Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction

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Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. Am. J. Physiol. Heart Circ. Physiol. 278: H643–H651, 2000.—Bilirubin is a potent antioxidant generated intracellularly during the degradation of heme by the enzyme heme oxygenase. The purpose of this study was to determine the role of increased cardiac bilirubin in protection against postischemic myocardial dysfunction. Rat hearts were isolated and perfused according to the Langendorff technique to evaluate the recovery of myocardial function after 30 min of global ischemia and 60 min of reperfusion. We found that upregulation of the inducible isoform of heme oxygenase (HO-1) by treatment of animals with hemin 24 h before ischemia ameliorated myocardial function and reduced infarct size (tetrazolium staining) on reperfusion of isolated hearts. Tin protoporphyrin IX, an inhibitor of heme oxygenase activity, completely abolished the improved postischemic myocardial performance observed after hemin-mediated HO-1 induction. Likewise, cardiac tissue injury was exacerbated by treatment with tin protoporphyrin IX. Increased cardiac HO-1 expression and heme oxygenase activity were associated with enhanced tissue bilirubin content and an increased rate of bilirubin release into the perfusion buffer. Furthermore, exogenously administered bilirubin at concentrations as low as 100 nanomolar significantly restored myocardial function and minimized both infarct size and mitochondrial damage on reperfusion. Our data provide strong evidence for a primary role of HO-1-derived bilirubin in cardioprotection against reperfusion injury.

exogenous antioxidants are mainly restricted to the interstitial space (3). The increase of cell defenses by inducing endogenous pathways that are selectively engaged in restoring cellular homeostasis represents a valid and complementary alternative. Heat shock proteins (HSPs), or stress proteins, exemplify this concept because of their function as molecular chaperones and their high inducibility in stress conditions (21). HSPs favor the correct folding of newly synthesized proteins, and they are actively involved in repairing denatured proteins or promoting their degradation (12). Studies on cardiac muscle have revealed a direct correlation between elevated expression of the 70-kDa heat shock protein (HSP 70) and improved contractile function after ischemia (19). Recent work (18) has confirmed the ability of HSP 70 to decrease infarct size in hearts of transgenic HSP 70 mouse models.

In contrast with the classic HSP family that facilitates the rearrangement of cellular components, some inducible stress proteins function as antioxidant enzymes. One of these, heme oxygenase-1 [HO-1 or heat shock protein 32 (HSP 32)], has attracted particular interest because of its unique ability to generate products that might have important biological activities (16). Heme oxygenase is the rate-limiting enzyme in heme degradation to iron, carbon monoxide (CO), and bilirubin (34). HO-1 is the stress-inducible isoenzyme and is very sensitive to upregulation by a variety of different stimuli, such as the substrate heme, endotoxin, various hemoglobin, heavy metal ions, NO donors, and peroxynitrite (2, 7, 8, 10, 16, 22, 29, 30); heme oxygenase-2 (HO-2) is the constitutive isozyme expressed under physiological conditions (16). The possible beneficial effects of HO-1 induction in stress situations are mainly ascribed to the vasoactive gas CO and the antioxidant bilirubin. In analogy to NO, constitutively generated CO by HO-2 has been suggested to act as a neurotransmitter and to participate in the control of vasorelaxation (15, 35, 36). Motterlini et al. (24) have recently shown that increased CO production by HO-1 in vascular tissue contributes to the suppression of acute hypertensive responses under stress conditions in vivo. Similarly, Sammut et al. (31) have reported that HO-1-derived CO significantly suppresses phenylephrine-mediated contraction in isolated aortic rings. The other catabolite of heme degradation, bilirubin, is usually regarded as a potentially cytotoxic waste
product when accumulated at abnormally high concentrations in biological tissues. However, Stocker and colleagues (28, 33) elegantly demonstrated that low concentrations of bilirubin are as effective as vitamin E in inhibiting lipid peroxidation in vitro, suggesting that the bile pigment is a physiological, chain-breaking antioxidant. Foresti et al. (10) recently reported that increased bilirubin levels after induction of HO-1 are associated with reduced apoptosis mediated by peroxynitrite in vascular endothelial cells. Despite these promising features, little has been done to elucidate the biological importance of bilirubin in the cardiovascular system. In particular, the inherent cytoprotective characteristics of endogenous bilirubin derived from HO-1 during stress situations have yet to be explored. Here, we investigate the induction of the HO-1-bilirubin pathway in cardiac tissue and its potential involvement in mitigating ischemia-reperfusion injury.

