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Beneficial effect of heme oxygenase-1 expression on myocardial ischemia-reperfusion involves an increase in adiponectin in mildly diabetic rats

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L'Abbate A, Neglia D, Vecoli C, Novelli M, Ottaviano V, Baldi S, Barsacchi R, Paolicchi A, Masiello P, Drummond GS, McClung JA, Abraham NG. Beneficial effect of heme oxygenase-1 expression on myocardial ischemia-reperfusion involves an increase in adiponectin in mildly diabetic rats. *Am J Physiol Heart Circ Physiol* 293: H3532–H3541, 2007. First published September 28, 2007; doi:10.1152/ajpheart.00826.2007.—Transient reduction in coronary perfusion pressure in the isolated mouse heart increases microvascular resistance (paradoxical vasoconstriction) by an endothelium-mediated mechanism. To assess the presence and extent of paradoxical vasoconstriction in hearts from normal and diabetic rats and to determine whether increased heme oxygenase (HO)-1 expression and HO activity, using cobalt protoporphyrin (CoPP), attenuates coronary microvascular response, male Wistar rats were rendered diabetic with nicotinamide/streptozotocin for 2 wk and either CoPP or vehicle was administered by intraperitoneal injection weekly for 3 wk (0.5 mg/100 g body wt). The isolated beating nonworking heart was submitted to transient low perfusion pressure (20 mmHg), and coronary resistance (CR) was measured. During low perfusion pressure, CR increased and was associated with increased lactate release. In diabetic rats, CR was higher, HO-1 expression and endothelial nitric oxide synthase were downregulated, and inducible nitric oxide synthase and O₂⁻ were upregulated. After 3 wk of CoPP treatment, HO activity was significantly increased in the heart. Upregulation of HO-1 expression and HO activity by CoPP resulted in the abolition of paradoxical vasoconstriction and a reduction in oxidative ischemic damage. In addition, there was a marked increase in serum adiponectin. Elevated HO-1 expression was associated with increased expression of cardiac endothelial nitric oxide synthase, B-cell leukemia/lymphoma extra long, and phospho activator protein kinase levels and decreased levels of inducible nitric oxide synthase and malondialdehyde. These results suggest a critical role for HO-1 in microvascular tone control and myocardial protection during ischemia in both normal and mildly diabetic rats through the modulation of constitutive and inducible nitric oxide synthase expression and activity, and an increase in serum adiponectin.

antiapoptotic signaling protein; superoxide anion; phosphoactivator protein kinase

ACCORDING TO CLASSICAL CONCEPTS of autoregulation, coronary microvessels dilate in response to a reduction in perfusion pressure so as to preserve flow. Beneath a certain pressure level, the exhaustion of this mechanism causes flow to decline

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and myocardial ischemia to ensue. Recently, both clinical and experimental studies (26, 48) have demonstrated a “paradoxical” coronary microvascular constriction during severe reduction in perfusion pressure and myocardial ischemia. It is not known whether this may be a naturally occurring response to severe hypotension and ischemia, possibly intended to preserve capillary pressure, or whether it may be caused or enhanced by the presence of endothelial dysfunction, hence, aggravating myocardial ischemia.

In the isolated mouse model, “paradoxical coronary vasoconstriction” has been reproduced by perfusion at low pressure and the endothelium appears to play a pivotal role in this vascular response, which involves the cooperative vasoconstrictive actions of the nitric oxide (NO) and endothelin systems (26). In particular, nitric oxide synthase (NOS) inhibition or the administration of reactive oxygen species (ROS) scavengers has been able to antagonize the paradoxical vasoconstrictive response to low pressure. This observation led to the hypothesis that, during ischemia, NO released in the vascular wall and possibly in the myocardium can be converted by ROS to a reactive species with constrictive properties, such as peroxynitrite, with a simultaneous reduction of NO availability and activation of the vasoconstrictive pathway of the endothelium.

Type 2 diabetes is a clinical condition, the prevalence of which is rapidly growing in Western countries. It has been estimated that by the year 2030 there will be almost 30 million Americans with diagnosed diabetes (60). Evidence suggests that diabetes and insulin resistance are major risk factors for cardiovascular disease (39) due to the associated endothelial dysfunction and increased oxidative stress (57). Insulin resistance is associated with abnormal endothelium-dependent coronary vasomotion (44), which progressively worsens with progression to overt Type 2 diabetes (43). In experimental diabetes, there is an overproduction of ROS in the vascular wall that is thought to enhance NO degradation and promote the formation of peroxynitrite. The excessive ROS burden may thus promote atherogenesis by causing endothelial dysfunction in concert with inflammatory reactions and LDL oxidation (30). It has recently been reported that the actions of adiponectin, an antidiabetic, antiatherogenic, and anti-inflammatory adipokine (7, 8), are mediated through adiponectin receptors and that genetic variations on the Adipo2 receptor are associ-

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ated with increased adiponectin levels and decreased triglyceride levels in patients with metabolic syndrome (6).

Adiponectin has been ascribed antioxidative properties (20); it improves endothelial function in patients with metabolic syndrome and also improves the beneficial effects of antihypertensive agents in hypertensive patients (40, 63). The concentration of adiponectin is reported to be decreased in coronary diseases and Type 2 diabetes (19). Some aspects of clinical insulin resistance and Type 2 diabetes can be reproduced experimentally in rats by streptozotocin administration (STZ) and nicotinamide (NA; Ref. 27). It can be hypothesized that the presence of coronary endothelial dysfunction and/or increased ROS formation could influence the coronary vasomotor response to low pressure ischemia in isolated hearts from STZ/NA rats compared with normal controls.

The recognition that heme oxygenase (HO)-1 is present in the heart and induced by various oxidants, including metals, signifies the importance of this enzyme in cardioprotection (3, 37). Cardioselective overexpression of HO-1 exerts a cardioprotective effect after myocardial ischemia-reperfusion in mice (55). The robust ability of HO-1 to protect against oxidative insult in cardiovascular diseases, including diabetes, suggests that HO-1 may be a target for pharmacological intervention in the alleviation of vascular diseases (1). The protective effects of HO-1 arise from its capacity to increase degradation of the heme moiety from destabilized heme proteins (36) and to generate biliverdin and bilirubin, which possess potent antioxidant properties (51) and display cytoprotective properties in the cardiovascular system (10, 18). Carbon monoxide (CO) is not an antioxidant (59) but has a beneficial effect on vascular relaxation and causes the induction of antioxidant genes, including superoxide dismutase and glutathione levels (1, 13, 46, 53).

