JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2010, 61, 1, 21-27
www.jpp.krakow.pl

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LOW CONCENTRATIONS OF AN NITRIC OXIDE-DONOR COMBINED WITH A LIPOSOLUBLE ANTIOXIDANT COMPOUND ENHANCE PROTECTION AGAINST REPERFUSION INJURY IN ISOLATED RAT HEARTS

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Nitric oxide (NO) and reactive oxygen species (ROS) are double-edged swords in reperfused hearts. The effects of a NO-donor and an antioxidant compound against ischemia/reperfusion were studied. The compounds were tested separately, as a mixture and as a new hybrid molecule containing both leads. Isolated rat hearts underwent 30 min global ischemia and 2 hrs reperfusion. Compounds were infused either at 1 or 10 μM concentrations during the first 20 min of reperfusion. Hybrid was also tested in the presence of mitochondrial K^+ ATP-sensitive (mKATP) channel blockade by 5-HD (100 μM). Reduction of infarct size and recovery of left ventricular developed pressure during reperfusion were evaluated. When given at 1 μM concentration, hybrid significantly improved all indices of protection; its beneficial effects were abolished by mKATP channel blockade. At the same concentration, mixture and NO-donor alone improved recovery of left ventricular developed pressure but did not reduce infarct size; antioxidant was ineffective. When given at 10 μM concentration, antioxidant and mixture improved all parameters of protection; NO-donor and hybrid were ineffective. Our data suggest that different signaling cascades could be elicited by low and high concentrations of antioxidant compound and/or NO-donor. It is likely that a different NO-induced release of reactive oxygen species *via* mKATP channel activation may play a pivotal role in affecting infarct size and post-ischemic contractile recovery.

Key words: *ischemia-reperfusion, myocardial protection, infarct size, nitric oxide, antioxidant, reactive oxygen species*

INTRODUCTION

Although required to allow myocardium to recover after an infarction, post-ischemic reperfusion produces several negative effects, *e.g.* myocardial stunning and increase in infarct size, which result in the so called reperfusion injury (1-3). Both a burst of reactive oxygen species (ROS) and an altered release of nitric oxide (NO) during reperfusion are believed to contribute to reperfusion injury (4-6). Thus, antioxidant therapy has been proposed as a possible intervention to limit myocardial ischemia/reperfusion (I/R) injury. However, clinical studies have been disappointing, since the various antioxidants employed in reperfusion produced limited or even negative effects (7-10). NO-donors have also been proposed as possible cardioprotective agents (7, 8), with conflicting results as well (11, 12).

Besides pharmacokinetics issues, the I/R scenario and its prevention are in fact more intricate than they appeared at a first glance. Paradoxically protection may in fact be achieved by ischemic pre- (IP) and post-conditioning (PostC), *i.e.* brief coronary occlusions performed either before or immediately after an infarcting ischemia, respectively. Even more paradoxically, ROS production during early reperfusion represents an important step toward the limitation of infarct size in both settings (13-16). In fact, protection by IP and PostC was suppressed by antioxidant administration right at the beginning of reperfusion (13-19). Thus, a better knowledge of the signaling cascades leading to protection suggests that a blanket reduction in ROS levels with a broad-spectrum antioxidant is highly unlikely to selectively target detrimental effects of oxidative stress.

In these protective cascades opening of mitochondrial ATP-sensitive K^+ (mKATP) channels is considered to be a key point towards protection (4, 13, 15, 19-21). In fact, activation of the NO-cGMP-protein kinase G (PKG) pathway may result in opening of mKATP channels and subsequent mitochondrial ROS production, which in turn activates protein kinase C (PKC) and induces protection (4, 5, 12, 13, 15, 22).

Yet, during reperfusion the role of ROS in the protective pathway is more subtle and delicate. For instance, before ischemia exogenous ROS may trigger preconditioning-like protection (23), while if given during the early reperfusion they are unable to trigger a postconditioning-like protection (15). It is likely that protective ROS in reperfusion are produced in a regulated and spatially confined manner (15, 22).

