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Chronic CO levels has a beneficial effect on vascular relaxation in diabetes ☆

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Abstract

Heme oxygenase (HO) has been shown to provide cytoprotection to the vascular system in diabetes. Isolated femoral arteries from diabetic rats treated with cobalt protoporphyrin (CoPP) exhibited increased relaxation to acetylcholine (ACh), which was markedly decreased in control diabetic rats. In control rats treated with either CoPP or with CO releasing molecules-3 (CORM-3), but not in rats treated with biliverdin, we observed an increased dilatory response to ACh. The inhibition of guanylyl-cyclase (GC) with 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) caused a contractile response to ACh in control rats and in biliverdin-treated rats, while in rats treated with CoPP and CORM-3, the ACh dilatory effect was only decreased. Moreover, the inhibition of HO with chromium mesoporphyrin did not change the response to ACh in rats treated with CoPP, suggesting that the improving effect of overproduction of CO on vascular reactivity is due to a decrease in iNOS and the beneficial effect on vascular function.
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Keywords: Heme oxygenase; CO-donors; Endothelial impairment; Bilirubin; Oxidative stress; Diabetes; Vascular reactivity

Endothelium-dependent vascular relaxation is predominantly mediated by nitric oxide (NO). Increased generation of reactive oxygen species (ROS) reduces NO bioavailability. Under oxidative conditions, superoxide anion (O_2^-) reacts with NO to generate peroxynitrite ($ONOO^-$), which is a potent oxidant interacting with biological regulatory systems, resulting in the attenuation of NO bioavailability [1]. This promotes vasoconstriction, platelet aggregation, and induction of neutrophil adhesion to the endothelium, which causes endothelial dysfunction. Furthermore, it has been shown that $ONOO^-$ oxidizes BH_4, resulting in endothelial nitric oxide synthase (eNOS) uncoupling and a decrease in NO bioavailability, which may contribute to the observed decrease in heme oxygenase (HO)-1 induction in diabetes.

The HO system has a pivotal role in protecting the endothelium from oxidative stress [2,3]. HO-1 and HO-2, two isoforms of HO, are viewed as having a major role in the formation of carbon monoxide (CO), and bilirubin and heme breakdown. The fact that HO-1 is strongly induced by its substrate heme and by oxidant stress in conjunction with the ability of HO-1 to guard against oxidative insult [3,4] supports the notion that the heme-HO system has a cytoprotective effect against oxidative stress injury [2–6]. The antioxidant effects of HO-1 arise from its capacity to increase reduced glutathione levels [7], to degrade heme,

☆ *Abbreviations:* ACh, acetylcholine; CO, carbon monoxide; CoPP, cobalt protoporphyrin; CORM-3, CO releasing molecules-3; CrMP, chromium mesoporphyrin; eNOS, endothelial nitric oxide synthase; GC, guanylyl-cyclase; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; $ONOO^-$, peroxynitrite; PE, phenylephrine; STZ, streptozotocin.

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and to elaborate biliverdin/bilirubin, which have potent antioxidant properties [8]. CO is a vasodilator that has been shown to improve endothelial function and to play an important role in the regulation of the basal- and constrictor-induced vascular tone [9,10]. The vascular interactions between CO and NO are complex [11]. Hara et al. and others [12–14] have shown that endogenous NO production regulates HO-1 in cells exposed to inflammatory cytokines, suggesting that these systems (NO and CO) may coexist under different control mechanisms.

The major objective of this study was to determine the effects of HO-1 on vascular function in diabetic rats. The administration of cobalt protoporphyrin (CoPP), an inducer of HO-1 protein and activity, increased eNOS protein expression while decreasing iNOS levels and improved vascular responses to acetylcholine (ACh) in femoral arteries. We also investigated which product of heme degradation by HO-1 (CO or biliverdin) was involved in the regulation of vascular reactivity. Our results demonstrate that CO is the main HO-1 derived regulator of vascular tone and that its chronic overproduction has a beneficial effect on vascular function and/or structure, thus improving vascular reactivity. This mechanism may explain the protective role of HO-1 overexpression in vascular function.

Materials and methods

Animals: induction of diabetes and treatments. Male Sprague-Dawley (SD) rats, 180–220 g, were divided into six groups: control, streptozotocin (STZ), CoPP, STZ plus CoPP, CORM-3, and biliverdin. Diabetes was induced by a single injection of STZ (65 mg/kg) via the tail vein. STZ was dissolved in 0.05 mol/l citrate buffer (pH 4.5). Age-matched control rats were injected with an equal volume of vehicle (sodium citrate buffer). Rats administered STZ developed hyperglycemia within three days, with glucose levels reaching 475 ± 65 mg/dl. To alleviate extreme hyperglycemia and to maintain normal body weight, insulin (Neutral Protamine Hagedon (NPH) 1-2 U/day/100 g bw) was administered three times a week to maintain glucose levels between 250 and 300 mg/dl in all diabetic rats [15]. Glucose determinations (Lifescan, Milpitas, CA) were assessed in blood obtained from the tail vein via capillary tube. All specimens were obtained after an overnight fast. CoPP (0.5 mg/100 g bw) was given subcutaneously (s.c.) once a week for six weeks, in diabetic rats starting from the day after diabetes developed. Biliverdin (100 μg/100 g bw) and CORM-3 (2 mg/100 g bw) were given subcutaneously (s.c.) every day for six weeks. All experiments were approved by the Institutional Animal Care and Use Committee and conducted under the guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health.

