Antioxidant activity of nitro derivative of aspirin against ischemia-reperfusion in hamster cheek pouch microcirculation

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Bertuglia, Silvia, Andrea Giusti, and Piero Del Soldato. Antioxidant activity of nitro derivative of aspirin against ischemia-reperfusion in hamster cheek pouch microcirculation. Am J Physiol Gastrointest Liver Physiol 286: G437–G443, 2004. First published October 16, 2003; 10.1152/ajpgi.00339.2003.—Aspirin has been chemically combined with a nitric oxide (NO) donor (NCX-4016) has been shown to inhibit cyclooxygenase and prostaglandin generation while maintaining the inhibitory effects of aspirin. The possible role of reactive oxygen species (ROS) in the action of NCX-4016 in ischemia-reperfusion (I/R) has not been studied. Furthermore, we were interested in comparing the effects of a conventional NO donor [2,2′-hydroxynitrosohydrazino-bis-etanamine (DETA/NO)] and NCX-4016 at the microvascular level in the hamster cheek pouch visualized by using an intravital fluorescent microscopy technique. Microvascular injury was assessed by measuring diameter change, the perfused capillary length (PCL), and leukocyte adhesion. Animals were treated with NCX-4016 (100 mg/kg or 30 mg/kg·day−1 for 5 days po) or DETA-NO (0.5 mg/kg). Mean arterial blood pressure increased slightly but significantly after NCX-4016 treatment. During 5- and 15-min reperfusion, lipid peroxides in the systemic blood increased by 72 and 89% vs. baseline, respectively, and were still higher than in basal conditions after 30-min reperfusion in the I/R group. Pretreatment with NCX-4016 maintained ROS at normal levels; increased arteriolar diameter, blood flow, and PCL; and decreased leukocyte adhesion (P < 0.05). DETA-NO decreased ROS during 30-min reperfusion; however, later there was a significant increase during reperfusion. DETA-NO decreased leukocyte adhesion (P < 0.05) but microvascular permeability increased after 30 min of reperfusion. In conclusion, NCX-4016 attenuates oxidative stress and prevents arteriolar constriction during I/R, whereas DETA-NO increases lipid peroxides in the systemic blood and permeability after reperfusion.

On restoration of blood flow during I/R, the production of reactive oxygen species (ROS) increases and is strongly implicated in microvascular dysfunction in experimental conditions, as well as in patients with acute myocardial infarction undergoing thrombolysis, coronary angioplasty, or coronary artery bypass grafting surgery (9, 14, 15, 18, 21). The interaction of ROS with membrane lipids and essential cellular biomolecule modification contribute to damage, leading to vasoconstriction and leukocyte adhesion on the endothelial surface (3, 12, 19). NO has been shown to be protective in I/R-induced damage by inhibiting platelet adhesion and aggregation and attenuating leukocyte adhesion (1, 5, 17, 24). However, despite recent advances in therapeutics and the potential of antioxidant intervention, both the efficacy of ROS scavengers and the role of NO in the clinical setting remain undetermined (2, 20, 41).

It is therefore interesting to study the role of oxidative stress in the action of NCX-4016 against I/R. Our aims were 1) to compare the properties of an NO donor and NCX-4016 against I/R-induced injury in the hamster cheek pouch visualized by an intravital fluorescent microscopy technique (3, 6); 2) to evaluate lipid peroxide formation in the systemic blood, an index of oxidative stress during I/R (5); 3) to evaluate the responses of arterioles during I/R after treatment with either NO donor or NCX-4016; and 4) to quantify the functional microvascular damage that occurs during I/R by changes in increased leukocyte adhesion and changes of PCL, namely the capillaries perfused by blood (3, 6).

MATERIALS AND METHODS

Male Syrian hamsters (80–100 g; Charles River, Calco, Como, Italy) were anesthetized by intraperitoneal injections of pentobarbital sodium (50 mg/kg body wt). Animals were tracheotomized, and the right carotid artery and femoral vein were cannulated to measure blood pressure and to inject the phosphorence probes and supplemental doses of anesthetic. Animal handling and care were carried out according to the procedures outlined in the Guide for the Care and Use of Animals in Laboratories of the Italian Research Council.