In addition, ROS may interact with NO leading to its inactivation and formation of nitrogen reactive species with either beneficial or detrimental effect (4, 22). Antioxidants may prevent NO degradation, further increasing its bioavailability, and therefore combined administration of antioxidants and NO-

donors has been put forward as a therapeutic strategy to prevent I/R-mediated myocardial injury. In this regard, in isolated rat hearts combined administration of a NO-donor and Tempol, a superoxide-dismutase mimetic, has indeed shown a protective effect, but if given before ischemia (24), *i.e.* when it has little clinical application (8, 22).

Taken together, the above reported data suggest that NO and ROS interaction during reperfusion may affect the outcome of I/R. We thus hypothesized that administration during early reperfusion of a novel hybrid compound with NO-donor (6) and cell permeable antioxidant leads may provide a better myocardial protection than either single lead given separately or as a mixture. We have evaluated two different concentrations of these compounds, which yield different levels of cell permeation, NO bioavailability and/or more complete ROS removal. Finally, we tested whether hybrid effects on I/R injury may be mediated by activation of mKATP channels.

MATERIALS AND METHODS

Animals

Six months old male Wistar rats (Harlan, S. Pietro al Natisone, Italy) were used for this study. The present investigation conforms with the Italian ethical guidelines (DL. 116, 27 Jan, 1996) and with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication N. 85-23, revised 1996). The purposes and the protocols of our studies have been approved by the Ministero della Salute (Ministry of Health), Rome, Italy and by the local ethical committee of the University of Turin.

Chemicals

All chemicals for the buffer solution, dimethyl sulfoxide (DMSO), 5-hydroxydecanoic acid (5-HD) and the antioxidant compound (AOX) 2,2,5,7,8-pentamethylchroman-6-ol, the phenol substructure present in vitamin E, were purchased from Sigma (USA) (*Fig.1*); the reagents necessary to assess myocardial infarction were purchased from Merck (USA). NO-donor (NOD) compound 4-[(dimethylamino)methyl]furoxan-3-carboxyamide, and the hybrid (HYB) 4-((N-((6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methyl)-N-methylamino) methyl)furoxan-3-carboxamide (*Fig. 1*) were synthesized and characterized as nitric oxide donors in the laboratories of the Department of Drugs Science and Technology, University of Turin (6). In particular, the release of NO by NOD and HYB was shown by the impairment of the relaxation of denuded rat aortic strip in the presence of a guanylyl cyclase inhibitor (6).

Isolated heart preparation

Ten minutes after intramuscular injection of heparin (200 UPS units), the animals were anaesthetised with urethane (1 g/kg) given intraperitoneally. The heart was rapidly excised and placed in ice-cold Krebs-Henseleit perfusion buffer. Aorta was then attached to the cannula of the perfusion apparatus, and the heart

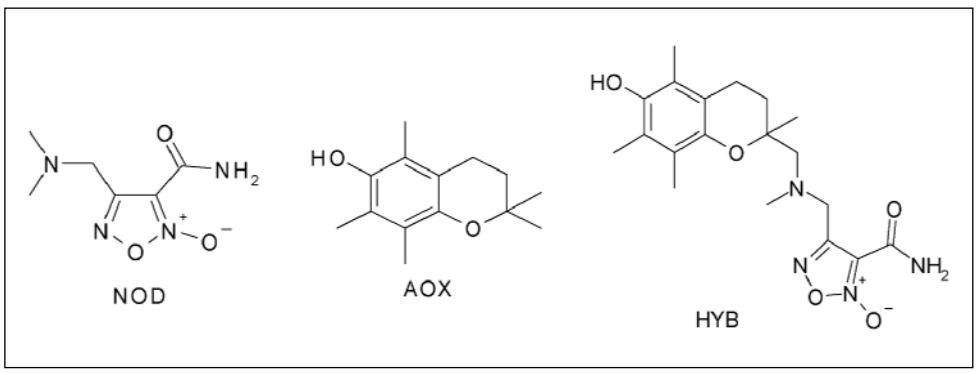

Fig. 1. Structures of the NO-donor/antioxidant hybrid (HYB) 4-((N-((6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methyl)-N-methylamino) methyl)furoxan-3-carboxamide, the parent antioxidant (AOX) 2,2,5,7,8-pentamethylchroman-6-ol, and the NO-donor (NOD) 4-[(dimethylamino)methyl] furoxan-3-carboxyamide.

