

# Hypertonic reperfusion preserves endothelial nitric oxide synthase expression after ischemia-reperfusion in isolated working rat hearts

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## Introduction

The development of myocardial edema has been clearly demonstrated in various clinical conditions, such as myocardial infarction or ischemia, orthotopic heart transplant rejection, uremic cardiomyopathy and myocarditis<sup>1-3</sup>. In experimental animals, edema formation has been induced by increasing hydrostatic pressure in the coronary vasculature, decreasing fluid removal by impairing myocardial lymph flow, altering the oncotic or osmotic pressure, or by coronary occlusion or reperfusion<sup>4-7</sup>. Previous studies have demonstrated, in ischemic-reperfused hearts, the direct contribution of myocardial edema in acute cardiac mechanical and vascular dysfunction<sup>8-10</sup>. In addition, the presence of chronic edema within the interstitium has also been shown to promote fibronectin deposition and stimulate fibrosis within the myocardial interstitial matrix<sup>11</sup>. Hypertonic reperfusion was successfully proposed as a therapeutic approach able to reduce ischemia-reperfusion damage in various experimental models<sup>8,9,12</sup>. However, information concerning the factors that control the rate and the magnitude of edema formation, is limited to a regulation of osmotic pressure and coronary resistance or permeability. Particularly, there are no data regarding potential effects of hypertonic reperfusion on endothelial function and nitric oxide (NO) production. These data may be of potential interest because NO is one of the most important modulators of en-

dothelial function, able to regulate various agents involved in myocardial edema after ischemia-reperfusion, such as microvascular hyperpermeability, vascular tone and platelet and/or leukocyte activation<sup>13-15</sup>.

We investigated the effects of hypertonic reperfusion on myocardial damage and cardiac function, vascular permeability and endothelial NO synthase expression induced by ischemia-reperfusion in a model of myocardial damage in isolated working rat hearts.

## Methods

**Animals and perfusion technique.** Sixty adult male Wistar rats (250-300 g) were anesthetized with a mixture of ether and air. After injection of 1000 IU heparin in the femoral vein, the hearts were quickly excised and weighed. A modified Krebs-Henseleit (KH) solution (108 mM NaCl, 25 nM NaHCO<sub>3</sub>, 4.8 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 11 mM glucose, 287 mOsm, pH 7.4, at 37° C) was used as perfusion medium. The solution was bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Preload and afterload were set at 20 and 72 cm H<sub>2</sub>O, respectively. Aortic and coronary flows (ml/min) were measured collecting aortic chamber overflow and heart chamber effluent into graded cylinders. Aortic pressure (mmHg) was monitored through a membrane transducer (TNF-R, Viggo-Spectramed, Oxnard, CA, USA) connected to the aortic cannula. Heart rate (b/min)

was determined with an epicardial electrocardiogram (Cardioline 350/1, Milan, Italy). Minute work (mmHg  $\times$  ml/min) was calculated as the product of cardiac output (aortic and coronary flow) and peak aortic systolic pressure. Coronary resistances (dynes  $\times$  s  $\times$  cm<sup>-5</sup>) were also calculated as mean aortic pressure  $\times$  80/coronary flow.

**Assessment of left ventricular function and myocardial tissue damage.** Hearts were submitted to 20 min stabilization followed by 15 min of global ischemia and 180 min of reperfusion (in the Langendorff perfusion mode for the first 10 min and in the working heart mode for the remaining 170 min). The hearts were subdivided into two groups: group A (n = 10, control group), untreated hearts perfused with KH solution, and group B (n = 10), hearts treated with hypertonic reperfusion. Hypertonic reperfusion was obtained by adding, during postischemic reperfusion, 80 mM sucrose (420 mOsm) to KH solution.

**Heart weight changes.** Hearts were weighed before and after the experiment on an analytical balance. Percent weight gain between the end and the beginning of the experiment was calculated in each group.

