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Role of nitric oxide and free radicals in cardioprotection by blocking Na^+/H^+ and Na^+/Ca^{2+} exchange in rat heart

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Abstract

Inhibition of Na^+/H^+ (NHE) and Na^+/Ca^{2+} (NCE) exchangers prevents myocardial ischemia/reperfusion injury by preventing cardiomyocyte Ca^{2+} overload. We hypothesized that it may influence ischemic/reperfused myocardium also indirectly by preventing endothelial Ca^{2+} accumulation, and thereby by attenuating reperfusion-induced formation of nitric oxide (NO) and/or oxygen free radicals. Langendorff-perfused rat hearts were subjected to 30-min ischemia and 30-min reperfusion. Myocardial outflow of NO (nitrite + nitrate) and hydroxyl radical (.OH, salicylate method), and functional recoveries were followed during reperfusion. In all groups, there was a transient rise in NO and .OH outflow upon reperfusion. An inhibitor of NHE, cariporide (10 μM) [(4-Isopropyl-3-methylsulfonyl-benzoyl)-quanidine methanesulfonate], and an inhibitor of the reverse mode of NCE, KB-R7943 (5 μM) (2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate), decreased NO and .OH formation, reduced contracture, and improved the recovery of mechanical function during reperfusion, compared to the untreated hearts. The formation of NO was reduced by 40% by 100 μM N^G-methyl-L-arginine acetate salt (L-NMMA, NO synthase inhibitor), and not affected by 50 μM L-NMMA. .OH formation, contracture, and the functional recoveries were affected neither by 50 nor by 100 μM L-NMMA. Also, the effects of cariporide and KB-R7943 were unaffected by 100 μM L-NMMA. This study shows for the first time that the inhibition of NHE and NCE attenuates post-ischemic myocardial formation of NO and .OH, suggesting that prevention of Ca^{2+} overload is cardioprotective via these mechanisms. The results indicate, however, that NO synthase pathway did not interfere with the protection afforded by NHE or NCE in our model.
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Keywords: Na^+/H^+ exchange; Na^+/Ca^{2+} exchange; Heart, isolated, rat; Ischemia/reperfusion injury; Cariporide; KB-R7943; Nitric oxide (NO); Free radical

1. Introduction

The contribution of the Na^+/H^+ exchanger type 1 (NHE) to ischemia/reperfusion myocardial injury has been indicated by consistent observation that administration of NHE inhibitors before ischemia reduces infarct size, myocardial stunning, arrhythmia, and endothelial dysfunction (Avkiran et al., 2001; Gumina et al., 2001; Karmazyn et al., 2001). Recently, clinical effectiveness of NHE inhibitors, cariporide, and eniporide in the setting of human ischemia/reperfusion has been verified in randomized multicenter studies (Theroux et al., 2000; Zeymer et al., 2001).

A current hypothesis proposes that the inhibition of NHE reduces cellular injury because it reduces intracellular Na^+ accumulation in ischemic/reperfused myocytes, which, in turn, results in less Ca^{2+} overload via the reverse mode of the Na^+/Ca^{2+} exchange (NCE) (Avkiran et al., 2001; Karmazyn et al., 2001). Indeed, an inhibitor of the reverse mode of NCE, KB-R7943, has been shown to reduce cellular Ca^{2+} accumulation in cardiomyocytes subjected to simulated ischemia and reperfusion, and to protect myocytes and whole heart against reperfusion injury (Ladilov et al., 1999; Schafer et al., 2001).

Ischemia/reperfusion and hypoxia result in cellular H^+, Na^+, and Ca^{2+} accumulation also in endothelial cells (Berna et al., 2001; Ladilov et al., 2000). We hypothesized, therefore, that the inhibition of NHE and NCE may also influence ischemic/reperfused myocardium indirectly, by preventing endothelial Ca^{2+} accumulation and attenuating reperfusion-induced formation of nitric oxide (NO) (Node et al., 1995; Wang and Zweier, 1996) and oxygen-derived free radicals (Sun et al., 1993; Zweier et al., 1987). The rationale for this hypothesis is twofold. First, the endothelial NO

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synthase (eNOS) shows strong requirement for calmodulin and Ca^{2+} (Govers and Rabelink, 2001). Also, the generation of superoxide ($.O_2^-$) by eNOS is a Ca^{2+}-dependent process, as is the generation of NO (Brovkovych et al., 2001). In addition, there is a number of other cellular systems, including mitochondria (Frantseva et al., 2000; Guarnieri et al., 1997), capable of producing free radicals in a Ca^{2+}-dependent manner. Second, there is convincing evidence that free radicals (Bernier et al., 1989; Bolli, 1998) and NO play an important role in the pathogenesis of various forms of myocardial reperfusion injury. Although it is still debated whether NO is protective (Dawn and Bolli, 2002; Jones et al., 1999; Lefer et al., 1993; Siegfried et al., 1992; Tosaki et al., 1998; Weyrich et al., 1992; Zhang et al., 2002) or injurious (Mori et al., 1998; Wang and Zweier, 1996; Yasmin et al., 1997) to the reperfused heart. Thus, the aims of this study were to examine: (i) whether NHE and NCE inhibition by cariporide and KB-R7943, respectively, affect post-ischemic formation of NO and free radicals in the isolated rat heart and (ii) if there is a relationship between the changes in NO and/or free radical production and the cardioprotection induced by NHE and NCE inhibition.