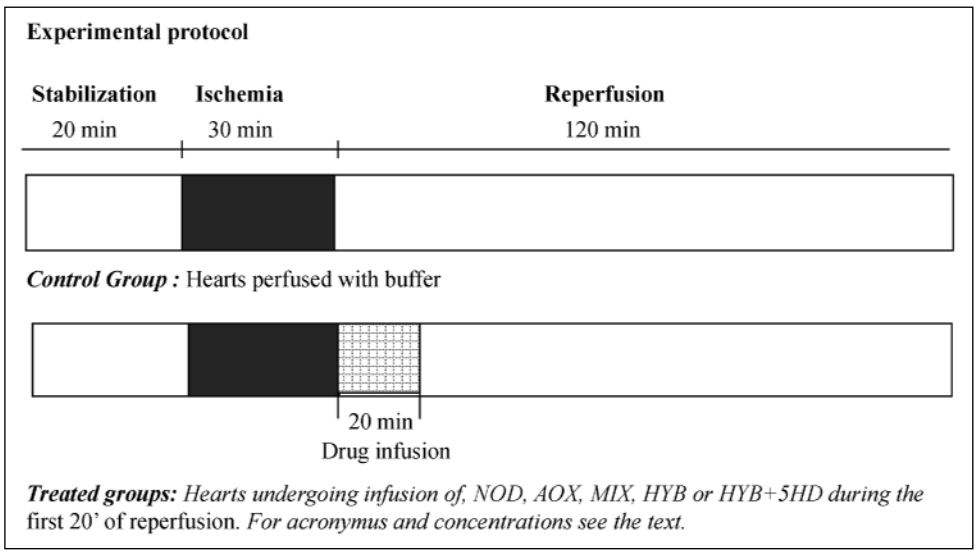

Fig. 2. Time-lines and protocols for the experimental groups.

was perfused at constant flow with a Krebs-Henseleit solution at 37°C containing NaCl (127 mM), $NaHCO_3$ (17.7 mM), $MgCl_2$ (1.26 mM), KCl (5.1 mM), $CaCl_2$ (1.5 mM) and glucose (11 mM). The perfusion buffer was saturated with a 95% O_2 and 5% CO_2 gas mixture as previously described (13, 15).

The constant flow was adjusted with a perfusion pump (Watson-Marlow 505DU, Falmouth, Cornwall, UK) to achieve a perfusion pressure of 80 mmHg during the stabilization. The mean flow to reach this value was 9±1 ml/min/g wet weight.

A small hole in the left ventricular wall allowed the drainage of the thebesian flow. To prevent possible changes in myocardial metabolism in response to changes in heart rate, the hearts were paced at 280 bpm by a Grass S11 stimulator (Grass Instruments, Quincy, Mass, USA).

Experimental protocol

After 20 minutes of stabilization, all hearts underwent 30 minutes of global ischemia at 37°C, obtained by arresting perfusion pump. Ischemia was followed by 2 hours of reperfusion. Pacing was stopped at the beginning of the ischemia and restarted after the third minute of reperfusion (13,15). Hearts were randomly assigned to one of the following groups (*Fig. 2*). Control Group 1 (n=7): hearts underwent ischemia and reperfusion only, without pharmacological interventions

The hearts of groups 2-9 were treated as follows: Group 2 (n=7): 1 μM NOD; Group 3 (n=6): 1 μM AOX; Group 4 (n=7): 1 μM AOX + 1 μM NOD (1 μM MIX); Group 5 (n=9): 1 μM HYB; Group 6 (n=7): 10 μM NOD; Group 7 (n=8): 10 μM AOX; Group 8 (n=9): 10 μM AOX +10 μM NOD (10 μM MIX); Group 9 (n=7): 10 μM HYB.

Since the extent of the protective effects brought about by ischemic postconditioning suggests that a great part of I/R injury occurs during reperfusion (8, 13), treatments were started at the end of ischemia to mimic the PostC and lasted 20 minutes to include the entire period during which most of the injury takes place (8). The two concentrations (1 and 10 μM) were chosen from the range in which the HYB showed both the NO-donating and antioxidant activities according to previous characterization (6).