**Creatine kinase release.** Creatine kinase (CK) activity in the coronary effluent was measured by a spectrophotometric commercial assay (Boehringer Mannheim Italia, Monza-MI, Italy) at fixed times: immediately before ischemia (at 20 min), during Langendorff mode perfusion (at 35, 37, 39 min), and during working heart mode reperfusion (at 55, 65, 75, 85, 95, 105 min). Data were reported as IU/ml/g wet weight.

**Assessment of microvascular permeability changes.** After ischemia, additional rat hearts (n = 5 each group) were perfused with fluorescein isothiocyanate (FITC)-albumin (Sigma, Milan, Italy) (75 mg dissolved in 200 ml KH solution) in order to assess microvascular permeability<sup>16,17</sup>. After FITC-albumin perfusion (mean perfusion time 20 min), hearts were reperfused for 2 min with KH solution (in the Langendorff mode) to eliminate intravascular fluorescence, and reweighed. Microvascular permeability changes were determined on FITC-albumin perfused hearts by fluorescence microscopy. Ventricles were cut transversally into 4-5 blocks. Tissue blocks were immediately fixed in O.C.T. (Miles, Elkhart, IN, USA) and stored at -80°C. Tissue blocks were mounted on a specimen holder in a Slee microtome-cryostat maintained at -35°C and oriented in order to cross-section capillaries and muscle fibers. Ten  $\mu$ m sections were obtained from each time block, placed on a pre-warmed dark box for at least 1 hour and then photographed at 40 $\times$  magnification under UV light (488 nm). FITC-albumin accumulation was quantified using a computerized image analysis system. Results were expressed as integrated optical intensity units<sup>18</sup>.

**Evaluation of endothelial nitric oxide synthase mRNA expression.** Quantitative multiplex reverse-transcribed polymerase chain reaction (PCR) was used to determine mRNA levels of the constitutive endothelial nitric oxide synthase (eNOS) isoform in rat ventricular tissue. Myocardial samples (n = 7 each group), fixed in liquid nitrogen and stored at -80°C, were homogenized in 800  $\mu$ l of RNA fast solution (Celbio, Milan, Italy). Total RNA was isolated as recommended by the manufacturer. RNA was dissolved in diethyl pyrocarbonate-treated water and quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding RNA (0.1  $\mu$ g) to a mixture containing 1 mM deoxynucleosidetriphosphates, 1 U/ $\mu$ l RNase inhibitor, 2.5 U/ $\mu$ l moloney murine leukemia virus reverse transcriptase, 2.5  $\mu$ M random examers, 5 mM MgCl<sub>2</sub>, 10  $\times$  PCR buffer in a final volume of 20  $\mu$ l. Reverse transcription was performed at 42°C for 1 hour followed by heat inactivation of reverse transcriptase at 99°C for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified from the same amount of RNA to correct for variation of different samples. The PCR solution contained 10  $\mu$ l of first-strand cDNA, 4  $\mu$ l 10  $\times$  PCR buffer, 2 mM MgCl<sub>2</sub>, 0.15 mM of both sense (5'-ACC ACA GTC CAT GCC ATC AC-3') and antisense (5'-TCC ACC ACC CTG TTG CTG TA-3') GAPDH primer, 0.15 mM of both sense (5'-CGA GAT ATC TTC AGT CCC AAG C-3') and antisense (5'-GTG GAT TTG CTG CTC TCT AGG-3') eNOS primer, 2U Taq DNA polymerase (Celbio, Milan, Italy), and water to a final volume of 50  $\mu$ l. These samples were overlaid with mineral oil and subject to 35 cycles at 95°C for 60 s, 60°C for 60 s, and to 1 cycle at 72°C for 7 min. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UV light. Bands on the gel were scanned and quantified using a computerized densitometric system (Bio Rad Gel Doc 1000, Bio-Rad, Milan, Italy)<sup>19</sup>.