**MATERIALS AND METHODS**

Isolated heart preparation. Langendorff heart preparations were performed using male Lewis rats (300–350 g) as previously described (26). Hearts were excised, and retrograde perfusion was established at a previously described (26). Hearts were excised, the aorta was removed from the aortic cannula and either dissected and fixed for electron microscopy analysis or stained for tissue viability. In an additional set of experiments, hearts from untreated animals were removed and perfused at constant flow for 10 min with oxygenated Krebs buffer containing bilirubin (0.05 or 0.1 µM) and subsequently subjected to ischemia (30 min) and reperfusion (60 min). Considering that hearts were perfused at a flow rate of 15 ml/min for 10 min, the total amount of bilirubin delivered to the organ was 7.5 or 15 nmol when the Krebs buffer contained 0.05 or 0.1 µM bilirubin, respectively. All the hemodynamic parameters were continuously monitored throughout the experimental protocol as reported above. Because both tin protoporphyrin IX and bilirubin are sensitive to light, all the experiments requiring these compounds were performed in the dark.

Heme oxygenase activity assay and Western blot analysis. Heme oxygenase activity and HO-1 protein expression were measured in the myocardial microsomal fraction. Microsomes were prepared as previously described (25). Hearts were perfused in situ with cold saline, removed, and immediately homogenized in 5 vol of 0.05 M Tris·HCl/0.25 M sucrose (pH 7.4). The homogenate was centrifuged at 27,000 g for 10 min, the supernatant was removed and centrifuged again at 105,000 g for 90 min, and the microsomal pellet was resuspended in 0.1 M phosphate buffer/2 mM MgCl2 (pH 7.4).

Heme oxygenase activity was determined in the microsomal fraction (1 mg of protein) by a spectrophotometric assay that measures the formation of bilirubin as a difference in absorbance between 464 and 530 nm (extinction coefficient for bilirubin 40 M⁻¹·cm⁻¹) (23). Expression of HO-1 protein was analyzed by Western blot as previously described (8).

Briefly, microsomes (60 µg of protein) from each sample were added to a gel loading buffer (0.5 M Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.03% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-PAGE using a 12% acrylamide resolving gel and transferred overnight onto a nitrocellulose membrane. Nonspecific binding of antibodies was blocked with 3% dried milk in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Membranes were then incubated for 2 h with a polyclonal rabbit anti-HO-1 antibody (StressGen, Victoria, Canada) using a 1:10,000 dilution in phosphate buffer containing 0.1% Tween 20. After being washed, blots were visualized using an amplified alkaline phosphatase kit from Sigma, and the relative density was analyzed using a GS-700 Densitometer (Bio-Rad). HSP 70 protein expression was determined in the myocardial cytosol by Western blotting using specific antibodies (Labvision).

**HPLC method.** Bilirubin was extracted from myocardial tissue and quantified by HPLC. Because bilirubin is highly sensitive to light, all the extraction procedures were performed in tubes covered with aluminum foil. Briefly, hearts were homogenized in 5 vol of 0.25 M sucrose and 0.05 M Tris·HCl (pH 7.4), mixed with 2 vol of chloroform, and shaken vigorously for 5 min. The chloroform phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 1,000 g for 20 min. This extraction step was repeated twice, and the extracts were pooled. The organic phase was transferred to a dry tube and evaporated under a stream of nitrogen gas, and the residue was resuspended in 1 ml of DMSO and stored at 4°C until analysis. All HPLC equipment and software were purchased from Gynotek (Macclesfield, Cheshire, UK). Chromatographic separations of bilirubin were performed using a Techsphere 5-µm C18 column (150 × 4.6 mm). The eluent used consisted of 40% ammonium acetate (100 mM, pH 5.0)-60% methanol running a linear gradient over 25 min to methanol (100%) at a flow rate of 1 ml/min. Peak identity for bilirubin was confirmed by
measuring the retention time, spiking the sample with commercially available standards, and determining absorbance spectra using an ultraviolet photodiode array detector (model VVD-340, Gynotec) set at 450 nm. Authentic standards of bilirubin were prepared in DMSO.