Recently, Nishikawa et al. (38) have shown that the HO-CO pathway is involved in ischemic vasodilation in the coronary microcirculation. Yet et al. reported the occurrence of severe right ventricular enlargement after chronic hypoxia in HO-1-deficient compared with wild-type mice (61) and that the cardiac-specific expression of HO-1 protects against ischemia and reperfusion (62). Further, HO-1 gene expression has been shown to reverse neointimal hyperplasia (21) and ischemic heart injury (16) and prevent vascular dysfunction in experimental diabetes (24) (for review see Ref. 1). These effects were related to an increase in the HO-CO pathway and to the associated increase in endothelial nitric oxide synthase (eNOS) and reduction in inducible nitric oxide synthase (iNOS) expression, thus both improving endothelial function and decreasing overall NO availability for peroxynitrite formation (1, 2, 13, 53).

The aims of this study were to assess the presence and extent of paradoxical vasoconstriction and its influence on ischemia in hearts from normal and mildly diabetic rats, to determine whether the upregulation of HO-1 accompanied by increased HO activity modulates coronary microvascular tone and myocardial ischemia, and to examine the possible mechanism(s) involved.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of CNR Institute of Clinical Physiology, Pisa, Italy, and conducted under the *Guidelines for the Care and Use of*

Laboratory Animals, published by the Office of Science and Health Reports, National Institutes of Health.

Induction of diabetes. Male Wistar rats, 2–3 mo of age, received 210 mg/kg of nicotinamide dissolved in saline (NA; Sigma, St. Louis, MO) intraperitoneally 15 min before an intravenous injection of 60 mg/kg STZ (Sigma) dissolved in citrate buffer (pH 4.5) immediately before use to obtain a mild and stable diabetes with reduced beta-cell mass (28). The resulting mild diabetes did not require insulin. This experimental model has been utilized as a model for noninsulin-dependent diabetes mellitus (NIDDM) syndrome and is regarded as similar to human Type 2 diabetes in that it has a significant response to insulin responsiveness to glucose, preserved sensitivity to tolbutamide, and provides partial pancreatic protection (9, 28). Each set of NA/STZ-treated animals was matched by one group of controls receiving vehicle. After 2 wk, the average plasma glucose level in NA/STZ rats was 149 ± 3.6 mg/dl and the corresponding plasma insulin level was 1.40 ± 0.14 ng/ml compared with 108 ± 2.6 mg/dl and 1.87 ± 0.40 ng/ml, respectively, in rats receiving vehicle ($P < 0.01$ and not significant, respectively). Cobalt protoporphyrin (CoPP; 0.5 mg/100 g) or the corresponding vehicle was given subcutaneously once a week for an additional 3 wk. Hence, four subgroups of animals were studied: control rats (C, $n = 18$), mildly diabetic rats (D, $n = 24$), control rats-CoPP (C-CoPP, $n = 8$), and mildly diabetic rats-CoPP (D-CoPP, $n = 11$).

Isolated heart preparation. Rats were anaesthetized with a mixture of ether and air and heparinized via the left femoral vein (250 units/kg) 72 h after the last CoPP or vehicle injection. The heart was rapidly excised and placed in perfusion medium. Within 30 s, the aorta was attached to a stainless steel cannula, the pulmonary artery was incised to permit adequate drainage, and the heart was perfused normothermically (37°C) by the method of Langendorff at a perfusion pressure equivalent to 80 mmHg. The perfusion medium was Krebs-Henseleit buffer (KHB) having the following composition in mM: 120.0 NaCl, 25.0 NaHCO₃, 4.8 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 11 glucose, and 1.4 CaCl₂·2H₂O (pH 7.4 when gassed with 95% O₂ and 5% CO₂).

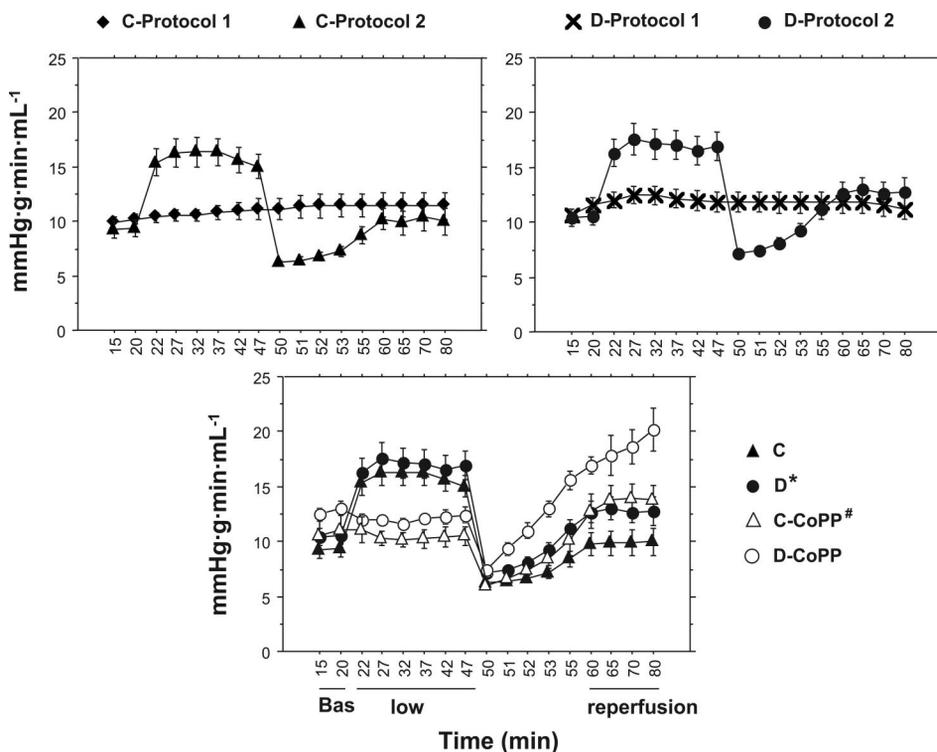
Experimental protocols. The time course of coronary resistance (CR) and lactate release were obtained according to the above protocol and were compared with those from isolated hearts continuously perfused with oxygenated KHB for 80 min at 80 mmHg pressure. Isolated hearts were perfused with oxygenated KHB for 20 min at 80 mmHg pressure, then perfusion pressure was decreased to 20 mmHg for 30 min, and then pressure was increased back to 80 mmHg for the remaining 30 min (reperfusion).