2. Materials and methods

2.1. Isolated heart preparation

The investigation conforms to the guide for the Care and Use of Laboratory Animals (US National Institute of Health publication no. 85-23, revised 1985).

Male Wistar rats (260–350 g) were injected with 500 units of heparin sulfate, i.p., 20 min before being anaesthetized with pentobarbital sodium (50 mg/kg, i.p.). Hearts were excised and perfused in the Langendorff mode at perfusion pressure of 70 mm Hg with prefiltered (5.0-μm Millipore filter) perfusion fluid containing, in mmol/l: 118 NaCl; 23.8 $NaHCO_3$; 4.7 KCl; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 1.2 $MgSO_4$, and 11 glucose, and gassed with 95% O_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 580–640 mm Hg at 37 °C. In the experiments aimed at studying myocardial .OH production, the buffer was supplemented with 1-mM sodium salicylate, which served as a specific trap for .OH (Floyd et al., 1984). In addition, glucose was reduced to 5.5 mM to limit .OH scavenging by this sugar (Halliwell and Gutteridge, 1986). The left ventricular-developed pressure (LVDP) (peak systolic pressure minus end-diastolic pressure), left ventricular end-diastolic pressure, and heart rate were recorded via a fluid-filled latex balloon inserted into the left ventricle and connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments) and a recorder (Siemens-Elema Mingograph-81 polygraph, Stockholm, Sweden). The volume of the balloon was adjusted to obtain end-diastolic pressure of ~ 5 mm Hg during the equilibration perfusion and it was not changed thereafter. The hearts were enclosed in a small, water-jacketed chamber. The temperature of the perfusate was thermostatically controlled and checked at regular intervals to ensure 37 °C. The hearts were not paced. Global ischemia was induced by clamping the aortic cannula and simultaneously immersing the heart in a small volume of the venous effluent (37 °C). The immersion was stopped when the cannula was unclamped to achieve reperfusion. Coronary flow was quantified by a timed collection and weighing of the perfusate leaving the right heart.

2.2. Agents used and selection of their concentration

N^G-methyl-L-arginine acetate salt (L-NMMA), sodium nitrate, sodium nitrite, and sodium salicylate were purchased from Sigma and KB-R7943 mesylate [2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate] was from Tocris Cookson (Avonmouth, Bristol, UK). Cariporide mesylate [(4-Isopropyl-3-methylsulfonyl-benzoyl)-guanidine methanesulfonate] was a generous gift from Aventis Pharma (Frankfurt, Germany). L-NMMA was dissolved directly in the perfusate. KB-R7943 was made up as a 33.4-mM stock solution in dimethyl sulfoxide, which was then diluted in the perfusate immediately prior to use to obtain 500-μM concentration. Cariporide was made up as a 1-mM solution in the perfusate immediately prior to use. These concentrated solutions of KB-R7943 and cariporide were infused via a side arm of the aortic cannula at a constant infusion rate of 1/100 of coronary flow with a digital infusion pump (Kwapisz, Poland).

The concentrations of KB-R7943 (5 μM) and cariporide (10 μM), selected for this study, are those reported to afford myocardial protection in isolated ischemic/reperfused rat heart (Schafer et al., 2001; Shipolini et al., 1997).

2.3. Experimental design

All the hearts were subjected to 105-min perfusion, and all had an initial 30-min equilibration perfusion followed by one of the three protocols designed to study:

(1) The effect of NHE inhibitor, cariporide (10 μM), NCE inhibitor, KB-R7943, (5 μM), and NO synthase inhibitor, L-NMMA (50 and 100 μM) on control non-ischemic hearts. After 30-min equilibration, the hearts were subjected to 50- or 55-min perfusion with or without the tested inhibitor, which afterwards was washed out for the remaining 20 min of the protocol. L-NMMA was infused between 30 and 85 min and cariporide and KB-R7943 between 35 and 85 min of the protocol (compare protocol 3).
(2) The effect of cariporide, KB-R7943, and L-NMMA on ischemic/reperfused hearts. After 30-min equilibration, the hearts were subjected to another 15 min of aerobic perfusion with or without cariporide, KB-R7943 or L-NMMA, as in protocol 1. Then, the hearts were subjected to 30 min of global ischemia followed by

30-min reperfusion. The infusion of the tested drug was discontinued after 10 min of the reperfusion.

(3) The effect of L-NMMA on ischemic/reperfused hearts treated with cariporide or KB-R7943. The study was aimed at examining whether the effects of L-NMMA and of cariporide or KB-R7943 are additive. The hearts were given cariporide or KB-R7943 and were subjected to ischemia/reperfusion as in protocol 2 and the infusion of L-NMMA (100 μM, via a side arm of the aortic cannula different from that used for the infusion of cariporide and KB-R7943) was started 5 min prior to the application of cariporide or KB-R7943 and it was continued till 10 min of the reperfusion.