Tissue preparation and HO activity. Frozen thoracic aorta segments 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% tergitol, pH 7.5). Homogenates were centrifuged at 27,000*g* for 10 min at 4 °C. The supernatant was isolated and protein levels were assayed (Bradford method). The supernatant was used for measurements of HO-1/HO-2 and eNOS/iNOS protein expression and HO activity [2].

Western blot analysis. Protein levels were visualized by immunoblotting with antibodies against rat HO-1 and HO-2 (Stressgen Biotechnologies, Victoria, BC), and eNOS and iNOS (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 20 μg of lysate supernatant was separated by 12% SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed as has been previously described [2]. Chemiluminescence detection was performed with the Amersham ECL detection kit, according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Preparation of tissues for immunohistochemistry. The fixed vessels were prepared for immunohistochemistry as previously described. Briefly, vessels were fixed in 4% paraformaldehyde, cut into small pieces, processed according to standard procedures, embedded in paraffin, and sectioned at 5 μm by microtome. Serial sections were then stained by hematoxylin–eosin and by HO-1, eNOS, and iNOS immunohistochemistry. Immunostaining was carried out using anti-HO-1, eNOS, and iNOS polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Serial sections were immersed in 3% hydrogen peroxide and diluted in methanol for 30 min to block endogenous peroxidase activity. The sections were incubated with goat serum (diluted 1:5) for 40 min and then successively with rabbit polyclonal antibodies for 2 h at the following dilution: anti-HO-1 was diluted 1/10 whereas anti eNOS and iNOS were diluted 1/100. They were then washed in Tris-buffered saline (0.1 M, pH 7.4), incubated with biotinylated goat anti-rabbit immunoglobulin and avidin-biotin-horseradish peroxidase complex, and stained by immersing the slides in a solution of 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide. All slides were counterstained with hematoxylin, dehydrated, and mounted. Negative controls for nonspecific binding, incubated with a secondary antibody only or Tris-buffered saline, were processed and revealed no signal.

Evaluation of iNOS And eNOS immunostaining. We evaluated immunostaining for HO-1, eNOS, and iNOS in the vascular tissues obtained from various groups. Staining intensity was computed as integrated optical density (IOD) and measured in seven samples for each experimental group. Digitally fixed images of the slices at 40× magnification were analyzed using an optical microscope (Olympus, Germany) equipped with an image analyzer (Image Pro Plus, Immaginie Computer, MiIan, Italy). Since the intima is a monolayer of cells, we used two different methods to measure IOD in the three different areas of the aorta. Intima IOD was measured cell by cell (assuming that each cell had a comparable area) for a total of 100 cells for each experimental group. In this measurement, the boundary of each individual cell was manually traced. The IOD was calculated for arbitrary areas, measuring five fields with the same area in the media or adventitia for each sample. The data were pooled to represent a mean value and ANOVA was applied to compare data from different experimental groups.

Measurement of force generation in vascular segments. Femoral arteries were isolated from the rats and connective tissue was removed. Rings measuring 2 mm in length were made from the arteries and incubated in individually thermostated (37 °C) DMT myograph baths (DMT, Atlanta, GA) with a passive tension of 1 g for 1 h in Krebs bicarbonate buffer (pH 7.4) containing the following (in mM): 118 NaCl, 4.7 KCl, 1.5 $CaCl_2$, 25 $NaHCO_3$, 1.1 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose, gassed with 21% O_2-5% CO_2 (balance N_2). Force was recorded from force displacement transducers via AD Instrument's Powerlab system, running Chart 5 software. After 1 h of equilibration, we calculated, through a cumulative curve, the concentration of phenylephrine (PE) that determined 70% contraction. Contracted vessels were then given increasing doses of ACh (1 nM–10 μM). The response to ACh was assessed also after selective inhibition of GC with ODQ 10 μM and after ODQ plus CrMP 5 μM.

Statistical analyses. The data are presented as means ± standard error (SE) for the number of experiments. Statistical significance ($p < 0.05$) between the experimental groups was determined by the Fisher method of analysis of multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor analysis of variance (ANOVA) for multiple groups or unpaired *t* test for two groups.

Results

Effect of diabetes on HO-1 and HO-2 expression and activity

To accurately determine the effect of hyperglycemia on HO-1 and HO-2, glucose levels were adjusted to be between 250 and 300 mg/dl by insulin treatment. Under

these conditions, diabetic rats did not lose weight during the period of the study, i.e., 6 weeks. Hyperglycemia did not stimulate HO-1 protein expression compared to control rats (Fig. 1). HO activity was significantly decreased in diabetic rats (0.59 ± 0.03 nmol/60 min/mg) compared to control rats (0.81 ± 0.03 nmol/60 min/mg) (Table 1). CoPP administration for 6 weeks caused an increase in HO-1 protein (Fig. 1) and an increase in HO activity (3.58 ± 0.15 nmol bilirubin/60 min/mg protein in aorta) (Table 1). No difference in HO-2 expression was observed between nondiabetic controls, untreated diabetic or CoPP-treated diabetic rats (Fig. 1).

