Myocardial protection by the nitroderivative of aspirin, NCX 4016: *in vitro* and *in vivo* experiments in the rabbit

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Key words:
Rabbit; Myocardial ischemia; Myocardial infarction; Nitroderivative of aspirin; NCX 4016; Aspirin.

Background. **A new family of nitroderivatives of conventional non-steroidal anti-inflammatory drugs capable of releasing nitric oxide has been synthesized. Among these compounds, a nitroderivative of aspirin (NCX 4016), which displays antiplatelet and vasodilating activities, appears to have clinical potential in cardiac pathology related to coronary insufficiency.**

Methods. **In this study the beneficial effects of NCX 4016 and aspirin were evaluated *in vitro* in a model of myocardial ischemia-reperfusion of the rabbit and *in vivo* in a model of acute myocardial infarction of the same animal species.**

Results. **The NCX 4016 (from 1×10^{-5} M to 3×10^{-4} M) caused dose-dependent cardiac protection in isolated rabbit hearts subjected to low flow ischemia-reperfusion. Inhibition of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) generation and proportional reduction of creatine kinase (CK) activity at reperfusion was observed. Aspirin (1×10^{-4} M) markedly worsened the post-ischemic ventricular dysfunction and this event was paralleled by a 63% increase in CK activity and abolition of 6-keto-$PGF_{1\alpha}$ formation. Perfusion of the hearts with N^G-monomethyl-L-arginine (1×10^{-5} M) worsened the ischemia-reperfusion damage in perfused hearts. This event was prevented by prior treatment with NCX 4016 (1×10^{-4} M) but not with aspirin (1×10^{-4} M). Ligation of the first antero-lateral branch of the left coronary artery in rabbits resulted in acute myocardial infarction with a mortality rate of 60% at 24 hours. NCX 4016 (0.5 mg/kg/min for 2 hours) significantly reduced the mortality rate by 10%, protected the rabbits against electrocardiogram derangement and almost abolished CK activity in plasma and myeloperoxidase activity in cardiac tissue. Aspirin was devoid of any protective activity.**

Conclusions. **In the rabbit NCX 4016 appears to exert a relevant cardioprotection likely mediated by nitric oxide donation. These results suggest that this nitroderivative of aspirin may lead to innovative therapy in myocardial ischemia and infarction.**

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Introduction

Recently, a new family of nitroderivatives of conventional non-steroidal anti-inflammatory drugs has been synthesized by the addition of a moiety capable of releasing nitric oxide (NO). This was accomplished with the aim of reducing gastric and renal side effects due to inhibition of prostaglandin synthesis resulting from constitutive cycloxygenase blockade[1,2].

Among these compounds, a nitroderivative of aspirin, NCX 4016 (2-acetoxy-benzoate 2-[1-nitroxy-methyl]-phenyl ester) which is characterized by antiplatelet and vasodilating activities, appears to be of potential use in the prevention of myocardial infarction in patients with coronary insufficiency[3]. The results reported in the literature support the hypothesis of a dual mechanism of action for NO-non-steroidal anti-inflammatory drugs involving inhibition of cycloxygenase and release of NO or NO complexes as active intermediates acting on guanilate cyclase in both platelets and vascular smooth muscle cells[4-6]. The continuous release of NO, which maintains a dilator tone, requires a functional endothelium. Any failure in the function of endothelial cells affects vascular thromboresistance and leads to the development of a variety of vascular diseases[7]. Therefore, the pharmacological effects of NO donor compounds play an important role in preserving the integrity of

organ function, and evidence is accumulating that this is particularly the case in the coronary and splanchnic vasculature. In these vascular beds, endothelial dysfunction, together with loss of NO and prostacyclin (PGI_2) production, is closely associated with damage of myocardial and splanchnic functions[8]. Therefore, NO donors represent likely candidates for the prevention and treatment of coronary vasospasm and myocardial ischemia[9,10].

All this information prompted us to investigate the protecting activity of the new nitroderivative of aspirin, NCX 4016, against post-ischemic ventricular dysfunction in perfused rabbit hearts. Furthermore, the ability of this compound to reduce the mortality rate caused in rabbits by 24 hour permanent ligature of the first anterolateral branch of the left coronary artery has been studied in comparison with aspirin.

Methods

All experimental procedures, both *in vitro* and *in vivo*, were approved by the Animal Care Committee of the University of Milan (Italy) 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).

***In vitro* experiments.** *Ischemia-reperfusion in the isolated rabbit heart.* Male New Zealand White rabbits (BMG-Allevamento, Cividate al Piano-BG, Italy) weighing 2.0-2.2 kg were used for these experiments. The hearts were excised and perfused retrogradely at 37 C through the aorta as previously described by Henry et al.[11] and slightly modified by Berti et al.[12]. The perfusion medium (Krebs Henseleit) contained (in mM): NaCl 118, KCl 2.8, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. The pH of the perfusate was 7.4 after a period of equilibration with a 5% CO_2 and 95% O_2 gas mixture, and the rate of perfusion was maintained at 20 ml/min with a roller pump (Minipuls 3, Gilson, Villiers-Le Bel, France). Coronary perfusion pressure (CP P) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett Packard, Waltham, MA, USA) connected with a Hewlett Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter (with a small latex balloon on the top) inserted into the left ventricular cavity. The balloon was filled slowly with saline solution until left ventricular end -diastolic pressure (LVEDP) stabilized in the range of 4-6 mmHg. Left ventricular developed pressure (LVDP; peak left ventricular systolic pressure minus LVEDP) was also evaluated. The hearts were electrically paced at a frequency of 180 b/min with rectangular impulses (1 ms duration, voltage 10% above threshold) by a Grass stimulator (model S-88; Grass Instr., Quincy, MA, USA). Ischemia was induced by reducing the flow rate from 20 to 1 ml/min for 40 min (ischemic period). A normal flow rate (20 ml/min) was then restored and the perfusion was continued for another 20 min (reperfusion period). After preliminary experiments, different molar concentrations of the compounds under investigation were selected and tested in groups of 8-10 hearts each. In particular, NCX 4016 (from 1×10^{-5} M to 3×10^{-4} M) and aspirin (from 1×10^{-6} M to 1×10^{-4} M) were perfused through the hearts for a period of 20 min before reduction of coronary flow. In these experiments, PGI_2 generation was measured in the heart effluent as its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) according to the enzyme immunoassay method (detection limit 3 pg/ml) described by Pradelles et al.[13]. Particularly, cardiac effluents were collected for 5 min immediately before flow reduction and during the first 10 min of reperfusion. The rate of formation of this arachidonic acid metabolite was expressed in ng/min.

