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Effects of iNOS-related NO on Hearts Exposed to Liposoluble Iron

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Inducible nitric oxide synthase (iNOS) protects heart against ischemia/reperfusion injury. However, it is unknown whether the beneficial effects of iNOS are mediated by the interaction of NO with radical oxygen species (ROS). To address this issue, we examined the effects of liposoluble iron-induced ROS generation in isolated perfused hearts from rats treated with lipopolysaccharide (LPS). LPS administration (10 mg/kg, i.p., 6 h before heart removal) induced iNOS expression and increased NO production as indicated by a 3-fold elevation of nitrite level in coronary effluents relative to control hearts. An enhanced expression of heme-oxygenase 1 protein was also observed in septic hearts compared to control. Iron-induced perfusion and contractile deficits were ameliorated by LPS with more important coronary than myocardial benefits. In iron-loaded hearts, oxidative stress as measured by the 2,3 dihydroxybenzoic acid/salicylic acid concentration ratio in cardiac tissue was 23% lower in septic than in control heart although the difference did not reach significance. In addition, the presence of the NO synthase inhibitor *N*-nitro-L-arginine in the perfusion medium totally blocked NO production but did not reverse the protective effects of LPS. The results indicate that LPS protects from iron-induced cardiac dysfunction by mechanisms independent on *ex vivo* NO production and suggest that NO acts as a trigger rather than a direct mediator of the cardioprotective effects of LPS in heart exposed to iron.

Keywords: NO; LPS; Iron; Isolated heart preparation; Hydroxyl radical

INTRODUCTION

Radical oxygen species (ROS) are produced in excess when the myocardium is reperfused after an episode of ischemia and they significantly contribute to manifestations of the reperfusion syndrome.[1,2] ROS toxicity in reperfused heart involves different mechanisms including direct oxidative damage to cell constituents, disruption of calcium homeostasis[3] and cellular energetics,[4] and activation of apoptotic processes.[5]

Several *ex vivo* and *in vivo* data are in favor of a beneficial role of inducible nitric oxide synthase (iNOS)-related NO in heart exposed to ischemia/reperfusion (I/R). Thus, treatment with lipopolysaccharide (LPS) or monophosphoryl lipid A (MLA), a non-toxin derivative of LPS, protects heart against I/R injury.[6–9] Furthermore, studies carried out with iNOS gene deleted mice have demonstrated the obligatory role of this enzyme in the cardioprotection afforded by the late phase of ischemic preconditioning[10] or by MLA treatment.[11] However, the exact mechanisms underlying the protective effects of iNOS have not been elucidated yet and it is still unknown whether an interaction of NO with ROS accounts for the beneficial effects.

NO can exhibit both prooxidant and antioxidant properties. Peroxinitrite that results from the reaction

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of NO with superoxide anion[12] has largely been implicated in the prooxidant effect of NO. In contrast, at high concentration, NO can act as an effective antioxidant,[13–16] thus supporting the hypothesis that NO can protect heart against I/R by decreasing ROS toxicity. Direct as well as indirect mechanisms may account for the antioxidant properties of NO. By itself, NO can decrease lipid peroxidation by scavenging lipid peroxyl radical[17] or by inactivating redox active iron through the formation of dinitrosyl iron complexes.[18] In addition, NO can indirectly decrease the sensitivity of cells to ROS through the induction of antioxidant defenses.[19] However, the effects of NO in heart exposed to ROS generating systems have never been investigated.

In the present study, ROS-induced contractile dysfunction and oxidative stress as measured by hydroxyl radical formation in cardiac tissue were examined in isolated perfused hearts from rats treated with LPS to induce cardiac iNOS expression. To study the implication of NO, the septic hearts were perfused with a NOS inhibitor. ROS production was induced by a transient exposure of heart to liposoluble iron[20] in order to mimic oxidative stress consecutive to I/R. In addition, data in favor of a control by NO of the inducible heme-oxygenase HO-1,[21] an enzyme which in other respects protects heart from I/R injury,[22] led us to examine the effect of LPS on heart HO-1 protein expression.