Electron microscopy and tissue viability. Heart muscle biopsies were fixed in 3% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1 M phosphate buffer for 2 h. After two washes, a second fixation with 1% osmium tetroxide in 0.1 M phosphate buffer was carried out for 1 h at room temperature. The specimens were then dehydrated through an increasing acetone series, infiltrated with acetone-araldite CY-212 resin (1:1) overnight in the specimen rotator, and embedded in araldite CY-212 resin, and blocks were polymerized at 60°C for 18 h. Ultra-thin sections (70–100 nm) were cut, collected on copper grids, and stained with 2% uranyl acetate (10 min) followed by Reynolds lead citrate (10 min). Mitochondrial integrity was analyzed in the stained sections using a JEOL-1200CX electron microscope. The analysis was performed in a blind manner on two sections of the left ventricle per group (~400 mitochondria/section), and a representative picture of mitochondrial integrity for each group is reported. A representative number of hearts from each experimental group (n = 3–4 hearts) were also stained for tissue viability. Hearts were perfused through a side arm of the aortic cannula for 20 min with tetrazolium red (3%) in Krebs-Henseleit buffer at 37°C. After they were stained, hearts were removed and stored in 2% Formalin in the dark before analysis. Hearts were carefully cut into 2-mm-thick sections and scanned into a computer using an AGFA Arcus II scanner, and the total ischemic size was determined by volumetric analysis software (Scion Image, Scion).

Statistical analysis. Statistical analysis was performed by the Student’s two-tailed t-test, and an ANOVA was performed when more than two treatments were compared. P values < 0.05 were considered statistically significant.

RESULTS

Hemin pretreatment induces HO-1 in cardiac tissue. We first examined whether treatment with hemin (75 µmol/kg ip), a substrate and a potent inducer of the heme oxygenase pathway, stimulates the expression of HO-1 in rat hearts. Figure 1A and B, shows the time course of HO-1 protein expression measured by Western blot. Hemin markedly increased HO-1 expression (fourfold, P < 0.05) in the microsomal fraction of hearts taken from animals 1 day after injection. The HO-1 protein levels gradually returned to control values within 4–5 days after treatment. Induction of HO-1 observed at 1 day after hemin administration was associated with increased heme oxygenase activity (P < 0.05 vs. control); this effect was suppressed by treatment with tin protoporphyrin IX (40 µmol/kg ip), a competitive inhibitor of heme oxygenase enzymes (Fig. 1A, inset). These data indicate that maximal upregulation of the HO-1 pathway occurs in the myocardium at 1 day after treatment of animals with hemin. Therefore, this time point was chosen to examine the possible involvement of the HO-1 pathway in protection against myocardial ischemia-reperfusion injury.

We also analyzed the expression of HSP 70, an inducible HSP, in cardiac tissue from hemin-treated animals. HSP 70 has been shown to be upregulated by ischemic preconditioning in the myocardium, resulting in protection of tissues against ischemia-reperfusion damage (17). We found that the level of HSP 70 did not change after hemin treatment because myocardial protein expression was comparable at all the time points examined (Fig. 1B). The results show that a functional role of HSP 70 in myocardial protection in hemin-treated animals can be excluded a priori and that our model provides a useful tool for investigating the potential effect of HO-1 in cardiac tolerance to ischemia.

Hemin pretreatment improves myocardial function after ischemia-reperfusion. To determine the effect of HO-1 induction on the functional recovery of hearts subjected to ischemia-reperfusion, cardiac performance (LVDP × HR) and other hemodynamic parameters were continuously monitored in the Langendorff preparation of hearts from vehicle-treated (control group) and hemin-treated animals. Figure 2 shows the time course of all the parameters measured during reperfusion in the groups examined. For simplicity and clarity, we will refer to the hemodynamic parameters evalu-
before heart isolation. Hearts were perfused according to Langendorff before removal of hearts. PP (40 µmol/kg) was given to animals 1 h plus PP (p hearts from animals pretreated with vehicle (EDP) (D and change in end-diastolic pressure (C were determined in group. The increase in CPP over the baseline value red or fixed for electron microscopy analysis at the end sess the damage caused by ischemia-reperfusion in the isolated organ for 2 h. The rate of bilirubin released into the perfusion buffer from hemin-treated hearts was also markedly increased by 55% (P < 0.05) compared with hearts removed from vehicle-treated animals (Fig. 4C).

