Participation of Oxidative Stress on Rat Middle Cerebral Artery Changes Induced by Focal Cerebral Ischemia: Beneficial Effects of 3,4-Dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2H)-benzopyran (CR-6)

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ABSTRACT

Cerebral ischemia followed by reperfusion alters vessel properties of brain arteries in rats, inducing an inflammatory response and excessive generation of reactive oxygen species. This study investigated the participation of oxidative stress on vessel properties after ischemia/reperfusion and the beneficial effects of 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2H)-benzopyran (CR-6). The right middle cerebral artery was occluded (90 min) and reperfused (24 h). Sham-operated animals were used as controls. Ischemic rats were treated either with CR-6 (100 mg/kg in 1 ml olive oil) or vehicle (1 ml olive oil) administered orally at 2 and 8 h after the onset of ischemia. The structural, mechanical, and myogenic properties of the middle cerebral artery (MCA) were assessed by pressure myography. Superoxide anion (O$_2^-$) production was evaluated by ethidium fluorescence, and protein tyrosine nitrosylation was determined by immunofluorescence. Infarct volume was smaller in rats treated with CR-6. In MCA, ischemia/reperfusion increased wall thickness, cross-sectional area, wall/lumen, and decreased wall stress. CR-6 treatment prevented all of these changes induced by ischemia/reperfusion. However, impaired myogenic response and larger lumen diameter in active conditions observed after ischemia/reperfusion were not modified by CR-6. Treatment with CR-6 prevented the increase in O$_2^-$ production and partially prevented the enhanced protein tyrosine nitrosylation that occurred in response to ischemia/reperfusion. Our findings suggest that oxidative stress is involved in the alterations of MCA properties observed after ischemia/reperfusion and that CR-6 induces protection.

Stroke is a complex neurodegenerative disorder characterized by interruption of blood flow supply to the brain. There are several mechanisms involved in ischemic brain injury. Increasing evidence indicates that cerebral ischemia and particularly reperfusion are responsible for oxidative stress caused by free radical production. After reperfusion, there is a surge in production of superoxide anion (O$_2^-$), nitric oxide (NO), and peroxynitrite (ONOO$^-$. Formation of these species in the vicinity of blood vessels plays an important role in reperfusion-induced injury (Doyle et al., 2008). In healthy individuals, antioxidant activity counterbalances free radical production, but in the case of ischemia, the balance between reactive oxygen species (ROS) and antioxidant activity is shifted toward free radicals, causing oxidative stress.

All vascular cells can produce ROS through different sources, including NADPH oxidase, mitochondria, cyclooxygenases, and...
uncoupled nitric-oxide synthase, among others (Lyle and Griendling, 2006; Chrissibolos and Faraci, 2008; Lee and Griendling, 2008), which then could participate in the pathogenesis of cardiovascular diseases. Changes in either vascular remodeling or myogenic tone have been reported in several animal models of vascular diseases such as diabetes (Sachidanandam et al., 2009), hypertension (Touyz, 2000), and cerebral ischemia (Cipolla et al., 1997; Jiménez-Altayo et al., 2007). Furthermore, vascular remodeling is improved by treatment with antioxidants, vitamins, or NADPH oxidase inhibitors in animal experimental models and in clinical trials of hypertension (Lee and Griendling, 2008).

Deficiency in some superoxide dismutase (SOD) isoforms results in increased levels of vascular $\text{O}_2^-$ and ONOO$^-$, as well as impaired endothelium-dependent relaxation and hypertension (Didion et al., 2002; Baumbach et al., 2006; Brown et al., 2007). ATP-sensitive K channels (KATP) limit myogenic depolarization and control myogenic reactivity, and voltage-dependent K channels contribute to resting vascular tone (Teramoto, 2006). Potassium channels have different sensitivity to oxidative stress, depending on the oxidant species and the types of channels. It has also been reported that superoxide anion inhibits the opening of KATP (Lee and Griendling, 2008). Thus, the formation of ROS could also participate in the myogenic response changes of the middle cerebral artery (MCA) induced by ischemia/reperfusion (I/R) (Cipolla and Curry, 2002; Jiménez-Altayo et al., 2007).

