

# Hyperglycemia in Streptozotocin-Induced Diabetic Rat Increases Infarct Size Associated With Low Levels of Myocardial HO-1 During Ischemia/Reperfusion

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This study investigated the role of heme oxygenase (HO)-1 in the cardiac tissue injury of acute ischemia/reperfusion (I/R) in diabetic streptozotocin (STZ)-induced hyperglycemic rats. The effects of 1) hemin, an inducer of HO expression and activity, and 2) zinc protoporphyrin IX (ZnPP-IX), an inhibitor of HO activity, have also been investigated on the tissue injury by I/R and some mediators released in these circumstances. STZ hyperglycemic rats had impaired levels of HO-1 within the cardiac tissue and increased myocardial infarct size (IS) following I/R, as compared with the nondiabetic rats. In these rats, administration of hemin 4 mg/kg 18 h before I/R increases the levels of HO-1 within the tissue. However, the values of HO-1 assayed in these circumstances were significantly lower ( $P < 0.01$ ) than those assayed in nondiabetic animals subjected to the same procedures; IS was much more extended ( $P < 0.01$ ) than in the parent nondiabetic group. STZ hyperglycemic rats also predisposed the heart to produce high levels of the cytokines interleukin (IL)-1 $\beta$  and CXCL8. Subsequent I/R further increased ( $P < 0.01$ ) the cytokine production, an effect partly prevented by hemin treatment. This recovered the huge number of infiltrated polymorphonuclear (PMN) leukocytes within the cardiac tissue associated with the STZ hyperglycemic state and I/R damage. *Diabetes* 54: 803–810, 2005

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Received for publication 13 September 2004 and accepted in revised form 29 November 2004.

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ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia inducible factor; HO, heme oxygenase; IL, interleukin; I/R, ischemia/reperfusion; IS, infarct size; LADCA, left anterior descending coronary artery; LV, left ventricle; MABP, mean arterial blood pressure; MFI, mean fluorescence intensity; MPO, myeloperoxidase; PMN, polymorphonuclear; PRI, pressure rate index; STZ, streptozotocin; ZnPP-IX, zinc protoporphyrin IX.

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**H**yperglycemia in diabetes leads to cardiovascular complications induced by oxidative stress (1). Overall, this independent risk factor worsens cardiac performance, cell survival, and tissue injury following myocardial ischemia/reperfusion (I/R) (2) via increased oxidants and reduced antioxidant defenses (3).

At the cardiac level, the enzyme heme oxygenase (HO)-1 is induced by several stimulants, such as hemolysis, inflammatory cytokines, oxidative stress, heat shock, heavy metals, and endotoxin (4–9), and it catalyzes the oxidation of heme into biliverdin, carbon monoxide (CO), and free iron (10), powerful cytoprotective agents. This enzyme has the purpose of being cardioprotective from cell damage induced by both diabetes and I/R (11–13). Moreover, it has potent antiinflammatory properties (14,15).

The present study focuses on the possibility that hyperglycemia in diabetes worsens cardiac tissue injury of I/R by involving HO-1 expression and activity. Moreover, we have investigated the effect of 1) hemin, an inducer of HO expression and activity, and 2) zinc protoporphyrin IX (ZnPP-IX), an inhibitor of HO activity, on the tissue injury of acute myocardial I/R and some mediators released in this circumstance.

## RESEARCH DESIGN AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Naples. Rats were randomly allocated to one of the six following groups: 1) nondiabetic rats (six sham-operated and six infarcted animals); 2) STZ hyperglycemic rats (six sham-operated and six infarcted animals); 3) nondiabetic rats pretreated 18 h before experimental procedure with hemin (4 mg/kg i.p.) (six sham-operated and six infarcted animals); 4) nondiabetic rats treated with ZnPP-IX (50  $\mu$ g/kg i.p.) 6 h before hemin (six sham-operated and six infarcted animals); 5) diabetic STZ rats pretreated 18 h before experimental procedure with hemin (six sham-operated and six infarcted animals); and 6) diabetic STZ rats treated with ZnPP-IX 6 h before hemin (six sham-operated and six infarcted animals). Overall mortality was 4% throughout the entire study.

**STZ hyperglycemic rats.** Experiments were conducted in male Sprague-Dawley rats (4–6 months old and weighing ~250 g) according to the procedure described by Marfella et al. (16). Particularly, STZ was administered at 70 mg/kg i.v. and a continuous intravenous infusion of regular insulin (1.5  $\pm$  0.5 units/day) was applied to rats in order to yield blood glucose levels of ~22 mmol/l (396 mg/dl) for 8 days (poor glycemic control rats).

### In vivo I/R injury of the rat heart

**Surgical procedure.** The procedure has been previously described (17). Briefly, ligation of the left anterior descending coronary artery (LADCA) close to its origin was applied to induce I/R. Coronary artery occlusion was done for a 25-min period and reperfusion occurred for 2 h. The mean arterial blood

pressure (MABP) was continuously recorded by a MacLab system (Ugo Basile, Comerio, VA). The heart rate was automatically calculated from the blood pressure, and the pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (18), was calculated as the product of MABP and heart rate and expressed in  $\text{mmHg} \cdot \text{min}^{-1} \cdot 10^{-3}$ .