MATERIAL AND METHODS

Animals and Chemicals

Experiments were performed on Wistar rats (Depré, Doulchard, France) weighing 350–450 g. LPS (from *Escherichia coli*, serotype 055:B5), N_ω-nitro-L-arginine (nitroarginine), L-arginine hydrochoride, sodium salicylate, $FeCl_3$ hexahydrate, 8-hydroxyquinoline (HQ, free base) and desferrioxamine mesylate were purchased from Sigma. The anti-HO-1 monoclonal antibody was obtained from Stressgen (Le Perray en Yvelines, France) and the anti-iNOS polyclonal antibody from Transduction Laboratories (Le Pont de claix, France). The horseradish peroxidase-labeled antirabbit and antimouse-IgG antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (Interchim, Montlucon, France). Liposoluble iron was prepared by mixing solutions of $FeCl_3$ and HQ to give a molar iron/HQ ratio equal to 1/2 as previously described.[20] LPS (10 mg/kg, i.p.) was administered to rats 6 h before heart removal.

Heart Perfusion and Experimental Design

Hearts were perfused according to the protocol previously used in our laboratory to investigate the effects of increasing concentrations of liposoluble iron on cardiac function.[20] Briefly, hearts were perfused orthogradely through the aorta in a non-circulating Langendorff apparatus with a standard buffer. The millimolar composition of the standard perfusion buffer was NaCl 118, KCl 5.9, $CaCl_2$ 3, $MgSO_4$ 1.2, $NaHCO_3$ 25, HEPES 1, glucose 5.5. The buffer was saturated with 95% O_2 − 5% CO_2 (pH 7.4, 37°C). The perfusion bottles were placed above the heart with an overflow to ensure a mean coronary perfusion pressure of 6.2 kPa (60 mmHg). Left ventricular end diastolic pressure was set between 5 and 8 mmHg throughout the experiment. After a 15 min of equilibration period, the coronary flow (CF) and the left ventricular developed pressure × heart rate (LVDP × HR), as an index of the contractile function, were measured every 5 min for 90 min. CF was evaluated by the collection of coronary effluents in graduated tubes. Hearts were excluded if a persistent arrhythmia was present during the equilibration period. They were exposed to the liposoluble iron complex Fe–HQ (10–20 μM) in the 45–60 min interval of perfusion.

Experiments were designed to study:

(1) The effect of LPS on heart function. Hearts from normal and LPS-treated rats were exposed or not to iron during the 45–60 min interval of perfusion. For septic hearts, L-arginine (100 μM) was added to the standard perfusion buffer because the extracellular supply of L-arginine is a limiting factor for NO production by iNOS in rats treated with LPS.[23]
(2) The role of *ex vivo* NO production in septic hearts exposed to iron. Hearts from LPS-treated rats were perfused with the standard perfusion buffer containing the NOS inhibitor nitroarginine but not L-arginine. These hearts were exposed to iron during the 45–60 min interval of perfusion. Two concentrations of nitroarginine were tested (10 and 100 μM).
(3) Hydroxyl radical production in hearts exposed to iron. Experiments were conducted according to (1) in the presence of 1 mM salicylate in the buffer perfusion.
(4) The effect of L-arginine on iron-induced cardiac dysfunction. Hearts from normal rats were perfused for 90 min with the standard buffer containing 1 mM arginine. They were exposed to iron during the 45–60 min interval of perfusion.

