The Nitroderivative of Aspirin, NCX 4016, Reduces Infarct Size Caused by Myocardial Ischemia-Reperfusion in the Anesthetized Rat

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ABSTRACT

NCX 4016, a nitro-ester of aspirin endowed with antithrombotic activity, appears to have clinical potential in treating cardiac complications related to coronary insufficiency. This compound has been shown to improve postsischemic ventricular dysfunctions and to reduce myocardial infarct size in the rabbit. The cardioprotection conferred by NCX 4016 (10, 30, and 100 mg/kg) and aspirin (ASA, 54 mg/kg) was evaluated in anesthetized rats subjected to 30 min of myocardial ischemia followed by 120 min of reperfusion (MI/R). Drugs were given orally for 5 consecutive days. NCX 4016 displayed remarkable cardioprotection in rats subjected to MI/R as was evident in the reduction of ventricular premature beats and in the incidence of ventricular tachycardia and fibrillation; they were reduced dose dependently and correlated with survival of all rats treated with the higher dose of NCX 4016. In these animals, infarct size was restricted proportionally to the dose of NCX 4016 associated with diminution of both plasma creatine phosphokinase and cardiac myeloperoxidase activities. ASA showed only a minor degree of protection against MI/R damage. Rats treated with N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg) demonstrated aggravated myocardial damage in terms of arrhythmias, mortality, and infarct size. Supplementation of nitric oxide (NO) with NCX 4016 (100 mg/kg) greatly reduced the worsening effect caused by L-NAME. The beneficial effects of NCX 4016 appear to derive in large part from the NO moiety, which modulates a number of cellular events leading to inflammation, obstruction of the coronary microcirculation, arrhythmias, and myocardial necrosis.

The major goal in the management of myocardial infarction is to reduce postmyocardial infarction complications and mortality by reversing myocardial ischemia and limiting the infarct size. The attempts to achieve these objectives are centered primarily on hemodynamic interventions and nitric oxide (NO) donors that have shown therapeutic potential (Jugdutt and Warnica, 1988).

Recently, a new family of nitroderivatives of conventional nonsteroidal anti-inflammatory drugs has been synthesized to reduce or abolish their gastrointestinal toxicity due to the well known inhibition of cytoprotective prostaglandins biosynthesis (Wallace and Granger, 1996). Among them, a chemical combination of aspirin (ASA) with an NO donor, 2-acetoxy-benzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016), has been shown to display antiaggregatory and antithrombotic activity by a dual mechanism of action involving inhibition of cyclooxygenase and release of NO, the latter acting on guanylate cyclase in both platelets and vascular smooth muscle cells (Minuz et al., 1998; Wallace et al., 1999). However, in contrast to ASA, the antithrombotic effect of NCX 4016 seems to require at least 5 consecutive days of treatment to be manifested “in vivo”, suggesting that the release of the NO moiety may play a pivotal role. In fact, pharmacokinetic studies demonstrated that the serum concentration of salicylate after a single oral administration of NCX 4016 increased slowly and to a lesser extent than that of an equimolar dose of ASA, which increased salicylate levels rapidly and to a greater extent (Wallace et al., 1999).

Moreover, NCX 4016 has been shown to reduce the susceptibility of the stomach to shock-induced damage through inhibition of neutrophil adherence to the vascular endothelium (Wallace et al., 1997). This property of NO donors is important since neutrophils are involved in generalized vascular disorders and participate in the ischemia-reperfusion-induced myocardial damage (Mullane, 1988). A recent report has shown that NCX 4016 is cardioprotective in the face of...
ischemia, producing marked improvement of postischemic ventricular dysfunction in the rabbit heart (Rossoni et al., 2000). Furthermore, this compound given by infusion to rabbits blunted cardiac arrhythmias and significantly reduced the mortality rate resulting from acute myocardial infarction produced by ligation of the left anterior coronary artery (Rossoni et al., 2000).

These findings suggest that with NCX 4016 it is possible to achieve an appropriate balance between platelet inhibition and neutrophil control. An NO-releasing compound can limit infarct size by inhibition of neutrophil invasion in the reperfused myocardium as well by decreasing formation of reactive oxygen species and injurious autacoids (Rossoni et al., 1996). However, the precise role of NO in this context is controversial; whether NO is harmful (Patel et al., 1993; Woolfson et al., 1995; Curtis and Pabla, 1997) or protective (Siegfried et al., 1992; Lefer et al., 1993; Rossoni et al., 2000) to the reperfused heart is still a matter of investigation.