In all experiments, coronary flow was measured continuously with the volume of effluent calculated with a calibrated pipette. CR was defined as input pressure divided by coronary flow per gram of myocardial tissue (mmHg·min·g⁻¹·ml⁻¹). For successive analyses, data relative to the first 10 min of perfusion (stabilization period) were discarded. At different times, coronary perfusate was collected and lactate concentration was determined in the perfusion medium. At the end of each experiment, the heart was rapidly frozen in liquid nitrogen and then stored at -80°C.

Lactate quantification. Lactate concentration was measured by the automated spectrophotometric enzymatic method on a Beckman CX4 Synchron Analyzer (Fullerton, CA) (17). The between assay coefficient of variation was 5%, and the detection limit was 0.05 μmol.

Malondialdehyde determination. Frozen hearts were pulverized under liquid nitrogen and homogenized in an acid solution (0.6 M perchloric-acetic acid). Homogenates were centrifuged at 13,000 g for 15 min at 4°C, supernatant was isolated, and protein levels were assayed by the Bradford method. One volume of supernatant was mixed with 1 volume of 0.66% (wt/vol) thiobarbituric acid, and the mixture was boiled for 15 min. After cooling in tap water, the mixture was read at 535 nm, and the thiobarbituric acid-malondialdehyde (MDA) adduct was calculated by using a molar absorption coefficient of 1.56×10^5 M⁻¹/cm (35).

Fig. 1. *Top*: time course of coronary resistance (CR) is shown in isolated hearts from control (C) and diabetic (D) rats during perfusion at constant pressure (P; 80 min at 80 mmHg; protocol 1) or transient reduction of perfusion pressure (30 min at 20 mmHg; protocol 2). *Bottom*: time course of CR during transient reduction of perfusion pressure (protocol 2) is shown for untreated (C and D) and cobalt protoporphyrin (CoPP)-treated (C-CoPP and D-CoPP) animals. D animals show higher coronary resistance in absolute values than C rats in all the study conditions except early reperfusion. Transient reduction of perfusion pressure causes a similar percent increase in coronary resistance in both groups that was prevented by CoPP treatment. For statistical significance, see Table 1. Bas, baseline perfusion pressure (80 mmHg); low, low perfusion pressure (20 mmHg); reperfusion, last 20 min of reperfusion at baseline perfusion pressure (80 mmHg). Time scale at the onset of low perfusion pressure and of reperfusion has been expanded. $P < 0.01$ D vs. C and D-CoPP vs. C-CoPP; * $P < 0.01$ vs. baseline; # $P < 0.01$ CoPP treated vs. corresponding untreated groups.



Determination of HO, eNOS, iNOS, pAKT, and serum adiponectin. Frozen hearts were pulverized under liquid nitrogen and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% tertigol, pH 7.5). Homogenates were centrifuged at 27,000 g for 10 min at 4°C, supernatant was isolated, and protein levels for HO-1/HO-2, eNOS, iNOS, and phospho activator protein kinase levels (pAKT), as well as O_2^- levels and HO activity, were measured as described previously (5, 24). Adiponectin assayed in serum obtained from venous blood samples obtained immediately before heart excision was determined using ELISA assay (Pierce Biotechnology, Woburn, MA).

Statistical analysis. Results are means \pm SE for the number (n) of replicate determinations. Statistical significance between experimental groups and between different study conditions was determined by using a two-way ANOVA followed by the Fisher's exact test; $P < 0.05$ was considered significant.

RESULTS

CR and effect of CoPP. In both C and D animals, CR tended to remain constant during the 80 min perfusion period at constant pressure. When the perfusion pressure was suddenly decreased to 20 mmHg, CR rapidly increased in both groups by 1.6-fold ($P < 0.01$ vs. baseline) and remained high for the entire period of low perfusion pressure. During early reperfusion (1st min), CR promptly decreased to below baseline values ($P < 0.01$ vs. baseline) and then progressively increased, stabilizing after 10 min at baseline values in C rats and slightly over baseline values in D rats (Fig. 1; Table 1). This demonstrates the presence of a vasoconstrictive response during transient low perfusion pressure (paradoxical vasoconstriction) followed by vasodilation (reactive hyperemia) at the onset of reperfusion in both groups. CR was higher in D rats than in C rats under all study conditions ($P < 0.01$), except during reactive hyperemia.

In both C-CoPP and D-CoPP animals, CR tended to remain constant during the 80 min perfusion period at constant pressure. During transient low perfusion pressure, CoPP was able to completely abolish paradoxical vasoconstriction in both C and D animals, leaving the maximal reactive hyperemic response (minimal resistance values) unchanged (Fig. 1). Compared with untreated animals, CoPP increased CR values at baseline ($P < 0.01$) and in the late phase of reperfusion ($P < 0.01$). CR was higher in D-CoPP than in C-CoPP rats under all study conditions ($P < 0.01$), except reactive hyperemia (Table 1), when the absolute values are considered. However, if the percentage increase is considered, then the difference between C and D animals is significantly decreased ($P < 0.01$).