Assessment of mKATP channel blockade

Since NO may lead to ROS production *via* mKATP channel activation (21), two groups of hearts received the novel HYB at the concentration of 1 μM (Group 10, n=5) or 10 μM (Group 11, n=5) during blockade of mKATP channels with 5-HD at the concentration of 100 μM, which "*per se*" does not alter the response to ischemia/reperfusion (13).

Due to their liposolubility, HYB, and antioxidant were dissolved in DMSO. In preliminary experiments DMSO, administered alone at 0.1% concentration in Krebs-Henseleit solution, did not exert any effect on myocardial I/R injury (data not shown). This is consistent with what observed in previous investigations (13, 14).

Cardiac function assessment

Left ventricular pressure was measured with the probe (Monitoring kit mk5-02 DTNVF, Abbott, Milan, Italy) of a pressure transducer (mod.: CL-810231, Gould inc., Instruments division, Cleveland Ohio, USA) connected *via* a catheter to a latex balloon placed in the left ventricle through the mitral valve. The balloon was filled with a sufficient volume of distilled water to achieve a left ventricular end diastolic pressure of 4-8 mmHg. No further changes of ventricular volume were made during the experiment.

Coronary perfusion pressure was measured with the probe of another pressure transducer connected to the perfusion line. Left ventricular pressure and coronary perfusion pressure were continuously monitored and recorded by a data acquisition system (Lab-View software, National Instrument Corporation, Austin, Texas, USA).

Hemodynamic variables were analysed using Chart Software (AD Instruments, Basile, Milan, Italy). *(LVDP) is expressed as percent recovery with respect to the basal values before ischemia.

Infarct size measurement

At the end of the experiments all hearts were rapidly removed from the perfusion apparatus. After isolation, the left ventricle was cut in 1 mm thick slices. After 20 minutes incubation in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, stained viable tissue was separated from unstained necrotic tissue and then weighed by an independent observer (13, 15). Infarct size is expressed as percentage of the left ventricular mass. Since global ischemia was performed, the total mass of the left ventricle mass corresponded to the risk area.

Statistical analysis

Data are expressed as means±SE. One-way ANOVA with post hoc Tukey test were used to assess the statistical significance of the changes of the studied variables.

RESULTS

1. Infarct size

In comparison with the control Group (54±3%), infarct size was significantly reduced by 1 μM of HYB to 20±6% of the left ventricle ($p<0.01$). No significant reduction was observed in response to NOD, MIX or AOX (37±7%, 30±6%, and 48±6% of the left ventricle, respectively; p=NS) (*Fig.3*, top panel).

2. Cardiac function assessment

A clear pulsatile activity (LVDP>15 mmHg) reappeared in 4 out of 7 hearts of the control group, in 6 out of 7 in NOD Group, in 4 out of 6 in AOX Group, in 7 out of 7 in MIX Group and in 8 out of 9 in HYB Group.

Bottom panel of *Fig. 3* shows the recovery of LVDP after 2 hrs of reperfusion. In comparison with controls (23±7%), recovery of contractile function was significantly improved after treatment with NOD (54±7%; $p<0.05$), MIX (61±6%; $p<0.05$) or HYB (50±8%; $p<0.05$), but not after AOX (28±11%).

1. Infarct size

In comparison with controls (54±3%), infarct size was significantly reduced by AOX to 23±3% ($p<0.01$) and by MIX to 25±8% ($p<0.05$), but not by HYB or NOD (42±9% and 39±7% of left ventricle, respectively) (*Fig. 4*, top panel)

2. Cardiac function assessment

A clear pulsatile activity (LVDP>15 mmHg) reappeared in 5 out of 7 hearts after both HYB and NOD, in 9 out of 9 after MIX and in 7 out of 8 after AOX. Bottom panel of *Fig. 4* shows the recovery of LVDP after 2 hrs of reperfusion. In comparison with controls (23±7%) a significant recovery was observed after AOX (50±4%; $p<0.05$) or MIX (49±4%; $p<0.05$), but not after the other treatments.

