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Heme Oxygenase-1 Enhances Renal Mitochondrial Transport Carriers and Cytochrome *c* Oxidase Activity in Experimental Diabetes*

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Up-regulation of heme oxygenase (HO-1) by either cobalt protoporphyrin (CoPP) or human gene transfer improves vascular and renal function by several mechanisms, including increases in antioxidant levels and decreases in reactive oxygen species (ROS) in vascular and renal tissue. The purpose of the present study was to determine the effect of HO-1 overexpression on mitochondrial transporters, cytochrome *c* oxidase, and anti-apoptotic proteins in diabetic rats (streptozotocin, (STZ)-induced type 1 diabetes). Renal mitochondrial carnitine, deoxynucleotide, and ADP/ATP carriers were significantly reduced in diabetic compared with nondiabetic rats ($p < 0.05$). The citrate carrier was not significantly decreased in diabetic tissue. CoPP administration produced a robust increase in carnitine, citrate, deoxynucleotide, dicarboxylate, and ADP/ATP carriers and no significant change in oxoglutarate and aspartate/glutamate carriers. The increase in mitochondrial carriers (MCs) was associated with a significant increase in cytochrome *c* oxidase activity. The administration of tin mesoporphyrin (SnMP), an inhibitor of HO-1 activity, prevented the restoration of MCs in diabetic rats. Human *HO-1* cDNA transfer into diabetic rats increased both HO-1 protein and activity, and restored mitochondrial ADP/ATP and deoxynucleotide carriers. The increase in HO-1 by CoPP administration was associated with a significant increase in the phosphorylation of AKT and levels of BcL-XL proteins. These observations in experimental diabetes suggest that the cytoprotective mechanism of HO-1 against oxidative stress involves an increase in the levels of MCs and anti-apoptotic proteins as well as in cytochrome *c* oxidase activity.

The heme-heme oxygenase (HO),[4] HO-1 and HO-2, isoforms, are viewed as having a major role in the formation of carbon monoxide (CO) and bilirubin, and in heme breakdown (1–3). The fact that HO-1 is strongly induced by its substrate, heme, and by oxidant stress, in conjunction with the robust ability of HO-1 to guard against oxidative insult (4, 5), suggests a countervailing system to oxidative stress injury. HO-1 is a regulator of endothelial cell integrity and oxidative stress (4–6). Up-regulation of HO-1 by pharmacological agents, including cobalt protoporphyrin (CoPP), has been shown to increase superoxide dismutase and to decrease reactive oxygen species (ROS) and NAD(P)H oxidase activity *in vitro* and *in vivo* (7–9). In earlier studies, we, as well as others, have demonstrated that overexpression of the *HO-1* gene in human, rabbit, and rat endothelial cells not only renders the cells resistant to agents that elicit oxidative stress but also enhances cell growth (6) and angiogenesis (10, 11) via HO-1-derived CO (12). More recently, up-regulation of HO-1 has been shown to prevent endothelial cell death and sloughing in diabetic rats (8).

Mitochondrially generated ROS have been well documented in diabetes (13, 14). Hyperglycemia-mediated local formation of ROS is considered to be a major contributing factor to renal and vascular dysfunction (14–20). An increase in ROS contributes to degenerative changes in mitochondrial function (13) and membrane depolarization (21). Mitochondrial ROS production associated with hyperglycemia is increased because of the lack of antioxidants, thus restoration of antioxidant levels is beneficial in decreasing mitochondrial membrane dysfunction (22, 23). Abnormalities in mitochondrial transport, either through a decrease or an increase in one of the carriers, have been shown to cause an increase in superoxide anion (O_2^-) production (24). Furthermore, inhibition of mitochondrial transport has been shown to decrease protein kinase C activity in cell cultures exposed to high glucose (13, 25). Kaplan *et al.* (26) have shown a decrease in the mitochondrial citrate carrier but an increase in both pyruvate and dicarboxylate transporters in streptozotocin-treated rats. In addition, a diabetes-induced decrease in the activity of the ADP/ATP transporter has been attributed to increased levels of long chain acyl-CoA, which is present in diabetic mitochondria. Decreased mitochondrial transport carriers will decrease pH gradient and impair the generation of ATP. Increased levels of the antioxidant proteins, catalase and metallothionine have been shown to restore mitochondrial function in diabetes (13, 18). The renal and vascular cytoprotective effect of HO-1, leading to increased cell survival *in vitro* and *in vivo*, has been previously described (6, 27); however, little is known about the mechanism involved. Mitochondrial transport of important substrates may play a significant role in the mechanism of cytoprotection.

Oxidative stress results in the activation of multiple signaling cascades that ultimately dictate the outcome for cell survival. Certain signals tend to favor the anti-apoptotic pathway, whereas others promote the apoptotic pathway. ERK and AKT are known for their activation in

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[4] The abbreviations used are: HO, heme oxygenase; AAC, ADP/ATP carrier; AGC, aspartate/glutamate carrier; CAC, carnitine/acylcarnitine carrier; CiC, citrate carrier; CoPP, cobalt protoporphyrin; DiC, dicarboxylate carrier; DNC, deoxynucleotide carrier; MC, mitochondrial carrier; NOS, nitric-oxide synthase; OAA, oxaloacetate; OGC, oxoglutarate carrier; PiC, phosphate carrier; ROS, reactive oxygen species; SnMP, tin mesoporphyrin; SOD, superoxide dismutase; STZ, streptozotocin; ERK, extracellular signal-regulated kinase.

response to increased HO-1 or CO generation and may play an important role in preventing apoptosis (28). The effect of *HO-1* gene expression and activity on the activation of AKT and BcL-XL proteins, which may be involved in the enhancement of mitochondrial carrier proteins in diabetes, remains to be determined.