Myocardial lactate release and effect of CoPP. During perfusion at constant pressure, no myocardial lactate release was measured in any animal. The absence of lactate release

Table 1. Effects of low perfusion pressure and HO-1 upregulation by CoPP on coronary resistance in control and diabetic rats

	Coronary Resistance, mmHg·g·min·mL ⁻¹			
	Bas	Low PP	Early RP	Late RP
Control	9.3 \pm 0.5	15.8 \pm 0.4*	6.2 \pm 0.2*	9.6 \pm 0.5
Diabetic	10.5 \pm 0.6	16.9 \pm 0.5*	7.1 \pm 0.3*	12.5 \pm 0.5
Control-CoPP	10.8 \pm 0.8†	10.4 \pm 0.3†	6.0 \pm 0.2*	12.8 \pm 0.6†
Diabetic-CoPP	12.7 \pm 0.2†	12 \pm 0.2†	7.4 \pm 0.3*	17.9 \pm 0.7†

HO-1, heme oxygenase-1; CoPP, cobalt protoporphyrin; Bas, baseline perfusion pressure (80 mmHg); Low PP, low perfusion pressure (20 mmHg); Late RP, last 20 min of reperfusion at baseline perfusion pressure (80 mmHg). $P < 0.01$ diabetic vs. control and diabetic-CoPP vs. control-CoPP at Bas, Low PP, and Late RP. * $P < 0.01$ vs. baseline; † $P < 0.01$ CoPP treated vs. corresponding untreated groups.

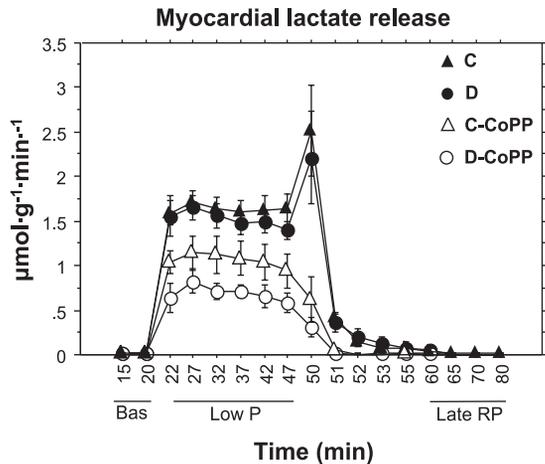


Fig. 2. Myocardial lactate release at different times during transient low perfusion pressure experiments in untreated (C and D) and CoPP-treated (C-CoPP and D-CoPP) animals. Time scale at the onset of low perfusion pressure and of reperfusion has been expanded. $P < 0.01$ C-CoPP and D-CoPP vs. untreated groups.

was also noted during baseline as well as during the late reperfusion period of the transient low perfusion pressure protocol. Conversely, during the low-pressure perfusion period, myocardial lactate release was documented in all groups (Fig. 2). Lactate release was similar in C and D animals, and it significantly decreased after CoPP in both (C-CoPP and D-CoPP) groups ($P < 0.01$). In D-CoPP animals, lactate release decreased to half the levels measured in the untreated D animals. At early reperfusion, hearts from C and D rats showed a significant peak of lactate release ($P < 0.01$ vs. baseline and vs. low-pressure perfusion), which was abolished after CoPP treatment (Fig. 2). The total amount of lactate released (integral of the curves

in Fig. 2) was 14.9 ± 1.3 $\mu\text{mol/g}$ in C and 12.8 ± 1.32 $\mu\text{mol/g}$ in D animals ($P = 0.29$). After CoPP, lactate release was reduced to 6.1 ± 0.92 $\mu\text{mol/g}$ in C-CoPP and to 4.57 ± 0.74 $\mu\text{mol/g}$ in D-CoPP animals ($P < 0.01$ C vs. C-CoPP and D vs. D-CoPP).

Cardiac HO-1 and effect of CoPP. In both C and diabetic hearts, pretreatment with CoPP caused an increase in HO-1 protein levels ($P < 0.01$, C-CoPP and D-CoPP vs. C and D, respectively) and an increase in HO activity ($P < 0.01$, C-CoPP and D-CoPP vs. C and D, respectively; Fig. 3). Diabetic hearts, compared with control hearts, displayed lower levels of HO-1 protein ($P < 0.05$ D vs. C). Diabetes did not influence the levels of HO-2 protein (Fig. 4). As shown in Fig. 3, HO activity was significantly lower in diabetic hearts compared with controls ($P < 0.05$ D vs. C).

Cardiac eNOS and iNOS levels and effect of CoPP. Compared with untreated controls, untreated diabetic hearts showed lower levels of eNOS protein ($P < 0.05$) and higher values of iNOS protein ($P < 0.05$; Fig. 5). The increases in HO-1 protein and total HO activity by CoPP resulted in enhanced expression of eNOS protein ($P < 0.05$ compared with control) and reduced expression of iNOS protein in both diabetic and control hearts (Fig. 6).

Cardiac MDA and serum adiponectin levels after transient reduction of perfusion pressure and effect of CoPP. MDA levels were similar in hearts obtained from D and C rats after transient low pressure. As a result of CoPP administration, MDA levels were decreased by 50% in C-CoPP and by 45% in D-CoPP rats ($P < 0.01$ CoPP-treated groups vs. corresponding controls; Fig. 7).

Adiponectin levels were lower in D (3.88 ± 1.04 $\mu\text{g/ml}$) compared with 7.1 ± 0.76 $\mu\text{g/ml}$ in C rats ($P < 0.05$). CoPP administration significantly increased serum adiponectin (to 12.7 ± 2.56 $\mu\text{g/ml}$) in D rats compared with untreated D rats