To define the role of NO supplementation, the efficacy of NCX 4016 in preventing the extension of infarct size in the anesthetized rats has been evaluated and compared with that of ASA. The contribution of endogenous NO production to infarct size and mortality was examined by inhibition of NO synthase. Blockade of NO production resulted in the highest mortality rate and largest infarct size that were reversible by coadministration of NCX 4016.

**Materials and Methods**

**Animals**

Male Wistar rats (300–450 g of body weight) obtained from Charles River Italia (Calco, LC, Italy) were used for all experiments. Animals were housed under standard conditions (four rats per cage; temperature, 22 ± 1°C; humidity, 55 ± 10%) and maintained on 12-h light/dark cycle with the light on from 7:00 AM. Rats were fed standard chow (code 014RF25C; Mucedola S.r.l., Settimo Milanese, MI, Italy), with water ad libitum. All experimental procedures were approved by the Animal Care Committee of the University of Milan and were in accordance with the principles set forth in the Italian guidelines for the Care and Use of Laboratory Animals, which conform with the European Communities Directive of November 1986 (86/609/EEC). Experiments were performed between September 1999 and June 2000.

**Surgical Preparation**

Rats were anesthetized with thiopentone sodium (60 mg/kg i.p.), placed in the supine position on a table, and the body temperature was maintained at 38 ± 1°C by means of a heating pad. Animals were tracheotomized, intubated, and ventilated with room air using a respirator for small rodents (model 7025; Ugo Basile, Comerio, VA, Italy) with a stroke volume of 10 ml/kg and a rate of 60 to 65 strokes/min to maintain normal pH (7.35–7.45), PO₂ (80–110 mm Hg), and PCO₂ (25–40 mm Hg) parameters. Catheters (polyethylene tubing; i.d. 0.58 mm, o.d. 0.965 mm) were inserted into the left femoral artery and right jugular vein for the measurements of blood pressure (BP) and drug/vehicle administration, respectively. A 2F micromanometer catheter with one high-fidelity pressure sensor (model SPR-249; Millar Instruments Inc., Houston, TX) was introduced via the isolated right carotid artery into the left ventricle and was used to measure the left ventricular pressure (LVP). The zero pressure baseline was obtained by placing the pressure sensor in 37°C physiological saline before measurements. Furthermore, subdermal platinum electrodes were placed to allow the determination of a lead II ECG. According to the procedure described by Himori and Matsuura (1989), the chest was opened by a left thoracotomy at the 4th or 5th intercostal space, the ribs were gently spread using a small-sized retractor, and the heart was exposed. After incision of the pericardium to allow access to the left main coronary artery (LCA), the heart was quickly removed from the thoracic cavity and inverted. An atraumatic needle (no. FS2; Ethicon, Pratica di Mare, Rome, Italy) with a thin silk thread (5-0) was used for the ligature. The needle was inserted (approximately 0.5 mm) into the myocardium 2 to 3 mm away from the origin of the LCA (just beneath the left auricular appendage). The thread was then made into an overhand knot (an occluder); two other threads were tied to the main knot (release). The heart was returned quickly to the thoracic cavity and the tips of the suture used to produce the coronary ligation were exteriorized through the chest wall. The whole surgical procedure described above took about 10 to 12 min and at the end the animals were allowed to stabilize for 30 min before LCA ligation. The coronary artery was occluded at time zero for 30 min by tightening of the occluder. This was associated with the typical electrocardiographic (ST-segment elevation and increase in R-wave amplitude) and hemodynamic (fall in BP) changes due to myocardial ischemia. After 30 min of LCA ligation, the occluder was reopened and the heart was reperfused for 120 min. At the end of this period the heart was removed for infarct size estimation and myeloperoxidase determination.

**Hemodynamic Measurements**

Throughout the experiments, using the signals of both BP and LVP transmitted continuously to the pressure modules (model Mc Lab/4E; AD Instruments, Hastings, UK), systolic arterial pressure, diastolic arterial pressure, and mean arterial pressure (MAP) were obtained. The following parameters related to cardiac mechanics were also determined: left ventricular systolic pressure, left ventricular end-diastolic pressure, left ventricular developed pressure (LVPd), and the first derivative of LVP. The ECG (lead II) recording used a Cardioline apparatus (model Delta-1; Remco Italia, MI, Italy); the signals were continuously transmitted into Mc Lab/4E ECG module (AD Instruments). All the data obtained from each module of the system were analyzed with computer software Chart3 Windows 3.5 (AD Instruments). The pressure rate index (PRI), an indicator of myocardial oxygen consumption (Buller et al., 1981), was also calculated as the product of MAP and heart rate.