The objectives of this study were to determine the effects of pharmacological and non-pharmacological interventions that either increase or decrease HO-1 on certain MCs and the anti-apoptotic proteins AKT and BcL-XL and pro-apoptotic proteins Bax. Our results demonstrate the restoration of six MCs, *i.e.* carnitine, citrate, phosphate, deoxynucleotide, ATP, and dicarboxylate, as a result of an increase in HO-1 protein and activity caused by CoPP administration to diabetic rats. In addition, specific human *HO-1* gene transfer to diabetic rats resulted in the restoration of MCs including ADP/ATP and dicarboxylate. Taken together, these results suggest that HO-mediated cytoprotection in diabetic rats involves restoration of MCs, a robust increase in the phosphorylation of AKT and an increase in BcL-XL signaling proteins.

EXPERIMENTAL PROCEDURES

Development of Diabetes—Diabetes was induced in Sprague-Dawley (S.D.) rats by a single injection, via the tail vein, of streptozotocin (STZ, 65 mg/kg, pH 4.5). Age-matched control rats were injected with an equal volume of vehicle (0.1 mM sodium citrate buffer, pH 7.8). Rats were divided into four groups: control, STZ, STZ plus CoPP, and STZ plus CoPP and SnMP (9). CoPP (0.5 mg/100 g/bw) were given subcutaneously once a week starting from the day after diabetes developed. SnMP (2 mg/100 g/bw) was given twice a week. Serum glucose levels increased from 128.3 ± 7.2 mg/dl to 491.7 ± 25.6 mg/dl after STZ treatment. Insulin administration (Neutral Protamine Hagedorn (NPH)) (3 times/week) decreased serum glucose to 298.6 ± 11.3, 295.3 ± 8.9, and 294 ± 2.5 mg/dl in animals receiving STZ alone, STZ+CoPP, and STZ+CoPP+SnMP, respectively. Insulin dose was individualized and thus dosages were different between the groups. Under these conditions body weight did not significantly change. The body weight of non-diabetic rats was 412 ± 10.7 g compared with 393 ± 20.7 g in diabetic rats. Similarly, body weights of diabetic rats treated with CoPP or CoPP+SnMP were 436.7 ± 16.1 and 400 ± 2.9 g, respectively, which was not significantly different from the control group. Blood for glucose determinations (Lifescan Inc., Milpitas, CA) was obtained from the tail vein; specimens were obtained after an overnight fast.

HO-1 transgenic rats were generated by retroviral gene transfer of human HO-1 in sense orientation to newborns, as has been previously described (27). Each experiment required nine pregnant S.D. rats to deliver about 80 littermates. Male rats (about 50%) were separated and used for viral delivery. The concentrated retroviruses (3–5 × 10^9 cfu/ml) were prepared as previously described (29, 30) and injected into the newborns twice intraventricularly at day 5 (20 μl) and day 12 (40 μl). Following injection, the rats were allowed to recover and were returned to their cages with the appropriate mothers for continued weaning. Transgenic rats overexpressing HO-1 or empty retroviral vector (LXSN) were kept in a pathogen-free environment. The animals were weaned at 21 days and housed in their own cages. Experiments were conducted in 12–13-week-old male rats (350–375 g body weight). At different time points, rats were taken to measure the expression of human HO-1 protein in various tissues. RT-PCR analysis demonstrated the functional expression of the gene of interest in all tissues, including kidney, aorta, heart, femoral artery, lung, and liver for up to six months (29–32) (data not shown). Hyperglycemia was induced in the transgenic rats as described above.

Animals were sacrificed using pentobarbital, and tissues were immediately excised, frozen in liquid nitrogen and stored at −80 °C. All experiments were approved by the Institutional Animal Care and Use Committee and conducted under the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

Mitochondrial and Microsomal Fraction—Mitochondria fractions were isolated from renal tissues as follows. Renal sections were diced and incubated in cold buffer containing sucrose (250 mM), Tris, pH 7.8 (100 mM), EGTA (1.0 mM), and PMSF (1.0 mM), and protease-phosphatase inhibitor cocktails. Diced tissues were homogenized in 8–10 vol of the same buffer by six strokes of a motor-driven Teflon pestle in a glass rotor homogenizer. The homogenate was centrifuged 10 min at 1000 × *g* to remove unbroken cell and large debris. The supernatant (kidney homogenate) was then centrifuged 10 min at 8000 × *g* to pellet the mitochondria. The supernatant was centrifuged at 40,000 × *g* to pellet the microsomal fraction. The mitochondrial fraction was washed twice by gentle homogenization and centrifuged for 15 min at 8000 × *g* and the final pellet was resuspended in ice-cold Sotarage buffer (Tris-sucrose buffer, pH 7.8) as previously described by our laboratory (33, 34). Expression of MCs and cytochrome oxidase activity were measured. Under these conditions, cytochrome *c* oxidase, a mitochondrial marker, was expressed at about 8–10% in the microsomal fraction. The samples were assayed for protein concentration using a Bio-Rad kit based on the Bradford dye binding procedure. Microsomal fractions were homogenized in 10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% tergitol, pH 7.5. The homogenate was centrifuged at 27,000 × *g* for 10 min at 4 °C. The supernatant was used for assessing HO-1/HO-2, AKT, BcL-XL, Bad protein levels, and HO activity. HO activity was measured as previously described (6).

