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Nitric oxide and prostacyclin pathways: An integrated mechanism that limits myocardial infarction progression in anaesthetized rats

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Abstract

Nitric oxide (NO) and cyclooxygenase-derived prostaglandins, such as prostacyclin (PGI_2), are involved in vascular homeostasis. To better understand the reciprocal role of both NO and PGI_2 on myocardial infarction in the rat, we have investigated the cardioprotective effect of nitro-naproxen, isosorbide dinitrate (ISDN), L-arginine, defibrotide and naproxen. In this study, male Wistar rats were treated orally once a day for 5 consecutive days with the compounds under investigation and then, under anesthesia, the animals were subjected to acute myocardial ischemia (30 min) and reperfusion (120 min). Systemic blood pressure, left ventricular pressure and related parameters of cardiac mechanics were recorded. Ventricular arrhythmias and infarct size of the left ventricular wall were also evaluated. Furthermore, cardiac myeloperoxidase (MPO) and plasma creatine phosphokinase (CPK) activities were determined. Defibrotide, nitro-naproxen, ISDN and L-arginine all provided a cardioprotection characterized by significant prevention of arrhythmias with high survival rate of the rats. Infarct size restriction was paralleled by reduction of both cardiac MPO and plasma CK. Cardioprotection of nitro-naproxen, ISDN and L-arginine involve nitrites/nitrates and PGI_2-increased in the circulation associated to a reduction of thromboxane B_2 (TXB_2) in the blood. Defibrotide displays a cardioprotection by increasing PGI_2 release and by reducing TXB_2 in the blood. Naproxen was devoid a lower protecting activity on myocardial infarction, and PGI_2 inhibition may have played a critical role in this context. The results suggested that the increase of both NO and PGI_2 brings about a cascade of integrated cellular and molecular events which are of paramount importance in prevention of myocardial ischemic insult.

Keywords: Nitric oxide; Prostacyclin; Myocardial infarction; Ischemia-reperfusion; Cardioprotection

1. Introduction

Over the last 2 decades, coronary reperfusion therapy with thrombolytic and fibrinolytic agents and coronary angioplasty has become established procedures for the management of acute myocardial infarction [1]. However, blood flow restoration to previously ischemic myocardium is responsible for injury to a viable myocardium and for a "mismatch" between flow and recovery of mechanical function even when no irreversible damage has been reported [2]. In this instance, the persistent ventricular dysfunction may lead to develop heart failure and progressive ventricular remodeling. Several studies have linked neutrophils to reperfusion injury since depletion of neutrophils is correlated with less cellular damage after ischemia-reperfusion [3]. Endothelial dysfunction is associated with a sequence of increased adhesiveness of neutrophils, increase in neutrophils–endothelial cell interactions, release of inflammatory mediators (e.g. oxygen free radicals and platelet-activating factor), activation of circulating neutrophils, release of a variety of harmful substances (cytokines, leukotrienes and proteases) and tissue injury and necrosis [4,5]. In the light of these events leading to a dysfunction of the coronary endothelium, research should focus on an adjunctive therapy that may limit post-ischemic ventricular dysfunction and speed functional recovery [6]. Potential candidates include nitric oxide (NO) or NO-donors such as nitroglycerine [7], and also the nitro-derivative of aspirin (nitro-aspirin) and naproxen (nitro-naproxen) should be considered of some therapeutical interest.

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These two nitro-derivatives of conventional non-steroidal anti-inflammatory drugs (NSAIDs), synthetized to reduce or abolish their gastrointestinal toxicity [8], have been reported to display cardioprotection in perfused rabbit heart submitted to global ischemia-reperfusion. Both nitro-aspirin and nitro-naproxen, by donation of NO, bring about a marked reduction of ventricular contracture during ischemia with remarkable improvement of left ventricular developed pressure at reperfusion [9–11].

Many of endothelium-derived factors like NO, prostacyclin (PGI_2) and endothelim-dependent hyperpolarization (EDHF) share a number of common functions, although they are part of different metabolic systems [12–16]. Pharmacological interventions of one pathway could result in a cross-modulation of the other, but the relevance of this interaction is often controversial [17,18]. Although the NO- and eicosanoid-producing pathways have been studied extensively, particularly in an inflammatory focus [19], the investigation on the ability of different NO-donors and the potent PGI_2-releaser defibrotide to control the myocardial infarction progression may further contribute to better understand the reciprocal role of NO and PGI_2 in cardioprotection.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Italia, Calco, LC, Italy), weighing 260–280 g were used. The animals were housed in a conditioned environment ($22 \pm 1\,^{\circ}C$, $55 \pm 5\%$ relative humidity, 12-h light and 12-h darkness cycle) and were given free access to food and tap water. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes for Health (NIH Publication No. 85–23, revised 1996).