We used four groups of animals subjected to I/R. The first group (I/R; n = 5) was subjected to I/R and used as the control group. The second group (NCX-10; n = 9) and the third group (NCX-3; n = 5) received NCX-4016 (NicOx, Sophia Antipolis, France) orally for 5 days at a dosage of 100 or 30 mg/kg body wt, respectively. NCX-4016 was dissolved in DMSO (100 μl/100 g) and suspended in carboxymethylcellulose 0.5% (Sigma, St. Louis, MO). The fourth group (DTN; n = 5) was treated with an NO donor, 2,2′-hydroxynitroso-hydrazino-bis-etanamine (DETA-NO; Inalco, Milan, Italy) (0.5 mg/kg body wt). DETA-NO was dissolved in 0.5 ml of saline and adminis-
tered intravenously over 30 min into the femoral vein. In a pilot study, DETA-NO was administered at a dosage of 1 mg/kg body wt (n = 3), but the decrease in MAP was too marked and the hamster cheek pouch was hypoperfused. NCX-4016 and the saline solution in I/R and DTN groups were administered by stomach tube.

Ischemia was induced by applyingatraumatic microvascular clips on the proximal part of the cheek pouch for a period of 30 min. The clamp was then removed, and the microcirculation was reperfused for 30 min.

The left cheek pouch was fixed to a Plexiglas platform of the microscope, and a thin black blade was inserted through a small incision between the upper and lower layers of the pouch (3, 5). Ringer solution (pH 7.35, 36°C, equilibrated with 5% CO2 in 95% N2) was perfused continuously on the cheek pouch. Observations were made with a Leitz Orthoplan microscope, operating in incident illumination with a Ploemopak filter block, fitted with a Leitz long-working-distance objective (4X, 0.14 numerical aperture; 20X, 0.25 numerical aperture). A X10 magnification eyepiece was used. Epiillumination was provided by a xenon 150-W lamp used in conjunction with the appropriate filters for fluorescein isothiocyanate bound to dextran 150,000 molecular weight (FD-150) (Leitz I2 Ploemopak filter block) and a heat filter (Leitz KGI). The tracer was injected intravenously (500 mg/kg body wt at 5% wt/vol solution in 5 min), televised with a COHU 5253 SIT low-light-level camera, observed from a Sony PVM 122 CE monitor, and recorded by a Sony U-Matic VO 5800 PS video recorder.

Images were stored in a computer. The hamster’s body temperature and cheek pouch temperature were maintained at 37°C with circulating warm water. An intravenous injection of pentobarbital sodium (300 mg/kg) was used as the method of euthanasia.

**Measurements of microvascular parameters.** To quantify the microvascular leakage of FD-150, the fluorescence intensity in the perivascular space was reported as normalized to baseline fluorescence NGL = ([I – I0]/I0), where NGL is normalized gray level, I is the average fluorescence intensity at baseline (average of 4–5 windows located outside the blood vessels, the same windows being used throughout the experimental procedure), and I0 is the same parameter after reperfusion (3, 6). Grey levels were determined by using fluorescence imaging software (Project Engineering, Florence, Italy). The size of the window used to measure fluorescence was set at 50 × 50 μm.

To stain leukocytes, the animals received an intravenous injection of acridin red (Chroma, Stuttgart, Germany) (10 mg/kg body wt in 0.3 ml) and a supplemental injection (final volume 3 ml kg–1 h–1) throughout the experiment (6). Adherence is operationally defined as the number of leukocytes that remains stationary for 30 s. The number of adherent leukocytes was reported as the mean number per 100-μm length of venules and was counted during baseline conditions (Ploemopak filter block, Leitz H2) and after reperfusion. Postcapillary venules with diameters of 15–30 μm and lengths >250 μm were selected for study.

Capillaries that have red blood cell (RBC) transit in at least a 30-s period (PCL) were assessed in a region of ~0.5 mm2. The total length of RBC-perfused capillaries divided by the area of the microscopic field of view (PCD; cm–1) was evaluated by measuring and adding the length of capillaries that had RBC transit. Relative change in PCD from baseline levels after the intervention is a relative indicator of capillary perfusion. PCL and normalized gray values were measured by using an imaging medical software system (MIP; Consiglio Nazionale delle Ricerce, Institute of Clinical Physiology, Pisa, Italy).

