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-β 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 vasodilatations 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, N•−-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.

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ABBREVIATIONS: MRA, mesenteric resistance artery(ies); MCA, middle cerebral artery; IL, interleukin; KHS, Krebs-Henseleit solution; PBS, phosphate-buffered saline; ACh, acetylcholine; SNP, sodium nitroprusside; l-NAME, N•−-nitro-L-arginine methyl ester; COX, cyclooxygenase; SOD, superoxide dismutase; PEG, polyethylene glycol; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; EC, extracellular; ANOVA, analysis of variance; ONOO−, peroxynitrite; ADV, adventitial layer; END, endothelial layer; MED, media layer; IEL, internal elastic lamina.
hence blood pressure. Disturbances of peripheral vascular resistance are basic contributors to different cardiovascular pathologies (Vila and Salaices, 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 (Triggle 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 for measurement of the content of interleukin (IL)-6 and IL-1β in plasma. Rats were then killed, and the brain and the mesenteric arcade were removed and placed in ice-cold Krebs-Henseleit solution (KHS; 112.0 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl2, 1.1 mmol/l KH2PO4, 1.2 mmol/l MgSO4, 25.0 mmol/l NaHCO3, and 11.1 mmol/l glucose) that was maintained at 4°C and continuously gassed with 95% O2 and 5% CO2. Afterward, the brain was sliced in 2-mm-thick sections that were stained with 2% 3,3',5-triphenyltetrazolium chloride for 10 min at 37°C followed by an overnight fixation with 4% paraformaldehyde. The infarcted and noninfarcted tissue was outlined with an image analysis system (Scion Image version 4.02; Scion Corporation, Frederick, MD), and the volume of infarction (corrected for edema) was calculated by integration of the lesion areas at the seven measured levels of the brain.

**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 [vascular reactivity, superoxide 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 Quimica Farmacéutica, Barcelona, Spain) containing Tissue-Tek OCT embedding medium (Sakura Finetek Europe, Zoeterwoude, The Netherlands) for 20 min, and then they were immediately frozen in liquid nitrogen for storage at −70°C until O2- measurement. For immunofluorescence studies, arterial segments were fixed with 4% phosphate-buffered paraformaldehyde, pH 7.4, for 1 h, and then they were washed in three 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. Trautwein, Aarhus, Denmark) filled with KHS that was kept at 37°C and following the protocol described previously (Briones et al., 2005a,b).

Endothelium-dependent and -independent vasodilatations were studied by evaluating the relaxation induced by Ach (0.1 nM–10 μM) and sodium nitroprusside (SNP; 1 nM–100 μM) performed in 10 μM phenylephrine-precontracted vessels from sham-operated and ischemic rats. The effect of the nonselective nitric-oxide synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME; 100 μM), the nonselective cyclooxygenase (COX) inhibitor indomethacin (10 μM), and the O2- scavenger superoxide dismutase (SOD; 150 U/ml) on Ach-induced vasodilatation was investigated by its addition 30 min before contraction by 10 μM phenylephrine in vessels from both groups of rats.

To investigate the influence of ischemia/reperfusion on contractile responses mediated by α1-adrenoceptor stimulation, concentration-response curves to phenylephrine (0.1–100 μM) were performed.

Vasoconstrictor responses were expressed as a percentage of the previous tone generated by phenylephrine. Vasoconstrictor responses were expressed as a percentage of the tone generated by 100 mM KCl. Location parameters for phenylephrine were estimated as pEC50 (the negative logarithm of the concentration required to cause 50% of the maximum response).

**Measurement of O2- Production.** The oxidative fluorescent dye dihydroethidium was used to evaluate production of O2- in situ, as described previously (Hernanz et al., 2004; Jiménez-Altayó et al., 2006). In brief, frozen tissue segments were cut into 14-μm-thick sections, and they were placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (130 mM NaCl, 5.6 mM KCl, 2 mM CaCl2, 0.24 mM MgCl2, 8.3 mM HEPES 8.3, and 11 mM glucose, pH 7.4). Fresh buffer containing 2 μM dihydroethidium (excitation, 488 nm; emission, 610 nm) was applied topically onto each tissue section, and then they were coverslipped and incubated for 30 min in a light-protected humidified chamber at 37°C. Sections were viewed by fluorescent laser scanning confocal microscope (63×; Leica TCS SP2; Leica, Heidelberg, Germany), using the same imaging settings in each case. Parallel sections were incubated with polyclonal antibodies against eNOS, iNOS, and COX-2 and 15-sodiumpyruvate membranes overnight. Western immunoblot 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, and then they were transferred to polyvinylidene difluoride membranes overnight. Western immunoblot was performed with monoclonal antibodies against eNOS (1:2000; BD Biosciences, Franklin Lakes, NJ) and iNOS (1:1500; BD Biosciences Transduction Laboratories, Lexington, KY) and polyclonal antibodies against COX-2 (1:500; Cayman Chemical, Ann Arbor, MI), Mn SOD (1:20,000; Nventa Biopharmaceuticals, San Diego, CA), Cu/Zn SOD (1:10,000; Nventa Biopharmaceuticals), and EC SOD (1:1000; Nventa Biopharmaceuticals). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgG; 1:2000; BD Biosciences). Immunocomplexes
were detected using an enhanced horseradish peroxidase/feminol 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 (1:50,000; Sigma-Aldrich) as a gel loading control. Data are expressed as the ratio between signals on the immunoblot corresponding to the primary antibody and processed as described above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

Quantitative analysis of nitrotyrosine fluorescence was performed with MetaMorph image analysis software (Molecular Devices). 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.

**Drugs and Solutions.** Drugs used were acetylcholine chloride, dihydroethidium, polyethylene glycol superoxide dismutase, sodium nitroprusside, and superoxide dismutase (Sigma-Aldrich). Assays included acetylcholine chloride, nitroprusside, and superoxide dismutase (Sigma-Aldrich, Madrid, Spain) as a gel loading control. Data are expressed as the ratio between signals on the immunoblot corresponding to the primary antibody and processed as described above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

**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.

**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.

**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).

**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 = 5; 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).

**Table 1**

<table>
<thead>
<tr>
<th>Body Wt.</th>
<th>Infarct Volume</th>
<th>IL-1β</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>mm³</td>
<td>pg/ml</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>398 ± 8.5 (14)</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>63.1 ± 10.2 (16)</td>
<td>29 ± 7.5 (5)</td>
</tr>
</tbody>
</table>

**Notes:**

- **p < 0.01 vs. ischemic by unpaired Student’s t test.**
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 displayed 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 O$_2^-$ is recognized as the major oxidant caus- ing 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 O$_2^-$ can interact with NO, forming ONOO$^-$, reducing the bioavailability of endothelium-derived NO and thus leading to vascular dysfunction (Félétou 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 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 O$_2^-$ on the observed vascular dysfunction after ischemia/reperfusion. The observed increase in 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 O$_2^-$.

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, Nogawa 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 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.,

![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 $\times$ 256 $\mu$m. *** $p$ < 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 isofrom 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 $\times$ 256 $\mu$m. * $p$ < 0.05 versus ischemic by unpaired Student’s $t$ test.]

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 (Emsl ey 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-Altabo et al., 2006) with IL-1β. In addition, vascular disturbances after short-term incubation (Orshal and Khalil, 2004a) or infusion (Orshal 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 vasoconstriction 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 noradrenaline 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 noradrenaline 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 noradrenerg ic 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 noradrenaline 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.

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References

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