**Measurement of area at risk and infarct size.** Measurement of area at risk (AR) and infarct size (IS) were assessed by staining with Evans blue dye (0.5 ml of a 1% wt/vol solution and 1.5% wt/vol triphenyltetrazolium chloride in PBS) and by computerized planimetry using an image analysis software program (National Institutes of Health Image, NIH Image Software), as described by Di Filippo et al. (19).

**Tissue assays.** Selected experiments were repeated twice with five rats per group. After the I/R injury procedure, the left ventricle (LV) was excised without performing any staining procedure, and tissue was half frozen at  $-80^{\circ}\text{C}$  before the analyses described below; a portion was fixed in 10% (wt/vol) PBS-buffered formaldehyde. A second series of experiments was performed for collecting cardiac tissue samples for HO-1 mRNA RT-PCR. This was performed as previously described (2,3) by using the following primers: HO-1 325: CCAGCCACACAGCACTAC; and HO-1 617: GCGGTCTTAGCCTCTCTCTG. Blood samples were also taken by the tail vein ( $\sim 0.2\text{--}0.25$  ml) into a heparinized eppendorf vial, and aliquots were used for peripheral blood counts (17).

**Tissue homogenates.** The frozen myocardial tissues (100–240 mg aliquot) were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mmol/l potassium phosphate buffer (pH 7.0) and centrifuged for 30 min at  $4,000g$  at  $4^{\circ}\text{C}$ .

**Cardiac HO-1 expression.** Immunohistochemistry was used to detect the expression of HO-1 within cardiac structures. At the end of the experiments, the tissues were fixed in 10% (wt/vol) PBS-buffered formaldehyde, and  $8\text{-}\mu\text{m}$  sections were prepared from paraffin-embedded tissues. After deparaffinization the sections were incubated overnight with anti-HO-1 (StressGen Biotechnologies, Victoria, BC, Canada) polyclonal antibody (1:500 in PBS, vol/vol). Sections were washed with PBS and incubated with secondary antibody. To confirm that the immunoreaction for the HO-1 was specific, some sections were also incubated with the primary antibody (anti-HO-1) in the presence of excess HO-1 (10 mmol/l) to verify the binding specificity. Immunohistochemistry photographs ( $n = 5$ ) were assessed by densitometry as previously described (20) by using Optilab Graftek software on a MacIntosh personal computer.

**Cardiac HO-1 quantitation.** This assay was done by using an enzyme-linked immunosorbent assay (ELISA) kit supplied by Stressgen Biotech. This kit shows sensitivity of 0.78 ng/ml and ranges up to 25 ng/ml as furnished by Stressgen. An aliquot (100  $\mu\text{l}$ ) of tissue supernatant was used.

**Myeloperoxidase activity.** The reaction was performed as previously described (17). Data are reported as units of myeloperoxidase (MPO) activity per gram of tissue protein (units/g). A standard curve was constructed with different numbers of rats neutrophils that were elicited into the peritoneal cavity at 4 h in response to application of 5 ml thioglycollate (21). Pellets were subjected to three cycles of freeze-thawing. Samples (100  $\mu\text{l}$ ) were obtained after a final centrifugation at 13,000 rpm for 30 min on a benchfuge. The MPO reaction was produced with a polymorphonuclear (PMN) number ranging from 0 to  $225 \times 10^3$ , obtaining a linear relationship with the absorbance at 620 nm following the equation:  $A_{620} = 4.504 \times \text{number of PMN} + 33.74$ , with a  $R^2 = 0.97983$ . Data are reported as equivalent numbers of PMN per milligram of tissue.

**Cytokine quantitation by ELISA.** Tissue interleukin (IL)-1 $\beta$  and CXCL8 levels in tissue homogenates (50  $\mu\text{l}$ ) were determined using a commercially available ELISA specific for the rats cytokine, purchased from R&D Systems (Abingdon, U.K.), as previously described (19).

#### Parameters of systemic activation

**Leukocyte profile.** Total white cell counts were determined using a standard in vivo protocol (17). Differential counts were obtained from blood smears stained with Turk's solution (crystal violet 0.01% wt/vol in acetic acid 3% vol/vol), and the total number of each leukocyte type was then calculated. Data are reported as  $10^6$  cells per milliliter of blood.

**CD11b levels on circulating neutrophils.** A whole-blood protocol was used (Harrison et al., 1995; D'Amico et al., 2000). Three distinct peripheral leukocyte populations (lymphocytes, monocytes, and PMN leukocytes) were discriminated using forward- and side-scatter characteristics. Data were analyzed as mean fluorescence intensity (MFI) units measured in FL1 (green) channel (wavelength of 530 nm).

**Drug treatment.** STZ, ZnPP-IX, and hemin were purchased from Sigma-Aldrich (Milan, Italy). These two latter compounds were dissolved in saline (vehicle) and used at doses according to previous studies (12).

**Statistical analysis.** All values are expressed as means  $\pm$  SEM of number ( $n$ ) of rats for the in vivo experiments. Statistical analysis was assessed either by

Student's  $t$  test (when only two groups were compared) or one-way ANOVA followed by Dunnett's test (more than two experimental groups). A  $P$  value  $< 0.05$  was considered significant to reject the null hypothesis.

## RESULTS

**STZ hyperglycemic rats.** Blood glucose averaged  $6.4 \pm 0.2$  mmol/l in basal conditions and rose to  $23.3 \pm 1.9$  mmol/l 15 h after STZ administration. In STZ rats, a continuous intravenous infusion of regular insulin ( $1.5 \pm 0.5$  units/day) was begun and adjusted to yield blood glucose levels of  $\sim 22$  mmol/l (396 mg/dl) for 8 days. Serum sodium and potassium concentrations and body weight did not significantly change after STZ treatment (data not shown).