**Assessment of Arrhythmias**

Using the ECG signals, ventricular arrhythmias (ensuing during the total 30 min of LCA occlusion and the first 10 min of LCA reperfusion) were assessed as described by Clark et al. (1980) and in accordance with the definitions reported in the Lambeth Conventions (Walker et al., 1988). In survivor rats the total number of ventricular premature beats (VPBs), including singles, bigeminy, salvos, and ventricular tachycardia (VT; defined as four or more consecutive VPBs) was counted. The incidence and duration of VT and ventricular fibrillation (VF) were also recorded along with the mortality (due to sustained VF, defined as continuous VF persisting for at least 3 min). Rats were excluded from the final analysis if any of the following occurred: arrhythmias before LCA occlusion; cardiac failure (defined as a profound reduction in arterial pressure, approaching zero within the first 5 min following LCA occlusion, usually accompanied by A-V block, which is probably due to the ligature being placed too deeply such that the septal branch of the LCA is also occluded); no evidence of ischemia after tying the ligature (changes in either the ST-segment or R-wave amplitude; arrhythmias); MAP <60 mm Hg before LCA occlusion. Any rats that were excluded were replaced immediately.

**Determination of Area at Risk and Infarct Size**

The area at risk and infarct size were evaluated with Evans blue dye and triphenyltetrazolium chloride, respectively (Clark et al., 1980). In brief, at the end of the 2-h reperfusion period, the ligature...
around the LCA was retightened and 1 ml of Evans blue dye (3% w/v) was injected intravenously into the jugular vein to delineate ischemic (area at risk) and nonischemic myocardium (area not at risk). The Evans blue solution stains the perfused myocardium, while the nonperfused myocardium remains uncolored. The rat was euthanized with a 15% KCl solution and the heart was rapidly excised, rinsed, and blotted dry. After removing the atria, right ventricle wall, and the major blood vessels, the left ventricle was sliced parallel to the atrioventricular groove in 3-mm-thick sections. The area at risk of the left ventricle (unstained portion) was separated from the area not at risk of the left ventricle (stained portion). The area at risk was again sectioned into 1-mm-thick slices and incubated in a 1% (w/v) solution of the triphenyltetrazolium chloride stain in 20 mM phosphate buffer (pH 7.4) at 37°C for 20 min. The tetrazolium dye forms a blue formazan complex in the presence of coenzymes and dehydrogenases (Klein et al., 1981). The irreversibly injured necrotic portion of the myocardium, which did not stain, was separated from the stained portion (i.e., ischemic but non-necrotic area at risk). All portions of the left ventricular myocardium were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity. Infarct size was expressed as a percentage of the area at risk.

Cardiac Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was evaluated as an index of neutrophil accumulation in jeopardized tissue because it correlates closely with the number of polymorphonuclear leukocytes present in the heart (Mullane et al., 1985). This enzyme was determined in the two portions of the left ventricle (area not at risk and area at risk) using a specific assay for this enzyme (Schierwagen et al., 1990). Myocardial tissue samples were first homogenized in a 0.5% (w/v) hexadecyltrimethyl ammonium bromide solubilized in 50 mM potassium phosphate buffer (pH 6.0) using a Polytron homogenizer (Ika Ultra-Turrax T25; Janke & Kunkel GmbH Co., Staufen, KG, Germany) for 30 s (15 s + 15 s) at 7000 rpm. Homogenates were centrifuged at 12,500 g at 2°C for 30 min on an Optima Ultra centrifuge (Beckman, Palo Alto, CA). The supernatant was collected and reacted with a solution of O-dianisidine dihydrochloride (0.167 mg/ml) and 0.0005% hydrogen peroxide in 50 mM potassium phosphate buffer (pH 6.0). The rate of change in absorbance was measured spectrophotometrically at 460 nm (model Lambda16; PerkinElmer Italia, Monza, MI, Italy). MPO standard curve (2.5–0.08 U/ml) was included in each assay. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μm of peroxide/min at 25°C and expressed in units per gram of tissue.

Plasma Creatine Phosphokinase Activity

Creatine phosphokinase (CPK) activity was determined in plasma collected immediately before LCA occlusion (time 0 min), at the end of 30 min of LCA occlusion period (time 30 min), and at the end of 120 min of LCA reperfusion period (time 150 min). In brief, samples (0.5 ml) of arterial blood were drawn from the carotid catheter. The blood was centrifuged for 15 min at 2400 g at 4°C and the plasma supernatant was removed and stored frozen at −20°C until assayed. Plasma was processed for CPK activity (Rosalki, 1967) using a commercially available kit and the total amount was determined on a spectrophotometer at a wavelength of 340 nm (model Lambda16; PerkinElmer Italia). CPK activity was expressed in units per liter of plasma.