2.2. Preliminary experiments

2.2.1. Experimental design

In these series of experiments different doses of naproxen (12.5, 25, 50 mg kg^{-1}), defibrotide (50, 100, 200 mg kg^{-1}), nitro-naproxen (20, 40, 80 mg kg^{-1}), isosorbide dinitrate (ISDN; 10, 20, 40 mg kg^{-1}) and L-arginine (6.25, 12.5, 25 mg kg^{-1}) were dissolved in polyethylene glycol 400 (PEG 400; vehicle) and administered orally by gavage (volume, 2 ml kg^{-1}) once a day for 5 consecutive days to 16 different groups of four animals each. Their capacity to reduce plasma thromboxane B_2 (TXB_2) levels, and to increase both NO (as nitrates/nitrites; NOx) and PGI_2 (as 6-keto-prostaglandin $F_{1\alpha}$, 6-keto-$PGF_{1\alpha}$) levels has been evaluated in the circulating blood.

2.2.2. Measurement of plasma TXB_2, 6-keto-$PGF_{1\alpha}$ and NOx levels

Blood samples for plasma TXB_2, 6-keto-$PGF_{1\alpha}$ and NOx measurements were withdrawn via cardiac puncture from the anaesthetized animals (thiopentone sodium, Pentothal®, mg kg^{-1} i.p.), collected with heparinized syringes and put into indomethacin (100 mmol l^{-1})-rinsed (for TXB_2 and 6-keto-$PGF_{1\alpha}$ samples) tubes. The samples were centrifuged for 15 min ($2500 \times g$ at $4\,^{\circ}C$) and aliquots of the supernatant were removed, and stored frozen at $-80\,^{\circ}C$ until assayed. After purification according to the manufacturer's instructions, plasma TXB_2 and 6-keto-$PGF_{1\alpha}$ levels were measured using enzyme immunoassay kits. Furthermore, plasma NOx levels were determined by a colorimetric microplate assay kit that uses the Greiss reagent.

2.3. Myocardial ischemia/reperfusion (MI/R) in anaesthetized rats

2.3.1. Experimental design

For MI/R experiments, rats were randomly assigned to seven different groups of at least 14 animals each and treated with naproxen (50 mg kg^{-1}), defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), ISDN (40 mg kg^{-1}) or L-arginine (25 mg kg^{-1}). All drugs, dissolved in PEG 400 (vehicle), were administered orally by gavage (volume, 2 ml kg^{-1}) once a day for 5 consecutive days. At the fifth day, the treatment was performed 1 h before anaesthesia. The doses used for naproxen, defibrotide, nitro-naproxen, ISDN or L-arginine were selected referring to the results obtained in preliminary experiment (see above section). At these doses the increase of NOx in the circulation was in the same range (see Table 1). Defibrotide has been utilized at 200 mg kg^{-1} since at this regimen a remarkable release of PGI_2 was observed. The length of treatment was established in terms of once a day for 5 consecutive days since the increase of both NOx and PGI_2 into the blood was maximal only after 4 days of treatment (data not shown).

2.3.2. Surgical preparation

Rats were anaesthetized with thiopentone sodium (Pentothal®, 60 mg kg^{-1} i.p.), placed in the supine position on a table and the body temperature was maintained at $38 \pm 1\,^{\circ}C$ 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, Varese, Italy) with a stroke volume of 10 ml kg^{-1} and a rate of 60–65 strokes min^{-1} to maintain normal pH (7.35–7.45), pO_2 (80–110 mmHg) and pCO_2 (25–40 mmHg) 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, Texas) 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 $37\,^{\circ}C$ physiological saline prior to measurements. Furthermore, subdermal platinum electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). According to the procedure described by Himori and Matsuura [20], the chest was opened by a left thoracotomy at the fourth or fifth intercostal space, the ribs were gently spread using a small-sized retractor and the heart was exposed. After