### In vivo myocardial injury

**Nondiabetic rats.** Occlusion of the LADCA and subsequent reperfusion produced a marked damage in the rat left ventricle as evidenced by histology (Fig. 1), which was reliably measured at the 2-h time point (Fig. 2). AR was 56% of the left ventricle in nondiabetic rats (Fig. 2), and  $\sim 52\%$  of this portion of the ventricle was infarcted (Fig. 2), giving an IS:LV between 42 and 47% (Fig. 2).

Intraperitoneal administration of the hemin 18 h before the I/R period afforded a marked protection against the myocardial injury measured 2 h later as estimated by the IS and the IS:LV (Fig. 2). This effect was 53.5% inhibition versus vehicle-treated rats ( $P < 0.01$ ). Moreover, ZnPP-IX (6 h before hemin) abolished the protective effect by hemin (Fig. 2).

**STZ hyperglycemic rats.** These rats showed increased histological damage compared with nondiabetic rats (Fig. 1). The infarct size as percentage of the left ventricle was significantly greater in poor glycemic controlled STZ hyperglycemic rats as compared with nondiabetic rats ( $61.5 \pm 2.2\%$  of the area at risk,  $P < 0.05$ ) (Fig. 2). Hemin pretreatment afforded a slight protection against the infarction; this protection was  $13.5 \pm 1.4\%$  (Fig. 2). ZnPP-IX given 6 h before hemin completely abolished the protection of hemin (Fig. 2).

**Hemodynamic measurements.** In STZ rats, the 8-day period of hyperglycemia resulted in a significant increase of MABP (from  $110 \pm 2$  to  $122 \pm 3$  mmHg,  $P < 0.01$ ) and heart rate (from  $392 \pm 14$  to  $482 \pm 18$  bpm,  $P < 0.01$ ). Coronary artery occlusion produced a significant ( $P < 0.01$ ) fall in MABP that was similar in all groups of rats (nondiabetic rats from  $106 \pm 4$  to  $82 \pm 7$  mmHg,  $P < 0.01$ , and STZ rats from  $122 \pm 3$  to  $94 \pm 10$  mmHg,  $P < 0.01$ ). However, recovery toward preocclusion values was impaired in STZ hyperglycemic rats only (120-min value  $101 \pm 10$  mmHg; baseline value  $122 \pm 3$  mmHg,  $P < 0.05$ ). In these rats, hemin produced a slight but significant increase of the recovery of MABP (from  $101 \pm 10$  to  $118 \pm 8$  mmHg,  $P < 0.05$ ).

**Cardiac HO-1 expression.** Immunohistochemistry showed intense and increased immunostaining for the HO-1 antigen in STZ hyperglycemic hearts compared with the groups of nondiabetic rats (Fig. 3). However, I/R procedure to both nondiabetic and STZ rats resulted in a superimposable staining for the HO-1 antigen between the two groups (Fig. 3). This was also confirmed by the RT-PCR for HO-1 mRNA expression (Fig. 4). Interestingly enough, treatment of both STZ and nondiabetic rats with hemin before I/R exerted different expression of HO-1