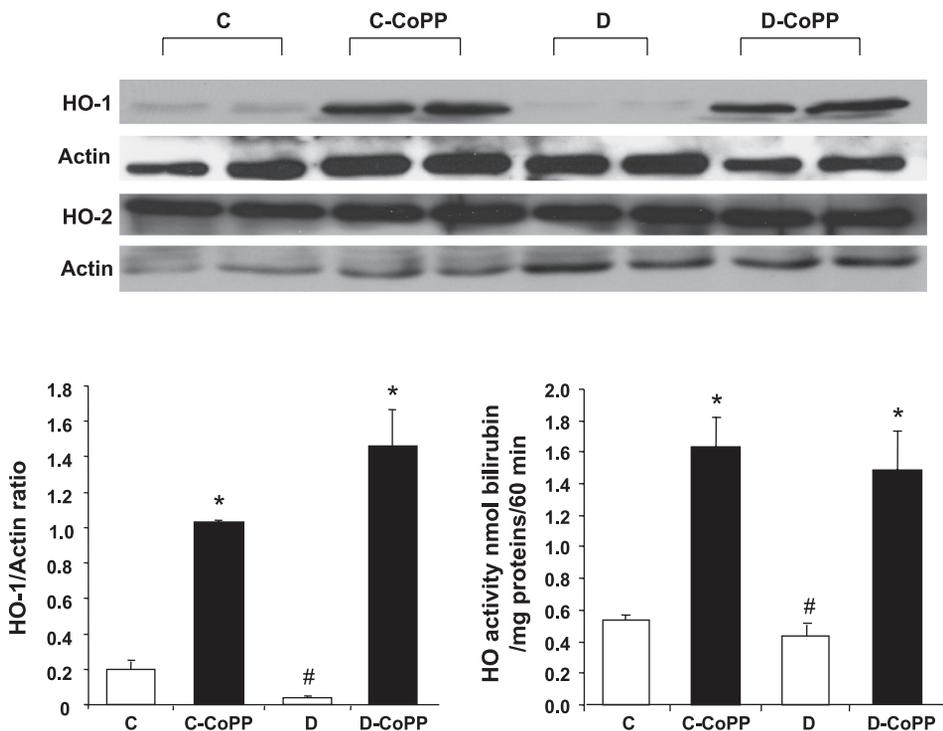


Fig. 3. Heme oxygenase (HO)-1, HO-2 protein levels, and HO activity in hearts from C and D rats with and without CoPP treatment after perfusion at transient low pressure. Mean band density was normalized relative to β -actin ($n = 4$ for each group). # $P < 0.05$ D vs. C rats; * $P < 0.01$ CoPP-treated groups vs. corresponding untreated groups.

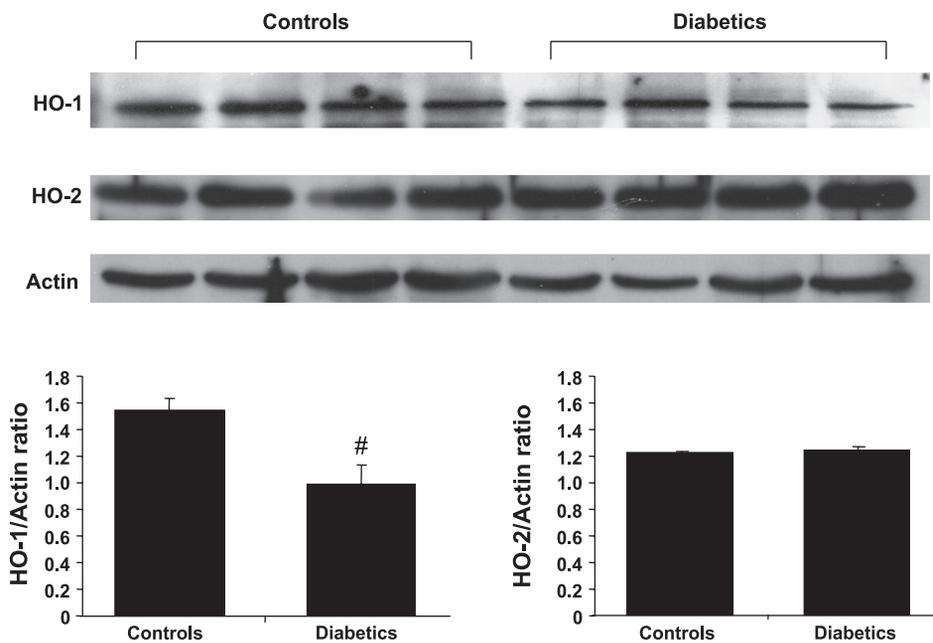


Fig. 4. Western blot analysis of HO-1, HO-2 protein, and β -actin in hearts from C and D rats after perfusion at transient low pressure. Mean band density was normalized relative to β -actin; $n = 4$; $\#P < 0.05$ D vs. C rats.

($P < 0.01$). To ascertain whether the CoPP-mediated increase in adiponectin levels was related to increased HO activity, we coadministered tin mesoporphyrin, an inhibitor of HO activity, to D-CoPP rats, which resulted in inhibition of HO activity and serum adiponectin release (Fig. 8).

Cardiac O_2^- levels and effect of CoPP. O_2^- measurements, using chemiluminescence, were performed at low concentrations of lucigenin ($5 \mu\text{M}$) in hearts obtained at the end of the transient low-pressure experiments. Diabetic hearts showed a significant increase in O_2^- compared with controls ($P < 0.05$ D vs. C). Treatment with CoPP caused a decrease in O_2^- in diabetic hearts ($P < 0.01$ D-CoPP vs. D), which is

in close association with the increase in HO-1 activity (Fig. 3).

B-cell leukemia/lymphoma extra long and pAKT expression and the effect of CoPP. The induction of diabetes resulted in no significant changes in B-cell leukemia/lymphoma extra long (Bcl-xL), AKT, and pAKT expression. CoPP administration had no effect on AKT expression; however, there was a significant increase in the expression of pAKT and Bcl-xL in both C-CoPP and D-CoPP animals (Fig. 9). The changes in protein expression of Bcl-xL and pAKT mirrored those seen with HO-1 protein expression.

