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Original Research Communication

Interdiction of the Diabetic State in NOD Mice by Sustained Induction of Heme Oxygenase: Possible Role of Carbon Monoxide and Bilirubin

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ABSTRACT

The aims of the present study were to assess whether sustained HO-1 expression could moderate or prevent diabetes in an animal model of the disease and, if so, to examine the possible mechanisms involved. Our results showed that HO-1 expression and HO activity were upregulated in the pancreas of non-obese diabetic (NOD) mice by the weekly administration of cobalt protoporphyrin (CoPP). Blood glucose levels in CoPP-treated mice decreased to normal, but continuously increased in untreated controls. Beta-cell numbers were preserved in the islets of CoPP-treated mice, whereas no beta cells were found in untreated diabetic mice. The number of $CD11c^+$ dendritic cells was significantly decreased in the pancreas of CoPP-treated NOD mice, but this effect was reversed by the inhibition of HO activity. Increased levels of HO-1 produced a new pancreatic phenotype, as reflected by increases in phosphorylated AKT, BcL-xL and RSK levels, and decreases in O_2^- and 3-NT levels. These novel findings provide a link between the increase in HO-1 activity, with its concurrent enhanced production of carbon monoxide (CO) and bilirubin, a decrease in infiltrated $CD11c^+$ dendritic cells and an increase in anti-apoptotic proteins, including RSK and BcL-xL, in the interdiction of the diabetic state.
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INTRODUCTION

THE DIABETIC STATE IN NONOBESE DIABETIC (NOD) mice results from autoreactive T cell–mediated autoimmune destruction of insulin-producing pancreatic beta cells due to defects in both negative selection and peripheral tolerance induction (16, 39). Because diabetes in the NOD mouse shares similarities to type 1 diabetes in humans, NOD mice have been used extensively as a preclinical model for the development of new therapeutic strategies. The majority of antidiabetic drugs are restricted to preventive use at the prediabetic stage, owing to their direct or secondary effects on immune homeostasis (36).

Recent reports have suggested that beta-cell destruction caused by elevated intracellular levels of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide, and nitric oxide, is a process that occurs through both apoptotic and necrotic mechanisms (19, 25, 30). T cell–mediated infiltration of the pancreas leads to the generation of ROS and proinflammatory cytokines (8, 15, 23). ROS are a consequence of hyperglycemia that can induce apoptosis via signaling or outright molecular damage or both (1).

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The HO-1 system has been shown to regulate T-cell proliferation and immune responses (6, 27). Studies have shown that $CD4^+$ T cells express HO-1, and that the lack of HO-1 modulates T-cell proliferation and maturation (3, 4). HO-1 upregulation has proven to be capable of providing cytoprotection to vascular function (1) and to pancreatic beta cells both *in vivo* (28) and *in vitro* (42). Recently, HO-1 upregulation has been shown to decrease dendritic cell infiltration into pancreatic tissues dramatically in diabetic mice (21). Further, HO-1 upregulation decreases ROS and iNOS in diabetic rats *via* an increase in biliverdin/bilirubin production and CO generation and an increase in extracellular superoxide dismutase levels (17, 37). CO, biliverdin, and iron are products of heme degradation. HO-1–derived biliverdin/bilirubin and CO have both antioxidant and antiapoptotic properties, respectively (1, 32, 33).

The current study was intended to determine whether upregulation of HO-1 expression in type 1 diabetic NOD mice is beneficial in preventing or delaying the appearance of this autoimmune disease. The results indicate that enhanced HO-1 activity, with its associated increased production of CO and biliverdin/bilirubin, can significantly interdict the development of this form of experimental diabetes.

METHODS

Animals and treatment protocol

Six-week-old female NOD mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These animals are born nondiabetic, but have a high (~90%) incidence of spontaneously developing late-onset (post ~180 days) diabetes (12). All animals were housed under special pathogen-free conditions in air-filtered and humidity- and temperature-controlled rooms with 12-h light and 12-h dark cycles. Urine glucose levels were measured daily, and blood glucose levels were measured twice a week. Because not every mouse would be expected to develop diabetes at the same time, our strategy was to use a large number of animals (90 mice) to have a sufficient number (12–15 mice/group) developing diabetes within a short time of each other. Once urine glucose levels were elevated, mice with blood glucose levels >300–350 mg/dl on two consecutive occasions were considered diabetic. Mice were grouped as nondiabetic NOD or diabetic NOD. If a mouse in the nondiabetic group developed diabetes at any time during the study, it was excluded. Diabetic NOD animals were administered either vehicle, cobalt protoporphyrin (CoPP) alone, CoPP in combination with tin mesoporphyrin (SnMP), or SnMP alone. CoPP (13 mg/kg, body weight) and/or SnMP (10 mg/kg, body weight) (Frontier Scientific Porphyrin Products, Logan, UT) was injected intraperitoneally (i.p.) twice per week for 3 weeks after the mice were considered to be diabetic. Control animals (nondiabetic and diabetic) were injected (i.p.) with a similar volume of vehicle (saline) twice per week for 3 weeks. All experiments were conducted under the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