Table 1
HO activity in control, diabetic, and diabetic rats administered CoPP

Treatment	HO Activity (nmol bilirubin/mg protein/h)
Control	0.81 ± 0.03
STZ	$0.59 \pm 0.03^{*}$
STZ + CoPP	$3.58 \pm 0.15^{**}$

HO activity was determined 24 h after last CoPP injection. Results are means ± SE, $n = 4$.
* $p < 0.05$ vs. control vehicle treated rats.
** $p < 0.001$ vs. diabetic rats.

HO-1 immunohistochemistry in aortic sections

In control animals (Fig. 2A, panel 1), consistent with Western blot analysis, immunohistochemistry staining showed that the HO-1 expression in diabetic rats was weak-to-moderate similar to the control levels. (Fig. 2A, panel 2 and Fig. 2, panel 3). CoPP administration to control and diabetic animals increased HO-1 immunoreactivity in the intima and in media of control (Fig. 2A, panel 3) and STZ rats (Fig. 2A, panel 4). The staining intensity of HO-1 was computed as integrated optical density (IOD) and measured in seven samples for each experimental group. As seen in Fig. 2B, HO-1 immunostaining did not increase with diabetes. In contrast, IOD analysis showed that CoPP-mediated increase in HO activity also increased HO-1 proteins in control and STZ-treated rats (Fig. 2B).

Effect of CoPP on responses to ACh (1 nM–10 μM) in femoral artery rings from diabetic rats

Diabetes is known to increase oxidant stress. We have previously observed an increase in vascular O_2^- [16], which has been suggested to decrease vascular reactivity to ACh. As expected, diabetic rats showed a decrease in vascular relaxation of the femoral artery to ACh (1 μM, $75.8 \pm 3.4\%$ in control vs. $30.6 \pm 8.2\%$ in STZ) ($p < 0.05$), which was reversed by inducing HO-1 with CoPP ($67.7 \pm 1.7\%$ in STZ + CoPP) ($p < 0.05$) (Table 2). These data show that the antioxidant enzyme HO-1 reverses vascular impairment in diabetes.

Effect of HO-1 expression on endothelial and inducible NOS expression

Since the vascular reactivity results showed that the upregulation of HO-1 was associated with an increase in vascular relaxation to ACh, we examined whether the increase in HO-1 protein and activity was associated with a modulation of eNOS and iNOS. Western blot analysis of aortas from diabetic rats demonstrated an increase in iNOS compared to control rats (Fig. 3) ($p < 0.05$). While eNOS protein was not significantly decreased in diabetic rats compared to control rats, CoPP administration resulted in a significant increase in eNOS protein ($p < 0.05$) compared to untreated diabetic rats.

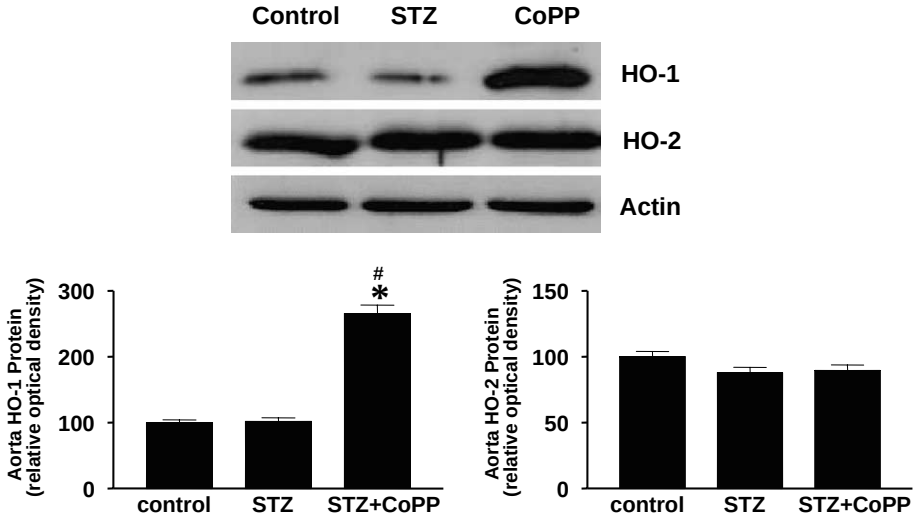

Fig. 1. Western blot and densitometry analysis of HO-1 and HO-2 protein in aorta from control, diabetic, and diabetic rats treated with CoPP once a week for six weeks. Rats were injected with the vehicle or STZ and 6 weeks later aorta were taken for analysis. Mean band density was normalized relative to α-actin ($n = 6$). Data are representative of six separate experiments. Values are means ± SE, $^{*}p < 0.05$ vs. control rats, $^{\#}p < 0.05$ vs. STZ rats.