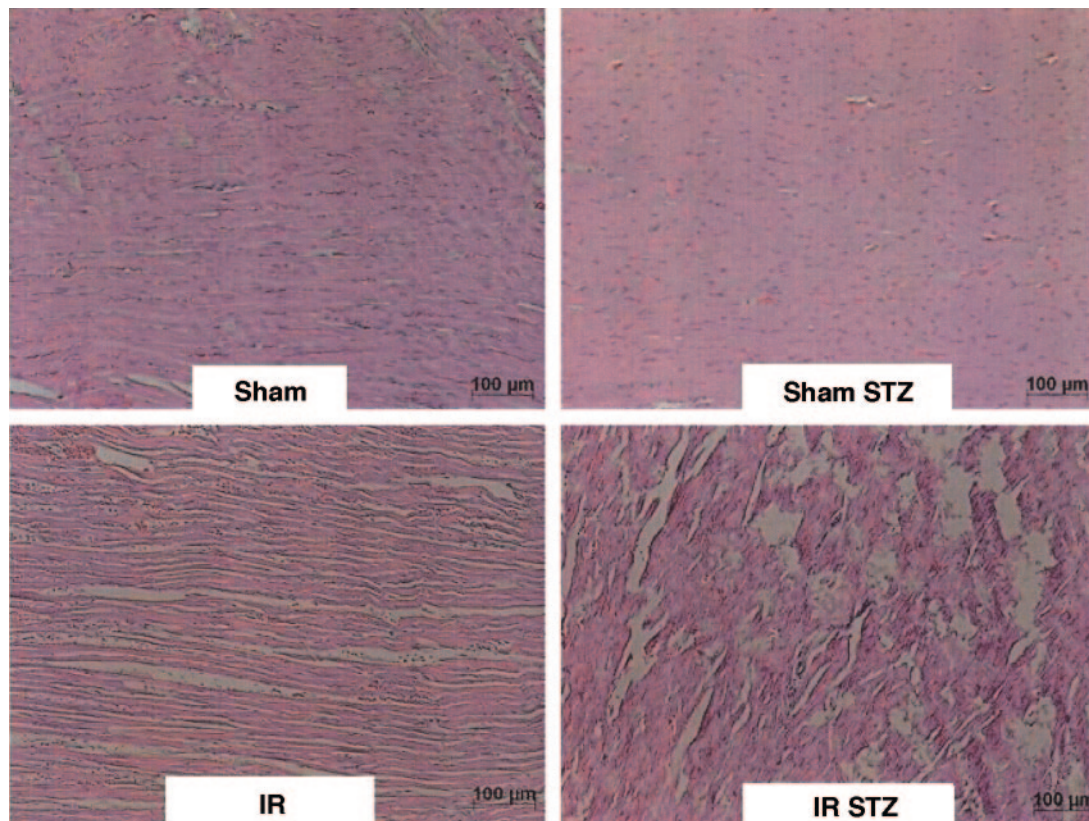


FIG. 1. STZ hyperglycemia causes increased damage in myocardial I/R. Depicted are the images of histological sections of both nondiabetic and STZ hyperglycemic rat hearts subjected to I/R procedure.

mRNA among the two groups at the end of the reperfusion period (Fig. 4).

**Cardiac HO-1 quantitation.** In basal conditions, cardiac HO-1 level was not appreciable in nondiabetic rats, as evidenced by ELISA (Fig. 5). HO-1 level increased after ischemia in nondiabetic rats, reaching a value of  $562 \pm 56\%$  relative to baseline ( $P < 0.001$ ). Administration of hemin before I/R produced a further significant increase of HO-1 (Fig. 5). A treatment of rats with ZnPP-IX plus hemin before I/R strongly restored the values of HO-1 detected after I/R alone (Fig. 5).

HO-1 was already present in rat hearts after 8 days of poor glycemic control (Fig. 5) as also previously evidenced by immunohistochemistry. The percent increase from baseline of HO-1 level after ischemia in STZ hyperglycemia was only  $66.3 \pm 14\%$  above baseline, that is  $11.7 \pm 4\%$  of the incremental expression seen in nondiabetic rats ( $P < 0.001$ ). Hemin treatment increased ( $P < 0.01$ ) the figure seen in STZ diabetic rats with I/R (Fig. 5); the values of HO-1, however, were still lower than those observed in I/R nondiabetic rats. ZnPP-IX plus hemin did not modify the levels observed in the I/R diabetic condition alone (Fig. 5).

**PMN-associated myocardial damage.** Myocardial injury was paralleled by an increase in MPO activity in the infarcted heart versus the noninfarcted hearts (noninfarcted  $184 \pm 20$  equivalent PMN number/mg tissue) collected from nondiabetic rats (Fig. 6). Administration of hemin, given here at the doses that produced a significant reduction in IS, produced a parallel attenuation in MPO activity as measured 2 h postreperfusion (Fig. 6). This

attenuation in MPO activity was absent in the rats treated with the hemin plus ZnPP-IX (Fig. 6).

STZ hyperglycemic rats showed an increase in MPO activity (noninfarcted  $346 \pm 32$  equivalent PMN number/mg tissue and infarcted  $762 \pm 27$  equivalent PMN number/mg tissue,  $P < 0.01$ ) (Fig. 6). Administration of hemin alone produced MPO levels still high with respect to nondiabetic rats measured 2 h postreperfusion (Fig. 6). However, the association with ZnPP-IX reduced the MPO values almost at the same values as the STZ I/R hearts (Fig. 6).

To find a causal relationship between PMN influx (MPO activity) and heart damage, we performed some separate experiments measuring the MPO values and the IS at the time interval of 45 min postreperfusion in both nondiabetic and STZ hyperglycemic rats. In nondiabetic experiments, MPO activity was  $543 \times 10^3$  PMN equivalent/mg protein ( $n = 5$ ), and the myocardial parameters were as follows: AR/LV  $52 \pm 2.9\%$ , IS/AR  $16.4 \pm 2.3\%$ , and IS/LV  $9.1 \pm 0.5\%$  ( $n = 5$  in all cases,  $P < 0.01$  vs. 2-h values shown in Fig. 2). STZ rats showed  $651 \times 10^3$  PMN equivalent/mg protein ( $n = 5$ ) and an AR of  $56 \pm 1.8\%$ , IS/AR  $21 \pm 3\%$ , and IS/LV  $10.8 \pm 0.7\%$  ( $n = 5$  in all cases,  $P < 0.01$  vs. 2-h values shown in Fig. 2).

**Cytokine levels in the infarcted myocardium.** At baseline, cardiac IL-1 $\beta$  and CXCL8 levels were slightly appreciable in nondiabetic rats ( $30 \pm 7$  pg/mg tissue and  $140 \pm 22$  pg/mg tissue, respectively). I/R of the heart showed levels of the IL-1 $\beta$  and CXCL8 up to values of  $132 \pm 14$  and  $680 \pm 43$  pg/mg tissue, respectively. Treatment with hemin before I/R produced a significant reduction in IL-1 $\beta$  and