Exogenously applied bilirubin increases functional recovery of the myocardium after ischemia-reperfusion. The effect of exogenously applied bilirubin on our ex vivo system was investigated after having demonstrated that upregulation of the heme oxygenase enzymatic pathway in cardiac tissue results in increased bilirubin production and enhanced myocardial function after ischemia-reperfusion. Hearts removed from untreated animals and perfused for 10 min with buffer containing low levels of bilirubin (0.1 µM) before ischemia showed an 87% recovery in cardiac performance at 60 min of reperfusion as opposed to 65% recovery in untreated hearts (P < 0.05, Fig. 5). The increased myocardial performance in hearts pretreated with bilirubin was associated with a significant reduction in ΔCPP (25 vs. 41 mmHg in untreated hearts, P < 0.05) and ΔEDP (19 vs. 44 mmHg in untreated hearts, P < 0.05) at 60 min of reperfusion. These data demonstrate that, at concentrations as low as 0.1 µM, exogenously applied bilirubin partially prevents reperfusion-mediated impairment of myocardial contractility.

Protective effect of hemin pretreatment and bilirubin against myocardial ischemia-reperfusion injury. To assess the damage caused by ischemia-reperfusion in cardiac tissue, hearts were stained with tetrazolium red or fixed for electron microscopy analysis at the end of the reperfusion period. The tetrazolium salt stains treated animals (41 mmHg) compared with those treated with hemin (14 mmHg) (P < 0.05). A similar pattern was seen for EDP changes (ΔEDP). Interestingly, the use of tin protoporphyrin IX (40 µmol/kg), a potent inhibitor of heme oxygenase activity, completely abolished the posts ischemic recovery of cardiac function of hemin-treated hearts (Fig. 3A), resulting in reduction of cardiac performance (70% of baseline) and increase in both ΔEDP (31 mmHg) and ΔCPP (33 mmHg). The doses of tin protoporphyrin IX used in our study (20 and 40 µmol/kg) did not have any apparent effect on the functional recovery of posts ischemic hearts removed from vehicle-treated animals (Fig. 3B).

Hemin pretreatment results in increased bilirubin production in the myocardium. Heme oxygenase-mediated heme degradation results in the production of CO and biliverdin. Biliverdin is promptly converted to bilirubin by the cytosolic enzyme biliverdin reductase, an enzyme present in most cell types. We detected a considerable biliverdin reductase activity in both control and hemin-treated hearts (data not shown). The bilirubin IX isomer was measured in cardiac tissue by HPLC as described in MATERIALS AND METHODS. A representative chromatogram and absorbance spectrum of bilirubin extracted from cardiac tissue of heme-treated animals are shown in Fig. 4A. An increase of 23% in bilirubin was measured in hearts from animals treated with hemin compared with vehicle-treated rats (P < 0.05, Fig. 4B). Bilirubin was also measured in the heart perfusate, which was circulated continuously through the isolated organ for 2 h. The rate of bilirubin released into the perfusion buffer from hemin-treated hearts was also markedly increased by 55% (P < 0.05) compared with hearts removed from vehicle-treated animals (Fig. 4C).

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viable myocardium brick red, whereas the damaged tissue remains unstained and appears white. Figure 6A shows the ischemic volume (infarct size) measured at 60 min of reperfusion in the experimental groups examined as well as a representative heart section from each group. Hearts from vehicle-treated animals that were subjected to ischemia-reperfusion showed a significantly larger infarct size compared with nonischemic, control hearts (11.7 vs. 0.4%, \( P < 0.05 \)). The infarct size was reduced to 6.3% in hearts from animals treated with hemin. The protective effect of hemin was abolished by treatment with tin protoporphyrin IX (infarct size 10.5%), which has been previously shown to inhibit heme oxygenase activity. Perfusion of the hearts with bilirubin (100 nmol) significantly reduced the infarct size to 3.9%. Figure 6B shows four electron micrographs of mitochondria from the left ventricular cardiac muscle. Compared with nonischemic tissue (control), mitochondria from hearts subjected to ischemia-reperfusion appeared swollen and the cristae structure was disrupted. However, both heme treatment and exogenous bilirubin perfusion preserved mitochondrial integrity after ischemia-reperfusion.