Western Blot Analysis—Protein levels were visualized by immunoblotting with antibodies against rat HO-1, HO-2 (Stressgen Biotechnologies Corp., Victoria, BC) and with antibodies against human AAC, AGC, CAC, DNC, against bovine OGC and PiC, and against rat CiC and DiC, which were generated in our laboratory. Cytochrome *c* oxidase subunit IV (Invitrogen) and AKT, P38, BcL-2, BcL-XL, and antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). 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 (6). Chemiluminescence detection was performed with the ECL detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Measurement of Cytochrome c Oxidase Activity—Mitochondrial cytochrome *c* oxidase activity was determined with a commercial enzyme assay kit (Sigma/Aldrich Inc.), according to instructions provided by the manufacturer. Briefly, the enzyme assay for cytochrome *c* oxidase is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome *c* caused by its oxidation to ferricytochrome *c* by cytochrome *c* oxidase.

Statistical Analysis—Data are presented as means ± S.E. (S.E.). Statistical significance ($p < 0.05$) between the experimental groups was determined by the Fisher method of analysis for 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 Student's *t* test for two groups.

RESULTS

Effect of HO-1 Inducers and Inhibitors of HO Activity on Mitochondrial Transport Carriers—To investigate the expression of HO in renal tissue of diabetic rats, we measured HO-1/HO-2 protein levels. As seen in Fig. 1*A*, HO-1 protein was not significantly changed in renal tissue of diabetic rats relative to controls. As seen in Fig. 1*A* (*lower panel*) densi-

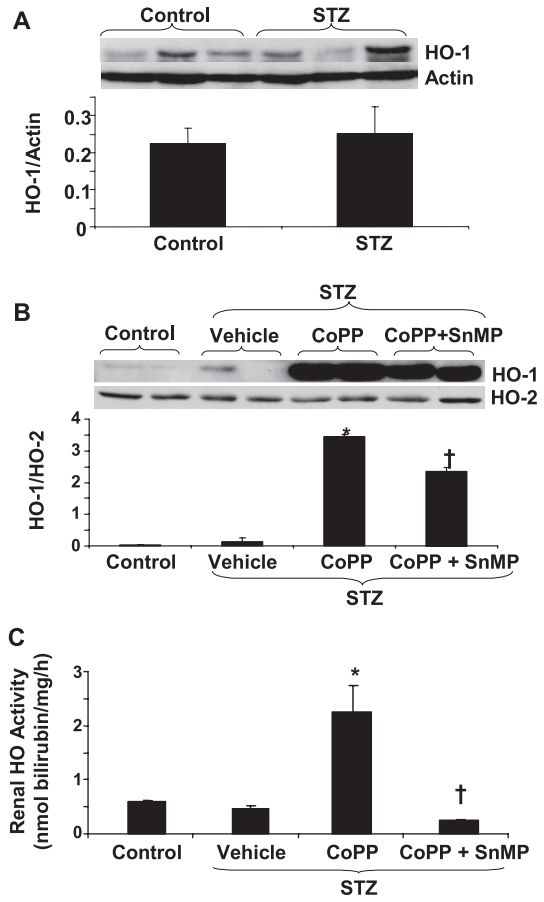

FIGURE 1. *A*, Western blot analysis of HO-1 protein expression in control and in STZ-induced diabetic rats. Rats were injected with STZ (65 mg/kg) and after 6 weeks, kidneys were removed and analyzed for HO-1 and α-actin as described under "Experimental Procedures." Quantitative densitometry evaluation of HO-1 and α-actin ratio in the kidneys was determined. Results are expressed as mean ± S.E.; $n = 6$. *B*, Western blot analysis of HO-1 and HO-2 proteins in rat kidney. Rats were injected with STZ (65 mg/kg) and further treated with either CoPP (0.5 mg/100 g/bw) once a week or in combination with SnMP (2 mg/100 g/bw, twice a week) for 6 weeks. Quantitative densitometry evaluation of HO-1 to HO-2 ratio in the kidneys was determined. Results are expressed as mean ± S.E. Data are representative of six separate experiments. *, $p < 0.001$ *versus* diabetic rats; †, $p < 0.005$ *versus* diabetic rats; and *C*, HO activity in control, diabetic rats, and diabetic rats administered either CoPP or CoPP+SnMP. HO activity was determined 2 days after the last SnMP injection. Results are mean ± S.E., $n = 4$, *, $p < 0.001$ *versus* diabetic rats; †, $p < 0.005$ *versus* diabetic rats.