Experimental Design

Study I. This study was designed to investigate the protective activity of NCX 4016 and ASA against MI/R damage. Rats were randomly assigned to six different groups of at least 14 animals each: group 1, sham-operated animals, not LCA occluded, treated with vehicle (Sham); group 2, vehicle-treated animals subjected to 30 min of LCA occlusion followed by 120 min of reperfusion (Vehicle + MI/R); groups 3 to 5, NCX 4016 (10, 30, and 100 mg/kg)-treated animals and subjected to MI/R (NCX + MI/R); and group 6, ASA (54 mg/kg)-treated animals and subjected to MI/R (ASA + MI/R).

All drugs, dissolved in polyethylene glycol 400 (PEG 400; vehicle), were administered orally by gavage (volume 2 ml/kg) once a day for 5 consecutive days. On the 5th day, the treatment was performed 1 h before starting the experiment. The length of the treatment has been established considering that the antithrombotic effect of NCX 4016 in the rat is evident only when this compound is administered orally for a 5-day period (Wallace et al., 1999) and the adequacy of the dose level/ regimen has been established by the results of preliminary experiments.

Study II. These experiments were performed to study the contribution of endogenous NO, and its supplementation via NCX 4016, to the prevention of MI/R damage. The rats were randomly divided in five groups of at least 10 animals each: groups 1 and 2, identical to groups 1 (Sham) and 2 (Vehicle + MI/R) in study I; group 3, NCX 4016 (100 mg/kg)-treated animals and subjected to MI/R (NCX + MI/R); group 4, Nω-nitro-l-arginine methyl ester (10 mg/kg)-treated animals and subjected to MI/R (l-NAME + MI/R); and group 5, NCX 4016 (100 mg/kg)-treated animals followed 2 h later by l-NAME (10 mg/kg) and subjected to MI/R (NCX + l-NAME + MI/R).

All drugs, dissolved in PEG 400 (vehicle), were administered orally (2 ml/kg) once a day for 5 consecutive days. On the 5th day, the treatment with NCX 4016 and l-NAME were performed 3 and 1 h before starting the experiment, respectively.

Plasma cGMP Determination

In these experiments, the effect of NCX 4016 on plasma cGMP levels was measured. For this study 30 rats (n = 5 rats/group) were treated orally with vehicle (PEG 400: 2 ml/kg) and NCX 4016 (10, 30, and 100 mg/kg) once a day for 5 consecutive days. On the 5th day, 1 h before the last treatment, the rats were anesthetized with thiopeptone sodium (60 mg/kg i.p.) and 5 ml of blood was collected from the abdominal aorta into plastic vessel containing 2% ethylenediamine-tetraacetic acid (1/20 volume) and kept on ice. The blood was immediately centrifuged for 15 min (2400g at 4°C) and the plasma supernatant was removed and stored frozen at −20°C until assayed. The cGMP level in this plasma was determined using a commercially available enzyme immunoassay kit and expressed in picomoles per milliliter.

Statistical Analysis

Except for the incidence of VT, VF, and mortality rate, all values are expressed as means ± S.E. Differences between means were compared by Student’s two-tailed unpaired t test with, when appropriate, a Dunnett’s multiple comparison procedure (GraphPad Prism; GraphPad, San Diego, CA). Incidences of VT and VF were compared by Fisher-Irwin (chi square with Yates correction) test. Analysis of mortality rate was carried out with the analysis of Log-Likelihood for categorical data and either Pearson or Likelihood-Ratio χ² tests (Snedecor and Cochran, 1989). Body weight, heart weight, left ventricular weight, area at risk, and infarct size were compared with a one-way analysis of variance followed, when appropriate, a Dunnett’s multiple comparison procedure (GraphPad). A value of P < 0.05 was considered statistically significant.

Drugs

The following drugs were used: NCX 4016 (NicOx S.A., Valbonne-Sophia Antipolis, France); aspirin, Nω-nitro-l-arginine methyl ester, triphenyltetrazolium chloride, Evans blue, human myeloperoxidase, hexadecyltrimethyl ammonium bromide, O-dianisidine dihydrochloride, hydrogen peroxide, polyethylene glycol 400, and ethylenedia- minetetraacetic acid (Sigma Chemical Co., St. Louis, MO); thiopeptone sodium (Abbott, Campoverde, Latina, Italy), kit for guanosine 3′:5′-cyclic monophosphate determination (RPN-226; Amersham Ita-
Results

Hemodynamics. Hemodynamic parameters measured throughout the experiments in rats treated orally with the highest dose of NCX 4016 (100 mg/kg), ASA (54 mg/kg), and vehicle (PEG 400; 2 ml/kg) are reported in Fig. 1 and Table 1. Baseline MAP values were in the same range in rats treated with vehicle, NCX 4016, and ASA. Following LCA occlusion the MAP values of the animals in the three experimental groups consistently and abruptly fell (peak effect at 5 min) and then progressively recovered within 30 min to levels of 95 to 100 mm Hg (Fig. 1). Cardiac mechanic parameters (LVP, LVDevP, heart rate, and PRI) of the animals treated with NCX 4016 and ASA, evaluated immediately before LCA occlusion (time 0 min), at the end of 30 min of LCA occlusion (time 30 min), and at the end of 120 min of LCA reperfusion (time 150 min) were not significantly different from those measured in sham-operated and vehicle-treated animals, indicating that, in spite of the surgical procedure, the circulatory support was well maintained (Table 1).