Table 1
Effect of naproxen, defibrotide, nitro-naproxen, isosorbide dinitrate (ISDN) and L-arginine on plasma nitrites/nitrates (NOx), thromboxane B_2 (TXB_2) and prostacyclin (as 6-keto-$PGF_{1\alpha}$) levels in male Wistar rats

Treatment (mg kg^{-1})	Plasma NOx (μM)	Plasma TXB_2 (pg ml^{-1})	Plasma 6-keto-$PGF_{1\alpha}$ (ng ml^{-1})
Vehicle			
–	32.7 ± 3.4	281.7 ± 23.4	0.38 ± 0.04
Naproxen			
12.5	25.9 ± 4.2	189.2 ± 14.5*	0.32 ± 0.05
25	31.0 ± 1.7	58.5 ± 11.2***	0.24 ± 0.03*
50	22.8 ± 5.5	29.6 ± 4.8***	0.11 ± 0.02**
Defibrotide			
50	42.3 ± 5.8	241.5 ± 26.4	1.45 ± 0.23**
100	50.6 ± 3.9*	179.3 ± 18.2*	3.97 ± 0.52***
200	82.3 ± 6.8**	135.4 ± 17.8**	5.72 ± 0.78***
Nitro-naproxen			
20	53.3 ± 4.2*	228.1 ± 31.2	0.45 ± 0.05
40	106.1 ± 9.4**	148.5 ± 15.6**	0.55 ± 0.08
80	143.6 ± 11.5***	69.6 ± 8.4***	0.53 ± 0.06
ISDN			
10	74.8 ± 9.5**	249.7 ± 20.1	0.45 ± 0.03
20	122.3 ± 16.3***	178.9 ± 36.5*	0.91 ± 0.06*
40	181.7 ± 15.4***	161.6 ± 24.3*	1.23 ± 0.04**
L-Arginine			
6.2	63.4 ± 9.4*	251.0 ± 23.6	0.79 ± 0.04*
12.5	111.7 ± 13.4**	197.6 ± 18.2*	1.40 ± 0.12**
25	176.2 ± 16.4***	148.2 ± 10.9**	1.84 ± 0.06***

Vehicle (PEG 400, 2 ml kg^{-1}), naproxen, defibrotide, nitro-naproxen, ISDN and L-arginine were administered orally once a day for 5 consecutive days. Data are expressed as mean ± S.E.M. ($n = 4$ animal each dose/group.)
* $P < 0.05$ vs. vehicle-treated rats.
** $P < 0.01$ vs. vehicle-treated rats.
*** $P < 0.001$ vs. vehicle-treated rats.

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. FS-2; Ethicon, Pratica di Mare, Rome, Italy) with a thin silk thread (no. 5-0) was used for the ligature. The needle was inserted (approximately 0.5 mm) into the myocardium 2–3 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 (releasers). The heart was returned quickly to the thoracic cavity and the tips of the suture used to produce the coronary ligation were exteriorised through the chest wall. The whole surgical procedure described above took about 10–12 min and at the end the animals were allowed to stabilize for 30 min before LCA-ligation. The coronary artery was occluded at time 0 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 re-opened and the heart was reperfused for 120 min. At the end of this period the heart was removed for infarct size estimation and myeloperoxidase (MPO) determination.

2.3.3. Hemodynamic measurement

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 and diastolic arterial pressure (DAP), and mean arterial pressure (MAP) were obtained. The following parameters related to cardiac mechanics were also determined: left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDevP) and the maximal velocity rate of pressure development of left ventricle ($LVdP/dt_{max}$). Furthermore, the ECG (lead II) was recorded with a Cardioline apparatus (model Delta-1; Remco Italia, Milan, Italy) and 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 Charter Windows 3.5 (AD Instruments). The pressure rate index (PRI), an indicator of myocardial oxygen consumption [21], was also calculated as the product of MAP and heart rate (HR).

2.3.4. Assessment of arrhythmia

Using the ECG signals, ventricular arrhythmia (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. [22] and in accordance with the definitions reported in the Lambeth Conventions [23]. 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 last 3 min). Rats were excluded from the final analysis if any of the following occurred: arrhythmias prior to 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; arrhythmia); MAP < 60 mmHg prior to LCA-occlusion. Any rats which were excluded were replaced immediately.

