOS, and COX-2) or 12% (Mn SOD, Cu/Zn SOD, and EC SOD) SDS-polyacrylamide gel electrophoresis (10% acrylamide, 4 mm H2O2). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgG; 1:2000; BD Biosciences). Immunocomplexes were detected by chemiluminescence (ECL, Amersham). Blotted membranes were washed with 3 changes of phosphate-buffered saline solution (PBS; pH 7.4). After clearing, arterial segments were placed in PBS containing 30% sucrose overnight, transferred to a cryomold containing Tissue-Tek OCT embedding medium, and frozen in liquid nitrogen. Tissues were kept at −70°C until the day of the experiments. Second- and third-order branches were frozen in liquid nitrogen, and they were kept at −70°C until the day of the protein expression assay.

Reactivity Experiments. Vascular function was studied in vessels mounted on an isometric wire myograph (model 410 A; J.P. Tracor, Aarhus, Denmark) filled with KHS that was kept at 37°C and pH 7.4. After clearing, arterial segments were placed in PBS containing 30% sucrose overnight, transferred to a cryomold containing Tissue-Tek OCT embedding medium, and frozen in liquid nitrogen. Tissues were kept at −70°C until the day of the experiments. Second- and third-order branches were frozen in liquid nitrogen, and they were kept at −70°C until the day of the protein expression assay.

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were detected using an enhanced horseradish peroxidase/streptavidin chemiluminescence system (ECL Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and film exposure (Hyperfilm ECL; GE Healthcare). The signal intensity of the bands on the film was quantified using a GS700 and Molecular Analyst 1.5 software (Bio-Rad, Hercules, CA). The same membrane was used to determine β-actin expression, using a monoclonal antibody anti-β-actin (1:5,000; Sigma-Aldrich) as a gel loading control. Data are expressed as the ratio between signals on the immunoblot corresponding to eNOS, COX-2, Mn SOD, Cu/Zn SOD, EC-SOD, and β-actin.

**Immunofluorescence.** Frozen transverse sections (14 µm) were cut onto gelatin-coated slides, and then they were air-dried for at least 60 min. After blockade, sections were incubated with a mouse monoclonal antibody against eNOS (1:100; BD Biosciences) and polyclonal antibodies against COX-2 (1:100; Cayman Chemical) and nitrotyrosine (1:50; Millipore, Billerica, MA) in PBS containing 2% bovine serum albumin for 1 h at 37°C in a humidified chamber. After washing, rings were incubated with the secondary antibodies, a donkey anti-mouse (eNOS, 1:200; iNOS, 1:50) or anti-rabbit (COX-2, nitrotyrosine, 1:200) IgG conjugated to cyanine 3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at 37°C in a humid box. After washing, immunofluorescent signals were viewed using an inverted Leica TCS SP2 confocal laser scanning microscope with oil immersion lens (63×/H11003) using an inverted Leica TCS SP2 confocal laser scanning microscope. Immunofluorescence signals were detected using an enhanced horseradish peroxidase/luminol reagent. The contractile responses to phenylephrine (0.1–100 µM) were similar in vessels from both groups (sham: pEC50 = 5.49 ± 0.08, n = 7; ischemic: pEC50 = 5.54 ± 0.1, n = 8). KCl (100 mM)-induced contraction was also similar between ischemic (2.8 ± 0.2 mN/mm; n = 8) and sham-operated (3.06 ± 0.4 mN/mm; n = 7) rats.

**Superoxide Anion Production.** Weak ethidium fluorescence was observed in media and endothelial layer of MRA from sham-operated rats (Fig. 3A). After MCA occlusion followed by reperfusion, the arteries showed a fluorescence signal prominent in all three layers of the vessel wall (Fig. 3B). Quantification of the ethidium bromide fluorescence showed a significant increase in vessels from ischemic rats reflecting an increase in O2− production (Fig. 3E). The permeable O2− scavenger PEG-SOD (500 U/ml) significantly reduced the observed fluorescence (Fig. 3E) in vessels from both sham (Fig. 3C) and ischemic (Fig. 3D) rats.