Vasopressor activity of angiotensin II and 6-keto-prostaglandin $F_{1\alpha}$ formation. In the same heart preparations, used for ischemia-reperfusion experiments, the vasopressor activity of angiotensin II was studied during the pre-ischemic and reperfusion periods and the activity of the autacoid on CP P was correlated with the rate of 6-keto-$PGF_{1\alpha}$ released in heart effluents. Angiotensin II was injected through the heart at the dose of 1 µg as a bolus since at this concentration this peptide does not cause mechanical changes in the perfused hearts. In order to establish the amount of 6-keto-$PGF_{1\alpha}$ released during the constrictive effect of angiotensin II, cardiac effluents were collected for 2 min starting immediately after the injection of the peptide.

Nitric oxide-synthase inhibition in the rabbit heart subjected to ischemia-reperfusion. In another series of experiments, the importance of the constitutive NO-synthase activity in the evolution of the ischemic process was studied. In these hearts, the endogenous NO generation was inhibited by infusing N^G-monomethyl-L-arginine (L-NMMA) 1×10^{-5} M for 10 min during the pre-ischemic period. In a previous paper[14] it has been proved that at this regimen L-NMMA causes an increase in CP P with aggravation of post-ischemic ventricular dysfunction. Therefore, the ability of NCX 4016 and aspirin to interfere with L-NMMA worsening activity on ischemia-reperfusion damage was investigated. Both NCX 4016 and aspirin were given through the hearts at the concentration of 1×10^{-4} M for 20 min just before L-NMMA treatment and changes in LVEDP, LVDP and CP P were recorded.

Creatine kinase determination. The heart effluents were collected every 150 s in an ice-cooled beaker before flow reduction and during reperfusion and the activity of creatine kinase (CK) was evaluated according to the method of Bergmeyer et al.[15]. The amount of the enzyme was determined on a Lambda16 spectrophotometer (Perkin Elmer Italy, Monza-MI, Italy) and expressed as mU/min/g⁻ wet tissue.

***In vivo* experiments.** *Animal preparation*. Male New Zealand White rabbits (BMG-Allevamento, Cividate al Piano-BG, Italy) weighing 2.5-2.7 kg were used in these experiments. The animals were anesthetized with 30 mg/kg i.v. of thiopentone sodium injected into the left marginal ear vein and the anesthesia was maintained with supplementary doses of thiopentone sodium as required. Under artificial respiration with air (rate 40-42 strokes/min; tidal volume 20-22 ml), the left side of the thorax was opened (2-3 cm) through the fourth-fifth intercostal space, to permit free access to the left ventricular myocardium. The first antero-lateral branch of the left coronary artery (1 cm distal from its origin) was ligated with a silk 6.0 suture, armed with an atraumatic needle. In contrast to other species, the left coronary artery of the rabbit supplies most of the left ventricular myocardium[16]. Care was taken not to include any veins draining blood from this area.

Experimental design. Rabbits were randomly divided into four experimental groups of 10 animals each. One group was subjected to sham-operation, including pericardiotomy without ligation of the left coronary artery. Of the three remaining groups of rabbits with permanent left coronary artery ligature (CAL), the first was considered as control, the second was treated with NCX 4016 and the third was treated with aspirin. Treatment was performed according to the following regimen: sham-operation and CAL groups were infused intravenously with vehicle, whereas NCX 4016 + CAL and aspirin + CAL groups were infused intravenously with NCX 4016 or aspirin, respectively, at the dose of 0.5 mg/kg/min for 2 hours. All the rabbits were infused, through the left marginal ear vein, at the flow rate of 50 µl/kg/min and this treatment started immediately after anesthesia and lasted for 2 hours. The second hour of infusion initiated at the beginning of the surgery procedure (time 0).

Electrocardiographic recording. The electrocardiogram (ECG) was performed according to the conventional method of Einthoven, setting the subdermal platinum electrodes on lead II and connecting them to the recording apparatus Cardioline Delta-1 (Remco Italia, Milan, Italy). The ECG was recorded immediately at the end of anesthesia (time 0) and repeated at 0.5, 1, 2 and 24 hours after CAL.

Creatine kinase assay in plasma. CK activity was determined in plasma obtained using 1 ml of peripheral venous blood drawn from the marginal ear vein, immediately before the ECG recordings. Plasma was processed for CK activity using a commercially available kit, following the spectrophotometric method described by Rosalki[17]. CK activity was expressed in U/l.