2.3.5. Determination of area at risk and infarct size

The area at risk and infarct size were evaluated with Evans blue dye and the triphenyltetrazoliun chloride, respectively [22]. In brief, at the end of the 2 h-reperfusion period, the ligature around the LCA was retightened and 1 ml Evans blue dye (3%, w/v) was injected intravenously into the jugular vein to delineate ischemic (area at risk) and non-ischemic myocardium (area not at risk). The Evans blue solution stains the perfused myocardium, while the non-perfused myocardium remains uncoloured. The rat was euthanized with a 15% potassium chloride 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 triphenyltetrazoliun 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 [24]. 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 MPO activity. Infarct size was expressed as a percentage of the area at risk.

2.3.6. Cardiac MPO activity

MPO activity was evaluated as an index of neutrophils accumulation in jeopardized tissue because it correlates closely with the number of polymorphonuclear leukocytes present in the heart [25]. 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 [26]. 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 + 15 s) at 7000 rev min^{-1}. Homogenates were centrifuged at 12,500 × *g* at 2 °C for 30 min on a Optima Ultra ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was collected and reacted with a solution of *O*-dianisidine dihydrochloride (0.167 mg ml^{-1}) 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, Perkin Elmer Italia, Monza, Milan, Italy). MPO standard curve (2.5–0.08 U ml^{-1}) was included in each assay. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μM of peroxide min^{-1} at 25 °C and expressed in U g^{-1} tissue.

2.3.7. Plasma creatine phosphokinase (CPK) activity

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 femoral artery 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 [27] using a commercially available kit and the total amount was determined on a spectrophotometer at a wavelength of 340 nm (Model Lambda16, Perkin Elmer Italia, Monza, Milan, Italy). CPK activity was expressed in U l^{-1} plasma.

2.4. 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 Dunnet's multiple comparison procedure (GraphPad Prism; GraphPad, San Diego, CA). Incidences of VT and VF were compared by Fisher-Irwin (chi-squared with Yates correction) test. Analysis of mortality rate was carried out with the analysis of LogLikelihood for categorical data and either Pearson or Likelihood-Ratio χ^2 tests [28]. Body weight, heart weight, left ventricular weight, area at risk and infarct size were compared with a one way analysis of variance (ANOVA) followed, when ANOVA was significant, by a Tukey-Kramer test for multiple comparison. A value of $P < 0.05$ was considered statistically significant.

2.5. Drugs

The following drugs were used: naproxen, L-arginine, Evans blue, PEG 400, hexadecyltrimethyl ammonium bromide, *O*-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, MO, USA); thiopentone sodium (Abbott, Campoverde, Latina, Italy), defibrotide (Crinos, Villaguardia, Como, Italy), nitro-naproxen (NicOx S.A., Valbonne, Sophia Antipolis, France), ISDN (ICN Biomedicals Inc., Irvine, CA, USA); kit for CPK determination (Boehringer-Mannheim Italia, Milan, Italy); kits for TXB_2, 6-keto-$PGF_{1\alpha}$ and NOx determinations (Cayman Chemical, Ann Arbor, MI, USA).

3. Results

3.1. Preliminary experiments

The results concerning the changes in plasma levels of NOx, TXB_2 and PGI_2 obtained with the compounds under investigation are reported in Table 1. Naproxen, at the maximal dose used (50 mg kg^{-1}), reduced by 89% ($P < 0.001$) and 71% ($P < 0.001$) the amount of blood TXB_2 and PGI_2, respectively, but it was devoid of any activity on NOx concentration. Defibrotide dose-dependently increased plasma PGI_2, and this effect was marked at 200 mg kg^{-1} (15.1-fold increment; $P < 0.001$). At this dose, defibrotide reduced TXB_2 (−52%; $P < 0.001$) and increased NOx (2.5-fold; $P < 0.001$). Nitro-naproxen did not influence the levels of PGI_2 in the circulation at all doses used. However, at the dose of 80 mg kg^{-1}, this compound markedly reduced TXB_2 (−75%; $P < 0.001$) and increased NOx (4.4-fold; $P < 0.001$). ISDN (40 mg kg^{-1}) and L-arginine (25 mg kg^{-1}) resulted equally effective in increasing the NOx concentration in the blood. At these doses, the amount of NOx was increased 5.6-fold ($P < 0.001$) and 5.4-fold ($P < 0.001$), respectively, as compared to that found in vehicle-treated rats. Furthermore, both ISDN and L-arginine significantly reduced the levels of plasma TXB_2 and this event was associated with an increase in plasma PGI_2.