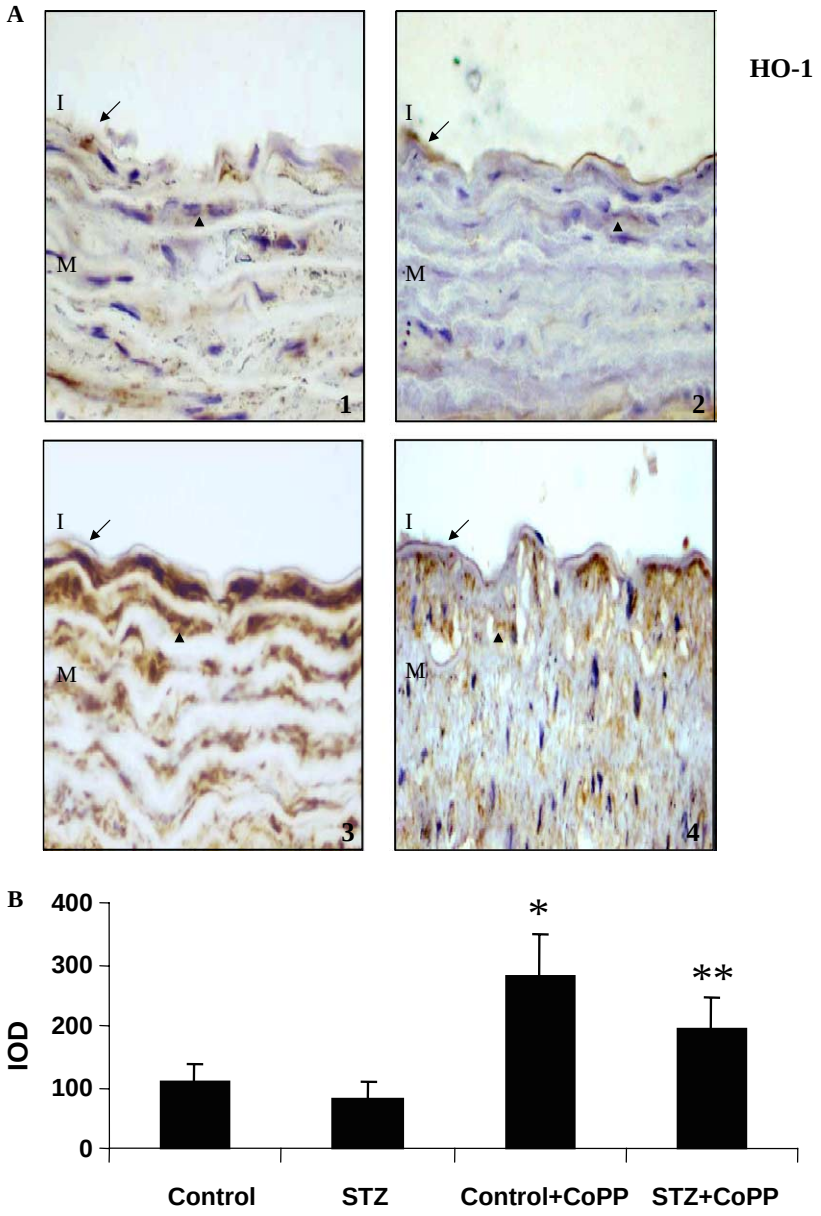

Fig. 2. (A,B) HO-1 immunohistochemistry of rat aorta. (A) (1) controls; (2) STZ; (3) Controls plus CoPP; (4) STZ plus CoPP. I, intima; M, media. Immunopositivity was dark-brown in the cytoplasm of cells. Nuclei were counterstained with hematoxylin. 300×. Arrows indicate intima immunoreactivity. Head arrows indicate media immunoreactivity. (B) Quantitative analysis of HO-1 immunostaining. Integrated optical density was plotted for each experimental group. Data are means ± SD, *$p < 0.05$ vs. controls, **$p < 0.05$ vs. diabetic rats.

Table 2
Dilation to ACh in femoral arteries from control, diabetic, and diabetic rats administered CoPP

	% of dilation with increasing concentrations of ACh (log [ACh] M)			
Treatment	−8	−7	−6	−5
Control	4.8 ± 0.3	45.4 ± 10.0	75.8 ± 3.4	80.4 ± 5.4
STZ	2.0 ± 0.8	20.8 ± 2.7 *	30.6 ± 8.2 *	41.6 ± 2.8 *
STZ + CoPP	2.4 ± 0.3	31.4 ± 10.0	67.7 ± 1.7 **	78.4 ± 5.9 **

Femoral arteries from control ($n = 4$), diabetic ($n = 4$), and diabetic rats administered CoPP ($n = 4$) were contracted with phenylephrine (PE) and then exposed to acetylcholine (ACh) in a dose- dependent manner. Results are means ± SE.

* $p < 0.05$ vs. control vehicle treated rats.