We designed experiments to study the participation of oxidative stress on the structural, mechanical, and myogenic alterations in the rat MCA induced by I/R. We also tested the beneficial effects of CR-6, a structurally simple derivative of vitamin E (Casas et al., 1992), with capacity to scavenge oxygen and nitrogen reactive species. CR-6 has previously shown some beneficial effects against oxidative stress in vivo (Miranda et al., 2007) and in vitro (Montoliu et al., 1999; Sanvicens et al., 2006).

Materials and Methods

Animals. Thirty male Sprague-Dawley rats (300–325 g body weight) obtained from Harlan (Barcelona, Spain) were housed under a 12-h light/dark cycle and provided with access to food and water ad libitum. All of the experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (NIH Publications 85-23, revised 1996) and to the Guidelines established by the Spanish legislation (RD 1201/2005). Experiments were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and the Universitat de Barcelona.

Experimental Cerebral Ischemia and Infarct Volume. Focal cerebral ischemia and the surgical protocol were similar to the methods described previously (Jiménez-Altayo et al., 2007). Rats were anesthetized with isoflurane (2–2.5%) vaporized in O2 and N2O (30:70). Afterward focal cerebral ischemia was produced by 90-min intraluminal occlusion of the right MCA with reperfusion (24 h). Sham-operated animals were also carried out by performing the full surgical procedure and by introducing the filament through the MCA for less than 1 min.

Sham-operated and ischemic rats were euthanized under deep isoflurane anesthesia, and the brain was removed and placed in cold Krebs-Henseleit solution (KHS) of the following composition: 112.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.1 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25.0 mM NaHCO$_3$, and 11.1 mM glucose, maintained at 4°C, and continuously gassed with 95% O$_2$ and 5% CO$_2$. The MCA from the right hemisphere (ipsilateral to ischemia) was dissected under a surgical microscope and kept in cold KHS. The brain was then sliced in 2-mm-thick coronal sections that were stained with 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) in saline solution for 10 min at 37°C. Sections were then immersed overnight in a 4% paraformaldehyde in phosphate buffer and washed in this buffer. Infarct volume was measured with an image-analysis system (Scion Image, Scion Corporation, Frederick, MD). The brain sections were digitized adjacent to a millimeter scale. The scale of the image was set in the image analysis program according to this scale before area measurements. The area was manually selected, and the infarcted zone was taken as the pale area in the image. The area of infarction (corrected for edema) in each section was calculated by multiplying the infarct area by the ratio of the contralateral area to the ipsilateral area. Areas were then integrated to calculate infarct volume. The infarct volume then was calculated by integration of the lesion areas at the seven measured levels of the brain.

Treatment. Ischemic rats were treated either with CR-6 (100 mg/kg in 1 ml of olive oil) or vehicle (1 ml of olive oil) administered orally at 2 and 8 h after the onset of ischemia. CR-6 was chemically synthesized as reported previously (Casas et al., 1992). The dose used was previously shown to protect against oxidative stress damage (Miranda et al., 2007).

Tissue Preparation. MCA from some animals was immediately used for pressure myography studies. Segments used for evaluation of superoxide anion ($\text{O}_2^-$) production were placed in KHS containing 30% sucrose overnight and transferred to a cryomold (Bayer Química Farmacéutica, Barcelona, Spain) containing Tissue Tek OCT embedment medium (Sakura Finetek Europe, Zoeterwoude, The Netherlands) for 20 min and then immediately frozen in liquid nitrogen for storage at −70°C until $\text{O}_2^-$ evaluation. For immunofluorescence studies, arteries were fixed with 4% phosphate-buffered paraformaldehyde (pH 7.4) for 1 h and washed in three changes of phosphate-buffered saline solution (pH 7.4). After clearing, arterial segments were placed in phosphate-buffered saline 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 immunofluorescence experiments.