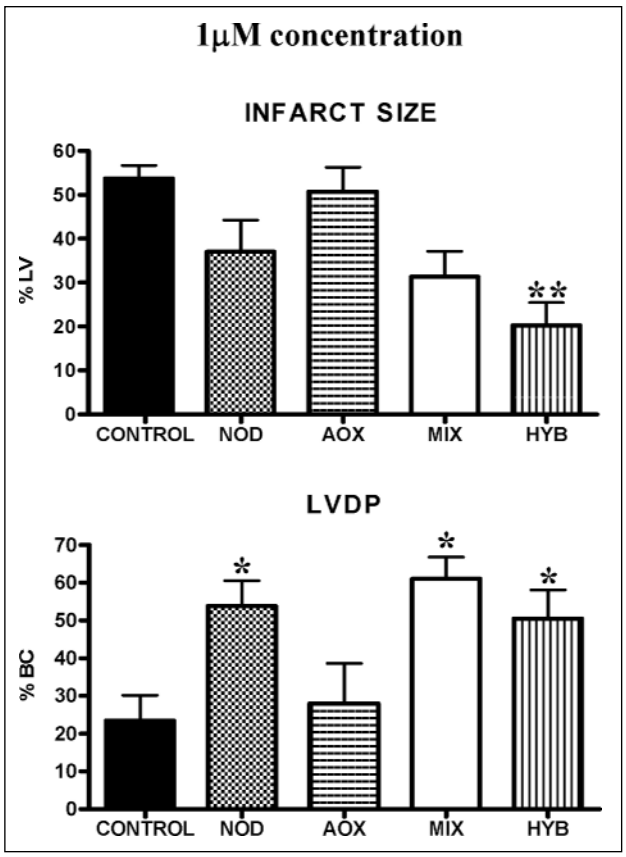

Fig. 3. Infarct size and recovery of left ventricular developed pressure (LVDP) when drugs were administered at 1 μM concentration. Top panel: Infarct size, measured as a percent of left ventricular mass (% LV). Bottom panel: LVDP at the end of reperfusion, expressed as percent of basal values before ischemia (% BC). *p<0.05; **p < 0.01 *vs*. controls.

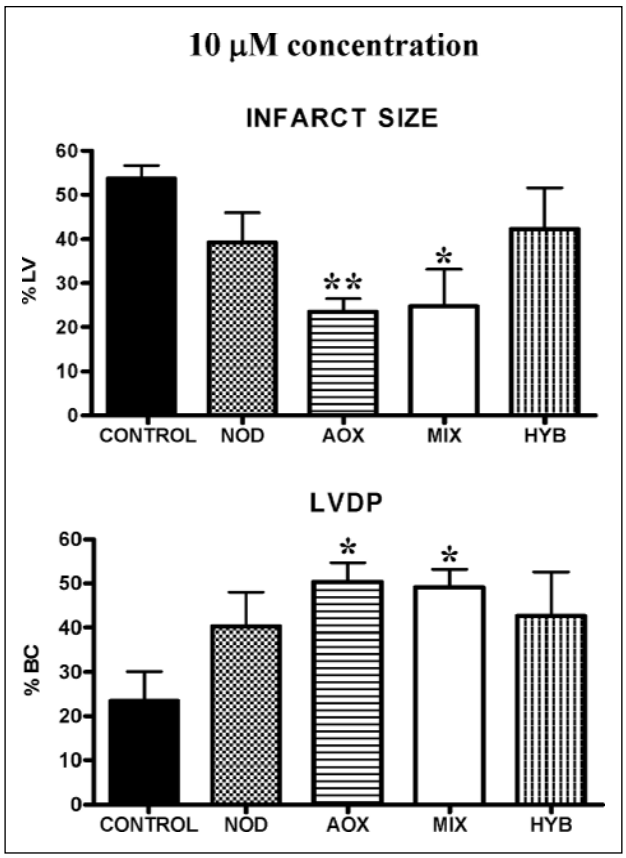

Fig. 4. Infarct size and recovery of left ventricular developed pressure (LVDP) when drugs were administered at 10 μM concentration. Top panel: Infarct size, measured as percent of left ventricular mass (% LV). Bottom panel: LVDP at the end of reperfusion, expressed as percent of basal values before ischemia (% BC). *p<0.05; **p < 0.01 *vs*. control.