tometry analysis showed no significant changes in HO-1 protein levels in diabetic rats ($n = 7$). Using the pharmacological agent CoPP, an inducer of HO-1, allowed us to examine the effects of HO-1-associated activity on mitochondrial transporters. As seen in Fig. 1*B*, weekly administration of CoPP for 6 weeks resulted in a robust increase in the amount of renal HO-1 protein compared with control ($p < 0.001$). HO-2 protein did not change with diabetes or following administration of HO-1 inducer. Although, the administration of SnMP, which has been shown to inhibit HO activity in animals and humans, resulted in activation of HO-1 protein expression, as has been observed previously (9), SnMP did not cause a significant change in HO-1 proteins in CoPP-treated rats. SnMP inhibited HO activity to 0.22 ± 0.004 nmol of bilirubin/60 min/mg protein when compared with control, *i.e.* 0.59 ± 0.05 nmol of bilirubin/60 min/mg protein (Fig. 1*C*). The repeated administration of CoPP resulted in a continuous sustained increase in HO activity (Fig. 1*C*) ($p < 0.001$), compared with diabetic rats treated with vehicle. HO activity in CoPP-treated diabetic rats was 2.25 ± 0.48 nmol of bilirubin/60 min/mg protein compared with 0.22 ± 0.04 nmol of bilirubin/60 min/mg protein in diabetic rats ($p < 0.001$).

Because HO-1 overexpression has been shown to increase antioxidant levels (6), presumably by suppressing O_2^-) (reviewed in Abraham and Kappas (4)), we examined if HO-1 can increase the levels of MCs. As shown in Fig. 2, the carnitine/acylcarnitine carrier protein and citrate carriers were decreased in the diabetic kidney compared with controls. As seen from the densitometry analysis, the protein levels of carnitine/acylcarnitine carrier was decreased in diabetic rats ($p < 0.05$). Weekly administration of CoPP for 6 weeks increased the levels of carnitine/acylcarnitine carrier protein ($p < 0.001$ *versus* STZ-vehicle). A similar result was observed on the citrate carrier proteins (Fig. 2*A*, *upper panel*). Densitometry analysis showed that the citrate carrier was significantly increased in diabetic rats treated with CoPP ($p < 0.005$ *versus* STZ-vehicle). The HO-1-mediated increase in both carriers was dependent on HO-1 activity because bi-weekly administration of SnMP for 6 weeks inhibited HO activity and prevented a CoPP-mediated increase in these carriers (Fig. 2, *upper* and *lower panel*).

The suppressive effect of diabetes on MCs involves three other carrier proteins, deoxynucleotide, dicarboxylate, and phosphate, which were decreased compared with control (Fig. 2*B*). As seen in Fig. 2*B* (*lower panel*), the ratio of deoxynucleotide, dicarboxylate and phosphate carrier protein to CYT IV was significantly decreased ($p < 0.03$, $p < 0.01$ and $p < 0.05$, respectively). Administration of CoPP to diabetic rats increased deoxynucleotide, dicarboxylate and phosphate carriers compared with diabetics receiving vehicle solution ($p < 0.04$, $p < 0.01$, and $p < 0.01$, respectively). Neither diabetes nor treatment with CoPP had an effect on oxoglutarate and aspartate/glutamate carrier proteins (Fig. 2, *A* and *B*). Simultaneous administration of SnMP to CoPP-treated diabetic rats decreased phosphate, deoxynucleotide, and dicarboxylate carriers, suggesting that increased expression of HO-1 and HO activity contributed to the activation of MCs. In addition, the ADP/ATP carriers were also decreased in diabetic rats, but were increased by CoPP administration (Fig. 2*C*). As seen in the lower panel of Fig. 2*C*, the ADP/ATP carrier ratio to oxoglutarate was significantly decreased in the diabetic kidney (0.99 ± 0.08 to 0.49 ± 0.93) when compared with control ($p < 0.05$).

Effect of Diabetes and HO-1 on Cytochrome c Oxidase Activity—Because MCs are considered important in regulating electron transport and cytochrome *c* oxidase activity (24), we measured the effect of up-regulation of HO-1 on cytochrome *c* oxidase activity. As seen in Fig. 3, cytochrome *c* oxidase activity was significantly decreased (45%) in kidneys from diabetic animals compared with kidneys from control rats ($p < 0.05$). In contrast, kidneys from diabetic rats treated with CoPP displayed a significant increase in renal cytochrome *c* oxidase activity compared with control and diabetic rats receiving vehicle ($p < 0.0\,5$ and $p < 0.01$, respectively). Cytochrome *c* oxidase activity in diabetic rats was 0.45 ± 0.01 units/mg compared with 1.33 ± 0.02 units/mg protein in diabetic rats preconditioned with the *HO-1* gene. As observed for the MCs, the HO-1-mediated increase in cytochrome *c* oxidase activity was abrogated by SnMP.

Effect of Human HO-1 Gene Transfer on HO Gene Expression—Because HO-1 converts heme to equimolar amounts of CO and bilirubin, we measured HO activity by the formation of bilirubin in vessels obtained from rats transduced with the human *HO-1* gene and compared with it to Ho activity in vessels of rats transduced with empty vector. HO activity in vessels transduced with empty viral vector was 0.59 ± 0.20 nmol of bilirubin formed/mg protein, similar to the control. However, HO activity was increased to 0.99 ± 0.16 nmol of bilirubin formed/mg protein in vessels obtained from rats transduced with the human *HO-1* gene ($p < 0.05$) (Fig. 4*A*). Functional expression of the human *HO-1* gene in control and diabetic rats was determined in rats