Microvascular diameters (D) and RBC velocity were analyzed online in arterioles and venules. The measured centerline velocity was corrected according to vessel size to obtain the mean RBC velocity (V). Blood flow (Q) was calculated from measured parameters as Q = V · π(D/2)2.

Vessel diameter was measured with an image-shearing system (digital video image shearing monitor model 907; IPM, San Diego, CA), whereas RBC velocity was analyzed by photodiodes (fiberoptic photo diode pick-up system; IPM) and cross-correlation technique (model 102 B velocity tracker; IPM).

**Measurement of lipid peroxides.** To measure plasma hydroperoxides, the analytic method of d-ROMs (Diacon, Parma Italy) was used (5). The d-ROMs assay is based on Fenton’s reaction or on radical formation during lipid peroxidation. The oxyradical species produced, whose quantity is directly proportional to the quantity of plasma hydroperoxides, are trapped by alchylene, a phenolic compound that forms a colored, stable radical detectable spectrophotometrically at 505 nm. The concentration of the colored complex is directly correlated to the concentration of hydroperoxides. Ten microliter of a chromogenic substance and 1 ml of kit buffer are mixed with 10 μl blood for 1 min at 37°C. Results are expressed in arbitrary units (1 arbitrary unit = 0.08 mg/100 ml H2O2). Blood samples were taken at baseline and at 5, 15, and 30 min of ischemia and reperfusion for each hamster from the cannulated carotid artery.

Mean arterial blood pressure (P10E2 transducer connected to a catheter in the carotid artery; Viggo-Spectramed, Oxnard, CA), and heart rate were monitored by a Gould Windograf recorder (model 13–6615–105; Gould). Data were recorded and stored in a computer.

All reported values were means ± SD. Paired or unpaired t-test was used when appropriate. For multiple comparisons, ANOVA and Scheffe’s post hoc test were performed. To compare the percent changes in PCL, we used the Mann-Whitney’s U-test and Kruskal-Wallis test. Statistical significance was set at P < 0.05.

**RESULTS**

Systemic parameters MAP and HR did not change significantly during I/R compared with baseline in all groups. In the NCX-10 and NCX-3 groups there was a slight but significant increase in MAP during baseline that persisted during reperfusion (P < 0.05). Conversely, in the DTN group, there was a significant decrease in MAP (P < 0.05). All of the systemic values were reported in Table 1. DMSO had no effect on MAP and heart rate.

**Effects of I/R on microcirculation.** In the I/R group, the arteriolar diameter decreased significantly after reperfusion compared with baseline (P < 0.05). Changes in the diameter of arterioles (baseline diameter: 25.8 ± 7.5 μm) during reperfusion are shown in Fig. 1. The diameter of venules (during baseline: 25 ± 4 μm for collecting venules and 73 ± 5 μm for large venules) did not change significantly after reperfusion. RBC velocity significantly decreased in arterioles during reperfusion (baseline: 1.98 ± 0.35 mm/s, reperfusion: 1.00 ± 0.45 mm/s).

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<th>I/R</th>
<th>NCX-10</th>
<th>NCX-3</th>
<th>DTN</th>
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<tr>
<td>MAP, mmHg</td>
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<td>110 ± 8*</td>
<td>100 ± 7*</td>
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*Data are means ± SD. Dosages were as follows: NCX-10 group, 100 mg/kg body wt NCX-4016; NCX-3 group, 30 mg/kg body wt NCX-4016; DTN group, 0.5 mg/kg body wt 2.2'-dihydroxyxystrohydrazino-bis-etanamine. I/R, ischemia-reperfusion (control). *P < 0.05 NCX group and DTN group vs. I/R group.
mm/s; \( P < 0.05 \). The blood flow was: 1.39 ± 0.95 and 0.70 ± 0.4 nL/s during baseline and reperfusion, respectively.