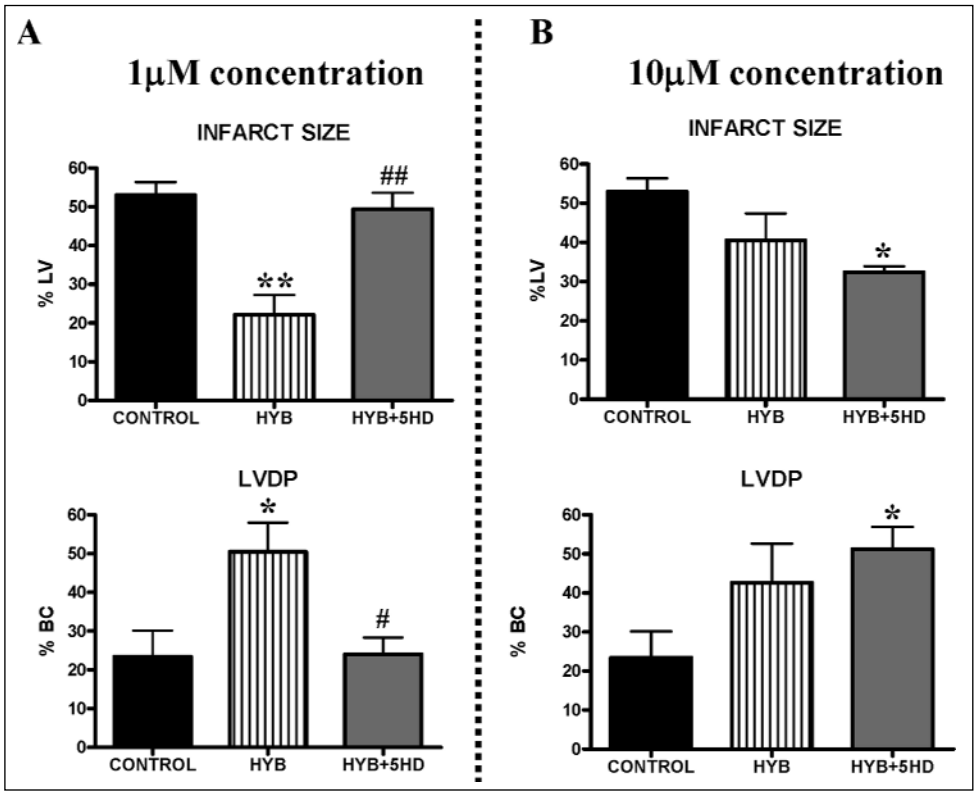

Fig. 5. Infarct size and recovery of left ventricular developed pressure (LVDP) at the end of reperfusion when HYB was administered during mKATP channel blockade by 5-HD. Infarct size was measured as a percent of the left ventricle mass (% LV); recovery of LVDP is expressed as percent of basal values before ischemia (% BC). Panel A: Infarct size (top) and LVDP (bottom) when HYB was administered at 1 μM, with and without 5-HD. Panel B: Infarct size (top) and LVDP (bottom) when HYB was administered at 10 μM, with and without 5-HD. *p<0.05, **p < 0.01 *vs*. control; #p<0.05, ##p<0.01 *vs*. HYB.

Effects with mKATP channel blockade

After blockade of mKATP channels by 5-HD, the effects of HYB on infarct size were reversed. When HYB was given at 1 μM concentration, protection was lost: in comparison with controls, infarct size was not significantly reduced (49±4% and 54±3%; p=NS) (*Fig. 5A*, top panel), and no significant recovery of LVDP was observed (*Fig. 5A*, bottom panel).

In sharp contrast, when HYB was given at 10 μM concentration, a significant reduction of infarct size from 54±3% to 32±2% was obtained ($p<0.01$) (*Fig. 5B*, top panel), accompanied by a significant recovery of LVDP (51±6%; $p<0.05$) (*Fig. 5B*, bottom panel).