Immunochemistry

Pancreases were fixed in 10% formalin solution for 24 h, processed, and paraffin-embedded. Sections, 2 μm thick, were stained in hematoxylin–eosin for histologic examination. Double immunostaining was performed to examine the residual glucagon- and insulin-producing cells. Sections were first incubated with a rabbit anti-human glucagon antibody (DAKO, Carpinteria, CA), followed by further incubation with an anti-rabbit horseradish peroxidase (HRP)-labeled polymer (DAKO) and developed using a peroxidase substrate kit (Vector Laboratories, Burlingame, CA). The same section was next incubated with a guinea pig anti-porcine pancreatic insulin antibody. Cryosections, 5 μm thick, were air dried and fixed in acetone. The sections were then treated for 20 min with 0.3% H_2O_2 in methanol, a protein-blocking agent, and then sequentially with biotinylated rat anti-mouse $B220^+$, $CD4^+$, $CD8^+$, and $CD11c^+$ antibodies (BD Biosciences, San Jose, CA) for 60 min at room temperature using the avidin-biotin complex (ABC) method. Immunoreactivity was visualized with diaminobenzidine (DAB). The sections were counterstained with Mayer's hematoxylin.

Infiltration of dendritic cells

Staining intensities of infiltrated dendritic cells were computed as integrated optical density (IOD) and measured in seven samples for each group. Digitally fixed images of the slices at ×40 magnification were analyzed using an optical microscope equipped with an image analyzer (Image Pro Plus; Immaginie, Milan, Italy). Two different methods were used to measure IOD in three different areas of the pancreas, measuring cell by cell (assuming each cell had a comparable area) for a total of 100 cells for each experimental group. Individual cell boundaries were manually traced. The IOD was calculated for arbitrary areas, measuring five fields in the same area for each sample, and the data were pooled.

Tissue preparation

Frozen pancreatic and aorta samples were pulverized under liquid nitrogen, placed in a homogenization buffer, and used for measuring HO activity, CO, HO-1, HO-2, BcL-xL, RSK, pAKT, and p47 phox.

Western blot analysis

Protein levels were visualized by immunoblotting with antibodies against rat HO-1 and HO-2 (Stressgen Biotechnologies Corp., Victoria, BC), BcL-xL (B-cell lymphoma survival factor-XL) (Santa Cruz Biotechnology, Santa Cruz, CA), RSK (phospho-p90RSK (phospho p90, Ribosomal S6 kinase), p47 phox, and phospho-Akt (serine-473 and threonine-308) kinase (pAkt) (Cell Signaling, Danvers, MA) were determined as previously described (17). In brief, 20 μg of lysate supernatant was separated by 12% SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Chemiluminescence detection of O_2^- was performed with the Amersham ECL detection kit, according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Measurement of HO activity

HO activity was assayed by the method of Abraham *et al.* (2), in which bilirubin, the product of HO degradation, was

extracted with chloroform, and its concentration was determined spectrophotometrically using the difference between absorbance at a wavelength of between 460 and 530 nm with an absorption coefficient of 40 m*M*/cm (5).