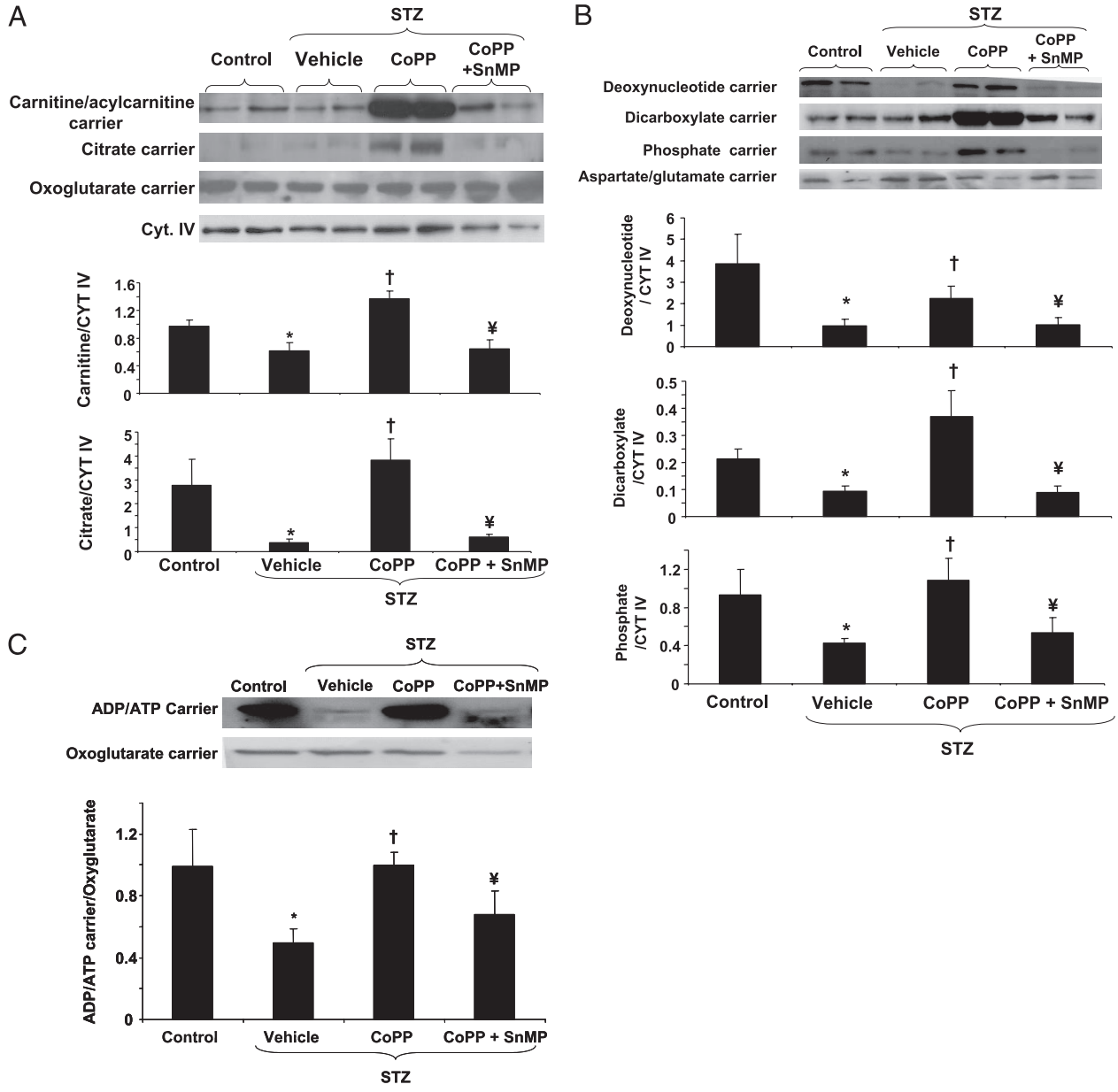

FIGURE 2. *A*, Western blot and densitometry analysis of carnitine/acylcarnitine, citrate, and oxoglutarate carriers from control, diabetic, CoPP-diabetic, and CoPP+SnMP-diabetic rat kidney. Quantitative densitometry evaluation of carnitine/acylcarnitine, citrate ratio to CYTO IV in the kidneys was determined. Each *bar* represents mean ± S.E. of the ratio of each carrier to CYTO IV for 3–4 experiments The significance for each carrier is as follows, carnitine/acylcarnitine, *, $p < 0.02$ *versus* control; †, $p < 0.001$ *versus* diabetic rats; ¥, $p < 0.001$ *versus* STZ-CoPP and citrate; *, $p < 0.04$ *versus* control; †, $p < 0.005$ *versus* diabetic rats; ¥, $p < 0.01$ *versus* STZ-CoPP. *B*, Western blots of deoxynucleotide, dicarboxylate, phosphate, and aspartate/glutamate carriers from control, diabetic, CoPP-diabetic, and CoPP+SnMP-diabetic rat kidney. Quantitative densitometry evaluations of deoxynucleotide, dicarboxylate, dicarboxylate ratio to CYTO IV were determined. The significance for each carrier is as follows, deoxynucleotide. *, $p < 0.03$ *versus* control rats; †, $p < 0.04$ *versus* diabetic rats; ¥, $p < 0.04$ *versus* STZ-CoPP, dicarboxylate, *, $p < 0.01$ *versus* control rats; †, $p < 0.01$ *versus* diabetic rats; ¥, $p < 0.01$ STZ-CoPP and phosphate; *, $p < 0.05$ *versus* control rats; †, $p < 0.01$ CoPP *versus* diabetic rats; ¥, $p < 0.04$ *versus* STZ-CoPP. *C*, Western blots of ADP/ATP and oxoglutarate carriers from control, diabetic, CoPP-diabetic, and CoPP+SnMP-diabetic rat kidneys. Quantitative densitometry evaluation of ADP/ATP and oxoglutarate carrier ratios were determined. Each *bar* represents mean ± S.E.; *, $p < 0.04$ *versus* control rats; †, $p < 0.01$ *versus* diabetic rats; ¥, $p < 0.04$ *versus* STZ-CoPP. The oxoglutarate carrier was not significantly altered by diabetes or by CoPP treatment.