** $p < 0.001$ vs. diabetic rats.

eNOS and iNOS immunohistochemistry in aortic sections

In control animals (Fig. 4A, panel 1), eNOS staining was well evident in the intima, while in the media we found a very weak immunoreactivity. In diabetic rats (Fig. 4A, panel 2), there was a nonsignificant decrease in eNOS immunoreactivity in all the vessel compartments. CoPP administration did not affect significantly the eNOS immunoreactivity in controls (Fig. 4A, panel 3), whereas it increased it in diabetic rats (Fig. 4A, panel 4). The staining intensity of eNOS was computed as described in the methods section and the IOD were measured in each experimental group. As seen in Fig. 4B, eNOS immunostaining

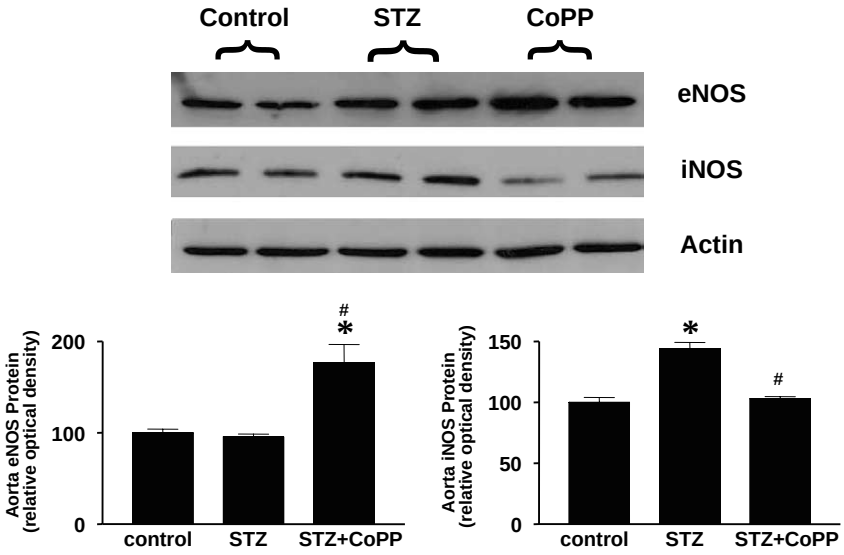

Fig. 3. Western blot and densitometry analysis of iNOS and eNOS in control, diabetic and diabetic rats treated with CoPP. Expression of actin is index of adequacy of sample loading. Data are representative of six separate experiments. Values are means ± SE, $^{*}p < 0.05$ vs. control rats, $^{\#}p < 0.05$ vs. STZ rats.

decreased with diabetes. In contrast, CoPP-mediated increase in HO activity increased eNOS levels (Fig. 4B).

The iNOS immunoreactivity was absent in control vessels (Fig. 5A, panel 1). In contrast, iNOS in diabetic rats (Fig. 5A, panel 2) showed a significant increase in both the intima and media, with a greater increase mainly in the media. CoPP administration in control rats (Fig. 5A, panel 3) did not show any effect compared to control but decreased iNOS immunoreactivity both in the intima and in the media. The staining intensity of eNOS was computed and the IOD were measured in each experimental group. As seen in Fig. 5B, iNOS immunostaining was increased in aorta of diabetic rats. In contrast, CoPP-mediated increase in HO activity decreased iNOS levels (Fig. 5B).

Effect of CoPP, biliverdin, and CORM-3 on responses to ACh

To understand the mechanisms by which HO-1 regulates vascular reactivity, we studied the response to ACh in femoral arteries of control rats and rats treated with the HO-1 inducer CoPP, the CO donor CORM-3, and biliverdin. In rats treated with CoPP, we observed an increased dilatory response to ACh compared to control rats (1 μM, 67.9 ± 7.6% in control vs. 92.9 ± 2.9% in CoPP) ($p < 0.05$). Although not significant, the same finding was present in rats treated with CORM-3 (1 μM, 74.2 ± 10.7%) (Fig. 6). On the other hand, biliverdin decreased the response to ACh (1 μM, 55.8 ± 11.4%) ($p < 0.05$). As the mechanism of action of ACh has been shown to be through activation of sGC and release of cGMP, we assessed how CoPP, biliverdin, and CORM-3 influenced the effect of inhibition of GC with ODQ on the vasodilator response to ACh. In control rats and in rats treated with biliverdin, ODQ determined a contractile response to ACh (1 μM, 12.4 ± 7.0% in control; 8.3 ± 1.7% in biliverdin) while in rats treated with CoPP and CORM-3, ODQ decreased the ACh dilatory effect ($p < 0.05$), but did not abolish it (1 μM, 4.0 ± 2.6% in CoPP; 18.9 ± 11.7% in CORM-3) (Fig. 7). To understand the underlying effect of CoPP on improved dilatory response to ACh, we subsequently inhibited HO with CrMP. CrMP did not change the response to ACh in rats treated with CoPP (1 μM, 4.3 ± 2.0%) (Fig. 8).