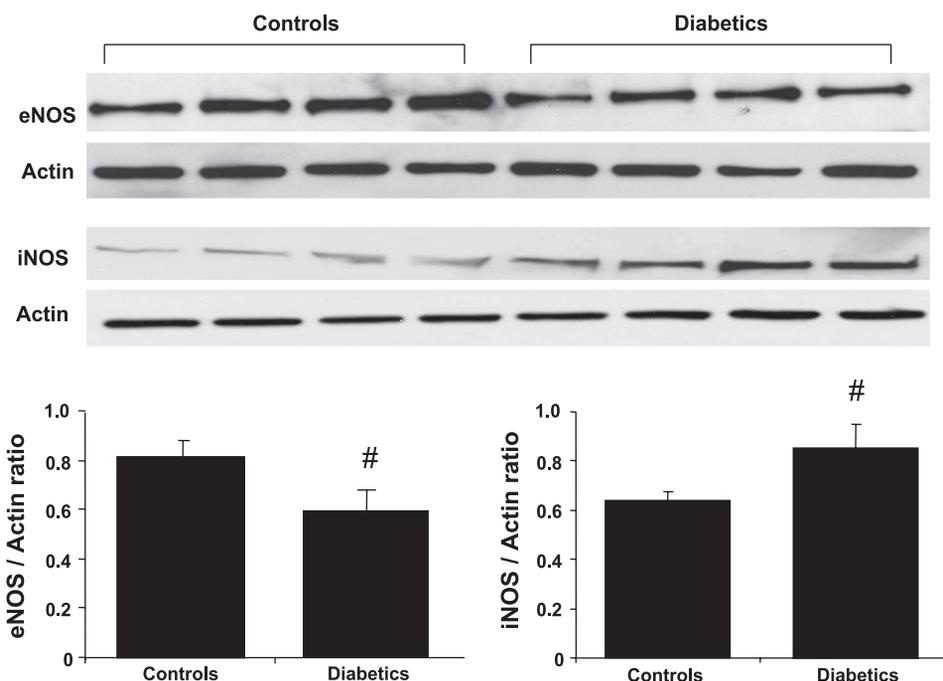


Fig. 5. Western blot analysis of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) protein, and β -actin in hearts after perfusion at transient low pressure from untreated C and D rats. Mean band density was normalized relative to β -actin; $n = 4$; $\#P < 0.05$ D vs. C rats.

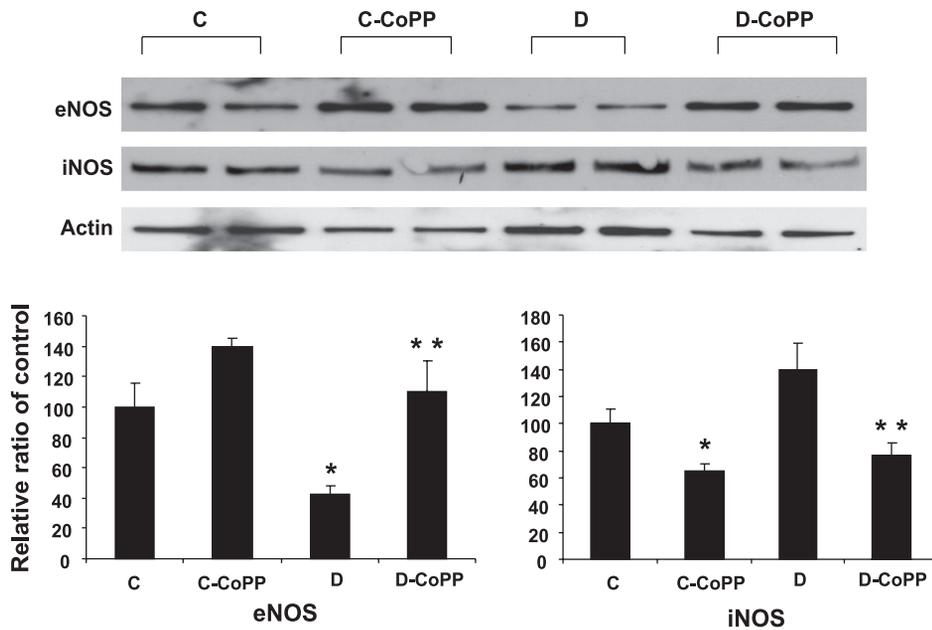


Fig. 6. Western blot analysis of eNOS, iNOS protein, and β -actin in hearts from C and D rats with and without CoPP treatment after perfusion at transient low pressure. Representative immunoblots are shown ($n = 4$). * $P < 0.05$ eNOS D vs. C and iNOS C-CoPP vs. C; ** $P < 0.001$ eNOS D-CoPP vs. D and iNOS D-CoPP vs. D.

DISCUSSION

This is the first study to demonstrate, in hearts isolated from normal and mildly diabetic rats, that paradoxical coronary vasoconstriction, occurring during low pressure ischemia, can be reversed by HO-1 induction through modulation of NOS isoforms and a decrease in superoxide (O_2^-) production. In addition, serum levels of the adipokine adiponectin increased as a result of HO-1 induction and increased HO activity.

We used coronary hypoperfusion in isolated rat hearts to reproduce a microvascular vasoconstrictive response similar to what has been documented by Kusmic et al. (26) in the isolated mouse heart. In this study, we also clearly demonstrated the occurrence of myocardial ischemia during low perfusion pressure, as evidenced by the prompt and consistent release of lactate. Thus, in spite of the documented ischemia, coronary microvascular tone increased, challenging the traditional view of maximal vasodilation during restricted flow and justifying the term “paradoxical vaso-

constriction” to define such a phenomenon. This is the expression of an active increase in vascular tone as previously shown by the prompt reversal via papaverine or adenosine and by manipulation with eNOS inhibitors, endothelin blockade, and antioxidants (26).

In the present study, paradoxical vasoconstriction was also observed, for the first time, in hearts isolated from mildly diabetic rats, which showed higher CR values compared with controls under all conditions studied and when absolute values were considered (Table 1). The changes decrease when considered as a percentage; however, this comparison also shows higher CR values in diabetic animals compared with controls. As judged by lactate release, no striking differences were apparent between control and diabetic animals in the severity of ischemia induced by perfusion at low pressure.

A major finding of the study is that pharmacological induction of HO-1 expression and increased HO activity by CoPP completely prevented paradoxical vasoconstriction in both control and diabetic rats and decreased lactate release,

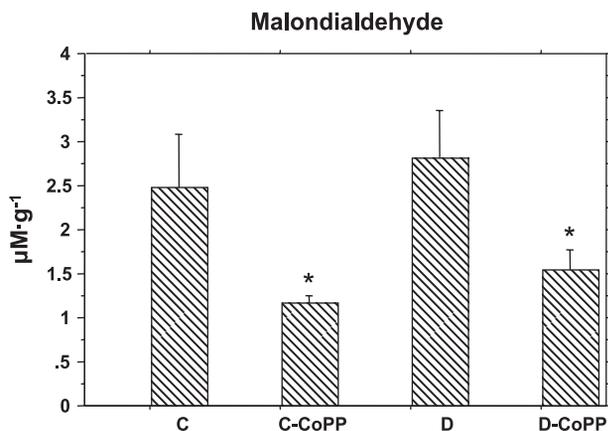


Fig. 7. Malondialdehyde tissue levels measured after transient low perfusion pressure in hearts from C and D rats with and without CoPP treatment. * $P < 0.01$ CoPP treated groups vs. corresponding untreated groups.