These changes were associated with a marked permeability increase in postcapillary and collecting venules during reperfusion (baseline: 0.09 ± 0.04 NGL; \( P < 0.05 \); Fig. 2). The number of leukocytes adhering to postcapillary venules increased significantly compared with baseline (Fig. 3). PCL decreased significantly when compared with baseline (baseline: 1.8 ± 0.4 cm⁻¹; \( P < 0.05 \); Figs. 4 and 6). ROS increased significantly at the beginning of reperfusion and after 15 min of reperfusion as shown in Fig. 5.

**Effects of NCX-4016 on I/R injury.** Both of the groups treated with NCX-4016 were protected against I/R injury. There was no arteriolar vasoconstriction during reperfusion as observed in the I/R group. Arterioles showed a significant increase in diameter compared with the I/R group (\( P < 0.05 \); Fig. 1). Many arterioles showed rhythmic diameter changes (vasomotion) during reperfusion in the group treated with 100 mg/kg of NCX-4016. In arterioles, RBC velocity and blood flow were maintained during reperfusion (baseline: 2.35 ± 0.25 mm/s and 1.66 ± 0.94 nL/s; after reperfusion: 2.48 ± 0.45 mm/s and 1.77 ± 0.90 nL/s). Both the velocity during baseline and after reperfusion were higher compared with the I/R group (\( P < 0.05 \)). Permeability did not increase after reperfusion in the groups treated with NCX-4106 (Fig. 2). The number of leukocytes adhering to postcapillary venules did not increase after reperfusion (Fig. 3).

PCL did not change during reperfusion compared with baseline (baseline: 1.6 ± 0.5 cm for the NCX-10 group; 1.6 ± 0.3 cm for the NCX-3 group; Fig. 4), but it increased significantly when compared with the I/R group (\( P < 0.05 \)). Microvascular network was immediately reperfused after ischemia (Fig. 7). PCL increased significantly by 6% (at the dosage of 10 mg) and by 3% (at the dosage of 3 mg/100 g) after reperfusion compared with baseline values in the NCX groups (\( P < 0.05 \)). ROS production did not increase and was not different from baseline during reperfusion (Fig. 5).
Effects of DETA-NO on I/R injury. In the DTN group, the diameter of arterioles did not change compared with baseline, but they were significantly higher when compared with the I/R group ($P < 0.05$). RBC velocity was significantly higher in arterioles after reperfusion compared with the I/R group ($P < 0.05$) (baseline: 1.95 ± 0.55 mm/s, 1.23 ± 0.92 nl/s; reperfusion: 1.90 ± 0.35 mm/s, 1.27 ± 0.87 nl/s).

These changes were associated with a slight increase in permeability in postcapillary and collecting venules after reperfusion (Fig. 2). However, there was a significant reduction in permeability compared with the I/R group (Fig. 3). PCL was significantly higher compared with the I/R group, whereas it did not change compared with baseline (baseline: 1.7 ± 0.7/cm, after reperfusion: 1.9 ± 0.5/cm) (Fig. 4). In the DTN group there was a significant increase in perfusion after 5–10 min of reperfusion that lasted until the end of reperfusion. Increase in ROS formation was significantly lower than in the I/R group. However, ROS formation was significantly higher than the values in the NCX-4016 group after 15–30 min of reperfusion (Fig. 5).

**DISCUSSION**

Our data show that the lipid peroxides increased significantly within 5 min of reperfusion and then declined until they reached the preperfusion levels in the I/R group. NCX-4016 had an effective and prolonged antioxidant activity during I/R, thus showing its inhibitory activity against lipid peroxidation. Conversely, in the hamster treated with the NO donor, the level of lipid peroxides and microvascular permeability started to increase after 30 min of reperfusion. DETA-NO restored capillary perfusion and reduced leukocyte adhesion on venules. NCX-4016 caused arteriolar dilation and immediate restoration of capillary flow at the beginning of reperfusion and significantly inhibited microvascular permeability and leukocyte adhesion on venules during reperfusion. It is likely that both the direct effects of NO and its antioxidant properties are responsible for its protective effects.

Recent evidence has suggested an intimate link between the overproduction of lipid peroxides in the blood and the development of endothelial cell damage early in reperfusion (5, 26). Production of lipid peroxidation products is associated with the loss of membrane integrity as well as the activity of membrane-associated protein after I/R.