2.4. NO measurement

NO formation by the heart was determined by measuring cardiac release of stable NO metabolites, nitrite, and nitrate (nitrite + nitrate = NO_x). The method involves conversion of the effluent nitrite and nitrate to NO gas and the chemiluminescent detection of the latter using Nitric Oxide Analyzer (Sievers, USA). Samples of the coronary effluent were collected at the preselected intervals at pre-ischemia and during reperfusion and were stored at −70 °C until the evaluation that was performed within 10 days after the experiment. A 50-μl aliquot of the unfrozen sample was injected into the glass purger. The apparatus was calibrated daily against a range of known concentrations of sodium nitrite and sodium nitrate.

2.5. Hydroxyl radical measurement

Hydroxyl radical (.OH) measurement was used here as an index of overall cardiac oxygen free radical production. The perfusate was supplemented with sodium salicylate, 1 mM, and .OH formation was determined by measuring hydroxylation products of salicylic acid, 2,5- and 2,3-dihydroxybenzoic acid (2,5-DHBA and 2,3-DHBA, respectively; 2,5-DHBA + 2,3-DHBA = DHBAs), as previously described (Maczewski and Beresewicz, 2000). 2,5- and 2,3-DHBA present in the effluent were separated by high-pressure liquid chromatography (HPLC) and quantitated by electrochemical detection, as originally described by Floyd et al. (1984). Samples of the coronary effluent were collected at the preselected intervals at pre-ischemia and during reperfusion, and were stored at −70 °C until the HPLC evaluation that was performed within 10 days after the experiment. A 20-μl aliquot of the unfrozen sample was injected into the HPLC Shimadzu System (Kyoto, Japan) consisting of a LC-6A Solvent Delivery Pump, a L-ECD-6A Electrochemical Detector, a SCL-6B Controller, a Chromatopac C-R4A data processor, and online ERC-3312 Degasser (Erma, Tokyo, Japan). A Macherey-Nagel Nucleosil C_{18} reverse phase column was used for separation. The eluent was 96% (v/v) 45-mM citrate/61-mM sodium acetate/47 acetic acid buffer (pH 3.6)/4% (v/v) 4% methanol). Retention times and heights for the peaks of 2,5- and 2,3-DHBA were verified by injecting authentic standards (Aldrich) every 14 samples.

2.6. Statistics

All data are expressed as mean ± S.E.M. Significant intergroup differences were calculated using one-way analysis of variance followed by Dunnett's post hoc test. Differences between groups were considered significant if the P value was <0.05.

3. Results

3.1. Sham experiments

There were no significant differences in baseline characteristics between any of the experimental groups studied (Table 1). Moreover, stability of our heart preparation was confirmed by the lack of any significant differences between baseline values and those obtained at the conclusion of the

Table 1
Effect of various treatments on coronary flow, heart rate, and left ventricular developed pressure in aerobically perfused rat heart

Treatment	N	Coronary flow (ml/min/g wet weight)			Heart rate (1/min)			LVDP (mm Hg)		
		Baseline	Treatment	Washout	Baseline	Treatment	Washout	Baseline	Treatment	Washout
Untreated	5	12.1 ± 1.1	11.8 ± 1.0	11.5 ± 1.1	245 ± 21	250 ± 11	247 ± 14	89.1 ± 5.1	87.6 ± 6.7	84.7 ± 5.1
Cariporide (10 μM)	5	13.2 ± 1.1	11.5 ± 1.2	12.2 ± 1.4	248 ± 24	229 ± 21	231 ± 19	87.8 ± 7.6	82.3 ± 7.1	81.4 ± 6.3
KB-R7943 (5 μM)	5	12.4 ± 1.3	10.2 ± 1.2	11.6 ± 1.3	249 ± 19	230 ± 25	229 ± 25	85.1 ± 4.6	81.6 ± 8.6	80.6 ± 6.9
L-NMMA (50 μM)	5	13.0 ± 1.4	10.0 ± 1.1[a]	12.0 ± 1.1	244 ± 22	231 ± 24	235 ± 18	86.1 ± 5.2	80.8 ± 8.1	81.2 ± 5.5
L-NMMA (100 μM)	5	12.8 ± 1.2	9.1 ± 1.0[a]	10.9 ± 1.3	240 ± 21	216 ± 25	221 ± 16	88.4 ± 6.6	79.7 ± 6.5	79.3 ± 6.6
Cariporide + L-NMMA (100 μM)	5	12.5 ± 1.3	8.9 ± 1.0[a]	10.2 ± 1.25	240 ± 20	230 ± 19	227 ± 23	88.6 ± 6.5	77.2 ± 6.6	79.0 ± 7.1
KB-R7943 + L-NMMA (100 μM)	5	12.9 ± 1.2	9.5 ± 1.2[a]	10.7 ± 1.3	247 ± 21	232 ± 20	226 ± 21	87.6 ± 8.8	76.5 ± 6.3	77.4 ± 5.6