Myeloperoxidase assay in the rabbit myocardium. The hearts obtained from the rabbits surviving at 24 hours belonging to the four experimental groups were used for determination of myeloperoxidase activity. Gross morphological examination revealed the presence of an area of coagulation necrosis of the myocardium with clear scar tissue in the left ventricular wall. Approximately 800 mg (wet tissue) pertaining to the necrotic area were dissected, frozen in liquid nitrogen, pulverized and used for determination of myeloperoxidase activity[18] which was expressed as U/g tissue.

Data analysis. The data were analyzed using both analysis of variance (ANOVA) for multiple group comparisons and the ANOVA for repeated measures. Differences between individual group means were tested using Student s t test with the Bonferroni correction with $p < 0.05$ considered significant. In all tables and figures, results are expressed as mean – SEM. For *in vitro* study, the area under the curve was assessed by using a computerized program Microcal Origin (Microcal Software Inc., Northampton, MA, USA). For *in vivo* study, analysis of mortality rate was carried out with the analysis of log-likelihood for categorical data and either Pearson or likelihood ratio χ^2 tests[19].

Drugs. The following drugs were used: NCX 4016 (NicOx S.A., Valbonne, Sophia Antipolis, France); aspirin, L-NMMA, L-arginine, human myeloperoxidase (Sigma, St. Louis, MO, USA); thiopentone sodium (Abbott, Campoverde, Latina, Italy), kit for 6-keto-$PGF_{1\alpha}$ determination (Amersham Italia, Milan, Italy); kit for CK determination (Boehringer-Mannheim Italia, Milan, Italy). NCX 4016 and aspirin were dissolved in dimethylsulfoxide at 0.5 M stock concentration and were further diluted with Krebs Henseleit (*in vitro* experiments) or saline (*in vivo* experiments) as necessary.

Results

***In vitro* experiments**. *Ischemia-reperfusion in the isolated rabbit heart*. When the rate of perfusion of electrically paced isovolumic left rabbit heart preparations was reduced from 20 to 1 ml/min for 40 min, LVEDP values increased progressively indicating that, after standstill, an ischemic process was occurring. In fact, at reper-

fusion ventricular function was impaired, LVDP values being significantly reduced and CP P considerably increased above baseline (Figs. 1-3). Furthermore, a consistent increase in CK activity released in the cardiac

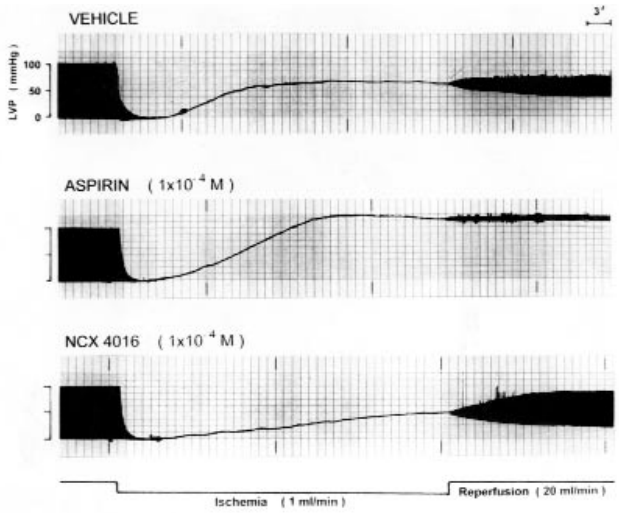

Figure 1. *Perfusion experiments with paced left rabbit heart preparations. Whereas aspirin does not have cardioprotective activity, but merely aggravates the ischemic damage, NCX 4016 reduces ventricular contracture and improves post-ischemic myocardial contractility. LVP = left ventricular pressure.*

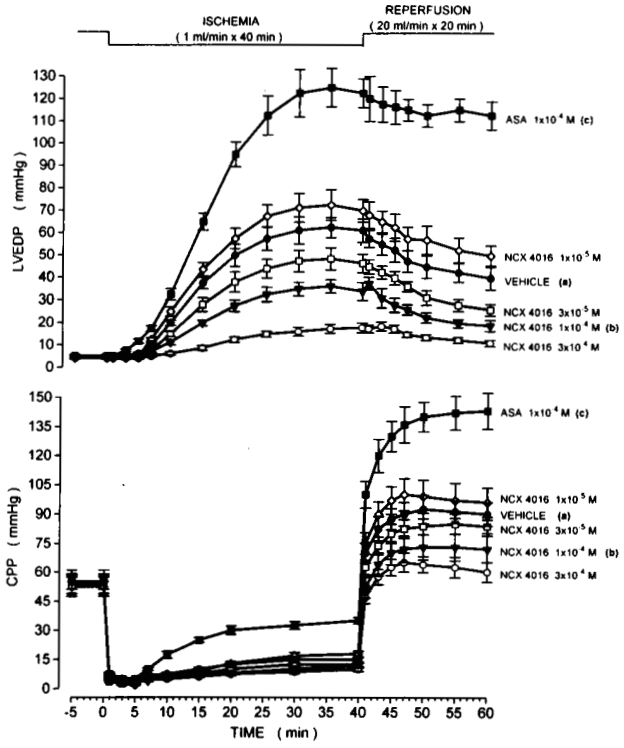

Figure 2. *Effects of NCX 4016 and aspirin (ASA) in perfused rabbit hearts subjected to ischemia-reperfusion: trend of left ventricular end-diastolic pressure (LVEDP) and coronary perfusion pressure (CPP). The data are mean – SEM of 8-10 different heart preparations per group. Statistical differences among the various curves related to LVEDP were evaluated as area under the curve according to the trapezoid method (in ordinate LVEDP in mmHg; in abscissa time from 0 to 60 min). The area under the curve values are: a = 2577 – 195; b = 1426 – 98; c = 5431 – 418. a vs b, p < 0.01; a vs c, p < 0.001.*