3.2. Myocardial ischemia/reperfusion in anaesthetized rats

3.2.1. Hemodynamics

The hemodynamic parameters measured in anaesthetized rats just before LCA-occlusion and treated orally with vehicle (PEG 400; 2 ml kg^{-1}), naproxen (50 mg kg^{-1}),

Table 2
Hemodynamic parameters measured in anaesthetized rats immediately before coronary artery occlusion

Treatment	MAP (mmHg)	HR (b min^{-1})	LVSP (mmHg)	LVEDP (mmHg)	LVdP/dt_{max} (mmHg s^{-1})	PRI (mmHg $min^{-1} \times 10^3$)
Vehicle ($n = 25$)	105 ± 6	406 ± 19	135 ± 7	5.3 ± 0.3	5095 ± 207	42.4 ± 1.2
Naproxen ($n = 18$)	107 ± 4	411 ± 21	134 ± 7	5.3 ± 0.5	5178 ± 206	43.7 ± 1.3
Defibrotide ($n = 17$)	103 ± 3	409 ± 18	136 ± 6	5.0 ± 0.4	5095 ± 273	44.5 ± 0.9
Nitro-naproxen ($n = 18$)	106 ± 4	417 ± 17	131 ± 6	5.4 ± 0.4	4926 ± 365	42.7 ± 0.9
ISDN ($n = 19$)	85 ± 3*	435 ± 25	108 ± 4*	6.1 ± 0.6	4797 ± 189	39.4 ± 1.4
L-Arginine ($n = 19$)	102 ± 8	424 ± 20	120 ± 5	6.0 ± 0.5	5035 ± 187	42.9 ± 0.6

Vehicle (PEG 400, 2 ml kg^{-1}), naproxen (50 mg kg^{-1}), defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), isosorbide dinitrate (ISDN, 40 mg kg^{-1}) and L-arginine (25 mg kg^{-1}) were administered orally once a day for 5 consecutive days. In brackets, number of experiments. Data are expressed as mean ± S.E.M. MAP, mean arterial pressure; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVdP/dt_{max} , maximal velocity rate of pressure development of left ventricle.

* $P < 0.05$ vs. vehicle-treated rats.

defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), ISDN (40 mg kg^{-1}) or L-arginine (25 mg kg^{-1}) for 5 consecutive days are reported in Table 2. At variance with ISDN, the values related to MAP and HR were all in the same range, and the values concerning cardiac mechanics (LVSP, LVEDP, LVDevP, LVdP/dt_{max} and PRI) did not show statistical differences. Following LCA-occlusion, the MAP-values of all the experimental animals fell consistently and abruptly (peak effect at 5–6 min) and then it progressively recovered within 30 min to 95–100 mmHg (data not shown).

3.2.2. Arrhythmia and mortality rate

When LCA was occluded in vehicle-treated rats, a consistent ventricular arrhythmias was observed. These events persisted significantly during reperfusion and it was associated with a relevant mortality rate (Table 3). During the 30 min of regional myocardial ischemia the VPBs recorded were 1547 ± 139 and the incidence of VT and VF was 100% and 61%, respectively. The dysrhythmias during the reperfusion period consisted of 303 ± 40 VPBs with an incidence of VT and VF of 47% and 12%, respectively. The total mortality rate in this group of 25 vehicle-treated animals submitted to MI/R was marked as being 36% ($P < 0.001$). Treatment of the rats submitted to MI/R with the compounds under investigation brought about protection against ventricular ectopic activity and reduction in mortality rate. Among them, the PGI_2-releaser defibrotide showed the highest antiarrhythmic effect during both the ischemic and reperfusion periods and all the animals in this group survived throughout the experiment. The rank order of potency in reducing the mortality rate was defibrotide ≥ L-arginine > nitro-naproxen and ISDN > naproxen ((Table 3).

3.2.3. Infarct size

The results obtained from the evaluation of the infarct size in rats submitted to MI/R are reported in Table 4. The mean values of the area at risk, expressed as a percentage of left ventricular wall, was similar in all animal groups studied. In vehicle-treated rats, LCA-occlusion for 30 min followed by 120 min reperfusion resulted in an infarct size of 64.5 ± 4.6% of the area at risk. Treatment of the animals with the compounds under investigation gave rise to a reduction of the infarct size as compared with that obtained in vehicle-treated animals. Defibrotide was the most effective since it reduced the infarct size of 13.8 ± 2.2% of the area at risk, which is 4.7-fold inferior ($P < 0.001$) to that determined in vehicle-treated rats. The rank order of potency of the remaining compounds under investigation in preventing the progression of ventricular necrosis was L-arginine ≥ nitro-naproxen > ISDN > naproxen (Table 4).