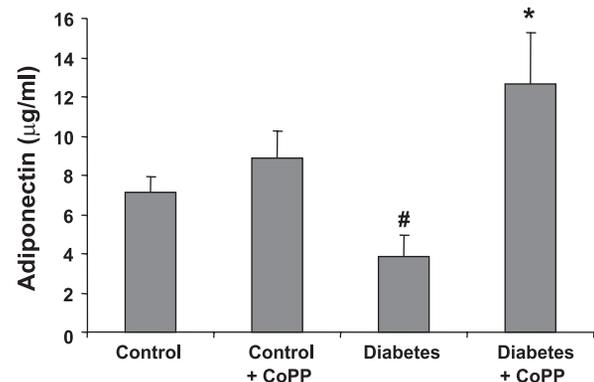
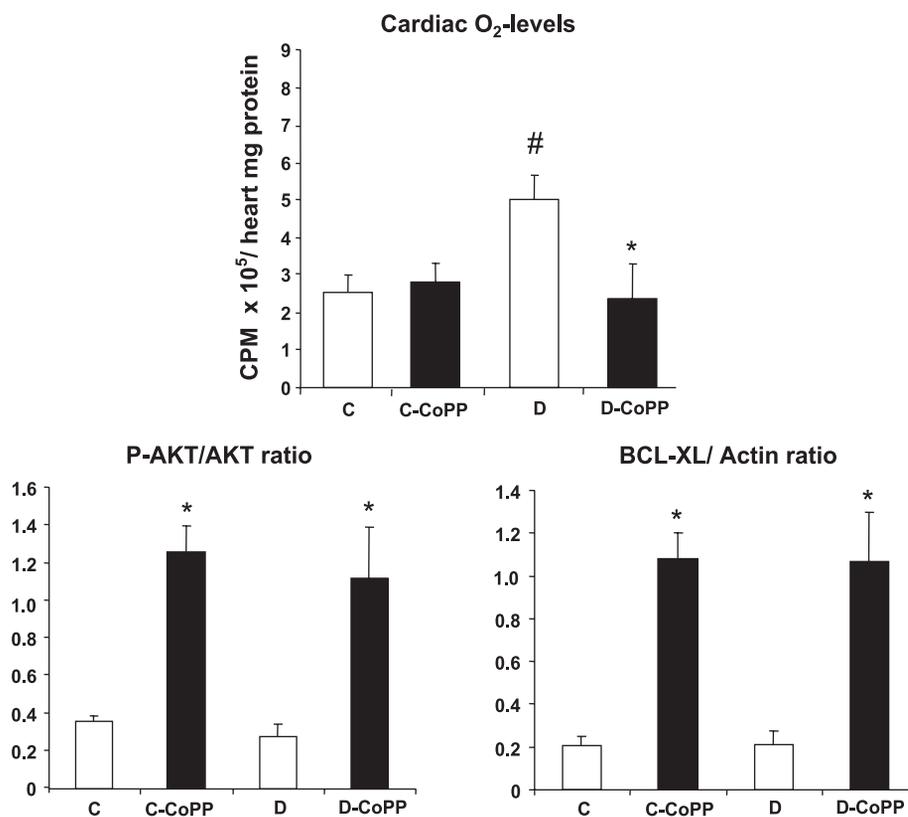


Fig. 8. Measurement of serum adiponectin levels in C and D animals before and after CoPP treatment. Adiponectin was determined as described in MATERIALS AND METHODS. Results are mean \pm SE; $n = 4$. # $P < 0.05$ D vs. C rats; * $P < 0.01$ D-CoPP vs. untreated D animals.

Fig. 9. Cardiac O_2^- levels (*top*) and expression of p-Akt and B-cell leukemia/lymphoma extra long (Bcl-xL; *bottom*) in C and D rats with and without CoPP treatment after perfusion at transient low pressure. Quantitative densitometry analyses of phospho activator protein kinase levels (p-AKT)-to-total AKT ratio and of Bcl-xL/actin ratio are shown; cpm, counts/min. # $P < 0.05$ D vs. C rats; * $P < 0.01$ CoPP-treated groups vs. corresponding untreated groups.



an effect that was particularly evident in the diabetic group. After CoPP administration, there is a clear reduction in both MDA and superoxide in diabetic hearts; conversely, MDA is reduced but superoxide is not reduced in the normal hearts. This may be due to several factors. For example, NADPH oxidase, which is a critical source of superoxide, may be present at low levels in the normal heart and CoPP may not further decrease superoxide production in control animals, as it is already very low. In diabetes, in contrast to normal groups, there is activation of NADPH oxidase and mitochondrial production of ROS, which is prevented by CoPP. Additionally, vasoconstriction may not come from superoxide only but from the inflammatory molecules transforming growth factor and angiotensin II under hyperglycemic conditions (for review see Ref. 1). Regardless, the CoPP-mediated increase in HO-1 supports the view that paradoxical vasoconstriction modulates the severity of ischemia and that its pharmacological control might be beneficial to the preservation of myocardial metabolic homeostasis.

Several potential molecular mechanisms involved in the striking effect of HO-1 induction on coronary microvascular response to low perfusion pressure were also explored. After the transient reduction of perfusion pressure, hearts from mildly diabetic rats showed, compared with controls, lower expression of both HO-1 and eNOS, higher expression of iNOS, and higher cardiac O_2^- levels, similar to those previously reported in hearts from severely diabetic animals not submitted to acute changes in coronary hemodynamics (24, 53). The CoPP-mediated induction of HO-1 significantly increased eNOS and decreased iNOS protein levels in both control and diabetic rats. In diabetic rats, CoPP increased HO-1 expression, HO activity, serum adiponectin levels, eNOS,

pAKT, and the antiapoptotic protein Bcl-xL compared with controls, and these changes correlated with a significant decrease in O_2^- levels. Taken together these results suggest that HO-1 involvement in the regulation of coronary microvascular tone is mediated by the modulation of NOS isoforms and control of oxidative stress.