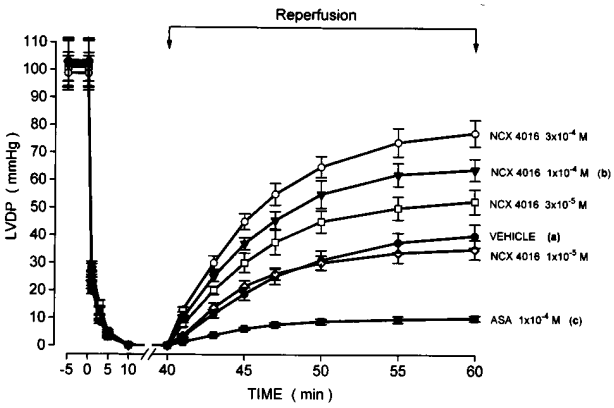

Figure 3. *Effects of NCX 4016 and aspirin (ASA) on left ventricular developed pressure (LVDP) in perfused rabbit hearts subjected to ischemia-reperfusion. The data are mean – SEM of 8-10 different heart preparations per group. Statistical differences among the various curves related to LVDP were evaluated as area under the curve according to the trapezoid method (in ordinate, LVDP in mmHg; in abscissa, time from 40 to 60 min). The area under the curve values are: a = 539 – 47; b = 944 – 65; c = 147 – 17. a vs b and c, p < 0.001.*

outflow was measured (Fig. 4). When the hearts were perfused for 20 min with graded concentrations of NCX 4016 (from 1 × 10^{-5} M to 3 × 10^{-4} M) in the pre-ischemic period, a dose-dependent myocardial protection against mechanical changes due to ischemia-reperfusion was recorded. In fact the characteristic ventricular contracture observed during the 40 min of ischemia was reduced and this event favored a better recovery of LVDP at reperfusion. At the same time, CP P and CK activity diminished as a function of the dose of NCX 4016 used (Figs. 1-4). In clear contrast with the results reported above, the perfusion of the hearts with aspirin (1 × 10^{-4} M) produced severe worsening of myocardial ischemic damage. In fact, LVEDP values were remark-

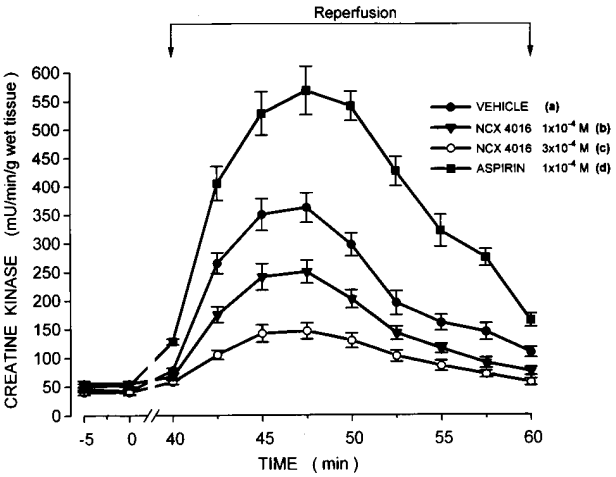

Figure 4. *Kinetic profile of creatine kinase activity released in perfusates of rabbit hearts subjected to ischemia-reperfusion. The data are mean – SEM of 8-10 different heart preparations per group. Statistical differences among the various curves related to creatine kinase activity were evaluated as area under the curve according to the trapezoid method (in ordinate, creatine kinase in mmHg; in abscissa, time from 40 to 60 min). The area under the curve values are: a = 3665 – 194; b = 2215 – 97; c = 1090 – 82; d = 7023 – 484. a vs b, p < 0.01; a vs c and d, p < 0.001.*

ably increased as compared with those of untreated preparations, and this phenomenon was associated with a marked depression of LVDP and an increase in CP P at reperfusion (Figs. 1-3). The severity of post-ischemic ventricular dysfunction was also marked by a notable increase of CK activity in heart effluents (Fig. 4).

In this set of experiments the rate of production of PGI_2 was measured as 6-keto-$PGF_{1\alpha}$ released into the cardiac effluents before ischemia and during reperfusion. The results obtained clearly indicate that NCX 4016 and aspirin inhibit the basal release of 6-keto-$PGF_{1\alpha}$. In particular, at the higher concentration used NCX 4016 and aspirin caused maximal inhibitory activity, since 6-keto-$PGF_{1\alpha}$ was undetectable (< 3 pg/ml) in heart effluents (Table I).

Vasopressor activity of angiotensin II and 6-keto-prostaglandin $F_{1\alpha}$ formation. When angiotensin II was injected through the hearts (1 μg as a bolus) during the pre-ischemic period, constriction of coronary vascular bed occurred and CP P increased 12.5 – 1.1 mmHg over the basal values (54 – 4 mmHg). This event was accompanied with a release in the effluents of 6-keto-$PGF_{1\alpha}$ (1.27 – 0.08 ng/min) 2-fold higher than basal values (2.68 – 0.16 ng/min), suggesting that the relaxing mechanism of vascular endothelium was operating (Fig. 5). In hearts perfused with NCX 4016 at concentrations of 1×10^{-4} M and 3×10^{-4} M, the vasopressor activity of angiotensin II was inhibited in a concentration-dependent manner in spite of a decreased PGI_2 metabolite generation. Moreover, when the formation of 6-keto-$PGF_{1\alpha}$ was abolished by NCX 4016 (3×10^{-4} M), the activity of angiotensin II on CP P was reduced to 45% ($p < 0.01$) as compared to that observed in untreated hearts. On the contrary, in aspirin-treated hearts (1×10^{-4} M), the vasopressor activity of angiotensin II was increased to 63% ($p < 0.01$) as compared to that observed in untreated preparations. The hyperreactivity of the coronary vascular bed to this autacoid was concomitant with suppression of 6-keto-$PGF_{1\alpha}$ generation since this PGI_2 metabolite was undetectable (< 3 pg/ml) in heart perfusates (Fig. 5).