Table 3
Arrhythmias in the anaesthetized rats during the 30 min of coronary artery occlusion followed by 120 min of reperfusion (MI/R)

Groups	Arrhythmias during occlusion			Arrhythmias during reperfusion			Mortality
	VPBs (total)	VT (% inc)	VF (% inc)	VPBs (total)	VT (% inc)	VF (% inc)	%
Sham ($n = 14$)	0	0	0	0	0	0	0
Vehicle + MI/R ($n = 16$)	1547 ± 1139	100	61	303 ± 40	47	12	36.0
Naproxen + MI/R ($n = 14$)	1010 ± 82*	74	40	192 ± 15*	30	8	22.2*
Defibrotide + MI/R ($n = 17$)	362 ± 76***	26	10	28 ± 8***	4	0	0***
Nitro-naproxen + MI/R ($n = 16$)	642 ± 51**	40	18	111 ± 23**	14	4	11.1**
ISDN + MI/R ($n = 17$)	775 ± 78**	48	26	108 ± 18**	12	0	10.5**
L-Arginine + MI/R ($n = 18$)	507 ± 62***	34	14	75 ± 10***	10	0	5.3**

Vehicle (PEG 400, 2 ml kg^{-1}), naproxen (50 mg kg^{-1}), defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), isosorbide dinitrate (ISDN, 40 mg kg^{-1}) and L-arginine (25 mg kg^{-1}) were administered orally once a day for 5 consecutive days. Data are expressed as mean ± S.E.M. In brackets, number of experiments. Sham, sham-operated animals treated with vehicle; VPBs, ventricular premature (ectopic) beats; VT, ventricular tachycardia; VF, ventricular fibrillation; % inc, percentage of incidence.

* $P < 0.05$ vs. vehicle + MI/R group.

** $P < 0.01$ vs. vehicle + MI/R group.

*** $P < 0.001$ vs. vehicle + MI/R group.

Table 4
Heart weight/body weight (HW/BW), left ventricle 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 (MI/R)

Groups	HW/BW (mg g^{-1})	LVW (mg)	AAR (% of LVW)	Infarct size (% of AAR)
Sham ($n=14$)	2.99 ± 0.10	515 ± 32	n.d.	n.d.
Vehicle + MI/R ($n=16$)	3.07 ± 0.08	504 ± 27	54.4 ± 2.5	64.5 ± 4.6
Naproxen + MI/R ($n=14$)	3.12 ± 0.13	527 ± 33	54.2 ± 3.1	47.9 ± 3.8*
Defibrotide + MI/R ($n=17$)	3.05 ± 0.11	498 ± 36	55.7 ± 3.4	13.8 ± 2.2***
Nitro-naproxen + MI/R ($n=16$)	2.97 ± 0.12	533 ± 25	53.1 ± 3.3	29.2 ± 3.7***
ISDN + MI/R ($n=17$)	3.06 ± 0.09	522 ± 30	53.3 ± 3.5	32.6 ± 4.8**
L-Arginine + MI/R ($n=18$)	3.09 ± 0.14	497 ± 51	55.0 ± 2.8	20.3 ± 2.6***

Vehicle (PEG 400, 2 ml kg^{-1}), naproxen (50 mg kg^{-1}), defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), isosorbide dinitrate (ISDN, 40 mg kg^{-1}) and L-arginine (25 mg kg^{-1}) were administered orally once a day for 5 consecutive days. In brackets, number of experiments. Data are expressed as mean ± S.E.M. Sham, sham-operated animals treated with vehicle. n.d., not detectable.
* $P<0.05$ vs. vehicle + MI/R group.
** $P<0.01$ vs. vehicle + MI/R group.
*** $P<0.001$ vs. vehicle + MI/R group.