Pieper et al. (30) showed that this NO-aspirin derivative caused a significant reduction of plasma isoprostanes in diabetic animals that is negatively linked to NO synthesis and ROS formation. Napoli et al. (26) showed that chronic treatment with NCX-4016 reduced oxidative stress in hypercholesterolemic mice. Therefore, the antioxidant activity of NCX-4016 may be responsible for the protection of endothelial damage, for the improvement of arteriolar blood flow, and for increased capillary perfusion during reperfusion.

NCX-4016 visualized by 4,5-diaminofluorescein diacetate is permeabilized inside the cells and releases NO at compartments near the plasma membrane, whereas DETA-NO appears only around the endothelial cell (10). Therefore, the protection...
exerted by pretreatment with NCX-4016 could have a significant potential because of the prolonged action against lipid peroxidation and penetration in the core of the membrane, maintaining its integrity during I/R.

During reperfusion, there is a period of low oxygenation characterized by competitive mechanisms between oxygen consumption by the tissue and the vascular endothelium (5). In fact, endothelial NO synthase (eNOS) activity is directly dependent on oxygen and is impaired in proportion to hypoxia. Furthermore, reoxygenation during reperfusion promotes increased ROS production, which would lead to enhanced NO degradation (5, 31). In view of these considerations, it is reasonable to propose that a potential mechanism of action of NCX-4016 could be related to the decreased destruction of NO by ROS.

Conversely, the treatment with DETA-NO decreased ROS formation at early reperfusion, whereas after 30 min of reperfusion ROS formation increased. It has been suggested that the cytotoxic levels of NO and other reactive nitrogen species may play a role in tissue injury (16, 34). An increased production of these reactive species also occurs when fluxes of NO are enhanced and peroxynitrite has been detected during inflammation after treatment with NO donors (40). Increase in lipid peroxidation after treatment with an NO donor could, therefore, be linked to uncontrolled NO formation during reperfusion.

NCX-4016 was shown to normalize arterial pressure in eNOS-deficient mice (8). Our findings also showed an effect on arterial blood pressure, because we observed a slight increase in mean blood pressure and an increase in vasomotor activity exerted by NCX-4016. Many anesthetics cause arterial vasomotion to disappear and affect cardiovascular dynamics such as heart rate, heart contractility, and mean blood pressure (7). Inhibition of COX activity due to aspirin might also explain differences observed during anesthesia in animals treated with NCX-4016 and DETA-NO. In fact, a mild increase in blood pressure can be attributed either to the common property of nonsteroidal anti-inflammatory drugs of inhibiting the formation of prostaglandins in the cardiovascular system or to direct actions on the tone and sensitivity of the resistance vessels in various regions. Moreover, NO metabolites appear to decrease during in vivo regional myocardial ischemia, whereas they are elevated during reperfusion after myocardial infarction and cardiac surgery (28). It was shown that HNO/NO− causes positive inotropic and lusitropic effects in failing hearts that were independent and additive to β-adrenergic stimulation. We therefore suggest that NCX-4016 could normalize the mean blood pressure during anesthesia, thus avoiding the hypertensive effects of pentobarbital anesthesia.

We obtained the same protective effects on capillary perfusion with both DETA-NO and NCX-4016. However, NCX-4016 increased capillary perfusion after reperfusion, immediately and more significantly. These effects could also be related to the antithrombotic effects of aspirin. It is an inhibitor of thromboxane A2 that promotes platelet aggregation, causes vasoconstriction, and increases infarct size, even if there is high residual incidence of cardiovascular events in patients with acute coronary syndromes treated with aspirin (36). Yamamoto et al. (40) showed a protective effect of NCX-4016 on the infarcted heart. These authors showed that NCX-4016 increased nitrate content and the formation of prostanoids from arachidonic acid in the infarcted heart muscle beyond that noted after ischemia alone. Conversely, NCX-4016 protected mice from pulmonary embolism, whereas aspirin was ineffective, thus showing a better antithrombotic effect of NCX-4016 compared with aspirin alone (22).