Discussion

This study provides the novel observation that activation of the HO system plays a significant role in counteracting diabetes-induced endothelial dysfunction. The observation that CO, either released by HO-1 or by CORM-3, improves relaxation to ACh [17], suggests that induction of HO-1 may improve vascular function through mechanisms other than increasing eNOS and NO bioavailability in type-1 diabetes. We have shown in a previous study that HO-1 serves as a multifaceted enzyme, increasing the antioxidant extracellular superoxide dismutase (EC-SOD) and simultaneously decreasing cellular O_2^- [16]. EC-SOD is considered a regulator of NO bioavailability [18]. In addition, induction of HO-1 using CoPP decreases heme as a result of an increase in heme degradation and the generation of bilirubin and CO. The increase in CO and bilirubin was shown to play an important role in attenuating the vasoconstrictor response and enhancing ACh-mediated relaxation in diabetic rats. We observed that ACh-induced relaxation in femoral arteries was reduced in diabetic rats compared to control rats. Treatment of diabetic rats with CoPP, an inducer of HO-1, significantly improved this reduction (Table 2). Therefore, overexpression of HO-1 reversed the effect of diabetes on

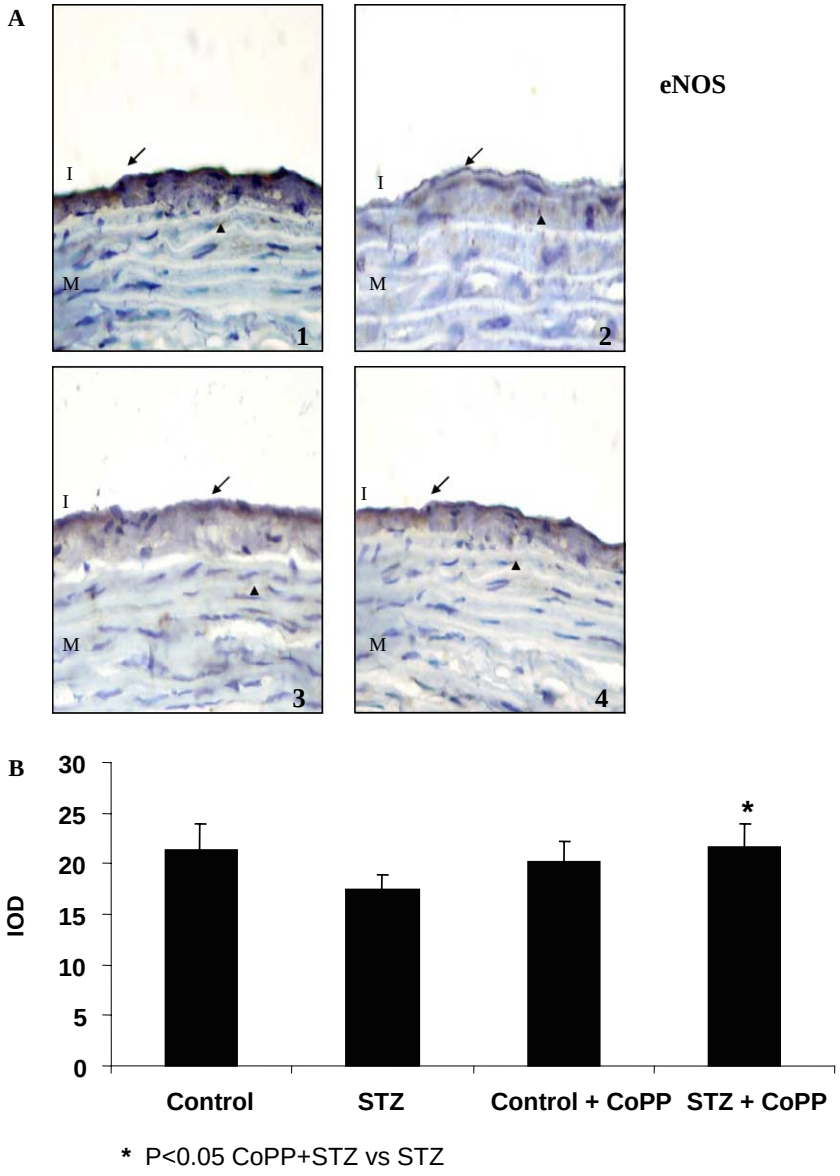

Fig. 4. (A,B) eNOS immunohistochemistry of rat aorta. (A) (1) controls; (2) STZ; (3) controls plus CoPP; (4) STZ plus CoPP. I, intima; M, media. Immunopositivity was a dark brown in the cytoplasm of cells. Nuclei were counterstained with haematoxylin. 300×. Arrows indicate intima immunoreactivity. Head arrows indicate media immunoreactivity. (B) Quantitative analysis of eNOS immunostaining. Integrated optical density was plotted for each experimental group. Data are means ± SD, $*p < 0.05$ vs. controls, controls + CoPP and STZ + CoPP groups.

the vascular wall; this was accompanied by a decrease in iNOS and an increase in eNOS as seen using Western blot and immunohistochemistry analysis (Figs. 3–5). Since most approaches to reduce oxidative stress in human diabetes have met with little success, HO-1 conditioning may represent a new alternative approach.