Values are means ± S.E.M. N, number of hearts.
After 30-min stabilization and baseline measurements (Baseline), the hearts were subjected to 50–55 min perfusion with the tested drug or their combination (Treatment), and then to 20-min washout perfusion (Washout). LVDP, left ventricular-developed pressure.
[a] $P<0.05$ vs. Baseline.

perfusion protocol for all measured parameters in the untreated hearts subjected to sham perfusion. Among the interventions studied, only 50 and 100 μM L-NMMA caused 23% and 29% ($P>0.05$) reduction in coronary flow and a small nonsignificant trend for a reduction in heart rate and LVDP, which were partially reversible upon washout. The coadministration of L-NMMA (100 μM) with cariporide or KBR resulted in the changes quantitatively similar to those produced by L-NMMA alone.

3.1.1. Post-ischemic NO generation

In all groups, a burst of NO_x outflow was observed upon reperfusion (Fig. 1A). The outflow peaked at 20–40 s, returned to the pre-ischemic values within the following 5

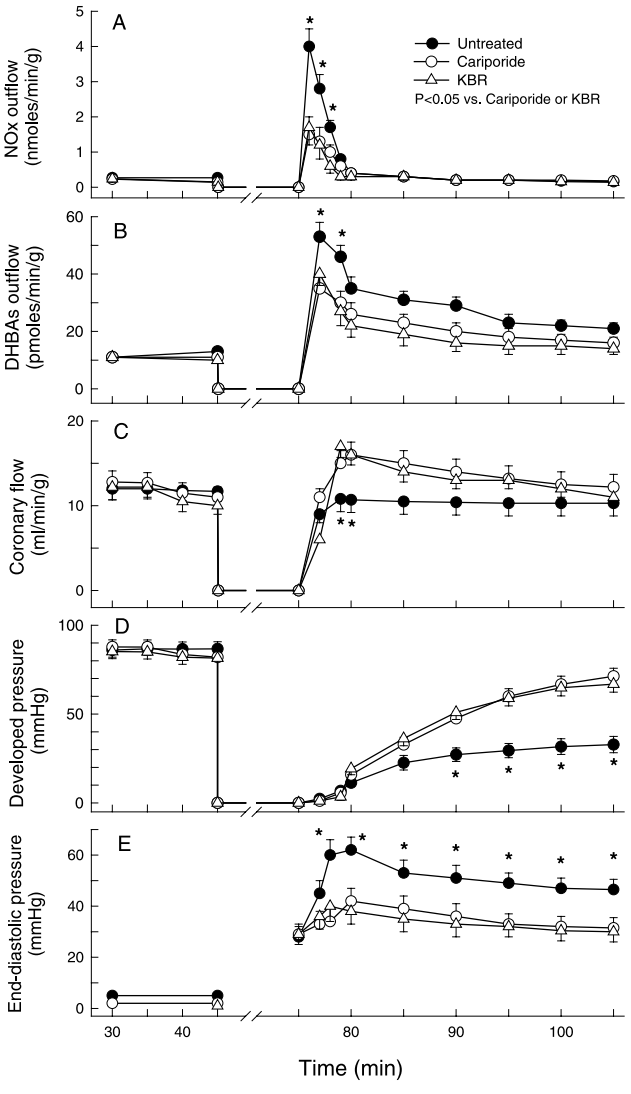

Fig. 1. Effect of cariporide and KB-R7943 on the time course of post-ischemic outflow of nitrite + nitrate (NO_x) and 2,5-DHBA + 2,3-DHBA (DHBAs), and recoveries of coronary flow, left ventricular-developed pressure, and left ventricular end-diastolic pressure in isolated rat heart subjected to 30-min ischemia and 30-min reperfusion. Values are means ± S.E.M. of eight experiments; *$P<0.05$ vs. cariporide or KB-R7943.

Table 2
Effect of various treatments on NO_x and on 2,5-DHBA and 2,3-DHBA outflow in ischemic/reperfused rat heart