**Protein Expression.** COX-2 protein expression was enhanced (p < 0.05) in vessels from ischemic rats compared with sham-operated animals (Fig. 4A). In contrast, eNOS (sham: 0.45 ± 0.07, n = 6; ischemic: 0.82 ± 0.27, n = 6), Mn SOD (sham: 1.06 ± 0.24, n = 7; ischemic: 1.14 ± 0.27, n = 6), Cu/Zn SOD (sham: 0.9 ± 0.25, n = 7; ischemic: 1.14 ± 0.27, n = 6), and EC SOD (sham: 0.25 ± 0.07, n = 5; ischemic: 0.16 ± 0.08, n = 4) protein expression was not modified by ischemia/reperfusion. In addition, iNOS protein expression was not detected in MRA from either sham or ischemic rats (data not shown).

**Immunofluorescence.** COX-2 protein expression was not detected in vessels from sham-operated animals, whereas COX-2 was clearly induced in all three layers (Fig. 4B) of the vessel wall after ischemia/reperfusion. The expression of eNOS was detected only in endothelial cells as expected (data not shown).

**Reactivity Experiments.** The influence of ischemia on endothelium-dependent and -independent vasodilatation is shown in Fig. 1. ACh (0.1 nM–10 µM; Fig. 1A) and SNP (1 nM–100 µM; Fig. 1B) relaxed the MRA precontracted with phenylephrine in a concentration-related manner. MCA occlusion followed by reperfusion decreased the vasodilator responses to ACh (Fig. 1A) without modifying the relaxation by SNP (Fig. 1B). After incubation with the COX inhibitor indomethacin (10 µM) or with the O2− scavenger SOD (150 U/ml) vasodilator responses to ACh were enhanced in MRA from ischemic rats (Fig. 2B), but they were not significantly affected in MRA from sham-operated animals (Fig. 2A). The nonselective nitric-oxide synthase inhibitor L-NAME (100 mM) inhibited to a similar extent the ACh responses in MRA from ischemic (Fig. 2B) and sham-operated animals (Fig. 2A).

**Results**

Table 1 shows the body weight, the infarct volume, and the cytokine plasma levels. Body weight was not affected by ischemia/reperfusion. Rats subjected to MCA occlusion followed by reperfusion showed a brain infarct, whereas sham-operated animals did not. After 24-h reperfusion plasma levels of IL-6 were significantly elevated (p < 0.01) compared with sham-operated animals. However, IL-1β levels were greater in plasma from ischemic rats, but the increase did not reach statistical significance.

**TABLE 1**

Body weight, infarct volume, and plasma levels of IL-1β and IL-6 in sham-operated and ischemic rats

<table>
<thead>
<tr>
<th></th>
<th>Body Wt.</th>
<th>Infarct Volume</th>
<th>IL-1β</th>
<th>IL-6</th>
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<tr>
<td></td>
<td>g</td>
<td>mm³</td>
<td>pg/ml</td>
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<tr>
<td>Sham</td>
<td>398 ± 8.5 (14)</td>
<td>63.1 ± 10.2 (16)</td>
<td>14.7 ± 2.36 (4)</td>
<td>74.1 ± 20 (4)</td>
</tr>
<tr>
<td>Ischemic</td>
<td>390 ± 9.5 (16)</td>
<td>29 ± 7.5 (5)</td>
<td>193.4 ± 24 (5)* *</td>
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**Statistics.** Results are expressed as mean ± S.E.M. of the number (n) of rats indicated in the figure legends. The dependence of relaxation response on concentration and treatment was assessed by a two-way (concentration, treatment) analysis of variance (ANOVA) with repeated measures on the concentration factor. For infarct volume, IL-6 and IL-1β content, O2− measurement, nitrotyrosine fluorescence, and Western blot, the differences between treatments were analyzed by unpaired Student’s t test. A value of p < 0.05 was considered significant.
NO can react with $O_2^-$, leading to peroxynitrite (ONOO$^-$), which can induce protein nitrosylation (Radi, 2004). Weak or no fluorescence for nitrotyrosine was found on MRA from sham-operated animals (Fig. 5A). However, a marked increase in nitrotyrosine immunofluorescence in the media and endothelial layer was observed after ischemia/reperfusion (Fig. 5A). Quantification of the fluorescent signal showed a significant increase in vessels from ischemic rats (Fig. 5B), indicating the formation of ONOO$^-$ after ischemia/reperfusion.

The negative control obtained with the secondary antibodies did not display fluorescence (data not shown).