**DISCUSSION**

In isolated perfused hearts, we found that reperfusion after 30 min of ischemia caused a significant decrease in myocardial contractility and impairment of hemodynamic function, as well as tissue damage and mitochondrial disruption. Myocardial ischemia-reperfusion is a well-documented pathological condition characterized by depletion of high-energy phosphates, loss of cellular integrity, and, ultimately, irreversible injury and cell death (14). It is also well established that oxidative stress resulting from increased production of reactive oxygen species plays a pivotal role in the progression of cell damage in postischemic tissue (20). Paradoxically, increased resistance to reperfusion injury has been shown to occur when brief ischemic episodes precede a prolonged ischemic insult, a phenomenon termed "preconditioning" (27). Two temporally distinct phases distinguish the cardioprotective effect of ischemic preconditioning: an early phase, which develops immediately after preconditioning and wanes within few hours, and a late phase associated with increased expression of inducible stress proteins or HSPs (17). In several experimental models of myocardial ischemia, cardioprotection directly correlates with the overexpression of either HSP 70, HSP 90, or HSP 60, the most widely studied molecular chaperones in the HSPs family (3). Because diverse stimuli can enhance HSPs levels, the possibility to pharmacologically or genetically manipulate the expression of these proteins to limit ischemic injury is attainable (21).

Our study shows that maximal upregulation of the stress protein HO-1 (or HSP 32), the inducible isoform of heme oxygenase, occurs in cardiac tissue 24 h after...
treatment of animals with hemin, both a substrate and inducer of HO-1. Interestingly, the recovery of postischemic myocardial function was markedly increased in hearts isolated after treatment of animals with hemin compared with hearts from vehicle-treated rats. Moreover, hearts displaying high HO-1 levels showed a considerably reduced infarct size and preserved mitochondrial integrity after ischemia-reperfusion. These data are indicative of a major role for HO-1 in cardioprotection. Although such a role for HO-1 has not been described in the context of ischemia-reperfusion yet, recent studies highlighted the importance of this stress protein in cardiac xenograft survival and prevention of chronic rejection in heart allografts (11, 32). Our results also exclude the possibility that HSP 70 may play

Fig. 4. Hemin pretreatment results in increased bilirubin production in myocardium. A: representative elution curve from HPLC analysis of myocardial bilirubin extracted from a hemin-treated rat. Chromatograms show peak and absorption spectra at 450 nm typical of bilirubin. B: rate of bilirubin released into the circulating buffer of isolated-perfused hearts. Hearts were removed from animals 1 day after pretreatment with vehicle (control) or hemin (75 µmol/kg) and perfused according to Langendorff technique as described in MATERIALS AND METHODS. After 20 min equilibration, 100 ml of buffer were recirculated through system for 2 h to allow bilirubin released from heart to accumulate in perfusion buffer. Bilirubin was extracted with chloroform at end of perfusion and measured spectrophotometrically as a difference in absorbance between 460 and 530 nm. C: tissue bilirubin content in hearts removed from animals 1 day after treatment with vehicle or hemin (75 µmol/kg). Tissue bilirubin was extracted with chloroform and measured by HPLC as described in MATERIALS AND METHODS. Results are expressed as means ± SE; n = 5–7 hearts. *P < 0.05; **P < 0.01 vs. vehicle.

Fig. 5. Exogenously applied bilirubin increases functional recovery of hearts after I/R. Hearts were removed from untreated animals and perfused according to the Langendorff technique as described in MATERIALS AND METHODS. After 10 min of equilibration, hearts were perfused with Krebs-Henseleit buffer containing 0.05 or 0.1 µM bilirubin for 10 min and then subjected to 30 min of ischemia plus 60 min of reperfusion. Cardiac performance (LVDP × HR) (A), ΔCPP (B), and ΔEDP (C) were measured at the end of reperfusion period and compared with untreated hearts (0 µM). Results are means ± SE; n = 6–7 hearts. *P < 0.05 vs. untreated control.
a role in protection against reperfusion injury in our system, because the levels of this HSP remained unchanged after hemin treatment. Pretreatment of animals with hemin could, however, have affected the levels of other HSPs, and at present we cannot rule out the involvement of other chaperones in the observed cardioprotection. Nonetheless, a direct contribution of the heme oxygenase pathway in minimizing reperfusion damage was ascertained in our study by using an inhibitor of its activity, tin protoporphyrin IX. Predictably, blockade of heme oxygenase activity completely abolished the improved postischemic myocardial performance observed after hemin treatment. Likewise, cardiac tissue injury was exacerbated by treatment of animals with tin protoporphyrin IX.