Treatment	N/n	NO_x outflow (nmol/5 min/g wet weight)		DHBAs outflow (pmol/5 min/g wet weight)		2,5-DHBA outflow (pmol/5 min/g wet weight)		2,3-DHBA outflow (pmol/5 min/g wet weight)		2,5-DHBA/2,3-DHBA ratio	
		Pre-ischemia	Reperfusion	Pre-ischemia	Reperfusion	Pre-ischemia	Reperfusion	Pre-ischemia	Reperfusion	Pre-ischemia	Reperfusion
Untreated	8/6	0.2 ± 0.0	8.2 ± 0.2[a]	10.2 ± 1.1	221.2 ± 12[a]	6.1 ± 1.4	121.2 ± 11[a]	4.1 ± 1.1	100.0 ± 9[a]	1.5 ± 0.5	1.2 ± 0.3
Cariporide (10 μM)	8/6	0.2 ± 0.0	3.8 ± 0.7[a,b]	14.4 ± 1.3	155.3 ± 18[a,b]	7.3 ± 1.6	89.2 ± 6[a,b]	7.1 ± 3.1	56.1 ± 4[a,b]	1.0 ± 0.5	1.6 ± 0.4
KB-R7943 (5 μM)	8/6	0.2 ± 0.0	4.1 ± 0.8[a,b]	13.4 ± 1.2	132.4 ± 13[a,b]	8.2 ± 1.3	80.4 ± 6[a,b]	6.2 ± 1.5	52.0 ± 4[a,b]	1.3 ± 0.5	1.5 ± 0.4
L-NMMA (50 μM)	8/0	0.2 ± 0.0	6.0 ± 0.5[a]								
L-NMMA (100 μM)	8/6	0.3 ± 0.0	3.8 ± 0.7[a,b]	9.6 ± 1.4	198.0 ± 14[a]	5.3 ± 1.2	109.2 ± 12[a]	4.3 ± 0.4	88.8 ± 9[a]	1.2 ± 0.5	1.2 ± 0.3
Cariporide+ L-NMMA	8/6	0.1 ± 0.0	3.1 ± 0.8[a,b]	12.1 ± 1.3	162.3 ± 14[a,b]	7.0 ± 1.1	96.1 ± 11[a,b]	5.1 ± 0.6	66.2 ± 8[a,b]	1.4 ± 0.7	1.5 ± 0.4
KB-R7943+ L-NMMA	8/6	0.2 ± 0.0	3.2 ± 0.7[a,b]	13.2 ± 2.1	142.1 ± 13[a,b]	8.1 ± 1.4	78.8 ± 9[a,b]	5.1 ± 1.5	63.3. ± 5[a,b]	1.6 ± 0.8	1.2 ± 0.4

Values are means ± S.E.M. N and n, number of hearts in which NO and .OH formations were studied, respectively.
After 30-min stabilization, the hearts were subjected to a further 15-min aerobic perfusion, 30-min ischemia, and 30-min reperfusion. In the treatment groups, the infusion of cariporide and KB-R7943 and of L-NMMA was started 10 and 15 min prior to the ischemia, respectively, and was continued through the initial 10 min of the reperfusion. Presented are the outflows measured during 5-min intervals preceding the ischemia (pre-ischemia) and during initial 5 min of the reperfusion (reperfusion). NO_x, nitrite + nitrate; DHBAs, 2,5-DHBA + 2,3-DHBA.
[a] $P<0.05$ vs. Pre-ischemia.
[b] $P<0.05$ vs. Untreated.

min, and was greatly reduced in the cariporide- and KB-R7943-treated vs. the untreated hearts. For statistical comparisons between groups, the total NO_x outflow and the total coronary flow during the initial 5 min of the reperfusion (hyperemic response) were estimated. The total NO_x outflow was attenuated by cariporide, KB-R7943, and 100 μM L-NMMA by approximately 40% and was not affected by 50 μM L-NMMA (Table 2). The effects of cariporide and L-NMMA, and of KB-R7943 and L-NMMA were not additive. This is evidenced by the fact that the outflow of NO_x was similarly attenuated when cariporide and KB-R7943 were given alone and when they were coadministered with 100 μM L-NMMA (Table 2). The hyperemic response was significantly greater in all cariporide-, KB-R7943-, and L-NMMA-treated groups vs. the untreated group (Table 3), indicating that the change in NO_x outflow reflected the reduced generation of NO_x, but not its reduced washout.

3.2. Post-ischemic hydroxyl radical production

The perfusate glucose was reduced from 11 to 5.5 mM and 1-mM salicylate was added to the perfusate in the experiments aimed at measuring myocardial .OH generation. Under these conditions, the basal coronary flow was slightly increased (by 14 ± 5%), the time course of the post-ischemic coronary flow hyperemic response was prolonged, and the amplitude of the hyperemic response was increased. Consequently, the .OH generation was studied in separate series of experiments.

A small outflow of 2,5- and 2,3-DBHA was detected in all examined groups during pre-ischemia. It was similar in the untreated and in all drug-treated groups, and in all groups the peak of 2,5-DHBA was more prominent than that of 2,3-DHBA (Table 2). In all groups, a burst of 2.5- and 2,3-DHBA outflow occurred upon reperfusion. The outflows peaked at 40–120 s, decayed systematically within the following 30-min reperfusion period, and were greatly reduced in cariporide- and KB-R7943-treated vs. the untreated hearts (Fig. 1B). Of note, the ratio of 2,5-DHBA to 2,3-DHBA during reperfusion was similar to that observed during pre-ischemia, and it was affected neither by cariporide and KB-R7943 nor by L-NMMA (Table 2). The total outflow of 2,5- and 2,3-DBHA during the initial 5 min of the reperfusion was attenuated in cariporide- and KB-R7943-treated hearts by 33% and 41%, respectively, as compared to the untreated group (Table 2). L-NMMA had no effect on the post-ischemic DHBAs outflow, whether it was given alone or together with cariporide or KB-R7943 (Table 2). The hyperemic response upon the reperfusion was similar in all experimental groups aimed at studying .OH generation (not shown).