receiving intracardiac delivery of retroviral-mediated human HO-1 vector (27). Western blot analysis of aorta obtained from retroviral human HO-1 rats demonstrated a functional expression of human HO-1 proteins in control and in diabetic rats up to the entire 6-month duration of the experiments (Fig. 4*B*) in both control and diabetic rats. Neither rat HO-1 nor HO-2 was increased.

Effect of Human HO-1 Gene Transfer on MCs—To define the effect of human *HO-1* gene transfer on mitochondrial transporters, we measured mitochondrial ADP/ATP and deoxynucleotide carrier proteins in the aorta of control and diabetic rats. As expected, the induction of diabetes resulted in a significant decrease in ADP/ATP and deoxynucleotide carrier levels (Fig. 5). The selective increase in HO-1 by delivery of human *HO-1* gene increased ADP/ATP and deoxynucleotide carrier proteins (Fig. 5). The ADP/ATP and deoxynucleotide to CYT IV protein ratios were significantly increased in transgenic rats overexpressing HO-1 ($p < 0.005$ and $p < 0.02$, *versus* STZ, respectively). The ADP/ATP and deoxynucleotide to CYT IV protein ratios were not significantly different in rats transduced with the retroviral vector (LXSN *versus* STZ).

Effect of Diabetes and HO-1 on AKT, BcL-XL, and Bax—We examined the phosphorylation of AKT and the levels of anti- and pro-apoptotic proteins, BcL-XL and Bax, respectively, in control, diabetic, and CoPP-treated diabetic rats. As seen in Fig. 6*A*, AKT phosphorylation

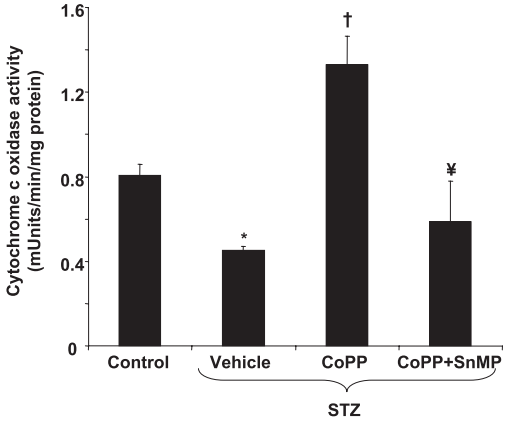

FIGURE 3. **Effect of CoPP and CoPP+SnMP administration on the specific activity of cytochrome *c* oxidase in kidneys of diabetic rats.** Results are expressed as the mean ± S.E. of three different experiments, each performed in duplicate. Activity is expressed in m units/min/mg protein where 1 unit is the amount of enzyme required to oxidize 1 μM ferrocytochrome c per min at pH 7.0 at 25 °C. *, $p < 0.05$ *versus* control rats,; †, $p < 0.05$ *versus* diabetic rats; ¥, $p < 0.05$ *versus* STZ-CoPP.

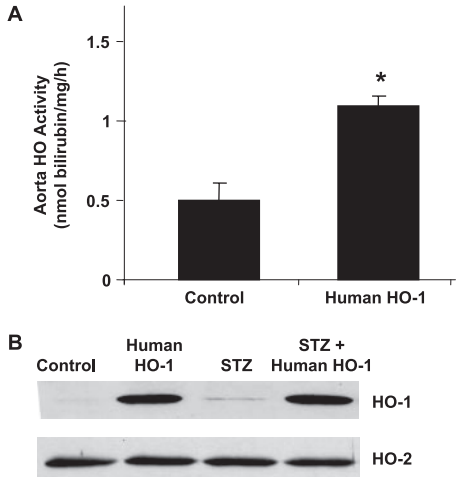

FIGURE 4. *A*, Western blot analysis of HO-1 and HO-2 in aorta of control and diabetic rats transduced with human HO-1: control rats, rats transduced with human HO-1, diabetic rats, rats transduced with human HO-1 and then administered STZ to develop diabetes as described under "Experimental Procedures." Representative immunoblots ($n = 3$) are shown. *B*, HO activity in aorta of control rats and rats transduced with human HO-1. HO activity was determined as described under "Experimental Procedures." Results are mean ± S.E., $n = 4$; *, $p < 0.05$ *versus* control.