Pressure Myography. The structural, mechanical, and myogenic properties of MCA were studied with a pressure myograph (Danish Myo Tech Model P100; J.P. Trading I/S, Aarhus, Denmark), as described previously (Jiménez-Altayo et al., 2007). In brief, the vessel was placed on two glass microcanulæ and secured with surgical nylon suture. After the small branches were tied off, vessel length was adjusted so that the vessel walls were parallel without stretch. Intraluminal pressure was then raised to 140 mm Hg, and the artery was un buckled by adjusting the cannulae. Afterward the artery was left to equilibrate (60 min) at 70 mm Hg in gassed KHS at 37°C. Intraluminal pressure was reduced to 3 mm Hg, and a pressure-diameter curve (3–120 mm Hg) was obtained in gassed KHB (37°C). Internal and external diameters (D$_{I\text{Ca}}$, D$_{E\text{Ca}}$) were measured for 3 min at each intraluminal pressure. The artery was then set to 70 mm Hg and allowed to equilibrate for 30 min at 37°C in gassed calcium-free KHS (0 Ca$^{2+}$: omitting calcium and adding 10 mM EGTA; Sigma-Aldrich). A second pressure-diameter curve was obtained in 0 Ca$^{2+}$-KHS, and D$_{I\text{Ca}}$ and D$_{E\text{Ca}}$ were measured.

Myogenic response percentages at each pressure were determined from 100 × D$_{I\text{Ca}}$/D$_{E\text{Ca}}$ where D$_{I\text{Ca}}$ and D$_{E\text{Ca}}$ are the internal diameters measured in active (2.5 mM Ca$^{2+}$-KHS) and passive conditions (0 Ca$^{2+}$-KHS), respectively. Slopes of the myogenic response-pressure curves for individual vessels were determined by linear regression.

Wall thickness (WT), cross-sectional area (CSA), and wall/lumen ratio (W/L) were calculated as follows: WT = (D$_{E\text{Ca}}$ − D$_{I\text{Ca}}$)/2; CSA = $(\pi r^4)/[2(D_{I\text{Ca}})^2 − (D_{I\text{Ca}})^2]$; and W/L = (D$_{E\text{Ca}}$ − D$_{I\text{Ca}}$)/2D$_{E\text{Ca}}$ where D$_{E\text{Ca}}$ is the external diameter observed for a given intravascular pressure in passive conditions.

Mechanical parameters were calculated as described by Baumbach and Heistad (1989). Circumferential wall strain ($\varepsilon$) was calculated as
\((D_{\text{OCA}} - D_{\text{OCA}})/D_{\text{OCA}}\) where \(D_{\text{OCA}}\) is the internal diameter at 3 mm Hg, measured under relaxed conditions. Circumferential wall stress \((\sigma)\) was calculated as \((P \times D_{\text{OCA}})/2WT\), where \(P\) is the intraluminal pressure (1 mm Hg = 133.4 nm \(^{-2}\)) and \(WT\) is wall thickness at each intraluminal pressure in 0 Ca\(^{2+}\) medium. Elastic modulus was calculated by fitting stress-strain data to \(\sigma = \sigma_{\text{orig}} \exp (\varepsilon)\), where \(\sigma_{\text{orig}}\) is the stress at the original diameter (3 mm Hg). The \(\beta\) value was used as an index of wall stiffness (Mulvany and Aalder, 1990).