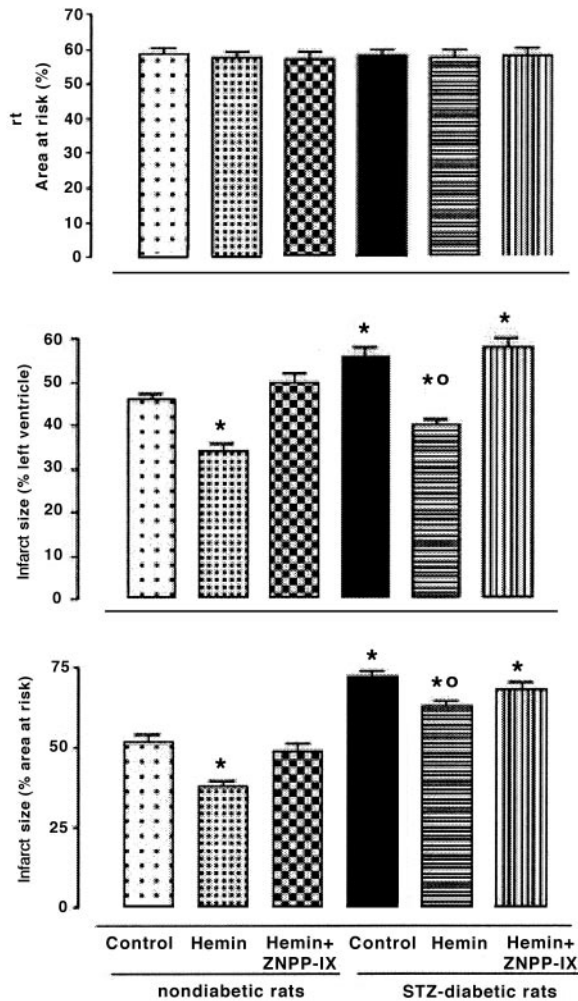


FIG. 2. HO-1 induction inhibits damage. Rats ( $n = 6$ ) were treated intraperitoneally with vehicle alone (control), hemin (4 mg/kg 18 h before I/R), and hemin plus ZNPP-IX (50  $\mu$ g/kg 6 h before hemin) before the I/R procedure. \* $P < 0.01$  vs. control and  $^{\circ}P < 0.05$  vs. STZ diabetic rats.

CXCL8 levels within the cardiac tissue (Fig. 7). This reduction was absent in the rats treated with hemin plus ZnPP-IX (Fig. 7).

STZ hyperglycemia predisposed the heart to produce high levels of both the cytokines IL-1 $\beta$  and CXCL8 (90  $\pm$  15 and 345  $\pm$  29 pg/mg tissue, respectively). Subsequent I/R further and significantly ( $P < 0.01$ ) increased the cytokine production (Fig. 7). Hemin decreased the cytokines. The levels were still higher than those in nondiabetic rats (Fig. 7). The association of ZnPP-IX and hemin report the cytokine levels to almost the values assayed in the hearts subjected to STZ treatment (Fig. 7).

**Effects of HO-1 activation on systemic PMN counts and CD11b expression.** I/R of the LADCA was associated with alterations in circulating leukocytes in nondiabetic rats. A significant ( $P < 0.01$ ) neutrophilia was observed at the end of the reperfusion period with respect to the values of PMN counted 5 min before the induction of the I/R procedure ( $-5$  min  $1.5 \pm 0.2 \times 10^6$ /ml, 120 min  $3.9 \pm 0.4 \times 10^6$ /ml). None of the treatments tested have modified the number of circulating PMNs at any of the time points tested (data not shown). STZ hyperglycemia did not mod-

ify the basal number of circulating leukocytes unless I/R procedure occurred. This caused a slight increase of the circulating PMNs when compared with the values obtained 5 min before ischemia ( $-5$  min  $2.1 \pm 0.4 \times 10^6$ /ml; 0 min  $3.3 \pm 0.3 \times 10^6$ /ml). Hemin and ZNPP-IX did not affect these figures (data not shown).

With respect to lymphocytes and monocytes, I/R induced a significant reduction in the number of circulating lymphocytes while not affecting the number of monocytes, when compared with the values obtained 5 min before ischemia in both STZ hyperglycemic and nondiabetic rats (Table 1).

I/R of nondiabetic rat hearts induced neutrophilia associated with PMN activation as indicated by the increase in cell surface CD11b. A marked upregulation (135  $\pm$  12 mean fluorescence intensity [MFI] units) of this  $\beta_2$ -integrin was detected at the end of the reperfusion period with an increase of 53  $\pm$  4% with respect to the 5-min preocclusion value (35  $\pm$  6 MFI units). Hemin treatment (4 mg/kg) before I/R did not modified CD11b expression with respect to the values of vehicle-treated rats at any time point considered (120 min 130  $\pm$  8 MFI units). CD11b expression after treatment with hemin plus ZnPP-IX was nearly at the same value observed in I/R (data not shown).

As expected, STZ treatment induced significant expression of CD11b ( $-5$  min 104  $\pm$  8 MFI) ( $P < 0.01$  vs. nondiabetic, 33  $\pm$  7 MFI). These values were further elevated by the application of the I/R procedure to the rats. They were at least 110% higher than the previous one (219  $\pm$  17 MFI at 2-h postreperfusion). Hemin treatment did not cause change of CD11b values either in basal conditions and after I/R (120 min 195  $\pm$  8 MFI units). Hemin + ZNPP-IX gave values of CD11b of 96  $\pm$  10 MFI units and 220  $\pm$  19 after I/R.