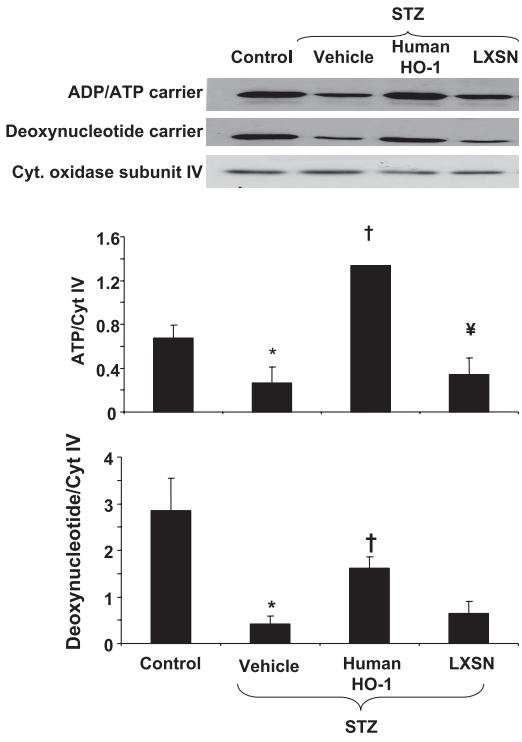

FIGURE 5. **Effect of HO-1 expression on ADP/ATP and deoxynucleotide carrier proteins.** Quantitative densitometry evaluation of ADP/ATP carrier to CYTO IV ratio in aorta was determined. Each *bar* represents mean ± S.E. of the ratio of each carrier to CYTO IV. *, $p < 0.03$ *versus* control; †, $p < 0.005$ *versus* diabetic rats; ¥, $p < 0.003$ *versus* human HO-1. Quantitative densitometry evaluation of deoxynucleotide carrier to CYTO IV ratio. *, $p < 0.05$ *versus* control; †, $p < 0.02$ *versus* diabetic rats. Representative immunoblots ($n = 3$) are shown.

was not significantly altered in diabetics compared with controls animals. However, the extent of AKT phosphorylation was significantly greater in CoPP-treated rats compared with diabetic controls. Densitometry analysis showed that CoPP treatment increased the ratio of P-AKT/AKT from 0.68 ± 0.14 in STZ to 2.69 ± 0.38 in STZ-CoPP tissue ($p < 0.02$). The increase in p-AKT was abolished by inhibition of HO activity with the co-administration of SnMP to CoPP-treated diabetic rats (Fig. 6*A*). The total amount of AKT protein was not different between groups, *i.e.* control, diabetic, diabetic-treated with CoPP, diabetic-treated with CoPP plus SnMP. The endogenous levels of the anti-apoptotic protein, BcL-XL were decreased in diabetic rats ($p < 0.05$) when compared with control rats (Fig. 6). Up-regulation of HO-1 expression by CoPP resulted in the stimulation of BcL-XL in diabetic rats, ($p < 0.001$), which was prevented by the co-administration of SnMP, suggesting that increased in HO activity (*i.e.* production of CO and bilirubin) contributed to the increase in BcL-XL. There was no effect on Bax, a pro-apoptotic signaling pathway member.

DISCUSSION

This study demonstrates, for the first time, that the heme-HO system participates in the regulation of renal and vascular mitochondrial transport carriers, presumably by regulating the levels of bilirubin and CO, secondary to an increase in HO-1 expression and activity. We have demonstrated that the increase in MCs, as a result of the increase in HO-1 expression, is associated with a robust increase in cytochrome *c* oxidase activity, presumably by influencing the levels of phosphorylation of the AKT and BcL-XL proteins. Three key findings substantiate this conclusion. The first is that hyperglycemia decreased five of the MCs examined, *i.e.* carnitine, deoxynucleotide, dicarboxylate, phosphate, and ADP/ATP, without affecting the aspartate and oxoglutarate carriers. This decrease was associated with a reduction in HO-1-derived CO and bilirubin formation.

The second key observation is that up-regulation of HO-1 by CoPP in diabetic and in control rats significantly increased the carnitine, citrate, phosphate, deoxynucleotide, dicarboxylate, and ADP/ATP carriers without affecting the aspartate and oxoglutarate carriers (Fig. 2). These observed effects of HO-1 on MCs appear to be dependent on the generation of CO and bilirubin, because an increase in HO-1 protein without an increase in HO activity, as seen in rats treated with SnMP, did not enhance MCs. Further, the increase in MCs following HO-1 expression was associated with an increase in cytochrome *c* oxidase activity. In addition, and more importantly, the direct delivery of HO-1 to diabetic rats resulted in the restoration of two of the MCs examined, ADP/ATP and deoxynucleotide (Fig. 5). This novel finding demonstrates the direct effect of HO-1 and HO activity on the enhancement of mitochondrial function and the electron transport system.

MCs play a crucial role in intermediary metabolism (24), as seen by their decrease in restricted diets, and by the fact that mitochondrial