We then attempted to determine which of the HO-1 products was the main regulator of the vascular reactivity, and in which way it could improve the dilatory response to ACh. The evidence that pretreatment with both CORM-3 and CoPP increased the dilatory response to ACh, while biliverdin slightly decreased it (Fig. 6), suggests that it is CO, and not biliverdin, that is involved in the regulation of vascular reactivity. This is consistent with a previous study [19] showing that biliverdin significantly reduces GC activity and the affinity of the NO-donor, DEA/NO, towards this enzyme. Furthermore, Kranc et al. [20] hypothesized that the oxidative degradation products of bilirubin exert biological activity, leading to vasospasm via an alkylation process, affecting cellular phosphatases or kinases.

Inhibition of GC in control rats and in rats treated with biliverdin caused a contractile response to ACh, while the dilatory effect was only impaired in rats treated with CoPP and CORM-3 (Fig. 7). Although it has already been shown that ODQ abolishes the dilatory effect of ACh [21], the contractile effect of ACh after the addition of ODQ to the arteries of control and biliverdin-treated rats was surprising.

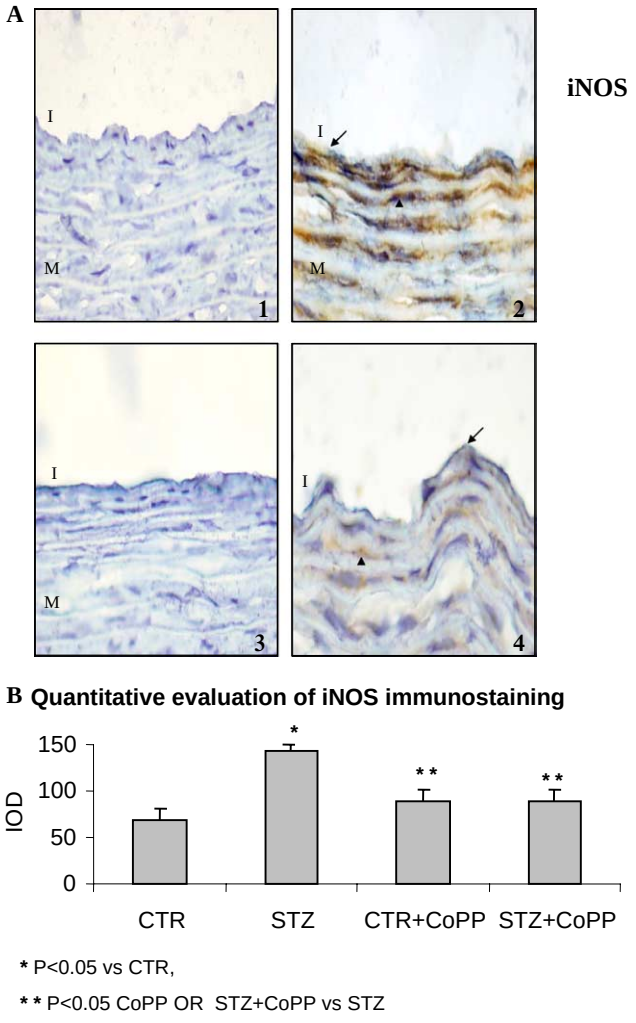

Fig. 5. (A, B) iNOS immunohistochemistry of rat aorta. (A) (1) Controls; (2) STZ; (3) Controls plus CoPP; (4) STZ plus CoPP. I, intima; M, media. Immunopositivity was a dark brown in the cytoplasm of cells. Nuclei were counterstained with haematoxylin. 300×. Arrows indicates intima immunoreactivity. Head arrows indicate media immunoreactivity. (B) Quantitative analysis of iNOS immunostaining. Integrated optical density was plotted for each experimental group. Data are mean ± SD.

A recent study [22] demonstrated that ACh caused an endothelium-dependent contraction in femoral arteries of eNOS−/− mice and wild-type mice after the inhibition of NOS with N(omega)-nitro-L-arginine methyl ester (L-NAME) [22]. The same authors reported that this contraction was inhibited by the cyclooxygenase inhibitor, indomethacin, or by a thromboxane A2/prostaglandin H2 receptor antagonist and was associated with endothelium-dependent generation of thromboxane A2. A previous study, performed in pulmonary microvessel endothelial cells, showed that long-term HO-1 gene overexpression increased cGMP levels but did not affect the level of sGC protein, suggesting that the effect of HO-1 overexpression on cGMP levels was due primarily to the direct effect on sGC activity [23]. In rats treated with CORM-3 and CoPP, the dilatory effect of ACh was still present, although impaired. This could be due to the increased CO

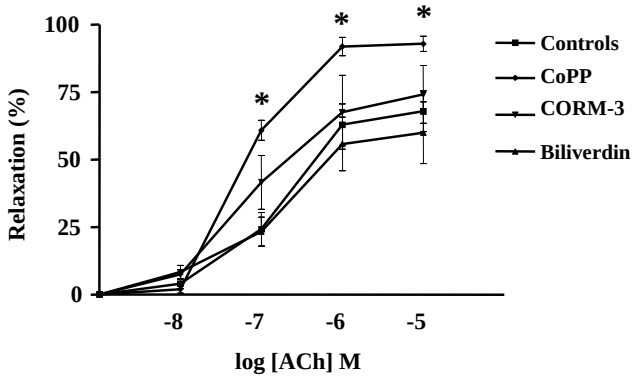

Fig. 6. Femoral arteries from normal ($n = 6$), biliverdin ($n = 6$), CoPP ($n = 6$), and CORM-3 ($n = 6$), treated rats were precontracted with phenylephrine (PE) and then exposed to acetylcholine (ACh) in a dose-dependent manner. Vessels from CoPP and CORM-3 animals demonstrated an increased response compared to control rats, while biliverdin rats showed a smaller response to ACh. Results are means ± SE, $*p < 0.05$ vs. control rats.