Evaluation of Hydroxyl Radical Formation

In experiments (3), hearts were stored at − 80°C until evaluation of hydroxyl radical formation by the salicylate trapping method.[24] Briefly, by using an Ultra-Turrax homogenizer, hearts were homogenized at 0°C in 10 volumes of standard buffer

containing desferrioxamine mesylate (100 μM) and $HClO_4$ 70% (10 μl/ml). After centrifugation, the supernatants (0.5 ml) were extracted for 45 s with diethyl-ether (2 ml). The organic phase was separated and evaporated until dryness. The residue was dissolved in mobile phase (1 ml) before HPLC separation of salicylic acid (SA) and 2,3-dihydroxybenzoic acid (2,3-DHBA). These compounds were detected by using coulometric and UV detectors, respectively. HPLC conditions and apparatus were previously described in detail by our laboratory.[25] Hydroxyl radical formation was estimated by the 2,3 DHBA/SA concentration ratio (pmol/nmol) in cardiac tissue.

Determination of NO Production and Protein Expression

Nitrite level in the coronary effluents was used as an index of cardiac NO production. The effluents were collected at time 35 min of perfusion and stored at −30°C until spectrophotometric measurement of nitrite by using the Griess reagent.

iNOS and HO-1 protein expression was studied 6 h after LPS administration. Total protein extracts of septum and ventricles were homogenized in 5 volumes of lysis buffer (PBS containing 1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 1 μg/ml pepstatin) and centrifuged at 10,000*g* for 10 min. An aliquot of the supernatant was kept for protein measurement according to Lowry *et al.*[26] Equal amounts of total proteins (40 μg/lane) were dissolved in 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue and were heated at 85°C for 10 min. Proteins were separated on 7–12% SDS-PAGE according to Laemmli.[27] Proteins were electrophoretically transferred to PVDF membrane (0.2 μm pore size, Pall) in cold transfer buffer (25 mM Tris–HCl, pH 8.3, 192 mM glycine, and 20% methanol for the detection of iNOS protein and 10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol, pH 9.9 for the detection of HO-1 protein). The blots were incubated overnight at 4°C in 5% non-fat dry milk in TBS buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween 20 to block unspecific binding. Blots were washed, incubated for 4 h at room temperature with the anti-iNOS or anti-HO-1 antibodies and for 1 h and 30 min with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs, respectively (1/30,000). Protein–antibody complexes were visualized using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham, Les Ulis, France). A computer-based imaging system was used to measure the relative optical density of each specific band.

Data Analysis

Results were expressed as mean ± SEM of *n* rats. Group comparisons were made using Student's unpaired *t* test or two-way analysis of variance (ANOVA). If statistical differences were indicated by ANOVA, group comparisons were made using the Student–Newman–Keuls test. Differences were considered to be significant if the *p* value was <0.05.

RESULTS

Effects of LPS on Heart Function and Protein Expression

The effects of LPS on heart function are summarized in Fig. 1. CF was significantly lower in LPS group than in control group at the end of the equilibration period (15.9 ± 0.7 vs. 11.0 ± 0.6 ml/min) and at time 40 min of perfusion (16.1 ± 0.7 vs. 10.8 ± 0.5 ml/min) but not thereafter (12.9 ± 0.9 vs. 10.4 ± 0.4 ml/min at 90 min of perfusion). For LVDP × HR, no statistical difference was observed between the two groups except at the end of the equilibration