Nitric oxide-synthase inhibition in the rabbit heart subjected to ischemia-reperfusion. Perfusion of the hearts with L-NMMA (1×10^{-4} M) for 10 min before flow reduction exacerbated ventricular dysfunction compared with untreated preparations. In fact, at the end of the ischemic period, LVEDP increased from 4.5 – 0.2 to 103 – 5 mmHg (1.7-fold over the corresponding values of untreated hearts, $p < 0.001$), and after 20 min of reperfusion the values were still in the range of 95 – 6 mmHg; in this instance, mechanical activity was severely impaired and associated with cardiac rhythm disturbance (data not shown). Furthermore during treatment of the hearts with L-NMMA, CP P rose (from 50 – 4 to 105 – 8 mmHg, $p < 0.001$) and at the end of reperfusion this value was in the range of 131 – 9 mmHg, that is 1.5 fold

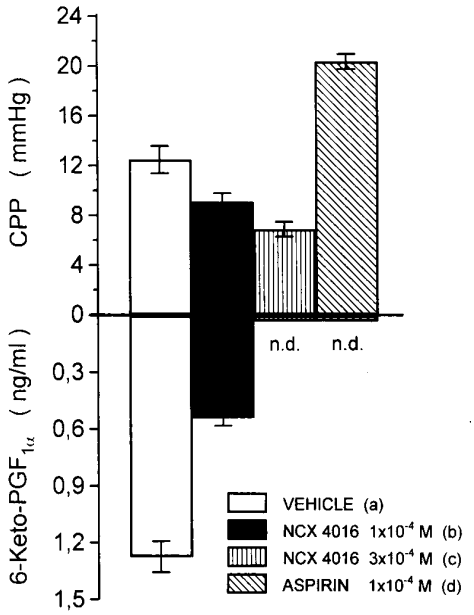

Figure 5. *Effects of angiotensin II (1 μg as a bolus) on coronary perfusion pressure (CPP) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) release in cardiac outflow of rabbit hearts perfused for 20 min in the pre-ischemic period with NCX 4016 or aspirin. Columns represent mean – SEM of 8-10 different heart preparations per group. The basal values for CPP and 6-keto-$PGF_{1\alpha}$ release were 54 – 4 mmHg and 2.68 – 0.16 ng/min, respectively. ND = not detectable (< 3 pg/ml). Statistical differences for CPP: a vs b, $p < 0.05$; a vs c and d, $p < 0.01$. Statistical differences for 6-keto-$PGF_{1\alpha}$: a vs b, $p < 0.01$.*

Table I. Rate of formation 6-keto-prostaglandin $F_{1\alpha}$ in perfused rabbit hearts subjected to low flow ischemia and reperfusion.

Treatment	6-keto-prostaglandin $F_{1\alpha}$ (ng/min)	
	Pre-ischemia	Reperfusion*
Vehicle	2.72 – 0.21	8.72 – 0.82
NCX 4016 (3×10^{-5} M)	1.84 – 0.15 (32.3)**	5.2 – 0.51 (42.4)**
NCX 4016 (1×10^{-4} M)	0.97 – 0.18 (64.3)***	2.48 – 0.32 (71.6)***
NCX 4016 (3×10^{-4} M)	ND	ND
Aspirin (1×10^{-4} M)	ND	ND

Data are mean – SEM of 8-10 different heart preparations per group. Drugs were infused for 20 min before flow rate reduction. ND = not detectable (detection limit 3 pg/ml). * data refer to the first 10 min of reperfusion. In brackets, percent of inhibition; ** $p < 0.01$ and *** $p < 0.01$ vs vehicle-treated hearts.

($p < 0.01$) over the corresponding values of untreated preparations (data not shown).

These mechanical changes (reduced compliance) of the hearts resulted in severely depressed left ventricular function during reperfusion. In fact, at the end of this period LVDP was confined to 10.5 – 2.2 mmHg (74% reduction, $p < 0.01$ vs pre-ischemic values) (Fig. 6). When the treatment with L-NMMA was preceded by a 20 min perfusion with 1×10^{-4} M of NCX 4016, a clear-cut protection against the marked ventricular contracture caused by the NO-synthase inhibitor was observed. As a consequence, in these hearts a significant amelioration of mechanical activity and regular pacing during reperfusion was recorded. In fact, LVDP recovered 65% ($p < 0.001$) of the baseline contractility, whereas the related values of untreated hearts only improved by 39% (Fig. 6). The 20 min perfusion of hearts with 1×10^{-4} M of aspirin in the presence of L-NMMA did not protect the hearts from ischemia-reperfusion damage. In this context, at the end of reperfusion LVDP (8 – 1.4 mmHg) was not different from that obtained in hearts treated only with L-NMMA (10.5 – 2.2 mmHg) (Fig. 6).