Arrhythmias and Mortality. In vehicle-treated rats, LCA occlusion and reperfusion caused consistent ventricular ectopic activity associated with a high degree of mortality (Table 2). During the 30 min of regional myocardial ischemia there were 1612 ± 186 VPBs and the incidence of VT and VF was 100 and 59%, respectively. The dysrhythmias in this period led to death in 7 of 29 rats (mortality rate 24%). Also during the first 10 min of reperfusion period the total number of VPBs was 287 ± 32 with an incidence of VT and VF of 45 and 21%, respectively, and with a mortality of 10%. In this group of 29 rats subjected to MI/R, 10 animals died (mortality rate 34%). In the groups of animals treated for 5 consecutive days with NCX 4016 (10, 30, and 100 mg/kg) a dose-dependent protection against dysrhythmias and mortality was obtained (Table 2). The protection was evident during both LCA occlusion and reperfusion periods. Particularly marked was the cardioprotection obtained with NCX 4016 when given at the dose of 100 mg/kg. In fact, during the 30 min of ischemia, this compound caused a 72% reduction of VPBs with a lower incidence of VT (36%) and VF (14%). All the 14 rats of this group survived throughout the length of the MI/R experiment.

Treatment of rats with ASA (54 mg/kg) brought about a certain degree of cardioprotection, which was evident during both ischemia and reperfusion periods. Compared with vehicle-treated animals, during the 30 min of LCA occlusion the total number of VPBs was reduced by 39% and the incidence of both VT (59%) and VF (35%) was less pronounced and associated with a 12% mortality. During the first 10 min of reperfusion dysrhythmias were less than those observed in vehicle-treated rats and mortality was 6%. Since the dose of 54 mg/kg ASA was equimolar to 100 mg/kg NCX 4016, the antidysrhythmic activity of ASA is significantly less than that observed with the highest dose of NCX 4016 although similar to that obtain with NCX 4016 at 30 mg/kg (Table 2).

Infarct Size. The results obtained from the evaluation of the infarct size in rats subjected to MI/R experiments are reported in Table 3. The mean value of the area at risk, expressed as percentage of the left ventricular wall, was similar in all animal groups studied. In rats that had received the vehicle, LCA occlusion for 30 min followed by 120 min of reperfusion resulted in an infarct size of 60.1 ± 2.6% of the area at risk. Treatment of animals with different doses of NCX 4016 caused a dose-dependent reduction of the infarct size compared with that obtained in vehicle-treated rats. Particularly marked was the cardioprotection obtained by 100 mg/kg NCX 4016; in this instance, the infarct size was limited to 22.7 ± 2.1% of the area at risk (P < 0.001 versus vehicle-treated rats). ASA given to the animals in a dose of 54 mg/kg was 2-fold less potent than equimolar dose of NCX 4016 (100 mg/kg) in reducing the infarct size (Table 3).

Cardiac MPO and Plasma CPK Activities. Results of cardiac MPO and plasma CPK enzymes related to all animal groups studied are reported in Table 4. The mean values of MPO activity obtained in the area not at risk of left ventricular wall were low and not significantly different among the various experimental groups (0.37 ± 0.03–0.42 ± 0.06 U/g of tissue). However, these values were increased 10.4-fold (P < 0.001) in the area at risk of vehicle-treated animals subjected to MI/R. This finding suggests neutrophil accumulation in jeopardized tissue, a phenomenon that is known to correlate well with the degree of damage to the myocardium (Mullane et al., 1985). Treatment of the rats with NCX 4016 at different doses (10, 30, and 100 mg/kg) caused dose-dependent inhibition of MPO activity in the area at risk of these hearts (Table 4). In the case of NCX 4016 given at 100 mg/kg the inhibitory effect on MPO activity was 76% (P < 0.001 versus vehicle-treated animals). In this respect, ASA given at 54 mg/kg reduced the MPO activity in the area at risk only by 28% (P < 0.05 versus vehicle-treated animals). The values of plasma CPK activity obtained immediately before LCA occlusion were in the same range for all experimental groups (Table 4). However, in vehicle-treated rats these values increased 3.5-fold at the end of 30 min of occlusion and 6.5-fold at the end of 120 min of reperfusion. The administration of 10, 30, and 100 mg/kg NCX 4016 caused a dose-dependent inhibition in plasma CPK activity and the effect was particularly evident when NCX 4016 was given at 100 mg/kg. In this instance, plasma CPK values increased only 1.3-fold and
almost 2-fold by the end of the occlusion and reperfusion period, respectively. In rats treated with 54 mg/kg ASA, plasma CPK activity was increased almost 5-fold at the end of the reperfusion period (Table 4).