**Measurement of \(O_2^\bullet\) Production.** The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate production of \(O_2^\bullet\) in situ, as described previously (Jimenez-Altabo et al., 2006; Martinez-Revelles et al., 2008). Frozen MCA were cut into 14-μm-thick sections and placed on a glass slide. Serial slices were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (130 mM NaCl, 5.6 mM KCl, 2 mM CaCl\(_2\), 0.24 mM MgCl\(_2\), 8.3 mM HEPES, 11 mM glucose, pH 7.4). Fresh buffer containing DHE (2 μM) (546-nm excitation and 610-nm emission) was applied topically on each tissue section, overslipped, and incubated for 2 h in a light-protected humidified chamber at 37°C, and then viewed by fluorescent laser scanning confocal microscope (×63; TCS SP2; Leica, Heidelberg, Germany) using the same imaging settings in either sham, vehicle, or CR-6 group. Parallel sections were incubated with polyethylene glycol sod (PEG-SOD; 500 U/ml, 2, 37°C). Fluorescence was detected with a 568-nm long-pass filter. For quantification, integrated optical densities were calculated from four sampled areas per ring for each experimental condition using MetaMorph Image Analysis Software (Molecular Devices, Sunnyvale, CA). The integrated optical densities in the target region were calculated. All measurements were conducted blind.

**Immunofluorescence.** Frozen transverse sections (14 μm) of MCA were processed as described previously (Martinez-Revelles et al., 2008). Sections were incubated with a rabbit primary polyclonal antibody against nitrotyrosine (1:150; Millipore, Billerica, MA). After washing, rings were incubated with the secondary antibody, a donkey anti-rabbit (1:200) IgG conjugated to Cy3 (Jackson Immuno-Research Laboratories Inc., West Grove, PA). After washing, immunofluorescent signals were viewed using an inverted Leica TCS SP2 confocal laser scanning microscope with oil immersion lens (×63). Cy3-labeled antibody was visualized by excitation at 568 nm and detection at 600 to 700 nm.

The specificity of the immunostaining was evaluated by omission of the primary antibody and processed as 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. Images of the natural autofluorescence of elastin were also taken. Autofluorescence was visualized by excitation at 488 nm and detection at 490 to 535 nm.

**Drugs and Solutions.** Drugs used were dihydroethidium, polyethylene glycol superoxide dismutase, EDTA, paraformaldehyde, 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich), and CR-6.

**Statistics.** Results are expressed as mean ± S.E.M. of the number of rats indicated in the figure legends. The dependence of structural, mechanical, or myogenic properties on treatment was assessed using two-way (pressure, treatment) analysis of variance (ANOVA), with repeated measures on the pressure factor. In the case of one single factor, unpaired Student’s \(t\) test (infracut volume) or one-way ANOVA followed by post hoc Tukey’s test (\(O_2^\bullet\) measurement and nitrotyrosine immunofluorescence) was used for two or more than two groups, respectively. Data analysis was carried out with PRISM software (GraphPad Software Inc., San Diego, CA). A value of \(P < 0.05\) was considered significant.

**Results**

**Effect of CR-6 on Infarct Size and Body Weight.** Body weight was measured at 0 and 24 h after I/R. A similar decrease on body weight (\(g\)) was observed at 24 h in sham-operated (333.11 ± 9; 318.1 ± 8.5, \(n = 8\), \(P < 0.001\)), ischemic vehicle (343.8 ± 6.9; 310.9 ± 6.8; \(n = 11\), \(P < 0.0001\)), and ischemic CR-6 (346.5 ± 9.3; 320.3 ± 7.9; \(n = 9\), \(P < 0.0001\)). Figure 1 shows that the infarct volume was significantly attenuated by CR-6.

**Structural, Mechanical, and Myogenic Properties of the MCA.** Arterial diameters were measured over the pressure range from 3 to 120 mm Hg both passively to assess the structural and mechanical properties and actively to assess the changes in myogenic activity. Figure 2 shows the structural changes induced by I/R as well as the influence of CR-6 on those parameters. After I/R, vessel diameter was unaffected (Fig. 2A), and lumen diameter from 60 to 120 mm Hg (Fig. 2B) showed a trend to decrease. Nevertheless, the influence of either ischemia or treatment on lumen diameter analyzed by two-way ANOVA did not reach statistical significance. WT (Fig. 2C), wall/lumen (results not shown), and CSA (Fig. 2D) were increased by I/R, and this effect was prevented by treatment with CR-6.