**Western blot analysis.** Ventricular samples were used for this purpose. Proteins (50  $\mu$ g) were separated on 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and electroblotted to a nitrocellulose membrane. eNOS was detected by monoclonal antibodies (Santa Cruz Biotech Inc., Santa Cruz, CA, USA).  $\beta$ -actin was used as internal control to correct for variations of different samples. Protein bands were visualized by a chemoluminescence detection system and quantified determining the change in integrated optical intensity using a computerized system (Kodak ISO Transmission Density, Eastman Kodak Company, Rochester, NY, USA).

**Assessment of the effects of nitric oxide synthase inhibition.** In order to confirm the involvement of NO in the cardioprotective effects of hypertonic reperfusion, 30  $\mu$ M N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME,

Sigma, Milan, Italy), a specific inhibitor of NO synthase, was used alone or in hypertonic reperfusion-treated hearts in the experimental protocols devoted to assess left ventricular function and myocardial tissue damage (n = 10) or microvascular permeability changes (n = 5).

**Statistical analysis.** Statistical analysis was performed using the Student's t test for two-group comparisons (heart's weight, ultrastructural data, vascular permeability, changes in gene expressions) and two-way analysis of variance (ANOVA) for multigroup comparisons (hemodynamic parameters, CK release), after the assessment of normality of distribution. The probability of null hypothesis < 5% (p < 0.05) was considered statistically significant. All results are reported as mean ± SD.