***In vivo* experiments.** *Mortality rate in rabbits with permanent ligature of the left anterior coronary artery*. CAL in the rabbit resulted in acute myocardial infarction of the left ventricular wall marked by a mortality rate of 60% at 24 hours ($p < 0.01$ as compared with sham-operated animals). In contrast, the intravenous infusion of rabbits with NCX 4016 (0.5 mg/kg/min for 2 hours) caused a clear myocardial protection with a remarkable reduction in the mortality rate, which at 24 hours was only 10% ($p < 0.01$ as compared with untreated rabbits with CAL). In the group of animals infused intravenously with aspirin (0.5 mg/kg/min for 2 hours) the mortality rate at 24 hours (50%) was in the range of that found in untreated rabbits with CAL. All these results are reported in table II.

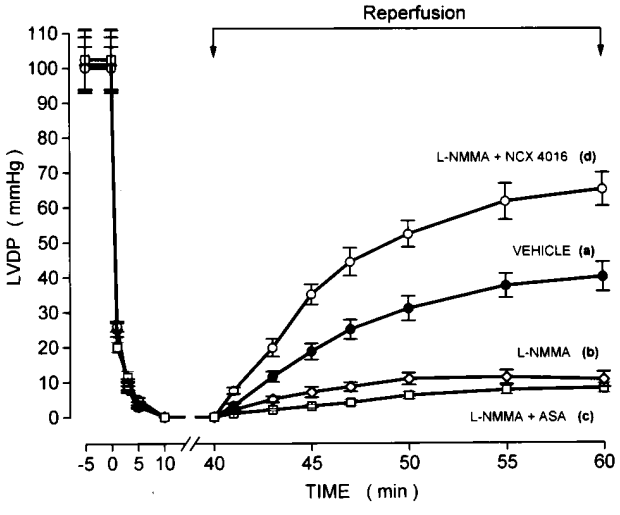

Figure 6. *Trend of left ventricular developed pressure (LVDP) in rabbit hearts subjected to ischemia-reperfusion. Treatments were performed during the pre-ischemic period. NCX 4016 (1×10^{-4} M) and aspirin (ASA, 1×10^{-4} M) were given through the hearts for 20 min followed by 10 min infusion with N^G-monomethyl-L-arginine (L-NMMA, 1×10^{-5} M). The data are mean – SEM of 8-10 different heart preparations per group. Statistical differences among the various curves were evaluated as area under the curve according to the trapezoid method (in ordinate, LVDP in mmHg; in abscissa, time from 40 to 60 min). The area under the curve values are: a = 539 – 47; b = 174 – 28; c = 103 – 14; d = 913 – 64. a vs b, c and d, $p < 0.001$.*

Table II. Mortality rate at 24 hours in rabbits with left coronary artery ligature.

Group	No. rabbits	Alive/dead	Mortality (%)
SH	10	10/0	0
CAL	10	4/6	60
NCX 4016 + CAL	10	9/1	10
ASA + CAL	10	5/5	50

CAL = animals with left coronary artery ligation and infused with vehicle; NCX 4016 + CAL and ASA + CAL = animals with left coronary artery ligation and infused with NCX 4016 or aspirin (0.5 mg/kg/min for 2 hours) respectively; SH = sham-operated animals infused with vehicle. All the rabbits were infused at the flow rate of 50 µl/min and this treatment started immediately after anesthesia and lasted for 2 hours. The second hour of infusion started at the beginning of the surgical procedure. Statistical differences: SH vs CAL and ASA + CAL, $p < 0.01$; NCX 4016 + CAL vs CAL and ASA + CAL, $p < 0.01$.

Plasma creatine kinase and myocardial myeloperoxidase activities. The results obtained from the analysis of CK activity are in line with the findings above. In fact, the permanent ligature of the left coronary artery in these animals caused a 11.5-fold increase in plasma CK activity at 24 hours ($p < 0.001$ as compared with sham-operated animals), whereas in the group of NCX 4016-treated rabbits the activity of this enzyme was increased only 1.8 fold ($p > 0.05$). Moreover, the amount of CK activity in the plasma of rabbits infused with aspirin was in the range of untreated animals with CAL, thus proving that aspirin was devoid of myocardial protective activity (Table III).

Several studies have shown that polymorphonuclear leukocyte migration in venules and at the periphery of an infarct after permanent coronary occlusion and myeloperoxidase activity are considered to be a reliable index of tissue neutrophil infiltration. As shown in table IV, the results obtained in these experiments clearly demonstrate that the myeloperoxidase activity, measured in the heart tissue corresponding to the central area of the ligature of the left anterior descending coronary artery of the survived animals at 24 hours after CAL, increased 3.6 fold when compared with sham-operated animals (from 3.24 – 0.21 to 11.78 – 0.74 U/g tissue, $p < 0.001$). In the rabbits infused with NCX 4016 the lev-

Table III. Creatine kinase activity measured in plasma immediately before (0 min) and 24 hours after left coronary artery ligation in rabbits.

Group	Creatine kinase (U/l)		Difference	Inhibition (%)
	0 min	24 hours		
SH	392 – 21 (10)	458 – 24 (10)	66	
CAL	374 – 18 (10)	1132 – 75 (4)	758	
NCX 4016 + CAL	385 – 14 (10)	502 – 36 (9)	117	92.6
ASA + CAL	358 – 19 (10)	995 – 62 (5)	637	7.9

Data are mean – SEM. In brackets the number of rabbits. Legend for group treatments as in table II. Statistical differences: SH vs CAL and ASA + CAL, $p < 0.001$; NCX 4016 + CAL vs CAL and ASA + CAL, $p < 0.001$.

Table IV. Myeloperoxidase activity measured in the heart tissue (ventricular wall) of the survived rabbits 24 hours after left coronary artery ligation.