**L-NAME Activity.** Results related to the relevance of endogenous NO and its supplementation with NCX 4016 in rats subjected to MI/R are reported in Table 5. In all animal groups studied values of body weight, heart weight, left ventricular weight, and the relative area at risk were not statistically different among them. In vehicle-treated rats the infarct size was 54.7 ± 2.0% of the area at risk associated with a 29% mortality. The administration of NCX 4016 (100 mg/kg) alone was effective in reducing the infarct size (P < 0.001 versus vehicle-treated animals); no rats died in this experimental group. Administration of L-NAME (10 mg/kg) to block NO synthase brought about a further worsening in terms of infarct size, which increased to 68.2 ± 4.5% when NCX 4016 (100 mg/kg) was given together with L-NAME (10 mg/kg), before subjecting animals to MI/R, the worsening effect determined by the inhibitor of NO synthase was reduced, from 68.2 ± 4.5% to 33.9 ± 2.3% of the area at risk (P < 0.01) associated with the death of only one rat. Animals treated with L-NAME showed a significant increase

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>0 min (Pre-Occlusion)</th>
<th>30 min (End-Occlusion)</th>
<th>150 min (End-Reperfusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVP (mm Hg)</td>
<td>LVDevP (mm Hg)</td>
<td>HR (beats/min)</td>
</tr>
<tr>
<td>Sham (n = 14)</td>
<td>134.7 ± 6.7</td>
<td>122.5 ± 5.8</td>
<td>206.8 ± 6.5</td>
</tr>
<tr>
<td>Vehicle + MI/R (n = 19)</td>
<td>132.3 ± 5.1</td>
<td>120.4 ± 5.6</td>
<td>204.6 ± 7.8</td>
</tr>
<tr>
<td>NCX + MI/R (n = 14)</td>
<td>133.7 ± 4.7</td>
<td>123.0 ± 7.2</td>
<td>205.3 ± 6.1</td>
</tr>
<tr>
<td>ASA + MI/R (n = 14)</td>
<td>136.1 ± 7.5</td>
<td>121.3 ± 6.4</td>
<td>205.9 ± 7.9</td>
</tr>
</tbody>
</table>

### Table 2

Effect of NCX 4016 (NCX) and ASA on the severity of arrhythmias induced by MI/R in the anesthetized rats

Vehicle (2 ml/kg PEG 400), NCX 4016 (NCX, 100 mg/kg), and ASA (54 mg/kg) were administered orally for 5 consecutive days. Data are expressed as mean ± S.E. In parentheses, number of experiments. Sham-operated animals were treated with vehicle.

<table>
<thead>
<tr>
<th>Arrhythmias during Occlusion</th>
<th>Total VPBs</th>
<th>VT</th>
<th>VF</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 14)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle + MI/R (n = 29)</td>
<td>1612 ± 186</td>
<td>100</td>
<td>59</td>
<td>24</td>
</tr>
<tr>
<td>NCX 10 + MI/R (n = 17)</td>
<td>1242 ± 93</td>
<td>82</td>
<td>41</td>
<td>18</td>
</tr>
<tr>
<td>NCX 30 + MI/R (n = 15)</td>
<td>901 ± 113**</td>
<td>60</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>NCX 100 + MI/R (n = 14)</td>
<td>459 ± 91***</td>
<td>36</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>ASA 54 + MI/R (n = 17)</td>
<td>983 ± 108*</td>
<td>59</td>
<td>35</td>
<td>12</td>
</tr>
</tbody>
</table>

### Table 3

Body weight (BW), heart weight/body weight ratio (BW/HW), left ventricular weight (LVW), area at risk (AAR), and infarct size in rats subjected to 30 min of coronary artery occlusion followed by 120 min of reperfusion

Vehicle (2 ml/kg PEG 400), NCX 4016 (NCX, 10, 30, and 100 mg/kg), and ASA (54 mg/kg) were administered orally for 5 consecutive days. Data are expressed as mean ± S.E. In parentheses, number of experiments. Sham-operated animals were treated with vehicle.