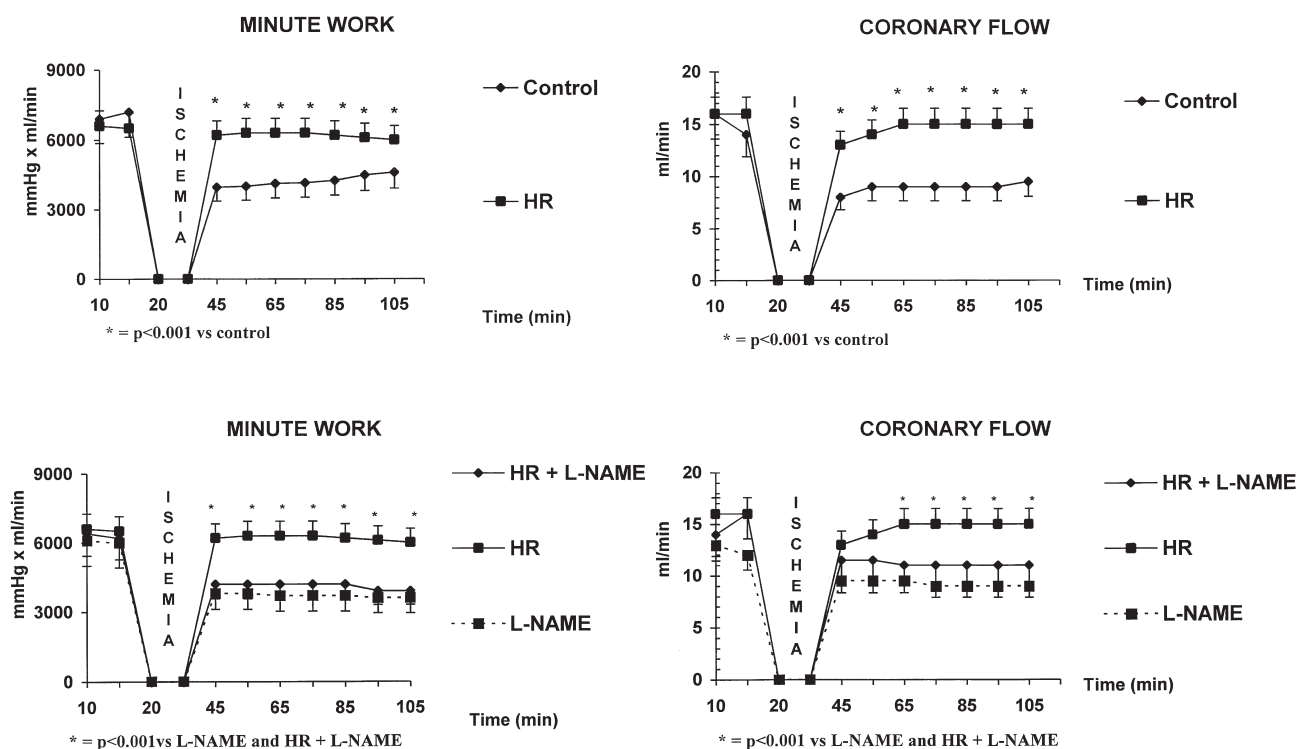
**Results**

**Effects of hypertonic reperfusion on left ventricular function, creatine kinase release and heart weight.** Hypertonic reperfusion determined a significant cardioprotection against mechanical dysfunction (Fig. 1). Parallel to these changes, a significant improvement in coronary flow and resistances was observed. In the same group, a significant reduction of early-reperfusion CK release and of reperfusion edema occurred (heart weight gain in group A was 30 ± 4.7, in group B 16 ± 5, p < 0.001 vs group A).

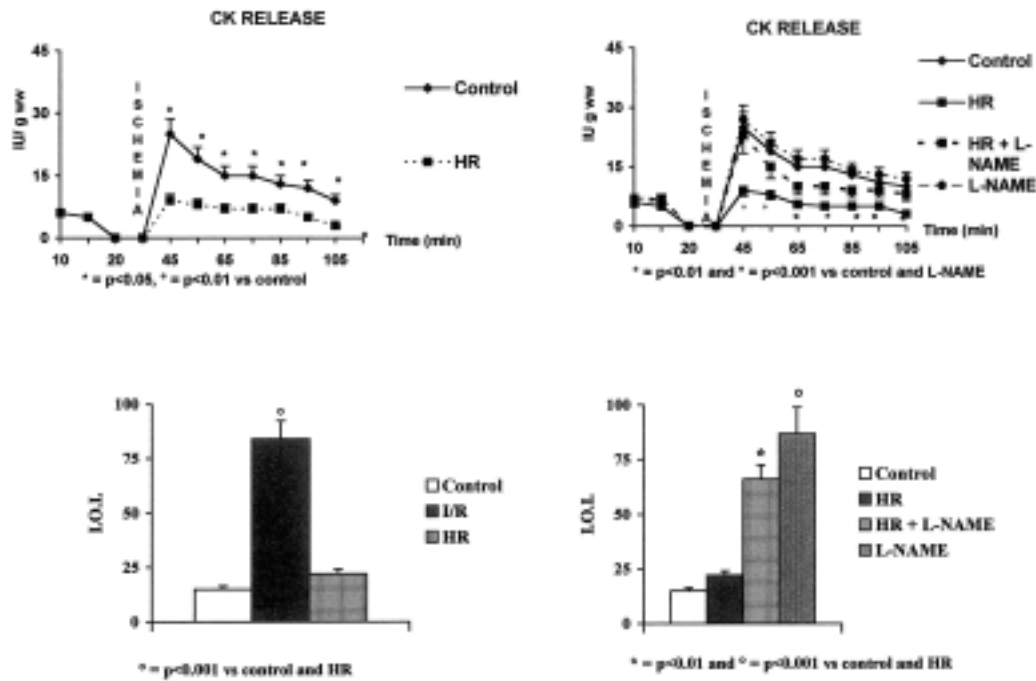
**Effects of hypertonic reperfusion on microvascular permeability.** In the control group, ischemia induced a marked FITC-albumin extravasation in perivascular and perimyocytic space. In hypertonic reperfused hearts, a significant reduction of FITC-albumin diffusion was detected (Fig. 2). In this experimental condition, albumin extravasation was limited to perivascular space, and no significant diffusion of the tracer was observed in the perimyocytic space.

**Effects of hypertonic reperfusion on endothelial nitric oxide synthase mRNA expression and protein levels.** Ischemia-reperfusion induced a significant (p < 0.02) reduction in eNOS mRNA and protein levels, while hypertonic reperfusion produced a significant (p < 0.03) increase in eNOS mRNA and protein levels compared with control hearts (Fig. 3).