Coronary artery disease is associated with deficiency in NO-mediated vasodilation with augmented responses to several vasoconstrictors (23). In agreement with our data, NCX-4016 reduced vasoconstriction due to transmural nerve stimulation and prevented the effects of different concentrations of norepinephrine in the perfused rat tail artery preparation only with intact endothelium (33). The vasodilatory effect of NCX-4016 was related to a direct and specific increase in cGMP in vascular smooth muscle (25). A direct effect of NO after NCX-4016 treatment may also prevent vasoconstriction and protect arterioles during vasospasm in the reperfusion period. However, NCX-4016 showed a more significant vasodilatory effect after reperfusion than in the group treated with DETA-NO. NO has been identified as responsible for arteriolar...
vasodilation and control of capillary perfusion; however, other factors linked to leukocyte and platelets could play a crucial role. We (3) have shown previously that there is a marked relationship between endothelial cell dysfunction and the coagulation system during I/R. Therefore, there is a possibility that aspirin’s antithrombotic effects could play a role in the vasodilation and in the inhibition of leukocyte adhesion exerted by NCX-4016. Indeed, many alterations occur simultaneously and independently during I/R injury and an antithrombotic, vasodilatory and antioxidative compound can provide protection against many aspects of I/R-induced injury.

Interestingly, during reperfusion, there was a significant effect on arteriolar vasomotor tone elicited by NCX-4016. These periodic changes in arteriolar diameter modulate the arteriolar tone and could explain the increase in capillary perfusion observed with NCX-4016 during reperfusion, because the onset of capillary perfusion after ischemia requires a higher pressure gradient at microvascular networks compared with the pressure gradient necessary to maintain perfusion through capillaries during baseline conditions.

The decrease in leukocyte adhesion exerted by NCX-4016 may be related to a direct effect of NO but also to the decreased oxidative stress. Our data show that there is a significant decrease in leukocyte adhesion on venules with both NCX-4016 and the NO donor.

Our previous observations (4) have shown an increased P-selectin expression at venular bifurcations in the hamster cheek pouch microcirculation in I/R. An increased oxidative stress before I/R blunted the protective effects of anti-P-selectin treatment with marked leukocyte adhesion on postcapillary venules, suggesting that the mechanism of leukocyte activation is due to dysfunctional endothelium after oxidative stress. These findings are in agreement with those obtained in rat mesentery circulation by Suematsu et al. (35), showing that NG-nitro-L-arginine methyl ester (L-NNAME) caused cellular oxidant formation mainly in venules with adhering leukocytes. The increased leukocyte adhesion to venules becomes a source of ROS during reperfusion (5, 12). The increased formation of ROS may also lead to decomposition of NO (19) and to endothelial cell activation, which is a potential mechanism for late systemic injury (29). Taken together, these findings and our data raise the possibility that, by decreasing ROS formation, NCX-4016 may inhibit leukocyte adhesion on venules and microvascular damage during reperfusion.

Increase in permeability caused by DETA-NO could be determined by an excessive increase in NO formation. NO is also known to be able to modulate microvascular permeability. Kurose et al. (13) used L-NNAME and monoclonal antibodies against CD11, CD18, and P-selectin in rat mesenteric vessels. Leakage was found to be related to platelet-activating factor and leukocyte-endothelial adhesion after NO production inhibition. However, the role of NO on vascular permeability is controversial, because some studies indicate that NO itself may be detrimental regarding microvascular barrier function. Generation of strong oxidants like peroxynitrite from the reaction of NO with superoxide may cause or aggravate endothelial and epithelial damage (31). We therefore suggest that the NO donors could limit increases in leukocyte-dependent permeability but may promote permeability in the absence of leukocyte adhesion during reperfusion.

In conclusion, our findings show that NCX-4016 is highly effective in preventing lipid peroxidation of endothelial cells against I/R-induced injury. We present functional evidence that the presence of NCX-4016 is critical against arteriolar constriction, the lack of capillary perfusion, and inhibition of leukocyte adhesion on venules during reperfusion. The complex mechanism by which NCX-4016 achieves this effect could be via the increase in NO or its scavenger effects combined with the antithrombotic effects of aspirin during reperfusion. However, the early inhibition of lipid peroxides could avoid initial damage to the endothelium, thus preserving endothelial barrier function and activation of leukocytes. The combined effects suggest that the inhibitory and antioxidant properties of NCX-4016 provide a protective therapy against the development of I/R injury in different pathologies.

REFERENCES