**Discussion**

The present study shows that MCA occlusion followed by reperfusion induced endothelial dysfunction in MRA. This peripheral vascular dysfunction is accompanied by an increase in the formation of $O_2^-$ and by an up-regulation of the COX-2 protein expression and protein tyrosine nitrosylation in MRA vessel wall. The vasodilatation disturbance in peripheral resistance could be ascribed, at least in part, to a systemic increase of IL-6 seen after ischemia/reperfusion. To our knowledge, this is the first study in which endothelial dysfunction after MCA occlusion followed by reperfusion is observed in vessels outside the brain.

Vasodilatations to ACh but not to SNP were impaired in vessels from ischemic rats, indicating an endothelium-dependent impairment. The observed endothelial dysfunction could be a consequence of a decrease in the synthesis, activity, or bioavailability of NO. Increases on iNOS expression occur in the brain after stroke (Park et al., 2006; Wang et al., 2007). Nevertheless, we did not find significant changes in either iNOS or eNOS protein expression after ischemia/reperfusion, excluding, although indirectly, alterations in the synthesis of NO. In addition, L-NAME inhibited to a similar extent vasodilator responses to ACh in vessels from sham-operated and ischemic rats. These results further support that the observed endothelial dysfunction was NO-independent. Endogenous and exogenous NO produces vasodilatation, by stimulating soluble gua-
nyl cyclase, which enhances cGMP levels in vascular smooth muscle. The observation that the vasodilatation induced by the NO-releasing agent SNP was unaffected by ischemia/reperfusion also excludes a disturbance on the activity of NO as responsible for the observed endothelial dysfunction.

Ischemia/reperfusion is associated with increases in reactive oxygen species, and $\text{O}_2^-$ is recognized as the major oxidant causing direct injury to ischemic brain (Wang et al., 2007). It is well established that oxidative stress play a major role in the pathogenesis of vascular dysfunction observed in vascular diseases (Cai et al., 2003; Wassmann and Nickenig, 2003). The newly formed $\text{O}_2^-$ can interact with NO, forming ONOO$^-$, reducing the bioavailability of endothelium-derived NO and thus leading to vascular dysfunction (Féletou and Vanhoutte, 2006). Moreover, ischemia/reperfusion also induced protein tyrosine nitrosylation, which is indicative of ONOO$^-$ formation. Our results also show that MRA from ischemic rats present an enhanced formation of $\text{O}_2^-$ that is absent in vessels from sham-operated animals. The fact that exogenous SOD reverted the observed impairment of the ACh vasodilatation induced by ischemia/reperfusion further supports the participation of $\text{O}_2^-$ on the observed vascular dysfunction after ischemia/reperfusion. The observed increase in $\text{O}_2^-$ could be a consequence of a decrease of endogenous SOD induced by ischemia/reperfusion. However, in our hands the protein expression of either the cytosolic Cu/Zn SOD and the Mn SOD or the EC SOD was similar in vessels from sham-operated and ischemic rats, indicating that dysfunctions of endogenous SOD are not responsible for the observed increase in $\text{O}_2^-$.

Fig. 3. Representative fluorescence photomicrographs (A–D) and quantification (E) of confocal microscopic sections of rat mesenteric resistance arteries from sham-operated and ischemic rats in the absence (A and B) or in the presence (C and D) of PEG-SOD (500 U/ml). Vessels were labeled with the oxidative dye hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide by superoxide anion. ADV, adventitial layer; END, endothelial layer; MED, media layer; IEL, internal elastic lamina; n = 5 to 6. Image size, 256 × 256 μm. ***p < 0.001 versus ischemic; ###p < 0.01 versus PEG-SOD-nonincubated vessels by Student’s t test.

Fig. 4. A, representative blot and densitometric analysis of the inducible isoform of COX-2 protein; n = 6. B, representative photomicrographs of COX-2 immunofluorescence in mesenteric resistance arteries from sham and ischemic rats; n = 4. Image size, 256 × 256 μm. *p < 0.05 versus ischemic by unpaired Student's t test.