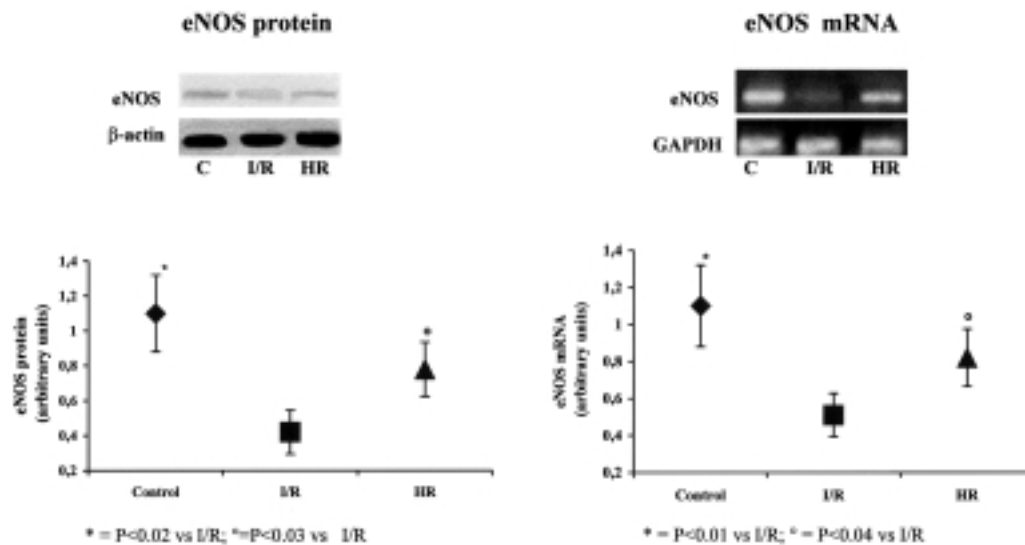
**Effects of nitric oxide synthase inhibition on hypertonic reperfusion effects.** The addition of 30 µM L-NAME to KH solution in presence of hypertonic reperfusion significantly reduced postischemic functional recovery and induced a significant increase of heart weight gain (+24 ± 4%; p < 0.01 vs hypertonic reperfusion) and CK release in the coronary effluent (Fig. 2). The addition of L-NAME also increased FITC-albumin extravasation both in the perivascular and in the perimyocytic space (Fig. 2). Thirty µM L-NAME perfusion alone did not significantly affect any of the parameters investigated.



**Figure 1.** Upper panels: time course of minute work (mmHg × ml/min) and coronary flow (ml/min) in the control and hypertonic reperfusion (HR) experimental groups. Lower panels: the effects of nitro L-arginine methyl ester (L-NAME). Data are expressed as mean ± SD (n = 10 each group).



**Figure 2.** The effects of hypertonic reperfusion (HR) on creatine kinase (CK) release in the coronary effluent ( $n = 10$ , top left panel) and microvascular permeability (fluorescein isothiocyanate-FITC-albumin extravasation) ( $n = 5$ , top right panel). Lower panels: the effects of nitro L-arginine methyl ester (L-NAME). Data are expressed as mean  $\pm$  SD. I.O.I. = integrated optical intensity units; I/R = heart subject to ischemia-reperfusion.



**Figure 3.** The effects of hypertonic reperfusion on endothelial nitric oxide synthase (eNOS) mRNA expression and protein levels after ischemia-reperfusion. Top left panel: original Western blot is reported. Levels of  $\beta$ -actin are shown for comparison. Top right panel: representative example of results of nitric oxide synthase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse-transcribed polymerase chain reaction amplifications of mRNA in different experimental conditions. GAPDH mRNA levels are used for densitometric normalization of nitric oxide synthase mRNA levels. Lower panels: eNOS protein and mRNA steady state results of densitometry. C = control heart in non-ischemic conditions; I/R = heart subject to ischemia-reperfusion; HR = heart subject to ischemia and hypertonic reperfusion. Data are expressed as mean  $\pm$  SD ( $n = 7$  each group).