3.3. Left ventricular function in ischemia and reperfusion

In all groups subjected to ischemia/reperfusion, 30-min ischemia caused a biphasic rise in left ventricular end-diastolic pressure, denoting the development of myocardial contracture. Neither the time course of the ischemic contracture (not shown) nor its amplitude at the conclusion of the 30-min ischemic period was affected by any of the interventions studied (Fig. 1E, Table 3). The contracture increased rapidly again during the initial 3–5 min of reperfusion, falling slowly thereafter. The reperfusion contracture was significantly, and to a similar extent, reduced by cariporide and KB-R7943 (Fig. 1E, Table 3), and it was not affected by L-NMMA, whether it was given alone or together with cariporide and KB-R7943 (Table 3). The post-ischemic recovery of left ventricular developed pressure was significantly more complete in cariporide and KB-R7943 groups vs. the untreated group (Table 3, Fig. 1D). The recovery was, however, not affected by L-NMMA, neither when it was given alone nor when it was coadministered with cariporide and KB-R7943 (Table 3).

Table 3
Effect of various treatments on hemodynamic recoveries in ischemic/reperfused rat heart

Treatment	N	LVDP (mm Hg)		Contracture (mm Hg)		Coronary flow (ml/min/g wet weight)		Hyperemic response (ml/5 min/g wet weight)
		30 min	105 min	75 min	105 min	30 min	105 min	
Untreated	8	88.1 ± 6.1	32.0 ± 5.1[a]	30 ± 6	46.5 ± 4.6[a]	12.1 ± 1.1	10.5 ± 1.3	43.4 ± 5.2
Cariporide (10 μM)	8	87.8 ± 6.7	69.9 ± 6.3[a,b]	32 ± 7	31.5 ± 4.0[a,b]	13.2 ± 1.1	13.1 ± 1.4	70.4 ± 6.3[a]
KB-R7943 (5 μM)	8	85.2 ± 5.6	66.8 ± 6.2[a,b]	29 ± 6	30.0 ± 4.1[a,b]	12.4 ± 1.3	12.3 ± 1.3	68.7 ± 5.5[b]
L-NMMA (50 μM)	8	85.5 ± 5.5	30.1 ± 5.2[a]	32 ± 5	43.5 ± 5.0[a]	14.5 ± 1.2	11.3 ± 1.2[a]	65.6 ± 5.8[b]
L-NMMA (100 μM)	8	89.4 ± 5.1	30.9 ± 5.2[a]	35 ± 8	48.5 ± 5.2[a]	12.8 ± 1.2	9.4 ± 1.3[a]	66.4 ± 4.3[b]
Cariporide + L-NMMA	8	88.6 ± 5.5	66.3 ± 5.3[a,b]	34 ± 5	31.5 ± 4.3[a,b]	12.5 ± 1.3	9.1 ± 1.2[a]	65.5 ± 3.3[b]
KB-R7943 + L-NMMA	8	89.6 ± 5.8	63.0 ± 5.8[a,b]	30 ± 4	33.2 ± 3.8[a,b]	12.9 ± 1.2	9.3 ± 1.3[a]	66.4 ± 5.7[b]

Values are means ± S.E.M. *N*, number of hearts. After 30-min stabilization and baseline measurements (30 min), the hearts were subjected to a further 15-min aerobic perfusion, 30-min ischemia, and 30-min reperfusion, and the hemodynamic measurements were repeated (105 min). In the treatment groups, the infusion of cariporide and KB-R7943 and of L-NMMA was started 10 and 15 min prior to the ischemia, respectively, and was continued through the initial 10 min of the reperfusion. The amplitude of left ventricular end diastolic pressure (Contracture) at 30-min ischemia (75 min) and at 30-min reperfusion was also measured (105 min). LVDP, left ventricular-developed pressure. Hyperemic response, the total coronary flow during initial 5 min of the reperfusion.

[a] $P < 0.05$ vs. 30 min.

[b] $P < 0.05$ vs. Untreated.

4. Discussion

The main finding of this study is that the putative inhibitor of NHE and of the reverse mode of NCE, cariporide and KB-R7943, respectively, attenuated the post-ischemic burst of NO and .OH formation in the isolated ischemic/reperfused rat heart. These results raise several questions as to the mechanism and functional meaning of these effects.

4.1. Activation of NHE and NCE and the post-ischemic generation of NO and .OH

Consistent with earlier reports (Avkiran et al., 2001; Karmazyn et al., 2001; Ladilov et al., 1999; Schafer et al., 2001), the inhibition of NHE and of the reverse mode of NCE appeared cardioprotective also in our study. Thus, post-ischemic recovery of left ventricular-developed pressure was significantly enhanced, and post-ischemic contracture was attenuated in isolated ischemic/reperfused rat hearts treated with cariporide and KB-R7943 when compared with the untreated hearts. It is believed that ischemia- and reperfusion-induced contractures are mediated by cellular energy depletion and Ca^{2+} overload, respectively (Piper et al., 1998). Cariporide and KB-R7943 reduced reperfusion-induced, but not ischemia-induced contracture, suggesting that, in our model, they also reduced Ca^{2+} accumulation in the reperfused cardiomyocytes.