Group	Myeloperoxidase (U/g tissue)	Difference	Inhibition (%)
SH	3.24 – 0.21 (10)		
CAL	11.78 – 0.74 (4)	8.54	
NCX 4016 + CAL	4.81 – 0.32 (9)	1.57	81.6
ASA + CAL	10.65 – 0.89 (5)	7.41	13.2

Data are mean – SEM. In brackets the number of rabbits. Legend for group treatments as in table II. Statistical differences: SH vs CAL and ASA + CAL, $p < 0.001$; SH vs NCX 4016 + CAL, $p < 0.05$; NCX 4016 + CAL vs CAL and ASA + CAL, $p < 0.001$.

el of myeloperoxidase in the myocardial tissue was reduced by 81.6% ($p < 0.001$ as compared with untreated animals with CAL), whereas the inhibitory effect of aspirin on this enzyme activity was only reduced by 13.2% ($p > 0.05$ vs untreated animals with CAL) (Table IV).

Electrocardiographic recordings. In these experiments the ECG was recorded in lead II. Normal sinus rhythm was present in all the rabbits before CAL, without significant differences in heart rate values, duration of the QRS complex and ST segment patterns. In sham-operated rabbits, serial ECG recordings showed no changes in ventricular repolarization, QRS morphology and heart rate during the recovery period of 24 hours. The ECG tracings of the rabbits after CAL showed typical evolutionary changes in acute transmural myocardial infarction characterized by upward or downward shifts of ST segment. The degree of ST segment and T wave abnormalities did not vary greatly between untreated rabbits with CAL and aspirin-treated rabbits with CAL. In some cases severe arrhythmias occurred resembling ventricular flutter and fibrillation. NCX 4016 clearly protected the myocardium by preventing cardiac ischemia or reducing infarct size. Indeed only 2 rabbits had slight ST segment shift consistent with infarction of the left ventricle. No significant repolarization abnormalities were found in the remaining 7 rabbits. The trend of these experiments is depicted in figures 7 and 8.

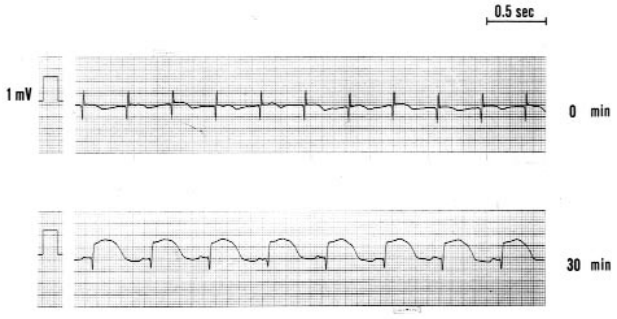

Figure 7. *Rabbit no. 29 infused with vehicle (50 l/kg-/min for 2 hours). The ECG tracings (lead II) were recorded before (0 min) and after left coronary artery ligation. At 30 min the ECG pattern reveals ST segment elevation of > 0.5 mV in the lead facing the area of infarction. The striking elevation obscures the downstroke of the R wave.*

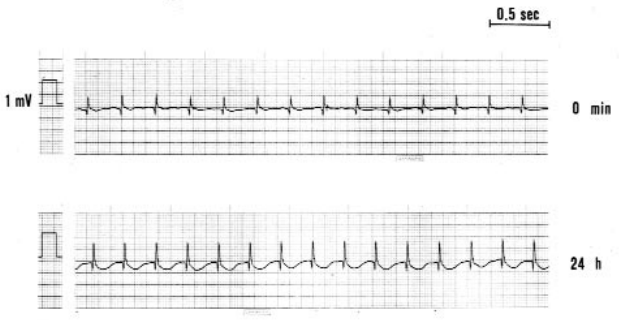

Figure 8. *Rabbit no. 39 infused with NCX 4016 (0.5 mg/kg-/min for 2 hours). In ECG tracings (lead II), no ventricular repolarization differences are noted before (0 min) and after left coronary artery ligation (24 hours).*

Discussion

The present results clearly demonstrate that the new nitroderivative of aspirin, NCX 4016, counteracts cardiac mechanical abnormalities induced by ischemia in perfused rabbit hearts and remarkably reduces the mortality rate in rabbits with permanent CAL.

In isolated rabbit heart preparations NCX 4016 reduced LVEDP values in a dose-related manner and, at reperfusion, significantly improved the recovery of heart contractility. These beneficial effects, accompanied by a strong inhibition of CK activity in cardiac effluents, were present in spite of inhibition of PGI_2 formation. In line with the present findings, it has already been reported that aspirin aggravates myocardial ischemia in perfused rabbit hearts[12]. This was attributed to the removal of cytoprotective prostaglandins, namely PGI_2. A role of this autacoid in the preservation of ischemic cardiac damage is well known and PGI_2 mimetics or releasers were found to be very effective in this respect[20-23].

However, it is reasonable to speculate that the beneficial effects of NCX 4016 may involve donation of NO[24], which in turn may neutralize the reduction in formation of cytoprotective prostaglandins in cardiac tissue. Indeed, the evaluation of NO in the heart perfusates is mandatory and we have no direct evidence of this crucial event. However, NO release by NCX 4016 has already been observed *in vitro*[25] by measuring nitrite/nitrate concentration in platelet suspension and plasma[5]. The pharmacokinetics of nitroaspirin has also been evaluated and the results obtained revealed both salicylate- and NO-releasing moieties[4,26]. Furthermore, *in vivo* intragastric administration of NCX 4016 to pylorus-ligated rats resulted in elevated NO levels in both gastric content and plasma[27].