## DISCUSSION

This study shows that 8 days of poor glycaemic control in STZ diabetic rats increases histological damage of myocardial I/R. The infarct size as percentage of the left ventricle was significantly greater in STZ hyperglycemic rats as compared with nondiabetic rats. The study also shows impaired levels of the HO-1 within the cardiac tissues associated with increased infarct size. We found HO-1 already present in rat hearts after 8 days of poorly controlled diabetes as evidenced by immunohistochemistry and ELISA. However, the application of I/R caused lower percentage increase of the HO-1 with respect to nondiabetic rats subjected to same procedure. To the best of our knowledge, this is the first demonstration that STZ hyperglycemia or poorly controlled glucose levels interferes with HO-1 expression and possibly function at the cardiac level.

Although the mechanism underlying the impairment of HO-1 is not disclosed here, the difference between the levels of HO-1 found in nondiabetic rats and those in STZ rats subjected to I/R argues that ischemic episode-mediated stress, which increases HO-1 in nondiabetic rats, when superimposed on preexisting hyperglycemia-mediated stress, would result in the impairment of the burst of HO-1 expression. This contention is based on preexisting evidence that the concomitant insurgence of both these situations, hyperglycemia-induced oxidative stress and



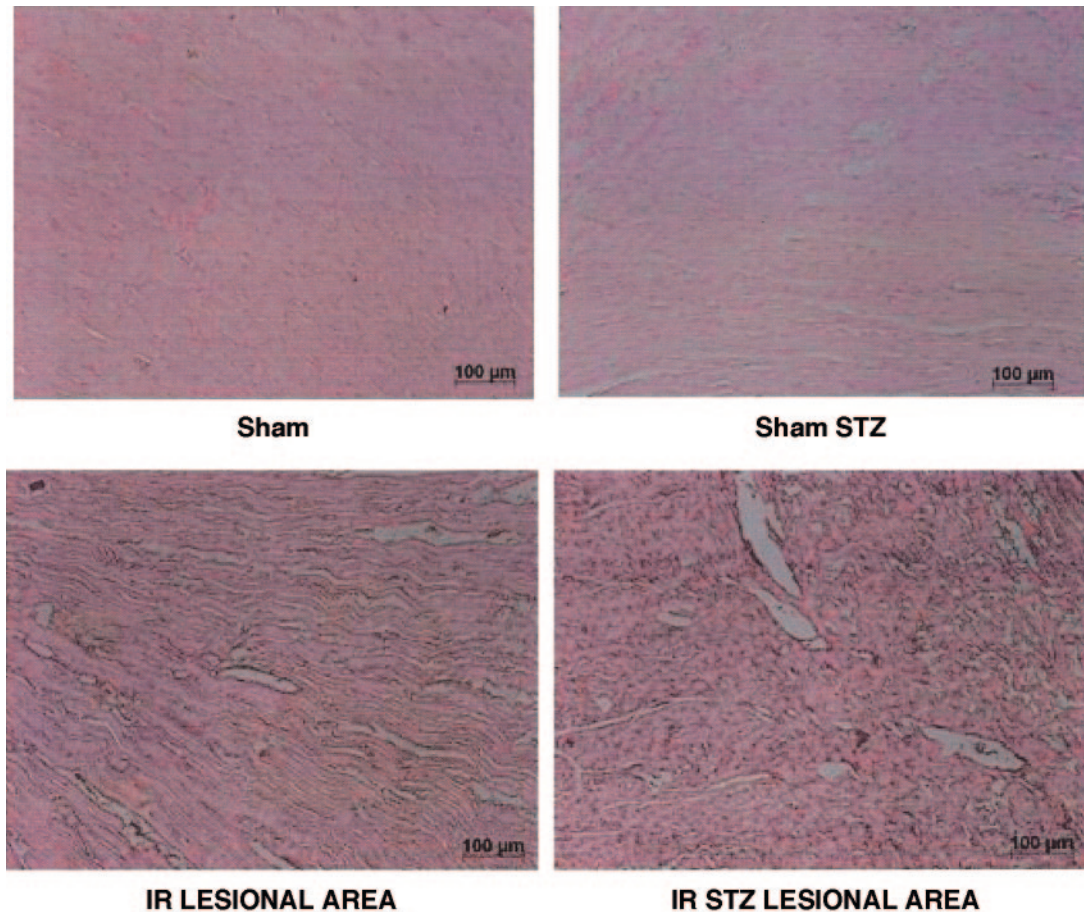


FIG. 3. Expression of the HO-1 following I/R in STZ diabetes. Immunostaining of HO-1 monoclonal antibody to both nondiabetic and STZ hyperglycemic rat hearts subjected to I/R procedure.

ischemia-derived stress, limits some transcriptional processes behind HO-1 expression within the myocardial tissue. For example, diabetic hyperglycemia limits I/R-induced expression of the hypoxia inducible factor (HIF)-1 $\alpha$  with respect to nondiabetic conditions (2). HIF-1 $\alpha$  is a transcriptional activator of the HO-1 gene in response to cellular hypoxia and stress (22,23). On another note, HO-1 expression may also be impaired by the fact that diabetes and I/R lead to high production of reactive oxygen species and reduced nitric oxide (NO) availability

via peroxynitrite formation into the heart (3,24). Because normally NO derived from inducible NO synthase (iNOS) enhances HO-1 expression in the tissues (25), the high peroxynitrite level formed into the heart may have impaired HO-1 expression.