Consistent with previous studies, reperfusion caused a burst of NO (Node et al., 1995; Wang and Zweier, 1996) and free radical generation (Sun et al., 1993; Zweier et al., 1987) also in our model. This was evidenced by the increased outflow from the hearts of nitrite and nitrate (stable metabolites of NO in crystalloid media) and of 2,5- and 2,3-DHBA (products of hydroxyl radical attack on salicylate). Although 2,5-DHBA, but not 2,3-DHBA, may arise from cytochrome *P*-450 (Ingelman-Sundberg et al., 1991), and although peroxynitrite, the reaction product of NO with $.O_2^-$ ($ONOO^-$), may mediate salicylate hydroxylation (Kaur et al., 1997), the following evidence suggests that 2,5- and 2,3-DHBA were a specific assay for .OH formation in our system.

Thus, cytochrome *P*-450 was not the likely source of 2,5- and 2,3-DBHA for two reasons. First, it has been demonstrated by other authors that .OH scavenger, mannitol, reduced the formation of 2,5-DHBA in in vitro .OH generating system as well as in isolated rat heart model of ischemia/reperfusion (Onodera and Ashraf, 1991). Second, the ratio of 2,5-DHBA to 2,3-DHBA formed in all our experimental groups amounted to 1.2–1.6 (cf., Table 2). Earlier, we have observed the similar ratio in an in vitro .OH generating system, and it was not affected by catalase, which otherwise attenuated the formation of 2,5- and 2,3-DHBA (Urbanski and Beresewicz, 2000).

Changes in the production of $.O_2^-$, and NO may be expected to influence DHBAs formation if $ONOO^-$ contributed to their production in our model. Indeed, in the cariporide- and KB-R7943-treated hearts, a parallel reduction in the post-ischemic outflow of NO_x and DHBAs was noted. However, a similar reduction of NO_x outflow was produced also by L-NMMA, that otherwise had no any effect on DBHAs outflow. These suggest that the reduction in DHBAs outflow in the present study reflected decreased myocardial production of .OH (perhaps generated form $.O_2^-$ and H_2O_2 in the Fenton reaction) rather than that of $ONOO^-$.

A new finding of this study is that, along with the functional protection, cariporide and KB-R7943 attenuated the burst of NO and .OH production in the post-ischemic hearts. Of note: (i) cariporide, KB-R7943, and L-NMMA, all reduced the post-ischemic NO generation by approximately 40%; (ii) the effects of cariporide and L-NMMA, and of KB-R7943 and L-NMMA were not additive and (iii) the production of .OH was reduced by cariporide and KB-R7943, but not by L-NMMA. These results suggest that the common mechanism, i.e., the inhibition of NOS, accounted for the reduction in NO generation afforded by cariporide, KB-R7943, and L-NMMA, and that .OH formation did not derive from NOS in our model.

At least four arguments suggest that the inhibition of NHE and NCE in the coronary endothelial, and/or smooth muscle cells, accounted for the effect of cariporide and KB-R7943 on the formation of NO. First, coronary endothelial cells and the underlying smooth muscle cells are coupled through myoendothelial gap junctions (Beny, 1997). It is therefore conceivable that the increase in cellular Ca^{2+}, like that resulting from ischemia/reperfusion, will be transmitted from smooth muscle cells to the endothelial cells and vice versa. In this context, it has been demonstrated that elevation of intracellular Ca^{2+} in smooth muscle mediates endothelial cell generation of NO in arterioles (Dora et al., 1997). Second, molecular targets for cariporide and KB-R7943, i.e., NHE and NCE exchangers, are expressed in the vascular endothelial and smooth muscle cells (Juhaszova et al., 1994; Teubl et al., 1999). Third, simulated ischemia/reperfusion results in cellular Ca^{2+} overload in isolated endothelial cells and the role NCE in this process have been suggested (Berna et al., 2001; Ladilov et al., 2000). Fourth, NO synthesis from eNOS and nNOS requires Ca^{2+} calmodulin (Govers and Rabelink, 2001). Furthermore, recent evidence suggests that NCE and eNOS proteins colocalize in caveolae in vascular endothelial cells, and that NCE facilitates Ca^{2+}-dependent activation of eNOS, suggesting functional interaction between these proteins (Teubl et al., 1999).

There is a number of cellular systems capable of producing free radicals in a Ca^{2+}-dependent manner (Frantseva et al., 2000; Guarnieri et al., 1997). Accordingly, by reducing cellular Ca^{2+} overload, the inhibition of NHE and NCE is expected to attenuate also free radical production. As already discussed, there is little evidence that Ca^{2+}-dependent NOS mediated the post-ischemic .OH generation in our system.