Indirect evidence in favor of the above hypothesis is supported by the fact that inhibition of cardiac NO-synthase with L-NMMA markedly enhanced the ischemia-reperfusion damage. However, to further substantiate that NO actually preserves the myocardium from ischemia damage, stimulation of NO synthesis in our experimental model with L-arginine is a more convenient approach and comparison of the beneficial effects of this aminoacid with those of NCX 4016 must be performed.

In view of the recent findings that exogenous NO exerts a direct relaxant myocardial effect independent of its vasodilator activity and without compromising systolic function[28,29], the anti-ischemic activity observed in rabbit hearts with NCX 4016 raises the question of its mechanism of action. According to Gergely[30] and Henry et al.[11], the accumulation of Ca^{++} in the mitochondrial fraction of cardiac myocytes and the increase of the undissociated crossbridges (actin-adenosine triphosphate-myosin complexes) are responsible for the cardiomechanical changes, such as incomplete or delayed myocardial relaxation and ventricular contraction typical of ischemia. Thus, it is tempting to reflect that NO donated by NCX 4016 to cardiac myocytes may have increased intracellular cyclic guanosine monophosphate (cGMP) and restricted the depletion of energy stores in ischemic cells, promoting the dissociation of crossbridges and reducing ventricular stiffness. In this respect, DeprŁ and Hue[31] reported that cGMP levels are increased during myocardial ischemia and that this phenomenon may control the phosphorylation status of several proteins and the intracellular content of Ca^{++} via cGMP-dependent protein kinase[32,33].

Another feature emerging from the present experiments is the hyperresponsiveness of the coronary vasculature to angiotensin II in preparations treated with aspirin at a dose fully inhibiting PGI_2 synthesis. The phenomenon has already been shown in perfused rabbit hearts treated with indomethacin where the vasopressor effect caused by endothelin-1 was significantly enhanced[34,35]. In sharp contrast with the above results NCX 4016, given through the heart at concentrations affecting PGI_2 synthesis, significantly antagonized the vasoconstriction induced by angiotensin II. This could be explained again with the donation of NO by NCX 4016, which in turn may have overcome the lack of PGI_2 formation in the vascular endothelium.

A parent compound of NCX 4016, the nitroderivative of aspirin NCX 4215 (nitroxybutyl-acetylsalicylate), has been proved to relax epinephrine-precontracted rat aortic rings with or without endothelium, an effect prevented by methylene-blue and therefore dependent on NO release and guanilate cyclase activation[36].

These promising *in vitro* findings with NCX 4016, which significantly protected the hearts from ischemia-reperfusion damage, mandated experiments *in vivo*. Thus, this compound was used in the rabbit in a well-established model of acute CAL with rapid progression to substantial necrosis of the subendocardium and to extensive transmural infarction[37,38]. In this experimental model, NCX 4016, perfused in a prophylactic regimen for 2 hours at 0.5 mg/kg/min, reduced the mortality rate by only 10% compared to untreated animals (60%).

In addition, CK activity, the measure of cardiac tissue damage, was remarkably elevated in the surviving untreated rabbits 24 hours after CAL whereas the level of this enzyme was almost normalized in rabbits treated with NCX 4016.

Although enzymatic estimates of infarct size based upon the amount of CK activity released into the plasma have been shown by Roberts[39] to closely correlate with short-term mortality, further studies may be required to evaluate directly the effect of NCX 4016 on the volume of the risk zone which is a critical determinant of both infarct size and arrhythmia.

A significant reduction in the myeloperoxidase content of the damaged cardiac area was also obtained with NCX 4016. Myeloperoxidase serves as a relevant marker for polymorphonuclear leukocyte involvement[18]. In this regard, according to Mullane et al.[40], neutrophils normally do not sequester in the heart, whereas they are activated by processes occurring during ischemia and reoxygenation that lead to their active accumulation. Moreover, activated neutrophils can release a variety of mediators that may contribute to ischemia-induced injury and ventricular dysfunction[41]. In order to explain the cardioprotective effect of NCX 4016 it may be hypothesized that NO released by this compound has prevented in some way neutrophil adhesion and activation[42,43]. Unlike aspirin, NCX 4016 was already found to inhibit neutrophil adhesion and activation in the hemorrhage shock model[44]. It is also known that endothelium-derived NO is an important endogenous modulator of polymorphonuclear leukocyte adhesion and activation in an inflammatory response[5,43]. Moreover, supplement of NO with NO donor in perfused rat hearts has been shown to attenuate the injury in which neutrophils were involved[45]. However, other mechanisms contributing to the cardioprotective effect of NCX 4016, such as inhibition of thromboxane synthesis and platelet aggregation cannot be excluded[5,24].

The most interesting findings were the ECGs of the surviving animals of the respective groups, showing that the severe upward and downward shifts of ST segment, which occurred 30 min after CAL, were significantly blunted by NCX 4016 but not by aspirin. ECG recordings taken at a later stage (24 hours after CAL) confirmed the existence of remarkable differences in the infarction process between control and NCX 4016-treated rabbits, as almost none of the surviving animals demonstrated severe abnormalities, i.e. ST segment elevation, cardiac arrhythmia which marked the untreated and all the aspirin-treated rabbits.

In conclusion, the results obtained from these studies provide evidence that an optimal balance of cycloxygenase inhibition and NO release can be achieved with NCX 4016; they also support the concept that the NO-releasing derivative of aspirin may bear therapeutic potential in the control of myocardial ischemia and infarction progression. However, since the lack of comparative analysis between NCX 4016 and other NO donors is the limitation of the present study, further experiments in this field are of utmost importance.

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