<table>
<thead>
<tr>
<th>Group</th>
<th>BW</th>
<th>HW/BW</th>
<th>LVW</th>
<th>AAR</th>
<th>Infarct Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 14)</td>
<td>306.6 ± 7.0</td>
<td>3.08 ± 0.08</td>
<td>509.2 ± 21.9</td>
<td>55.5 ± 1.7</td>
<td>60.1 ± 2.6</td>
</tr>
<tr>
<td>Vehicle + MI/R (n = 19)</td>
<td>307.3 ± 6.9</td>
<td>3.06 ± 0.06</td>
<td>519.3 ± 18.5</td>
<td>55.5 ± 1.7</td>
<td>60.1 ± 2.6</td>
</tr>
<tr>
<td>NCX 10 + MI/R (n = 13)</td>
<td>304.8 ± 4.8</td>
<td>3.06 ± 0.05</td>
<td>529.3 ± 27.1</td>
<td>53.3 ± 2.0</td>
<td>40.5 ± 2.1</td>
</tr>
<tr>
<td>NCX 30 + MI/R (n = 13)</td>
<td>308.8 ± 7.2</td>
<td>3.05 ± 0.03</td>
<td>510.6 ± 20.7</td>
<td>53.9 ± 2.7</td>
<td>34.5 ± 2.7**</td>
</tr>
<tr>
<td>NCX 100 + MI/R (n = 14)</td>
<td>311.0 ± 5.5</td>
<td>3.06 ± 0.06</td>
<td>521.7 ± 24.2</td>
<td>53.8 ± 1.7</td>
<td>22.7 ± 2.1***</td>
</tr>
<tr>
<td>ASA 54 + MI/R (n = 14)</td>
<td>305.2 ± 11.2</td>
<td>3.07 ± 0.05</td>
<td>515.2 ± 24.8</td>
<td>54.3 ± 2.9</td>
<td>43.9 ± 3.8**</td>
</tr>
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</table>

* P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle + MI/R group.
of basal MAP values ($P < 0.01$ versus vehicle-treated rats), which was antagonized when NCX 4016 administration was combined with l-NAME (Table 5).

**Plasma cGMP.** Treatment for 5 consecutive days with NCX 4016 (10, 30, and 100 mg/kg) caused a dose-dependent increase of plasma levels of cGMP, particularly marked with the higher dose of this compound because the plasma level of the nucleotide was 3-fold higher (29.10 ± 2.28 pmol/ml) than that of vehicle-treated rats (10.16 ± 0.95 pmol/ml) (Fig. 2).

### Discussion

These experiments clearly indicate that the nitroderivative of aspirin NCX 4016 can provide substantial cardioprotection when given orally for 5 consecutive days to rats subjected to the marked insult of 30 min of regional myocardial ischemia followed by 120 min of reperfusion. The beneficial effects conferred by this compound are evident in the prevention of cardiac and biochemical abnormalities observed in vehicle-treated rats. The occurrence of VPBs and the incidence of VT and VF were reduced in a dose-dependent way by the administration of NCX 4016, which correlated with the diminished mortality of rats in these experimental groups. The extension of the infarct size of the left ventricular wall of these animals was lessened proportionally to the dose used of NCX 4016 associated with a diminution of plasma CPK activity; reduction in MPO activity in the tissue of the area at risk suggested that neutrophil infiltration was restricted. However, other events can lead to enhanced MPO activity such as adherence of polymorphonuclear leukocytes to vascular endothelial cells. Relative to this point, Mullane et al. (1984) have demonstrated that extension of the infarct size is accompanied by neutrophil accumulation in the jeopardized tissues; they proposed that cardioprotection could be attained by suppressing neutrophil activation, thereby inhibiting biosynthesis of cardiotoxic autacoids.

Regarding the mechanism(s) involved in the cardioprotective action of NCX 4016 in rats, the dominant mechanism appears to be the ability to donate NO. This crucial point has been addressed in vitro (Wallace et al., 1995) in human platelets by measuring NO generation by chemiluminescence and in vivo (Takeuchi et al., 1998) in pylorus-ligated rats. For
the latter, NCX 4016 administration resulted in elevated levels of NO both in gastric contents and plasma. More recently nitrosyl-hemoglobin, a marker of NO release, has been detected by electroparamagnetic resonance analysis in plasma of rats treated orally with NCX 4016 (Aldini et al., 2000). Although the role of NO in the progression of myocardial infarction is controversial (Patel et al., 1993; Woolfson et al., 1995; Curtis and Pabla, 1997), the majority of studies support the idea of NO mimicry/supplementation that limit infarct size and production of dysrhythmias (Siegfried et al., 1992; Sato et al., 1995). In normal conditions, NO released by endothelial cells of coronary arteries exerts potent vasodilator actions, antiplatelet activity, and inhibition of neutrophil aggregation and adhesion. Johnson et al. (1991) have demonstrated that authentic NO, given at subsaturator concentration, significantly reduced myocardial necrosis in cats subjected to myocardial ischemia and reperfusion. This finding supports the concept that addition of NO reduces the infarct size since, in the pathogenesis of myocardial reperfusion injury, the decisive event is likely a diminished release of NO.