DISCUSSION

The present study demonstrates that the protection involves both a better recovery of post-ischemic systolic function (improved LVDP) and a reduction of infarct size when hearts are treated with the novel HYB at 1 μM. A similar protective effect is observed with higher concentration (10 μM) of either AOX or MIX. On the other hand at 1 μM concentration either NOD or MIX can improve the LVDP recovery without causing a significant reduction of the infarct size. While NOD and HYB were ineffective at 10 μM concentration, AOX alone was ineffective at 1 μM concentration. Thus, with the exception of HYB, the treatments are either partially protective (better recovery of systolic function only) when contain low NOD concentration or fully protective (infarct size reduction and recovery of LVDP) when include a high AOX concentration. The novelty of the present study consists in the synergy between the NO-donor and antioxidant effects in 1 μM HYB. This synergy seems to consist mainly in the enhancement of the partially beneficial effect of a low concentration of NOD by the contribution of antioxidant at an otherwise ineffective concentration. It is likely that at 1 μM both leads of HYB reach the appropriate intracellular concentration to achieve both limitation of infarct size and improvement of contractile recovery. In fact HYB molecule may allow the NO-donor portion to enter the cell easily, thus enhancing is availability in the right moment in the right intracellular site.

It seems surprising that HYB at a higher concentration (10 μM) induced neither reduction of infarct size nor significant recovery of LVDP. The different results obtained at 1 and 10 μM of HYB may be explained by the different effects of low and high NO concentration on ROS release and myocardial contractility. The protective role of low levels of ROS has been confirmed in a number of studies (13, 19, 25, 26). In particular, in the survival pathway of myocardial protection, ROS have been shown to be produced in response to opening of mKATP channels elicited by a signaling cascade triggered by NO (21). Since we gave different amounts of HYB with different NO release capacities, we suggest that the protective role of NO-triggered ROS release is likely to be related to the limited quantity and the proper timing of their release, as it occurred when the compounds containing NOD were given at 1 μM concentration. It is also possible that, if the amount of NO-triggered ROS release exceeds a certain, though unknown, value, their damaging effect counteracts the protective role, as it may have occurred when NOD or HYB was administered at 10 μM concentration. This is in keeping with the previous studies reporting that both a burst of ROS and an impaired availability of NO contribute to I/R injury (9, 27). This is also in line with the observation that diazoxide given after ischemia induces a ROS burst which can (15) or cannot (4, 15) be protective, depending on modality of administration. As regards the contractile recovery, it has been reported that, while low concentrations of NO induce a positive inotropic effect, high concentrations cause a loss of contractility (18, 28). The former effect is mainly attributed to prevalence of the cGMP-induced inhibition of phosphodiesterase III and accumulation of cAMP (28), the latter to prevalence of the effect of PKG activation and ROS formation (18, 28, 29). This explanation seems to be corroborated by ongoing experiments with different hybrid compounds, bearing the same antioxidant moiety but with a stronger NO-donor substructures. The results suggest that an excess availability of NO prevents myocardial protection (unpublished observations).

Data obtained with MIX are also in line with this explanation. In fact, unlike HYB, MIX is protective at 10 μM concentrations. At this concentration, the effect of the high concentration of AOX seems to prevail over the detrimental effect of the high concentration of NOD. The two different effects of HYB and MIX at 10 μM concentrations may also be attributed to the different liposolubility of NOD and AOX present in the MIX, which could result in greater concentration of AOX in comparison with NOD inside the cell. On the other hand, as said above, since HYB contains both pharmacophors in the same molecule, it brings the NO-donor portion inside the cell easily, and therefore the high intracellular NO concentration limits protection.

In the present investigation, the dual effect of NO is in keeping with the behavior of NOD alone: 10 μM concentrations of this compound did not show any effect on infarct size and LVDP recovery (*Fig. 4*), while at lower concentrations (1 μM) a trend towards a protective effect was observed, as evidenced by the significant recovery of LVDP only (*Fig. 3*).