There are numerous possible mechanisms by which the HO-1/HO-2 pathway may improve vascular function. Nishikawa et al. (38) have suggested that HO-2 activation may occur in ischemic hearts in dogs and that inhibition of the HO system by SnMP inhibits vasodilation during ischemia in the presence of NO and COX inhibitors. CO-releasing molecules, i.e., CORM-3, prevent ischemic damage (16). CO also protects isolated hearts against ischemia-reperfusion (11). Ischemic and nonischemic cardiomyopathy patients exhaled CO levels lower than those of healthy controls at rest and after exercise. Guo et al. (16) have presented evidence that CO, one of the products of HO-1-derived activity, was able to reduce infarct size. Di et al. (13) and others (26a) have shown that increased CO levels have a beneficial effect on vascular relaxation and prevent endothelial cell death. In addition, Kruger et al. (24) and Turkseven et al. (53) have shown that upregulation of HO-1 may improve vascular function by increasing superoxide dismutase and catalase activity and reducing tissue O_2^- levels. Hyperglycemia and ischemia enhance endothelial O_2^- production, leading to increased vascular formation of the NO/superoxide reaction product peroxynitrite (12, 22, 34). Peroxynitrite oxidizes the active NOS cofactor tetrahydrobiopterin to cofactor inactive molecules, such as dihydrobiopterin (32). This uncouples the enzyme, which then preferentially increases O_2^- production over NO production (32, 58). The HO-1 gene expression-mediated decrease in O_2^- may, in turn, lead to

protecting eNOS from uncoupling. Bilirubin, another heme degradation product, may have vascular protective effects as well. Numerous studies indicate that a higher serum bilirubin level is related to a decrease in lipid peroxidation and is associated with a decrease in the risk for coronary artery disease in humans (49, 54). The benefits of bilirubin as an antioxidant and cytoprotective agent have been reviewed (1).

In the present study, CoPP administration to diabetic rats resulted in a significant increase in serum adiponectin levels, which was blocked by the administration of the HO inhibitor SnMP. This increase in adiponectin may precondition the heart to be more resistant to oxidative stress. The existence of an adiponectin-HO-1 axis may explain the direct effect of adiponectin alone on the observed increase in pAKT. Serine/threonine protein kinase AKT/PKB is recognized as a key regulator of cell endothelial cell survival, growth, and migration (15, 33, 47). More recently, activation of pAKT was reported to promote the survival/proliferation of endothelial cells (50, 64). Kumada et al. (25) and Ouchi et al. (41, 42), in a series of elegant studies, have reported that adiponectin is critical for endothelial cell survival and function. A decrease in circulating adiponectin levels was reported in patients with metabolic syndrome (6) and was linked to higher mortality in ischemic congestive heart failure patients (52). Adiponectin levels increased after rosiglitazone treatment in nondiabetic individuals with metabolic syndrome with a resultant improvement of endothelial function (4, 63). Polymorphisms at the adiponectin locus have been reported to be predictors of circulating adiponectin levels, insulin sensitivity, and atherosclerosis (31). Adiponectin has been reported to protect against apoptosis via increased expression of superoxide dismutase and catalase and regulation of Bcl-2 and Bax expression (20).

Thus, the effect of CoPP treatment increasing adiponectin levels in diabetic animals, which we report here, suggests the possible beneficial role of adiponectin in diabetes. This conclusion stems from the fact that the HO-1-mediated increase in adiponectin provides the heart with a tolerance to oxidants, thus enabling the isolated heart to be resistant to stress situations. It is therefore possible that, in addition to increased levels of bilirubin (anti-oxidant) and CO (antiapoptotic), compounds regarded as cytoprotective, observed after HO-1 induction, increased levels of adiponectin also function to protect the cell against toxic insults. This is the first study to demonstrate that increased levels of adiponectin are associated with the induction of HO-1 and, as such, offer a new therapeutic approach to the treatment of diabetes.

The present study establishes the direct action of HO-1 in enhancing eNOS while downregulating iNOS cardiac protein levels in this model of low-pressure ischemia. These effects were closely associated with an improvement of coronary microvascular response to a transient reduction in perfusion pressure, as shown by the abolition of "paradoxical vasoconstriction." These findings highlight the role of HO-1 in modulating, via multiple mechanisms, coronary vascular tone during ischemia. In circumstances associated with a marked decrease in antioxidant capabilities, such as in ischemia-reperfusion or diabetes (for review see Ref. 1), increased levels of O_2^- scavenge NO. As a result, NO bioavailability in the vascular system decreases and peroxynitrite formation increases, potentially leading to reduced vasodilating properties.

High levels of HO-1 in blood vessels and in the myocardium may, therefore, antagonize vasoconstriction by modulating NOS isoforms expression in favor of eNOS vs. iNOS and by diminishing extracellular O_2^- through an enhancement of antioxidant systems. In our study, the overall effects of CoPP in counteracting myocardial oxidative stress were particularly evident in diabetic animals where both myocardial O_2^- and MDA levels were significantly reduced after treatment. In control animals, CoPP still reduced MDA levels but did not change O_2^- levels. We do not have an explanation for this discrepancy. It is possible that detectable changes in O_2^- require large differences, as in diabetes, while changes in MDA (an integrated measure of oxidative stress) are more sensible. Additionally, vasoconstriction may not come from superoxide but from the inflammatory molecules transforming growth factor and angiotensin II under hyperglycemic conditions, which is not increased in control animals.

The role of Bcl-xL and pAKT may also be crucial in mediating the observed favorable effect of CoPP treatment in this model of low-pressure ischemia. Fujio et al. (14) and Matsui (29) have shown that AKT signaling is essential to protecting against apoptosis, limiting infarct size induced by ischemia-reperfusion injury, and promoting contractility and glucose uptake (14, 29). Additionally, an increase in pAKT may cause the acceleration of glucose uptake via glucose transporter-4 translocation to plasma membranes (56), subsequently decreasing the rate of glucose oxidation and attenuating cardiac damage (23, 56). These results are in agreement with the present data in which CoPP causes a significant increase in pAKT and Bcl-xL levels, mirroring a marked reduction in the myocardial metabolic effects of low pressure induced ischemia. Finally, for the first time, HO-1 induction was associated with a parallel increase in the serum levels of the antidiabetic, anti-inflammatory, and antiathrogenic adipocytokine adiponectin. These findings suggest that HO-1-adiponectin regulatory axis serves to define that some of the key mechanisms involved in the maintenance of microvascular tone and to offer a possible approach as to how these mechanisms might be therapeutically manipulated.

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