Table 5
Cardiac myeloperoxidase (MPO) and plasma creatine phosphokinase (CPK) activities measured in rats subjected to 30 min of coronary artery occlusion followed by 120 min of reperfusion (MI/R)

Groups	Cardiac MPO (U/g tissue)		Plasma CPK (U/l)		
	Area not at risk	Area at risk	0 min (Pre-occlusion)	30 min (End-occlusion)	150 min (End-reperfusion)
Sham ($n=14$)	0.45 ± 0.08	n.d.	242 ± 30	291 ± 27	321 ± 37
Vehicle + MI/R ($n=16$)	0.43 ± 0.05	5.01 ± 0.43#	260 ± 27	934 ± 33	2012 ± 108
Naproxen + MI/R ($n=14$)	0.39 ± 0.07	3.94 ± 0.27*	239 ± 12	702 ± 44*	1564 ± 75*
Defibrotide + MI/R ($n=17$)	0.42 ± 0.05	0.72 ± 0.16***	221 ± 32	355 ± 38***	535 ± 46***
Nitro-naproxen + MI/R ($n=16$)	0.37 ± 0.03	2.32 ± 0.14**	257 ± 25	527 ± 52**	1023 ± 51**
ISDN + MI/R ($n=17$)	0.41 ± 0.09	2.11 ± 0.20**	238 ± 32	453 ± 24**	904 ± 71**
L-Arginine + MI/R ($n=18$)	0.43 ± 0.07	1.85 ± 0.24***	251 ± 30	405 ± 28***	701 ± 52***

Vehicle (PEG 400, 2 ml kg^{-1}), naproxen (50 mg kg^{-1}), defibrotide (200 mg kg^{-1}), nitro-naproxen (80 mg kg^{-1}), isosorbide dinitrate (ISDN, 40 mg kg^{-1}) and L-arginine (25 mg kg^{-1}) were administered orally once a day for 5 consecutive days. In brackets, number of experiments. Data are expressed as mean ± S.E.M. In brackets, number of experiments. Sham, sham-operated animals treated with vehicle; n.d., not detectable.
# $P<0.001$ vs. sham.
* $P<0.05$ vs. vehicle + MI/R group.
** $P<0.01$ vs. vehicle + MI/R group.
*** $P<0.001$ vs. vehicle + MI/R group.

3.2.4. Cardiac MPO and plasma CPK activities

The results of cardiac MPO and plasma CPK enzymes related to all animal groups are reported in Table 5. In vehicle-treated rats the mean values of MPO activity obtained in the area not at risk of the left ventricular wall was low and not significantly different among the seven experimental groups (from 0.37 ± 0.03 to 0.45 ± 0.08 U g^{-1} tissue). On the contrary these values determined in the area at risk of vehicle-treated animals increased 11.7-fold ($P<0.001$) as compared to that obtained in the area not at risk, indicating infiltration and activation of inflammatory cells such as neutrophils in the jeopardized ventricular tissue. Treatment of the rats with the compounds under investigation resulted in a reduction of MPO activity in the area at risk of different degree with a maximal activity for the PGI_2-releaser defibrotide. However, the rank order in the control of MPO enzymes is: defibrotide ≥ L-arginine > ISDN and nitro-naproxen > naproxen. In Table 5 results concerning plasma CPK activity are reported. In vehicle-treated rats it increased 7.7-fold ($P<0.001$) at the end of reperfusion period, being this value 260 ± 27 U l^{-1} before coronary occlusion. Among the compounds used in this study, defibrotide was the most effective in the control of plasma CPK activity. The rank order in the control of plasma CPK activity is: defibrotide ≥ L-arginine > ISDN and nitro-naproxen > naproxen.

4. Discussion

The results obtained with the present study clearly indicate that nitro-naproxen, ISDN, L-arginine and defibrotide provide a substantial cardioprotection when given orally for 5 consecutive days to rats submitted to a marked insult of 30-min regional myocardial ischemia and 120-min reperfusion. The beneficial effects disclosed by these compounds are evident in the prevention of cardiac and biochemical abnormalities observed in vehicle-treated rats. The occurrence of VPBs was noticeably reduced and this was strictly correlated with the high incidence of the survived animals and the reduction of infarct size. This may be explained by the results obtained with preliminary experiments showing a significant increase of NOx-levels in nitro-naproxen, ISDN and L-arginine treated animals. This indicates that NO availability was first augmented, broken dawn in nitrite, and subsequently oxidized to nitrate by red blood cells

[29,30]. A small but a significant increase in blood PGI_2 concentration was also observed in these experiments with both ISDN and L-arginine. This may be due to a direct interaction of NO and cyclooxygenase to cause stimulation of enzyme activity [31,32]. In this respect, the cyclooxygenase enzymes are a potential target for NO because they contain an iron-heme center at their active site [19]. However, with defibrotide the remarkable increase of PGI_2 production may have played a prominent role in the cardioprotection observed during ischemia-reperfusion. The basal levels of blood TXB_2 were also reduced by various degrees. These data may support the concept that the well-known prothrombotic effect of this autacoid may have been antagonized during the ischemic insult by the current assets of NO and PGI_2 in the circulation caused by the four compounds under investigation.