NO-donors may differently influence the activity of antioxidant enzymes, depending on the protocol of treatment and the studied tissues. For instance, various NO-donors have been seen to increase superoxide dismutase (SOD) activity in gastric tissue and to protect it against the injury by water immersion restraint (30). In myocardium of spontaneously hypertensive rats (SHR) NO-donor *pentaerythrityl tetranitrate* (PETN) improved SOD activity and reduced cardiac hypertrophy (31, 32). However, Kristek *et al*. concluded that PETN "does not result in a beneficial effect on the myocardium and on the geometry of carotid and coronary arteries" (31).

It may then be argued that, to protect the heart also against infarct size, the beneficial NOD effect should be integrated by an exogenous antioxidant activity as it occurs with 1 μM HYB. In addition to the availability of NO, the protection offered by HYB is likely to be due to intracellular scavenging of excessive ROS with prevention of membrane lipid peroxidation (33).

The involvement of ROS in the different effect of low and high concentrations of NO released by HYB has been also suggested by the experiments in which mKATP channels were blocked and NO cascade was prevented from inducing ROS production by mitochondria. In these experiments, when HYB was given at 1 μM concentration, infarct size was not reduced because the protective NO cascade was interrupted by mKATP blockade in the presence of an otherwise ineffective antioxidant concentration. Conversely, at the concentration of 10 μM, the dangerous effect of the high concentration of NO was prevented by mKATP blockade, so that the strong antioxidant activity of the compound at this concentration was not impaired. These results bring further support to the hypothesis that NO effect is mediated by mKATP channels, which can limit or increase infarct size *via* the production of low or high ROS levels, respectively (21, 22).

Our conclusions about the role of NO-ROS pathway in myocardial protection are based on the previous demonstration, by our and other groups, that in isolated hearts and cardiomyocytes protection induced by NO occurs *via* ROS release from mitochondria after opening of mKATP channels both in pre- and post-conditioning (4, 13, 15, 21). Nevertheless, ROS may exert divergent effects, depending on the site, time,

and amount of their generation; they are thus double-edged sword and our data with mKATP channel blocker suggest a pivotal role for NO-triggered ROS release in both protection and reperfusion injury, depending on the amount of available NO-donor. This hypothesis deserves future studies to be demonstrated.

After ischemia a clear pulsatile activity was observed in only 4 out of 7 experiments of the control group, while it reappeared in most of the protected hearts (1 μM of HYB, 10 μM of AOX and 10 μM of MIX); this different recovery might also be considered an additional marker of protection.

Compared with the other effective treatments, the effect of HYB at 1 μM concentration is important not only because of the significant reduction of infarct size, recovery of LVDP and increased rate of reappearance of pulsatile activity, but also because of the small dose required to produce all features of protection. In addition, the effect of HYB was obtained with one molecule only that allows the same pharmacokinetics for the two leads.

Overall, our data support the idea that the timing and intracellular compartmentalization of NO release and ROS scavenging are important issues. These might explain the lack of effects of antioxidant therapies only and the seemingly deleterious effect of antioxidant pretreatment in clinical setting (9, 10). Yet, since HYB is effective if given at low concentration at the time of reperfusion, it might represent a promising clinical tool, because it could be administered to patients after the onset of infarction, at the time of reperfusion.

CONCLUSION

In conclusion, administration of an hybrid compound with both NO-donor and antioxidant properties may represent a novel therapeutic strategy against ischemia-reperfusion injury if infused in the coronary stream at the beginning of reperfusion, and at a dose which causes the appropriate balance between NO donor and antioxidant activity. Our experiments suggest that the antioxidant moiety may help the cell permeation and the activity of the low concentration of NO released by the HYB at 1 μM concentration. Finally, our data suggest that the protective effects of 1 μM of HYB are mediated at least in part by mKATP channel activation.

Work was performed at Dipartimento di Scienze Cliniche e Biologiche, Facolta di Medicina e Chirurgia "S. Luigi Gonzaga", Regione Gonzole 10, 10043 Orbassano (TO), Italy.

Acknowledgments: This research was funded by the Italian Ministry of University (2006061988_003), the local Government of Regione Piemonte (CIPE 2004), the Istituto Nazionale per le Ricerca Cardiovascolare (INRC) and Compagnia di San Paolo, Torino.

Conflict of interests: None declared.

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Received: April 14, 2009
Accepted: January 12, 2010

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