## Discussion

This study shows that the administration of a hypertonic buffer after myocardial ischemia significantly reduces myocardial dysfunction and vascular endothelial and myocardial damage (postischemic increase in microvascular permeability, and changes in microcirculatory resistances) occurring after ischemia-reperfusion in the isolated work-

ing rat heart. Concurrent with these beneficial effects, hypertonic reperfusion partially prevented eNOS mRNA reduction induced by ischemia-reperfusion. The action of hypertonic reperfusion on the mRNA expression for the enzymes catalyzing NO production appears to be causally linked to the here reported beneficial effects, since totally abolished by the simultaneous treatment of the heart with the NO synthase inhibitor L-NAME.

The modulation of plasmatic and interstitial osmolarity appears fundamental in preventing cellular damage after ischemia and reperfusion. Myocardial ischemia causes endothelial dysfunction and increases of coronary artery permeability<sup>20</sup>. The dysfunction depends upon membrane damage, activation of transmembrane transport system, and hypercontracture of the endothelial cells<sup>21</sup>. Sunnergren and Rovetto<sup>16</sup> reported that these alterations are not modified by oxygen-free radicals but can be ameliorated by calcium channel blockers. The hypertonic reperfusion results in a significant reduction of edema extension, microcirculatory impairment, and reperfusion damage. The observed reduction of myocardial enzyme leakage is in agreement with previous reports<sup>9,12</sup> describing a reduction of the infarct size using hyperosmotic mannitol. However, other authors failed to demonstrate protective effects of mannitol infusion after ischemia both in pigs or dogs and baboons<sup>22,23</sup>. These differences can be attributed to the different animal models used, the time of ischemia and degree of osmolarity.

Associated with the favorable effects on osmotic load, such effects might be implicated in the preservation of endothelial function during hypertonic reperfusion, as well as in preventing the acute myocardial and vascular damage accompanying ischemia and/or reperfusion. NO release is an important factor in the regulation of endothelial function. Besides its well known vasodilatory effects, NO is known to modulate endothelial permeability under basal and ischemic conditions, reduces postischemic hyperpermeability, decreases platelet adhesion and aggregation, and leukocyte adherence and emigration<sup>13-15</sup>. Because of these multiple actions, we hypothesized that decreased NO is implicated in myocardial damage induced by ischemia-reperfusion<sup>16-24</sup>, and that at least part of the cardioprotective effects of hypertonic reperfusion might be mediated by NO. Indeed, in our experimental model, hypertonic reperfusion could modulate NO synthesis, by increasing eNOS mRNA. On a theoretical ground, it is likely that eNOS up-regulation has positive effects after ischemia-reperfusion because of the host of endothelial protective effects of NO interferon- $\gamma$ , and could induce myocardial damage or deterioration of myocardial performance<sup>16-24</sup>. The importance of NO in hypertonic reperfusion-induced myocardial and vascular endothelial protection is indeed supported by our findings of the loss of these favorable effects in the presence of the NO synthase inhibitor L-NAME.

We recognize intrinsic limitations in our experimental setup, making the *in vivo* transferability of our experimental data open to some questions: the oxygen content in our perfusion buffer is low, with consequent high coronary flow; in addition, glucose is practically the only energy source in our model, contrary to the preferential fatty acid utilization of the intact heart. This method, however, allows the primitive endothelial alterations in the absence of cardiac load variations.

The use of sucrose as hyperosmotic agent is well recognized as it lacks scavenging effects; in the experimental model reported, the absence of whole blood largely reduced the importance of the free radicals on myocardial reperfusion damage.

In conclusion, we have shown that hypertonic reperfusion could exert NO-dependent cardioprotection against ischemia-reperfusion injury in the isolated rat heart. These results suggest a novel spectrum of potentially favorable effects of hypertonic perfusion in coronary artery disease syndromes.

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