Analysis of the stress-strain curve (Fig. 3A) showed that stiffness (sham: \(\beta: 9.9 ± 1.0, n = 6\) was not altered by I/R in either ischemic vehicle (\(\beta: 11.8 ± 0.8, n = 7\)) or ischemic CR-6 (\(\beta: 10.6 ± 1.2, n = 7\)-treated rats. However, ischemic vehicle vessels showed a decrease in wall stress (Fig. 3B) that was prevented by treatment with CR-6.

Lumen diameter in active conditions (2.5 mM Ca\(^{2+}\)-KHS) was enlarged from 20 to 120 mm Hg perfusion pressures in ischemic vessels irrespective of the presence or absence of CR-6 (Fig. 4A). Myogenic response as a function of pressure revealed the extent of the constrictor tone. This parameter is calculated by internal diameter reductions in active relative to passive (0 Ca\(^{2+}\)-KHS) conditions (Fig. 4B). In ischemic vehicle vessels, myogenic response was decreased at all of the perfusion pressures tested (Fig. 4B). Treatment with CR-6 did not influence the changes of myogenic response due to I/R (Fig. 4B).

**Effect of CR-6 on \(O_2^\bullet\) Production.** Figure 5 shows the formation of \(O_2^\bullet\) on MCA from sham and ischemic rats. Ischemia-reperfusion induced the formation of \(O_2^\bullet\) that was apparent in the three layers of the vascular wall (Fig. 5, B and...
D). The increase of DHE fluorescent signal induced by I/R was significantly prevented by CR-6 treatment (Fig. 5, C and D). The permeable O$_2^-$ scavenger, PEG-SOD (500 U/ml), dramatically reduced the observed fluorescence in vessels from either ischemic vehicle (Fig. 5, E and D) or ischemic CR-6 (Fig. 5, F and D)-treated rats, confirming the specificity of the fluorescent signal for superoxide anion.

**Effect of CR-6 on Protein Tyrosine Nitrosylation.** NO can react with O$_2^-$ leading to peroxynitrite (ONOO$^-$), which can induce protein tyrosine nitrosylation. Weak or no fluorescence for nitrotyrosine was found on MCA from sham-operated animals (Fig. 6, A and D). Ischemia-reperfusion (Fig. 6, B and D) induced an increase in nitrotyrosine immunofluorescence. After treatment with CR-6, there was a decrease (Fig. 6, C and D) on nitrotyrosine immunofluorescence that did not reach statistical significance. The negative control obtained with the secondary antibodies did not display fluorescence (results not shown), demonstrating specificity of the reaction.
Discussion

When focal cerebral ischemia is induced experimentally in rats, changes occur in whole MCA structure, mechanics, and myogenic behavior as described previously (Cipolla et al., 1997; Cipolla and Curry, 2002; Coulson et al., 2002; Jiménez-Altayó et al., 2007). The present study shows that treatment with CR-6 prevents the increase in wall thickness, cross-sectional area, and wall/lumen relationship and the decrease in wall stress that occurs in response to I/R. We have also shown that in MCA the impairment of myogenic response and the lumen diameter increases in active conditions seen after I/R is similar in ischemic rats treated with either CR-6 or vehicle. Finally, we have dem-

Fig. 4. Influence of CR-6 treatment on myogenic properties after I/R. A, lumen diameter (2.5 mM Ca\(^{2+}\))-intraluminal pressure. B, myogenic response (DiCa\(^{2+}\)/H11001/Di0Ca\(^{2+}\)/H11001)-intraluminal pressure. DiCa\(^{2+}\) and Di0Ca\(^{2+}\), internal diameters measured in active (2.5 mM Ca\(^{2+}\)-Krebs-Henseleit solution) and passive conditions (0 Ca\(^{2+}\)-Krebs-Henseleit solution), respectively. Values are means ± S.E.; n = 6–7; *** P < 0.001 versus sham by two-way ANOVA.