The control of ventricular necrosis extension in rats submitted to ischemia-reperfusion obtained with nitro-naproxen, ISDN, L-arginine and particularly defibrotide is corroborated by a significant reduction of plasma CPK and MPO activity in the area at risk of the ventricular wall. A number of events can lead to the enhanced MPO activity such as adherence of polymorphonuclear leukocytes to vascular endothelial cells. With regard to this point, it has been already shown that cardiac necrosis can be significantly attenuated by treatment with NO-donors, an organic NO-donor and L-arginine or by specific blockers of adhesion molecules, particularly P-selectin [33,34]. Neutrophils have been implicated as a primer mechanism underlying ischemic-reperfusion injury and cardiac necrosis progression. The propensity to injure the myocardium and its component cells, notably coronary vascular endothelium, microvasculature and myocytes, stems from the myocardium's primary responses to inflammatory mediators which lead to a redirection of a normal inflammatory response. The process involved in inducing tissue injury by neutrophils includes free radical generation and release of proteases, and other proinflammatory mediators [35].

The relevant increase of PGI_2, in the circulation such as that obtained with defibrotide (15-fold as compared to vehicle-treated animals) should be considered a key point in limitating polymorphonuclear leukocytes activation during ischemia-reperfusion. In fact, it has been already proved that, in perfused rabbit heart with human polymorphonuclear leukocytes under recirculating conditions, defibrotide, via PGI_2 release, prevents leukocyte–vascular wall interaction and inhibits sulfidopeptide leukotrienes generation [36]. These lipidic mediators affect the major component of the cardiovascular system, i.e. they can constrict small and large coronary vessels, thus contributing to the manifestations of ischemia-reperfusion damage [37,38].

On the other end, the increased availability of NO provided by nitro-naproxen, ISDN and L-arginine, is of paramount importance in cardioprotection since NO has been shown to reduce polymorphonuclear leukocytes-mediated endothelial dysfunction in myocardial ischemia-reperfusion, and this is very likely to have happened because of a specific interaction with adhesion molecules [39]. NO may also affect reperfusion and reoxigenase injury by augmenting coronary dilation and coronary blood flow reducing leukocyte and platelet interaction with vascular endothelium and scavenging superoxide radicals [40]. Furthermore, other known physiological effects of NO, such as reduction of ventricular filling pressure, augmentation of collateral coronary flow and inhibition of platelet aggregation, may have contributed to restrain infarct size. However, excessive production of NO during ischemia-reperfusion has been shown to cause myocardial injury where peroxinitrite-increased generation is involved [41]. On this ground, Parlakpinar et al. [42] and Sahna et al. [43] demonstrate that inhibition of inducible NO synthase with aminoguanidine reduced NO's side effect in ischemia-reperfusion damage. Although the role of NO in the progression of myocardial infarction is controversial [44,45], the present results are in line with most other studies supporting the idea that NO mimicry/supplementation limits infarct size and production of dysrhythmias [46,47]. The results obtained with naproxen, which are in agreement with those recorded with aspirin in the same experimental procedure [10], suggest that blockade of TXB_2 formation is not sufficient to provide cardioprotection particularly when in parallel PGI_2 generation is inhibited. Recent data indicate that chronic use of traditional NSAIDs is associated with risk of non-fatal myocardial infarction in the general populations [48]. Particularly, in healthy subject, naproxen appears to interfere with the effect of aspirin on cyclooxygenase-1 (COX-1) activity and function, under miming the sustained inhibition of COX-1 in platelets [49].

Although, experimental conditions adopted in the present study are very different from the clinical situation, where pretreatment is most often not possible and where ischemia-periods are frequently more protracted, the results obtained further emphasize that the increase of both NO and PGI_2 in the circulation brings about an orchestrated sequence of cellular and molecular events which lead to a limitation of myocardial damage ensuing from ischemia-reperfusion.

In conclusion, for patients who needs an adequate control of cardiovascular risk factors, the therapeutic management with compounds that insure a good balance between NO/PGI_2 production and TXB_2 inhibition represent a favorable option.

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