4.2. Reduction in the post-ischemic generation of NO and .OH and the cardioprotection

It is believed that inhibitors of NHE and NCE attenuate myocardial reperfusion injury because they prevent cardiomyocyte Ca^{2+} overload (Avkiran et al., 2001; Karmazyn et al., 2001; Ladilov et al., 1999; Schafer et al., 2001). An intriguing finding of this study is that these inhibitors attenuated also post-ischemic burst of myocardial .OH and NO production, raising a question as to the functional meaning of this effect.

There is considerable evidence that oxygen free radicals, and particularly .OH, mediate cellular injury in various models of myocardial ischemia/reperfusion injury (Bolli, 1998; Onodera and Ashraf, 1991; Sekili et al., 1993; Sun et al., 1993). In this context, we have also found .OH scavengers, like dimethylthiourea and desferrioxamine, to be cardioprotective in a model of ischemia/reperfusion injury in isolated working rat heart (Karwatowska-Prokopczuk et al., 1992). We postulate that in the present investigation, .OH contributed to myocardial injury and that cariporide and KB-R7943 conferred protection, at least partially, via the inhibition of the post-ischemic .OH production.

The role of the endogenous NO in the phenomenon of reperfusion injury has been studied intensively, but it is still debated whether NO is protective or injurious to the reperfused heart Accordingly, the inhibition of NO formation by the inhibitors of NHE and NHC may also be hypothesized to be injurious or protective. The issue may be complicated even further by the fact that NHE may be inhibited by NO (Gill et al., 2002).

NO is thought to be detrimental to the myocardium either due to direct toxicity or through $ONOO^-$ (Beckman and Koppenol, 1996). In apparent agreement with this notion, the blockade of either NOS or $.O_2$ has been found to prevent post-ischemic $ONOO^-$ formation and reperfusion injury in isolated rat heart (Wang and Zweier, 1996; Yasmin et al., 1997) and in in vivo dog heart (Mori et al., 1998). Nevertheless, as in the case of NO, a debate continues whether $ONOO^-$ is deleterious or beneficial for the ischemic/reperfused heart (Ferdinandy and Schulz, 2001; Vinten-Johansen, 2000).

In the majority of studies, however, reperfusion injury has been attributed rather to the reduced formation or activity of NO because the NO precursor L-arginine (Weyrich et al., 1992), several NO donors (Lefer et al., 1993; Siegfried et al., 1992), and lipopolysaccharide, which stimulates myocardial expression of inducible NO synthase (Tosaki et al., 1998), have all been found to protect the heart. In addition, NO is known to serve as the trigger as well as the mediator of the late phase of ischemic preconditioning (Dawn and Bolli, 2002). NO has also been demonstrated to mediate protection afforded by opiates (Zhang et al., 2002). In line with the cardioprotective role of NO, myocardial ischemia/reperfusion injury has been found to be exacerbated in eNOS-deficient mouse (Jones et al., 1999). If the cardioprotective role of NO is assumed, then inhibition of NHE and NHC might be speculated to have simultaneously beneficial as well as deleterious action on ischemic/reperfused myocardium. The former would occur through the inhibition of cardiomyocyte Ca^{2+} overload, and perhaps, through the inhibition of free radical formation, and the latter through the inhibition of NO formation.

However, neither injurious nor beneficial effect of NO could be demonstrated in our experimental model. Although the post-ischemic NO formation was similarly attenuated (by ca. 40%) by cariporide, KB-R7943, and L-NMMA, only the inhibition of NHE and NCE appeared to be cardioprotective, at least in terms of the improved post-ischemic functional recovery and reduced post-ischemic contracture. Furthermore, cariporide and KB-R7943 appeared equally protective, whether they were administered alone or together with L-NMMA. This was the case, although basal coronary flow was similarly reduced (by ca. 30%) in all L-NMMA-treated hearts. Accordingly, the difference in the ability of cariporide and KB-R7943 and of L-NMMA to protect the heart could not be related to alterations in myocardial metabolism resulting from L-NMMA-induced flow reduction. In fact, the literature is controversial as to whether NOS inhibition is harmful (Weiland et al., 2000), neutral (Yang and Mehta, 1997) or protective (Wang and Zweier, 1996; Yasmin et al., 1997) in the isolated ischemic/reperfused rat heart. It may be argued that a bell-shaped concentration dependence of the protective action of L-NMMA, reported in this preparation (Yasmin et al., 1997), provides a clue to these discrepancies. However, neither 50 nor 100 μM L-NMMA appeared to influence ischemia/reperfusion injury in our model.

In summary, this study shows for the first time that the inhibition of NHE and NCE attenuated post-ischemic myocardial formation of NO and .OH. Theoretically, both these effects may be expected to play a role in the myocardial protection provided by NHE and NCE inhibition. The results indicate, however, that the NO synthase pathway does not interfere with the protection afforded by NHE or NCE inhibition in our model of ischemia/reperfusion in the isolated rat heart. Further studies are needed to establish if similar response is seen in other models of ischemia/reperfusion injury, particularly in the in vivo hearts.

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