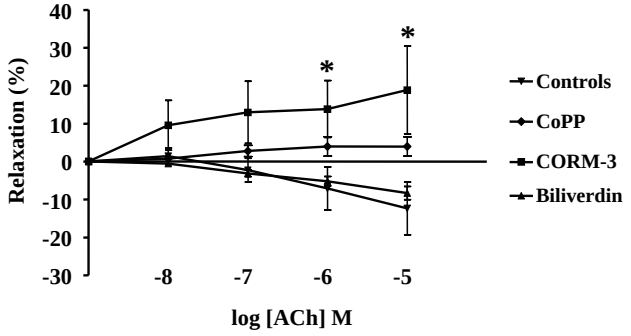

Fig. 7. The cumulative curve to acetylcholine (ACh) was assessed after addition of ODQ (10 μM) in the chambers. In control rats and in rats treated with biliverdin, ODQ caused a contractile response to ACh while in rats pretreated with CoPP and CORM-3, ODQ decreased the ACh dilatory effect, but did not abolish it. Results are means ± SE, $*p < 0.05$ vs. normal rats.

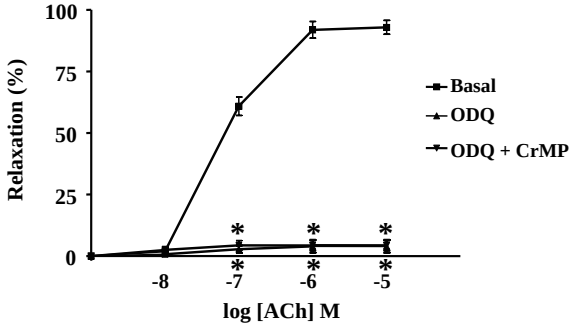

Fig. 8. In femoral arteries from CoPP-pretreated rats, we analyzed the response to acetylcholine (ACh) after the addition of chromium mesoporphyrin (CrMP; 5 μM). Comparing to the curve of dilation to ACh obtained after the incubation of ODQ alone, the addition of CrMP in the chambers did not alter the response to ACh. Results are means ± SE, $*p < 0.05$ vs. basal conditions.

bioavailability in these two groups. CO overproduction may balance the effect of ACh on TXA2 production, acting on K^+ channels, or defy the inhibitory effect of ODQ on cGMP. Nevertheless, in rats treated with CoPP, inhibition of HO with CrMP did not modify the dilating effect of ACh (Fig. 8). This may mean that the improving effect of overproduction of CO on vascular reactivity was due to a chronic effect on vascular function and/or structure and not to the release of CO by ACh. The same mechanism may explain the protective role of HO-1 overexpression in the vascular function of diabetic rats.

It has been proposed that endothelial dysfunction, leading to imbalances in the release of, or sensitivity to, endothelial-derived vasoconstrictors and vasodilators, may contribute to diabetes-induced vascular complications [24]. Under normal conditions, NO is produced by eNOS; however, during pathological conditions, iNOS is also expressed in the vascular smooth muscle cells (VSM). iNOS induction has been reported in cardiomyocytes from diabetic rats [25] and in platelets from patients with type-1 and type-2 diabetes [26]. iNOS can be induced by cytokines [27] stimulated by advanced glycosylation end products (AGEs) and can release 10 to 50 times more NO than eNOS. This increase in NO, by reacting with oxygen-derived free radicals, leads to the formation of $ONOO^-$, which is responsible for cell damage. HO-1 induction, by decreasing iNOS, may reduce peroxynitrite formed by ROS and thus improve vascular relaxation to ACh. Restoration of vascular function may be related to HO-1-mediated increase in GSH, increase in EC-SOD, and or decrease in cellular heme which is associated with a decrease in O_2^-[7,16,28].

In summary, the results of the present study demonstrate that the upregulation of HO-1 can prevent diabetes-induced vascular dysfunction. This effect is related to an increase in eNOS and a reduction in iNOS expression. Treatment with the HO-1 inducer CoPP restored the vascular response to ACh in diabetic rats. In wild-type rats, treatment with CoPP and CORM-3 increased the dilatory response to ACh, while biliverdin decreased it. In control rats and in rats treated with biliverdin, the inhibition of GC with ODQ determined a contractile response to ACh. In rats treated with CoPP and CORM-3, ODQ decreased the ACh dilatory effect. In rats treated with CoPP, further inhibition of HO with CrMP did not change the response to ACh. Thus, the effect of CO overproduction on improving vascular reactivity in vessels obtained from diabetic rats may be due to decrease in iNOS which has a beneficial effect on vascular function, suggesting a mechanism through which HO-1 overexpression may improve the vascular response in diabetes.

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