Thus, tissue damage parallels the molecular alterations in HO-1. This trend was also copied on cardiovascular parameters. Indeed, there were systemic hemodynamic consequences of HO-1 induction evidenced here by monitoring MABP and PRI. Of note, STZ hyperglycemia and I/R

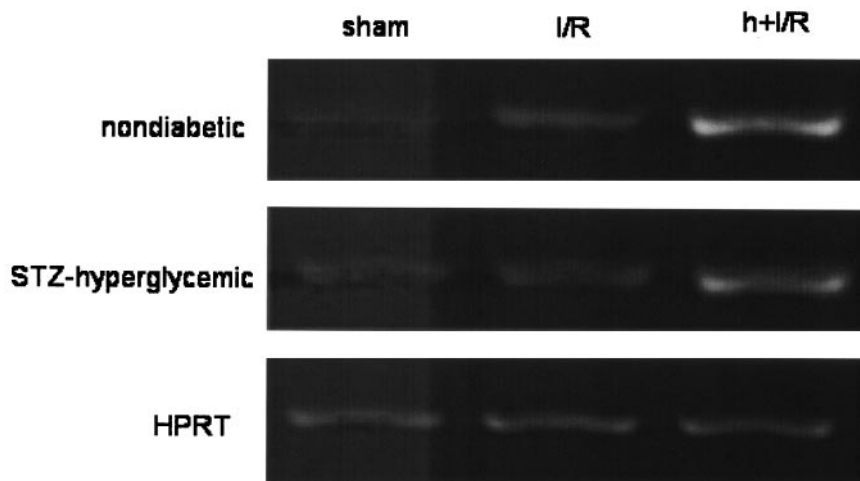


FIG. 4. HO-1 mRNA after I/R. Semiquantitative RT-PCR traces of the HO-1 mRNA of nondiabetic and STZ hyperglycemic rats subjected to I/R and treated or not with hemin (h).

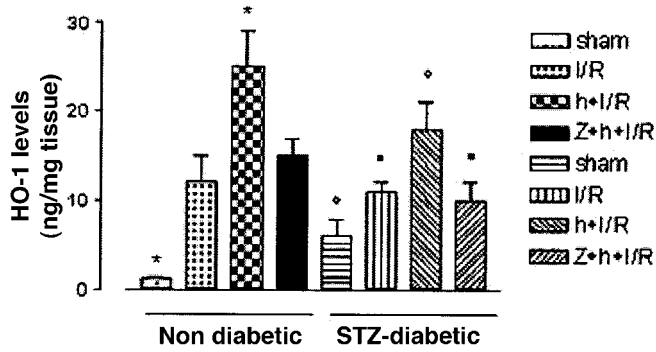


FIG. 5. STZ diabetes and HO-1 levels after I/R. Treatments as in Fig. 2 with  $n = 6$  for each group. h, hemin; Z, ZNPP-IX. \* $P < 0.01$  vs. I/R and ° $P < 0.01$  vs. nondiabetic rats.

fall within these cardiovascular parameters throughout the entire period of our experiments (see RESULTS); HO-1 induction within the heart was associated to a much greater recovery of MABP and PRI toward the physiological values of nondiabetic condition. This is probably due to an increased endogenous CO release that may play a role in the maintenance of vascular and cardiac tone during the reperfusion of ischemic hearts. The recent report of Motterlini et al. (26) sustains this hypothesis by demonstrating that HO-1-derived CO is a major regulator of pressor responses in vivo and in vitro.

Our and other previous studies have shown that myocardial infarction is associated with a local and systemic inflammatory response that may expand tissue damage that followed I/R by activating processes such as cytokine/chemokine release and recruitment of neutrophils into the injured tissue (17,19,27,29). The latter is generated by free radicals (superoxide anion, peroxynitrite, peroxyhydrogen, etc.) produced by activated PMNs, which determine membrane lipid peroxidation, DNA injury, and other modifications (30).

It is well known that tissues exposed to stress show a correlation between HO-1 and inflammation (31,32). The presence of HO-1 in injured tissues represents an adaptive and defensive response to the inflammatory process (31–32). Here we show that the impairment of the HO-1 levels

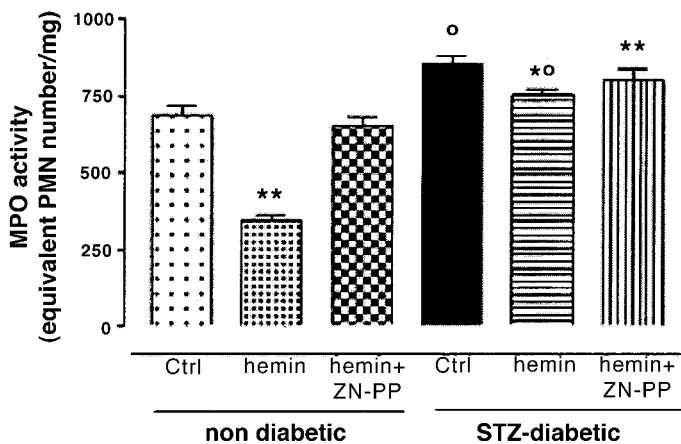


FIG. 6. Myeloperoxidase activity (MPO) in infarcted hearts. Rats ( $n = 6$ ) treated as described in Fig. 1 had hearts collected at the end of the reperfusion period and MPO activity determined in the AR. \* $P < 0.05$ , \*\* $P < 0.01$  vs. vehicle control, and ° $P < 0.05$  vs. nondiabetic rats same treatment.