COX-2 (Kontos et al., 1985) and iNOS (Xia et al., 1998), among others, have been reported as sources of superoxide anion formation. In cerebral ischemia, COX-2, a rate-limiting enzyme for prostanoid synthesis, was up-regulated not only in regions of ischemic injury but also in regions remote from the infarcted area (Sairanen et al., 1998). In contrast, Nagawa et al. (1998) found COX-2-positive neurons and iNOS-positive neutrophils in the ischemic territory but only COX-2-immunoreactive neurons were observed in regions remote from the infarct. In our study, only COX-2 was found expressed in MRA from ischemic rats. These results suggest that COX-2, but not iNOS, participates in the formation of $\text{O}_2^-$ observed after ischemia/reperfusion. This view is further supported by the finding that indomethacin improved ACh-mediated vasodilatation in ischemic animals.

It is known that cytokines are rapidly up-regulated in response to injury, infection, and inflammation (Lucas et al.,
After cerebral ischemia, there is an inflammatory response in which several pro- and anti-inflammatory cytokines are up-regulated (Allan and Stock, 2004; Chamorro, 2004; Huang and Vita, 2006; Huang et al., 2006; Wang et al., 2007). In clinical and experimental studies, discrepancies have been reported on the time course of induction of inflammatory cytokine protein and mRNA expression (Allan and Stock, 2004; Huang et al., 2006) and on the increase in cytokine plasma levels (Emsley et al., 2003; Smith et al., 2004; Offner et al., 2006). We found that IL-6 but not IL-1β plasma levels were significantly increased at 24 h after ischemia/reperfusion. These results confirm the previous observation in mice that IL-6 plasma levels were increased, but IL-1β plasma levels were unchanged after 6 and 24 h after MCA occlusion (Offner et al., 2006). Several studies have demonstrated a link between cytokines and endothelial dysfunction. Thus, disturbances on NO relaxation or phenylephrine contraction were observed in rat MRA after injection of LPS (Virdis et al., 2005) that increases plasma levels of several cytokines, or after 14-h incubation (Briones et al., 2005b; Jiménez-Altayó et al., 2006) with IL-1β. In addition, vascular disturbances after short-term incubation (Orshol and Khalil, 2004a) or infusion (Orshol and Khalil, 2004b) with IL-6 were induced in the aorta of either pregnant or hypertensive rats. In addition, in vivo administration of IL-6 to wild-type mice induced the formation of O₂⁻ and a marked impairment of endothelial-dependent relaxation to carbachol, whereas the vasoconstrctor to phenylephrine or KCl was not affected (Wassmann et al., 2004). These results are in good agreement with ours in which increases of O₂⁻ and endothelial dysfunction were observed, but KCl- and phenylephrine-mediated vasoconstriction was unaffected.

Although most of the patients after acute stroke present an increase in blood pressure that returns to normal levels in a few days after the onset, one third become hypertensive (Varon and Marik, 2000). Among the mechanisms underlying hypertension after a cerebral accident, activation of sympathetic nervous system but also other disturbances on the mechanisms that control vascular tone may be involved. MCA occlusion-induced (Boselli et al., 2002) and photochemically induced (Boselli et al., 2007) focal cerebral ischemia were reported to markedly impair responses to exogenous noradrenalin in the rat vas deferens, an organ richly innervated by sympathetic nervous system. MRA is innervated by the sympathetic nervous system and their contractile response is mainly due to noradrenalin acting on α₁-adrenoceptors as in vas deferens. In contrast with the results observed in rat vas deferens, responses to the α₁-adrenoceptor agonist phenylephrine in MRA were unaffected by ischemia/reperfusion, suggesting that postjunctional noradrenergic responses are not affected. Because responses to electrical field stimulation were not tested in our study, we cannot exclude changes on the release of endogenous noradrenalin after ischemia/reperfusion.

Based on all these findings, we hypothesize that MCA occlusion followed by reperfusion induces an inflammatory response accompanied by an increase of systemic cytokines that, in turn, could produce an excess of O₂⁻ partly due to COX-2. Thus, an increase in NO breakdown induced by O₂⁻ in peripheral vessels could be relevant for the blood pressure disturbances observed after stroke.

Acknowledgments

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References


Fig. 5. Representative photomicrographs (A) and quantification (B) of nitrotyrosine immunofluorescence of confocal microscopic sections of mesenteric resistance arteries from sham-operated and ischemic rats; n = 4. Image size, 256 × 256 μm. *, p < 0.05 sham versus ischemic vessels by Student’s t test.


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