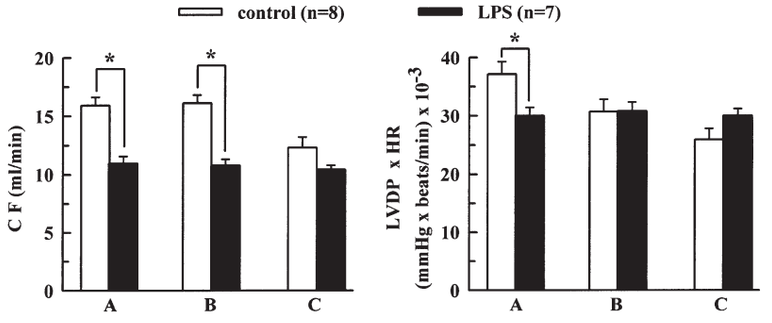

FIGURE 1 Effect of LPS on heart function. CF and contractile function (LVDP × HR) were measured in hearts from normal rats (control group) and LPS-treated rats (LPS group). Hearts were perfused for 90 min after a 15-min equilibration period. Data indicate the values obtained at the end of the equilibration period (A) and at times 45 min (B) and 90 min (C) of the perfusion period. LPS (10 mg/kg, i.p.) was administered 6 h before heart removal. LVDP: left ventricular developed pressure, HR: heart rate, *n* = number of rats. * Different from control group (Student's unpaired *t* test).

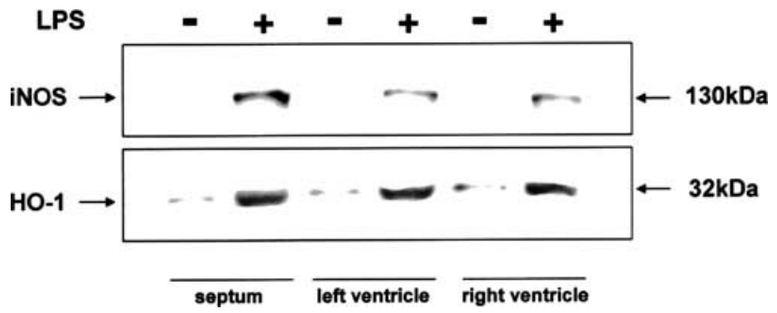

FIGURE 2 Immunoblots of heart homogenates. iNOS and HO-1 protein expression were examined in normal rats (LPS −) or 6 h after treatment of rats with LPS (LPS +).

period (37.1 ± 2.2 in control group vs. 30.0 ± 1.4 mmHg × beats/min × 10^{-3} in LPS group).

Protein expression was examined in heart from control rats ($n = 2$) or LPS-treated rats ($n = 2$). As shown in Fig. 2, immunoblots displayed evidence of iNOS protein expression only in septic hearts. HO-1 protein expression was slightly observed in control hearts and increased by about 2.5-fold after LPS challenge. Thus, HO-1 expression after LPS increased from 2.4 ± 1 to 5.4 ± 0.1 in the septum, from 2.1 ± 0.9 to 5.5 ± 0.7 in the left ventricle and from 2.3 ± 0.8 to 5.4 ± 0.3 in the right ventricle (arbitrary units).

Effects of LPS on Iron-induced Cardiac Dysfunction

The effects of iron on control and septic hearts are summarized in Fig. 3. In control hearts, iron induced a severe and long lasting decrease in both CF and LVDP × HR. LPS pretreatment totally inhibited the iron-induced perfusion deficit. In addition, the iron-evoked contractile deficit was less severe in septic hearts than in control hearts although the difference did not reach significance (F − ratio = 4.46, $p = 0.052$). Because arginine (1 mM) did not ameliorate the iron-induced dysfunction in control hearts ($n = 6$, data not shown), it is unlikely that arginine present at 100 μM in the perfusion medium of septic hearts accounted for the protection afforded by LPS.

In Fig. 4, are summarized the effects of nitroarginine and salicylate in septic hearts exposed to iron. Neither CF nor LVDP × HR were modified by 10 μM nitroarginine whatever the time point examined ($n = 3$, data not shown). The two parameters were also not affected by 100 μM nitroarginine, except during iron exposure. During this period, both CF and LVDP × HR were lowered by nitroarginine. Salicylate was without effect on CF and LVDP × HR whatever the time point examined.

Effects of LPS on Hydroxyl Radical Production

Thirty minutes after iron removal, the 2,3-DHBA/SA concentration ratio in cardiac tissue was 14.3 ± 0.9 in control group and 11.0 ± 0.5 in LPS group. However, the difference did not reach significance.