Fig. 5. Representative fluorescence photomicrographs and quantification of confocal microscopic sections of MCA from sham-operated (A), ischemic-vehicle (ISC-VHC; B and E), and ischemic-CR-6-treated (ISC-CR6; C and F) rats in the absence (top) or in the presence (bottom panel) 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 = 5–7. Image size, 256×256 μm. *, P < 0.05; **, P < 0.01 versus sham; +, P < 0.05, ++, P < 0.01 versus PEG-SOD-incubated vessels by one-way ANOVA followed by post hoc Tukey’s test.
onstrated that CR-6 treatment prevents the increase in O$_2^-$ production and partially prevents the enhanced protein nitrotyrosination observed in the MCA after I/R. Therefore, antioxidant treatment with CR-6 prevents MCA structural and mechanical changes but not myogenic changes observed after experimental focal cerebral ischemia. The effect of CR-6 on MCA may contribute to explain its protective action against the ischemic injury.

Abnormal small artery structure is a risk factor for later cardiovascular events (Mulvany, 2008). Thus, structural alterations in cerebral blood vessels may compromise the blood supply to the brain and increase the risk of stroke (Iadecola and Davison, 2008). In the present study, although passive diameters of the MCA from vehicle-ischemic rats were not significantly different from sham-operated control animals, values of lumen diameter in passive conditions were decreased after I/R. As a consequence, wall thickness, cross-sectional area, and wall/lumen were significantly increased. Consistent with these findings, previous studies have observed hypertrophy of the MCA in rat models of focal cerebral ischemia (Cipolla et al., 1997; Jiménez-Altayó et al., 2007). In the present study, antioxidant treatment with CR-6 prevented the hypertrophic response induced by I/R, suggesting a relationship between structural properties and generation of free radical species in the rat MCA. Reactive oxygen species trigger multiple cellular events eliciting specific cellular responses, including growth (Li et al., 1997; Zafari et al., 1998; DeMarco et al., 2008). For instance, hypertrophic response was present in cerebral arterioles of mice deficient in one or both genes encoding Cu,Zn-SOD (Baumbach et al., 2006). Alterations in autoregulation increase the risk of cerebral ischemia when cerebral blood vessels fail to compensate changes in perfusion pressure (Immink et al., 2004). Prevention of hypertrophy by increasing the vascular lumen and decreasing vascular resistance may result in an augmented ability of the MCA to autoregulate (Barry, 1985; Baumbach and Heistad, 1988). Changes in mechanical properties of arteries have been reported after focal cerebral ischemia in rats (Coulson et al., 2002; Dorrance et al., 2006; Jiménez-Altayó et al., 2007). It is commonly accepted that changes in wall stiffness may take part in the cerebral disruption caused by I/R (Coulson et al., 2002; Jiménez-Altayó et al., 2007). An increase in vascular stiffness, indicated by an increase of the $\beta$-coefficient, may exacerbate infarct size. However, in the present study, MCA occlusion followed by reperfusion did not alter vascular wall stiffness, discarding a prominent role of this parameter on the deleterious effects caused by I/R in our experimental conditions. This is in contrast with other studies that reported an increase (Coulson et al., 2002) or decrease (Jiménez-Altayó et al., 2007) in wall stiffness in ischemic compared with sham-operated rats by use of different rat strains (Wistar and Wistar-Kyoto, respectively). An important finding of the present study is that wall stress was diminished after I/R. Hypertrophy is an adaptive response aimed at reducing stress on the vessel wall and protecting downstream vessels from the effect of increased blood pressure (Baumbach and Heistad, 1988). Thus, the observed reduction in wall stress correlates with the observed increase in the cross-sectional area after I/R. Accordingly, a decrease in wall stress and an increase in the cross-sectional area were observed in MCA of normotensive rats after I/R (Jiménez-Altayó et al., 2007). The administration of CR-6 during reperfusion normalized wall stress, suggesting that this property may play an important role as indicator of risk for brain injury. In agreement with this idea, wall stress is altered in cerebral arteries from several models of hypertension (Cipolla et al., 2006; Chan et al., 2008).