In line with this view are the results obtained with inhibition of NO synthase in the present study, namely, chronic treatment of the rats with l-NAME caused a notable augmentation of the infarct size associated with a remarkable increment of the mortality rate compared with that obtained in vehicle-treated animals. Moreover, supplementation of NO with NCX 4016 considerably lowered the worsening effect of l-NAME on MI/R damage. These findings are also consonant with the results obtained by Pernow and Wang (1999) in isolated rat hearts subjected to global ischemia followed by reperfusion. These authors reported that l-arginine, but not d-arginine, enhanced the recovery of myocardial performance and coronary flow, and reduced the area of no reflow and creatine kinase outflow. This protective effect of l-arginine has been ascribed to NO production by Ca²⁺-dependent/NO synthase in the heart.

Another point of interest emerging from the present experiments is the increased levels of cGMP in plasma of rats treated with NCX 4016. Cyclic GMP, formed from activation of soluble guanylate cyclase, is a cellular second messenger that mediates vasodilation to a variety of drugs and endogenous substances such as atrial natriuretic factor (Luscher, 1991) and nitroso agents (Wood and Ignarro, 1987). Kita et al. (1994) reported a close correlation between the cardioprotective effect of FK409, a spontaneous NO releaser, with an increase in plasma cGMP and claimed that this event may serve as an indicator of the beneficial effect of spontaneous NO-releasing drugs. A possible explanation of the elevation of plasma cGMP may involve activation of platelet guanylate cyclase, especially because NCX 4016 had been administered to the animals for 5 consecutive days. In fact, it has been shown that the NCX 4016, but not ASA, when incubated with platelets significantly elevated cGMP levels in parallel with the release of NO, suggesting that NCX 4016 is likely metabolized by platelets to yield NO (Del Soldato et al., 1999).

The salutary activity produced by NCX 4016 in the present experiments, therefore, seems to derive primarily from increased availability of NO to counterbalance the marked decrease of its generation from the coronary endothelium damaged by ischemia and reperfusion. This interpretation is supported by the negative impact of L-NAME treatment on infarct size and survival as well as by blunting of the beneficial effects of NCX 4016 by concurrent treatment with L-NAME (Table 5). Furthermore, treatment with ASA has a much lesser effect on infarct size, incidence of arrhythmias, and survival than equimolar dose of NCX 4016 (Tables 2 and 3), suggesting that the NO donor function of NCX 4016 is the major determinant of its beneficial effects (Tables 2 and 3). Additionally, the ascending benefit of NCX 4016, which is related to dose (Table 2), can be correlated with increasing plasma levels of cGMP that reflect the NO donor function of NCX 4016 (Fig. 2).

The ASA moiety released by NCX 4016 appears to play a minor role in the overall cardioprotective mechanism(s) of this compound in view of the fact that ASA was much less effective than an equimolar dose of NCX 4016 in the present MI/R experiments. The number of VPBs, the incidence of VT and VF, the mortality rate, and the infarct size in animals treated with ASA were only slightly reduced compared with NCX 4016-treated rats. However, NCX 4016 may cause effects other than cyclooxygenase impairment; for example, inhibition of T-lymphocyte activation, cytokine release, apoptosis stimulation (Fiorucci et al., 1999), and nuclear factor-κB activation (Minto et al., 1997) also have been reported for this drug. Therefore, even if NO donation by NCX 4016 assumes a predominant function in the mode of action of this compound, as appears to be the case, a contribution of the ASA moiety to the beneficial effects of NCX 4016 must be considered.

In conclusion, these results indicate that NCX 4016 has a greater cardioprotective activity than that shown by ASA in rat MI/R experiments. These beneficial effects of NCX 4016 probably derive mainly from the NO moiety, which in turn interferes with and modulates a variety cellular events leading to inflammation and obstruction of the coronary microcirculation associated with arrhythmias and myocardial tissue necrosis. The lack of gastrointestinal side effects of NCX 4016 in preclinical studies (Elliott et al., 1995; Wallace and Granger, 1996) opens the possibility for a broad range of therapeutic applications in cardiovascular diseases, particularly in treatment of myocardial ischemia and infarct progression.

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References


A Nitroderivative of Aspirin Reduces Myocardial Infarct Size