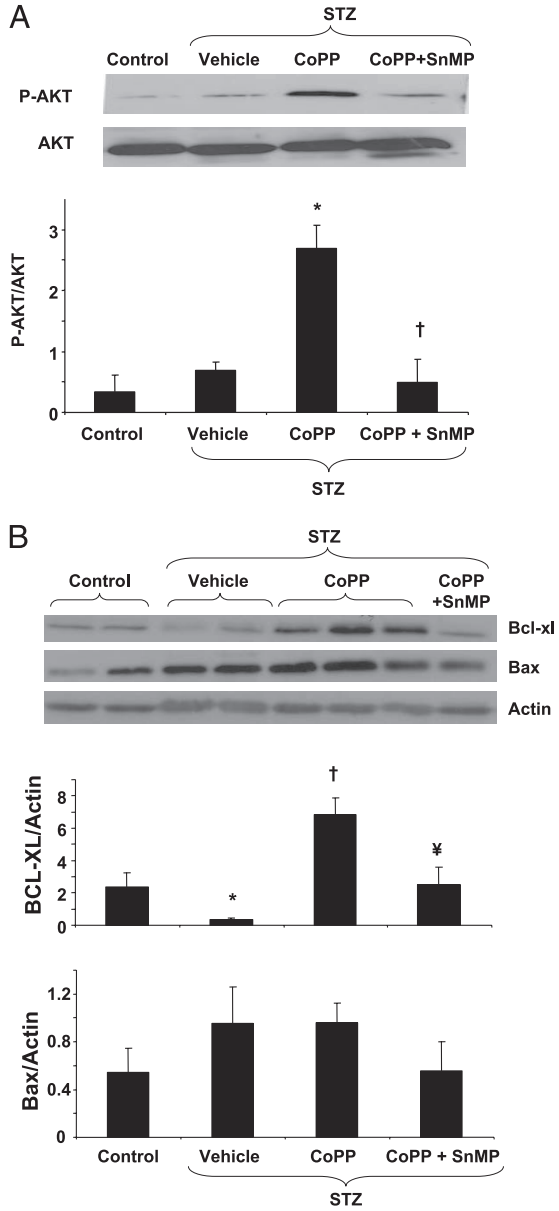

FIGURE 6. *A*, effect of diabetes and HO-1 expression on phospho (*p*) p-AKT and total AKT in renal proteins. Quantitative densitometry evaluation of p-AKT and total AKT proteins ratio was determined. *, $p < 0.02$ *versus* diabetic rats; †, $p < 0.02$ *versus* STZ-CoPP. *B*, effect of diabetes and HO-1 expression on BcL-XL and Bax, in renal proteins. Quantitative densitometry evaluation of BcL-XL and β-actin protein ratio were determined. *, $p < 0.05$ *versus* control; †, $p < 0.001$ *versus* diabetic rats; ¥, $p < 0.01$ *versus* STZ-CoPP. Representative immunoblots ($n = 6$) are shown.

dysfunction can be restored by refeeding (35). The present study shows that the increase in HO activity by induction of HO-1 can restore the citrate transport carrier. Therefore, the observed changes in mitochondrial transporters following *HO-1* gene expression, by pharmacological or genetic means, may attenuate diabetes-induced ROS by improvement in cytochrome *c* oxidase activity and in the mitochondrial transport systems.

The third key observation is that the up-regulation of HO-1 in diabetic rats by CoPP restored the diabetes-mediated decrease in BcL-XL (Fig. 6) and greatly increased AKT phosphorylation; both are important factors in cell survival. Others have shown that an increase in AKT phosphorylation is critical to cell survival in diabetes (36, 37). More recently, increases in AKT phosphorylation and BcL-XL levels have been shown to prevent the loss of β-cells in diabetes (38, 39). It is interesting to note that the alteration in mitochondrial function *in vitro* and *in vivo* has been shown to correlate with the levels of activation of AKT and BcL-2 family protein (23, 40–42). A decrease in BcL-2 family members has been suggested to contribute to apoptosis and the translocation of cytochrome *c* from the mitochondria to cytosol (23, 41, 43). Activation of AKT has been shown to augment ATP synthesis (44), promote association of hexokinase with the VDAC channel and, in so doing, promote VDAC closure, thus blocking release of cytochrome *c* (45). However, the cause of the diabetes-related decline in certain MCs and BcL-XL proteins remains to be clarified.

The HO-1 product, bilirubin, has been shown to inhibit protein kinase C and NADPH oxidase activities (46). Recently, up-regulation of *HO-1* gene expression was shown to decrease the availability of the heme-containing gp91 subunit necessary for NADPH oxidase activity and O_2^-) generation and to increase bilirubin formation (7). Furthermore, a decrease in mitochondrial heme as a result of the increase in mitochondrial HO-1 activity may reduce fatty acid lipid peroxidation and enhance mitochondrial membrane resistance to oxidative stress.

Cardiac mitochondrial damage, as seen in type I diabetes, was the result of a decrease in reduced glutathione and decreased state 3 in mitochondrial respiration (14). In other studies, a deficiency in the deoxynucleotide carrier was associated with abnormal brain growth (47), and a deficiency in carnitine-acylcarnitine was shown to cause muscle weakness and cardiomyopathy (48). Diabetic complications have been shown to be related to abnormalities in mitochondrial function (14, 49, 50) as well as to increased endothelial cell death and detachment (51). Therefore, up-regulation of HO-1 in the mitochondria or in the vicinity of mitochondrial membranes may be essential to modulate the redox state in favor of antioxidants and to enhance mitochondrial transport of substrates and metabolites.

Our findings indicate, for the first time, that HO-1 prevents the hyperglycemia-mediated decrease in six mitochondrial transporters and restores cytochrome *c* oxidase activity. The mechanism by which up-regulation of HO-1 expression and activity stimulates the MCs may be via an increase in phosphorylation of AKT and in anti-apoptotic molecules such as BcL-XL. Thus, pharmacological or genetic probes to enhance *HO-1* gene expression enable the vascular system to mount an important host defense response to resist diabetes-mediated increases in oxidative stress.

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