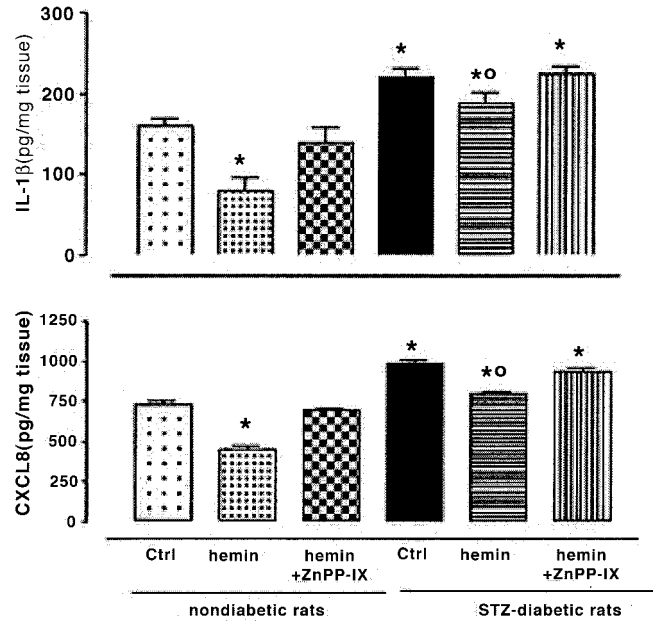


FIG. 7. HO-1 induction reduces cytokine levels in infarcted myocardium. IL-1 $\beta$  or CXCL8 were determined by ELISA ( $n = 5$ ). \* $P < 0.01$  vs. vehicle control (Ctrl) and ° $P < 0.05$  vs. nondiabetic same treatment.

induced by hyperglycemia increases the inflammatory component associated with I/R damage. In fact, STZ rats showed higher levels of MPO activity, a marker used to monitor tissue infiltration by PMN (21), with respect to nondiabetic normoglycemic rats subjected to I/R. Interestingly enough, hemin treatment showed less reduction in cardiac MPO levels as compared with nondiabetic rats, in accordance with the levels of the protein found in the ELISA assay. This latter observation supported by previous evidence indicating that induction of HO-1 activity results in reduced expression of adhesion molecules and reduced adhesion and migration of leukocytes following an inflammatory stimulus (14,15,33).

The casual correlation between cardiac HO-1 expression following I/R and white blood cell recruitment into myocardium was confirmed on local generation of leukocyte activators, cytokines, and chemokines, which are known to promote leukocyte-endothelium interaction (34). For this purpose, we investigated a cytokine that is able to increase the adhesive properties of the endothelial wall (35,36) and that is implicated in the pathology associated with experimental myocardial infarction (37,38)—IL-1 $\beta$ . We chose the CXCL8 chemokine because it is a chemokine able to recruit neutrophils in rodent species during experimental inflammation (39,40). The results of our study showed that the levels of these two cytokines copy the trend observed with PMN.

The effect of the HO-1 inducer hemin was not mirrored by any alteration of the systemic leukocyte profile induced by the I/R procedure. As expected, the reperfusion phase was characterized by systemic leukocytosis (41) in nondiabetic rats. Changes in neutrophil numbers and in their CD11b expression on the plasma membrane (as a marker of generalized cell activation) occurred in parallel over the time course analyzed. In fact, ischemia itself produced a modest but significant increase in both parameters that was greater marked to the end of the reperfusion period

TABLE 1  
Number of lymphocytes and monocytes in rats subjected to myocardial I/R

Experimental group	-5 min		120 min	
	Lymphocytes	Monocytes	Lymphocytes	Monocytes
Nondiabetic control	3.7 ± 0.3	1.3 ± 0.3	1.9 ± 0.3*	1.0 ± 0.3
Nondiabetic + hemin	3.5 ± 0.2	1.4 ± 0.4	1.9 ± 0.4*	1.3 ± 0.3
Nondiabetic hemin + ZnPP-IX	3.7 ± 0.2	1.6 ± 0.4	2.0 ± 0.3*	1.2 ± 0.4
STZ diabetic	3.5 ± 0.4	1.3 ± 0.3	1.6 ± 0.5*	1.2 ± 0.2
STZ diabetic + hemin	3.2 ± 0.2	1.4 ± 0.3	2.2 ± 0.3*	1.1 ± 0.2
STZ diabetic + hemin + ZnPP-IX	3.6 ± 0.3	1.2 ± 0.4	2.0 ± 0.4*	1.1 ± 0.3

Data are means ± SE ( $n = 5$  per group) and are intended as  $10^6$  cell/ml blood. Blood samples were collected 5 min (-5) before ischemia and after 120 min of reperfusion. \* $P < 0.01$  vs. the respective values at -5 min.

(41). Hemin treatment did not alter the number of leukocytes and the expression of CD11b molecules on circulating neutrophils. These latter were significantly increased after treatment of rats with STZ and were still not altered by hemin. Therefore, hemin-derived HO-1 seems to be capable of inhibiting tissue leukocyte recruitment without affecting their activation, both in nondiabetic and in STZ hyperglycemic rats.

In conclusion, our studies showed that streptozotocin-induced hyperglycemic rats had increased infarct size associated with low levels of myocardial HO-1 during I/R. Impairment of this stress-sensitive enzyme increases the inflammatory response to I/R injury of the myocardium. It involves a local increase of the expression of cytokines, increased recruitment of PMNs, and cell damage.

#### ACKNOWLEDGMENTS

This work was supported by the Italian MIUR. We acknowledge the help of Mauro Perretti and Emanuela Mazzon.

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