How can heme oxygenase activation provide protection against the cytotoxicity caused by ischemia-reperfusion? Because heme oxygenase is the rate-limiting step in heme degradation, increased HO-1 protein is expected to result in augmented production of its catabolites, bilirubin and CO. Bilirubin is regarded as a waste product but has been known to possess antioxidant properties for a long time (4). This peculiarity of bilirubin has been examined by Stocker and colleagues (33), who showed that physiological levels of the bile pigment suppress the oxidation of lipid membranes more than α-tocopherol, which hitherto had been regarded as the best antioxidant against lipid peroxidation. Bilirubin is normally present in plasma as either free bilirubin or bound to serum albumin to...
form a bilirubin-albumin complex. Interestingly, free bilirubin protects low-density lipoproteins from oxidation more efficiently than albumin-bound bilirubin (28). Motterlini and colleagues (10, 23) also reported that both exogenous and endogenously generated bilirubin can effectively prevent endothelial cell death mediated by hydrogen peroxide and peroxynitrite, respectively. Remarkably, while this paper was in preparation, Dore et al. (6) demonstrated that accumulation of bilirubin due to enhancement of HO-2 catalytic activity by phosphorylation is also protective against hydrogen peroxide-induced cytotoxicity in neuronal cultures. The results of the present study provide strong evidence for a direct involvement of HO-1-derived bilirubin in reducing reperfusion injury. Indeed, consistent with the significant recovery of postischemic myocardial function and preserved tissue viability, we found that hearts displaying high levels of HO-1 and increased heme oxygenase activity exhibited elevated intracellular bilirubin content. In addition, the rate of bilirubin released into the circulating buffer was higher in hearts showing increased HO-1 expression. The notion that bilirubin is most likely a candidate for cardioprotection was further corroborated by our findings showing that bilirubin, exogenously delivered to isolated hearts before ischemia at concentrations as low as 100 nM, significantly restored myocardial function and minimized both infarct size and mitochondrial damage on reperfusion.

Our findings on increased intracellular bilirubin as a consequence of HO-1 induction imply that production of CO, the other catabolite of heme degradation, is also enhanced. CO, as a vasoactive molecule, could be partially responsible for the beneficial effects mediated by activation of the heme oxygenase pathway. Notably, we found that the rise in CPP, a parameter indicative of coronary vessel contractility, was less pronounced in postischemic cardiac tissue expressing high levels of HO-1. These results suggest that increased endogenous CO may play a role in the maintenance of vascular tone during the reperfusion of ischemic hearts. The recent reports of Motterlini and colleagues (24, 31) sustain this hypothesis by demonstrating that HO-1-derived CO is a major regulator of pressor responses in vivo and in vitro.

Our studies delineate a plausible mechanism by which HO-1 protein confers protection against reperfusion injury in the myocardium. The induction of this stress-sensitive enzyme, which is triggered by a range of oxidant-related stimuli, appears to act as a ubiquitous defensive system in a variety of cell types (1). In addition, a fundamental role for heme oxygenases in the maintenance and restoration of cellular homeostasis has been recently postulated (9). The results presented here substantiate this concept by direct assessment of the cardioprotective action of HO-1-derived bilirubin. The possibility that the protection provided by bilirubin in the ischemic myocardium could be physiologically significant is indicated by reports showing an inverse correlation between plasma bilirubin levels and the risk of coronary artery disease (5, 13).

Therefore, manipulation of the HO-1 pathway to raise endogenous bilirubin levels may represent a feasible strategy to counteract oxidative stress and, ultimately, may have relevant clinical implications in the prevention of cardiovascular disease.

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