A change on arterial function is considered a traditional
cardiovascular risk factor. Myogenic behavior is a vital response that helps to maintain cerebral blood flow and thereby assures tissue perfusion. After I/R, myogenic response of the MCA is compromised (Cipolla and Curry, 2002; Jiménez-Altayó et al., 2007). Consistently, in the present study, a decrease in myogenic response was observed in ischemic rats. Ischemia-reperfusion reflects the interplay of different processes, transient ischemia that produces damage to the brain, and consequent reperfusion that exposes damaged tissue to re-establish blood flow, inducing an inflammatory response and excessive generation of ROS (Rubin and Strayer, 2007; Martinez-Revelles et al., 2008). Peroxynitrite is produced from the interaction of NO and O$_2^-$ and present during focal cerebral ischemia not only in brain tissue (Warner et al., 2004; Romanos et al., 2007) but also in blood vessels (Maneen et al., 2006; Martinez-Revelles et al., 2008). Increasing evidence indicates that ONOO$^-$ can have deleterious effects on cerebral blood vessels (Faraci, 2006; Iadecola and Davison, 2008). For example, exposure to exogenous ONOO$^-$ dilates spontaneous tone, impairs myogenic reactivity in posterior cerebral arteries (Maneen et al., 2006), and reduces vasodilator responses to progressive reductions in intravascular pressure in MCA (DeWitt et al., 2001) of rats. CR-6 is an antioxidant known to scavenge oxidant and nitrating species such as O$_2^-$, NO, and ONOO$^-$ (Montoliu et al., 1999; Sanvicens et al., 2006). Our results show that MCA from ischemic rats presents an enhanced formation of O$_2^-$ and ONOO$^-$ that is prevented completely (O$_2^-$) or partially (ONOO$^-$) by treatment with CR-6. It is surprising that CR-6 treatment was unable to prevent I/R-induced decrease in myogenic response. Impaired vascular tone is normally accompanied by vasodilatation. Accordingly, lumen diameter of the MCA in active conditions was increased after I/R. However, CR-6 treatment did not prevent the increase in lumen diameter induced by I/R. Previous studies have suggested that diminished myogenic tone resulting from I/R injury is associated with an increase of ONOO$^-$ production causing loss of F-actin in rat posterior cerebral artery (Maneen et al., 2006; Maneen and Cipolla, 2007). In these studies, the authors observed increased nitrotyrosine content after either 1 h or MCA occlusion with 30 min of reperfusion (Maneen et al., 2006) or short exposures (<1 h) to ONOO$, which in turn caused dilation (Maneen and Cipolla, 2007). In our study, we found that treatment with CR-6 did not fully prevent the ONOO$^-$ formation. Therefore, it is feasible that early production of ONOO$^-$ during ischemia or immediately after reperfusion (before treatment) promotes long-lasting vascular alterations leading to impaired myogenic tone and subsequent vasodilatation. The present study also gives insights into the different actions of reactive species after I/R. It suggests a link between O$_2^-$ and alteration of structural and mechanical properties, whereas the alteration of myogenic properties can be ascribed to an early ONOO$^-$ production after ischemia/reperfusion.

Taken together, these findings suggest that antioxidant treatment with CR-6 during reperfusion may reduce infarct size caused by cerebral ischemia through a mechanism that involves prevention of vessel hypertrophy and normalization of wall stress. The inability of CR-6 to prevent myogenic impairment and vasodilatation of MCA after I/R may also be involved. These results could help to understand the mechanisms by which antioxidants exert their beneficial actions in the brain and the involvement of the vascular response in brain damage. Finally, this study may offer important therapeutic perspectives for the treatment and prevention of the adverse effects caused by cerebral ischemia/reperfusion.

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References

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