Effects of Nitroarginine and Salicylate on LPS-Induced NO Production

Table I gives the nitrite concentration values in coronary effluents collected at time 35 min of perfusion. LPS administration was associated with a 3-fold increase in nitrite level compared to control. When septic hearts were perfused with 100 μM

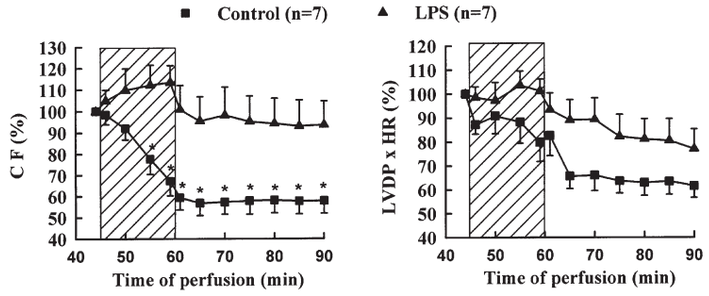

FIGURE 3 Effect of iron on heart function. Heart from normal (control group) and LPS-treated rats (LPS group) were perfused for 90 min and exposed to iron within the 45–60 min interval of perfusion (dashed line). LPS (10 mg/kg, i.p.) was administered 6 h before heart removal. CF and contractile function (LVDP × HR) were expressed as percentage of values obtained just before iron exposure. LVDP: left ventricular developed pressure, HR: heart rate, n = number of rats. * Different from control group (ANOVA followed by Student–Newman–Keuls test).

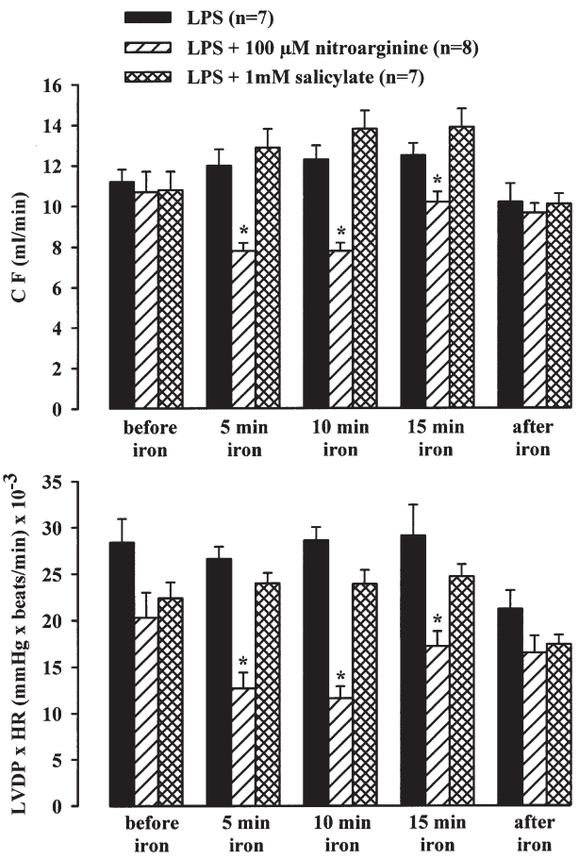

FIGURE 4 Effect of nitroarginine and salicylate on iron-induced dysfunction in septic hearts. Graphs show CF and contractile function (LVDP × HR) before, during iron exposure (5, 10, 15 min) and 30 min after iron removal in hearts from LPS-treated rats (10 mg/kg, i.p., 6 h before heart removal). See "Materials and methods" section for the composition of the perfusion buffer. LVDP: left ventricular developed pressure, HR: heart rate, n = number of rats. * Different from LPS group (Student's unpaired t test).

nitroarginine, the blockade of NO production by hearts was total. At time 85 min of perfusion nitrite was still below the detectable level (data not shown) indicating long-lasting NO synthesis inhibition by nitroarginine. As shown in Table I, salicylate like nitroarginine inhibited LPS-induced NO production, probably as a result of iNOS activity inhibition.[28.29]

TABLE I Effects of LPS administration on nitrite level in coronary effluent

Group	Nitrite level (nM)
Control ($n = 7$)	192 ± 55
LPS ($n = 11$)	682 ± 90*
LPS+100 μM Nitroarginine ($n = 8$)	Not detectable
LPS+1 mM Salicylate ($n = 7$)	149 ± 45**

Nitrite level was measured in coronary effluent collected at time 35 min of perfusion. LPS (10 mg/kg, i.p.) was administered 6 h before heart removal. See "Materials and methods" section for buffer perfusion composition. n = number of rats. *$p < 0.05$ vs. control group, **$p < 0.05$ vs. LPS group.

DISCUSSION

The present study reports the effect of *in vivo* LPS challenge (10 mg/kg, i.p.) on isolated perfused heart exposed for 15 min to liposoluble iron. Hearts were treated with iron 6 h after LPS administration, a time at which septic hearts exhibited iNOS protein expression and high HO-1 protein expression. The results indicated that iron-induced perfusion and contractile deficits were ameliorated by LPS with more important coronary than myocardial benefits. The cardioprotection afforded by LPS was associated with a 23% decrease in iron-evoked hydroxyl radical formation although the difference did not reach significance. Furthermore, the blockade of *ex vivo* NO production did not reverse the protection afforded by LPS challenge.

In ischemic heart, reoxygenation is associated with excessive formation of superoxide anion, hydrogen peroxide and hydroxyl radical.[30–33] Because the first two species are poorly reactive in aqueous solution, it is generally considered that their toxicity requires the presence of redox-active iron which transforms them into the most toxic ROS, hydroxyl radicals.[34] Disruption of iron handling occurs in heart exposed to I/R. Thus, cardiac ischemia causes a progressive increase in the tissue level of low molecular weight (LMW) iron,[35] likely as a result of iron delocalization from intracellular ferritin. Furthermore, during reperfusion, LMW iron is secreted by injured cells with a burst-like manner. Indeed, the serial collection of coronary effluents during the reperfusion period following global ischemia showed the mobilization of LMW from the cardiac tissue with the highest level of LMW iron in the first CF fraction.[36] Interestingly, the pattern of hydroxyl radical appearance and disappearance in the coronary effluents and the extracellular space is also burst-like,[37,38] suggesting a causal link between secreted iron and extracellularly formed hydroxyl radicals. Consistent with such a link, the cell-impermeable iron chelator desferrioxamine inhibits reperfusion-induced extracellular hydroxyl radical formation.[38] However, the failure of desferrioxamine to attenuate myocardial injury despite an inhibition of hydroxyl radical formation strongly suggests that hydroxyl radicals produced outside the cells do not mediate the postischemic myocardial injury. Conversely, the cardiac reperfusion abnormalities were decreased by the introduction of a liposoluble iron chelator at the onset of the reperfusion.[39] Taken together, these data suggest that (1) despite an iron washout during the reperfusion period, reperfused cardiac cells still exhibit substantial toxic amount of redox-active iron, (2) the availability of redox-active iron at critical intracellular sites is essential to I/R injury. Therefore, liposoluble iron appears to be a reliable

ROS-generating system to mimic oxidative stress induced by I/R to the heart as argued from a previous study in which a liposoluble (the Fe^{3+}-hydroxyquinoline complex, Fe-HQ) and a hydrosoluble form (the ferric citrate ammonium complex, FAC) of iron were compared on isolated rat hearts.[20] Whereas Fe-HQ induced a concentration-dependent decrease in both CF and contractile function, FAC was without effect on the heart function. Furthermore, whereas only Fe-HQ induced lipid peroxidation, both Fe-HQ and FAC increased extracellular hydroxyl radical formation without difference between the two complexes. Thus, as observed in hearts exposed to I/R, intracellular but not extracellular oxidative stress is largely involved in the abnormal cardiac dysfunction of hearts exposed to liposoluble iron. In the present study, hearts were exposed for 15 min to 10–20 μM Fe-HQ because such a concentration induced a marked but not lethal cardiac dysfunction.

LPS given 6 or 24 h before heart removal was previously reported to ameliorate I/R injury in isolated perfused rat heart.[7–9,40] In the present study, we examined for the first time the effects of LPS in heart exposed to iron. LPS was found to decrease iron-induced perfusion and contractile deficits. This suggests a decreased vulnerability of septic heart to iron-induced oxidative stress. However, the participation of the *ex vivo* NO production in LPS effects is unlikely for two reasons. First, in septic heart, despite a long-lasting blockade of NO production, nitroarginine only restored the effect of iron during iron exposure. Second, salicylate like nitroarginine abolished LPS-induced NO formation but, conversely to nitroarginine, did not blunt LPS effects. Consistent with these findings, the decreased responsiveness of septic coronary vessels to vasoconstrictors was not reversed after inhibition of iNOS activity.[41] Nevertheless, an obligatory role for iNOS in mediating the decreased responsiveness of vessels to constrictors[42,43] and in mediating the cardioprotective effects of MLA against I/R[11] was demonstrated by using pharmacological inhibition and iNOS gene knockout mice. It is therefore possible that iNOS-produced NO in septic hearts may initiate some processes that, once developed, are no longer dependent on NO or sensitive to acutely administered NOS inhibitors.

Many genes are directly controlled by NO.[44] However, some genes such as gene encoding HO-1 may be modulated only in the presence of the inflammatory stimulus that increase the expression of iNOS.[45] Our results indicating a 2.5-fold increase in heart HO-1 expression 6 h after LPS administration are consistent with previous data.[46] Evidence begins to accumulate identifying HO-1 as a beneficial protein in heart submitted to I/R-induced oxidative stress. Thus, by using Langendorff preparation, hearts from transgenic mice overexpressing HO-1 showed improved recovery of contractile performance during reperfusion after ischemia in a HO-1 dose-dependent manner.[47,48] Similarly, in isolated hearts subjected to I/R, HO-1 upregulation by treatment of rats with hemin ameliorated myocardial function on reperfusion and reduced infarct size.[22,49] Furthermore, treatment with a HO-1 inhibitor completely abolished the cardioprotective effects of MLA against I/R.[50] Given the similarity of the oxidative stress (source and effect) generated in hearts either subjected to I/R or exposed to liposoluble iron, iNOS-mediated HO-1 expression is an attractive mechanism to explain the cardioprotective effects of LPS challenge against iron toxicity. HO-1 is known to confer protection against various kinds of oxidative stimuli.[51] Moreover, HO-1 is the major cellular source of carbon monoxide (CO), a product of heme degradation. According to the vasodilatory effect of CO,[52] it is therefore possible that CO accounts for the decreased responsiveness of coronary vessel to iron. Furthermore, an inhibition of antiapoptotic processes by HO-1[47] cannot be excluded in the cardioprotection afforded by LPS against iron as suggested by a study in which a cardioselective HO-1 overexpression was reported to decrease I/R-induced apoptosis.

In conclusion, the ability of iNOS to protect hearts from I/R injury and that of NO to decrease ROS-mediated toxicity led us to examine in rats the effect of LPS pretreatment in isolated perfused hearts exposed to iron. LPS was found to ameliorate iron-induced perfusion and contractile deficits by mechanisms independent of *ex vivo* NO production. The marked induction of HO-1 by LPS observed in our experimental conditions, as well as the HO-1 protective effects reported in the literature in hearts exposed to I/R emphasize the importance of this enzyme in the decreased vulnerability of septic hearts to iron and suggest that NO acts as a trigger rather than a direct mediator of the cardioprotective effects of LPS in hearts exposed to iron.

Acknowledgements

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