Measurement of CO

Pancreatic tissue homogenates were transferred into amber glass vials (2 ml), containing 0.5 ml HO buffer system and containing NADPH generating system in the presence of heme (30 μ*M*). The blanks were assayed in the absence of NADPH but in the presence of heme (30 μ*M*) for 30 min. The amount of CO in the headspace was measured using an HP-5989A mass spectrometer interfaced to an HP-5890 gas chromatograph. CO separation from other gases was carried out on a GS-Molesieve capillary column (30 m; 0.53 mm inside diameter; J & W Scientific, Folsom, CA) kept at 40°C. Helium was used as the carrier gas with a linear velocity of 0.3 m/sec. CO eluted at 3.6 min and was fully separated from N_2, O_2, H_2O, and CO_2. The mass spectrometer parameters were as follows: ion source temperature, 120°C; electron energy, 31 eV; transfer line temperature, 120°C. Aliquots (100 μl) of the headspace gas of either the standard solutions or experimental samples were injected, using a gas-tight syringe, into the spitless injector having a temperature of 120°C. An abundance of ions at *m*/*z* 28, 29, and 31 corresponding to $^{12}C^{16}O$, $^{13}C^{16}O$, and $^{13}C^{18}O$, respectively, was acquired by means of selected ion monitoring. For the measurement of CO concentration, the sample in 1 ml of solution was prepared in an amber glass vial (2 ml), and then capped tightly with a Teflon/silicone septum. One microliter of the [^{13}C]carbon monoxide saturated solution (1 m*M*) was added to the sample, resulting in the internal standard concentration of 1 μ*M*. After sample equilibration, 100 μl of the headspace gas was taken from the vial and injected into the gas chromatograph. The amount of CO in pancreatic tissue samples was calculated from standard curves constructed with an abundance of ions *m*/*z* 28 and *m*/*z* 29 or *m*/*z* 31, as previously described (14, 29). Both standard curves were linear over the range from 0.05 to 5.0 μ*M*, and both yielded comparable results when used for determining the concentration of endogenous CO (14).

Heme determination

Pancreatic homogenate heme was determined as the pyridine hemochromogen using the reduced minus oxidized difference in absorbance at 400 and 600 nm with an absorption coefficient of 32.4 m*M*/cm (9).

Measurement of nitrotyrosine

Nitrotyrosine (3-NT) was quantitated using a rabbit anti-nitrotyrosine antibody (Cayman, Ann Arbor, MI).

Statistical analyses

The data are presented as mean ± standard error of the mean (SEM) for the number of experiments. 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 *t* test for two groups.

RESULTS

Glucose levels

Blood glucose levels began to decline in CoPP-treated mice 1 week after treatment commenced and returned to normal levels (before the onset of diabetes) by 3 weeks (Fig. 1A). Treatment with SnMP alone, CoPP in combination with SnMP, or vehicle did not halt the steady increase in blood glucose levels (Fig. 1C, D, and B, respectively). Moreover, urine glucose levels, which declined in CoPP-treated animals, did not decline in the other groups (data not shown).

Pancreatic infiltration of dendritic cells

No significant difference was found in the number of infiltrated $B220^+$ and $CD4^+$ cells between the CoPP-treated group and the untreated control group. However, the number of infiltrated $CD11c^+$ dendritic cells was significantly increased in diabetic mice compared with NOD mice before the development of diabetes. The upregulation of HO-1 by CoPP decreased the number of $CD11c^+$ dendritic cells in diabetic mice to levels seen in prediabetic animals ($p < 0.01$). Conversely, administration of the HO inhibitor SnMP to CoPP-treated NOD mice reversed the CoPP-mediated protective effect and increased the level of infiltrated $CD11c^+$ cells (in the range of 1.0–3.0 infiltrated $CD11c^+$ cells in CoPP-treated NOD *vs*. 19–25 infiltrated $CD11c^+$ cells in NOD treated with CoPP and SnMP; $p < 0.05$), suggesting a link between HO-1 expression and the level of $CD11c^+$ dendritic cell infiltration.

Immunohistochemical analyses of insulin-producing cells

As seen in Fig. 2, beta cells (brown) were detected in nondiabetic animals, but the number decreased at the onset of diabetes, and none was detected 2 weeks after the development of diabetes. CoPP administration at the onset of diabetes provided considerable protection with regard to the number of beta cells present. The distribution of beta cells in CoPP-treated mice was the same as that in NOD animals before the onset of diabetes. In contrast, the inhibition of HO activity by SnMP caused the destruction of beta cells. Moreover, when SnMP was administered in combination with CoPP, the CoPP-mediated cytoprotection was reversed. The clustered insulin-producing beta cells, seen after CoPP treatment, were not detectable. Glucagon-producing cells (black) were detected in nondiabetic NOD animals and in NOD mice 3 weeks after diabetes commenced. CoPP treatment restored insulin levels to those seen in prediabetic animals (21–32 and 15–22 μg/ml, respectively). No insulin was detectable in untreated diabetic mice or in mice treated with SnMP alone or CoPP in combination with SnMP.

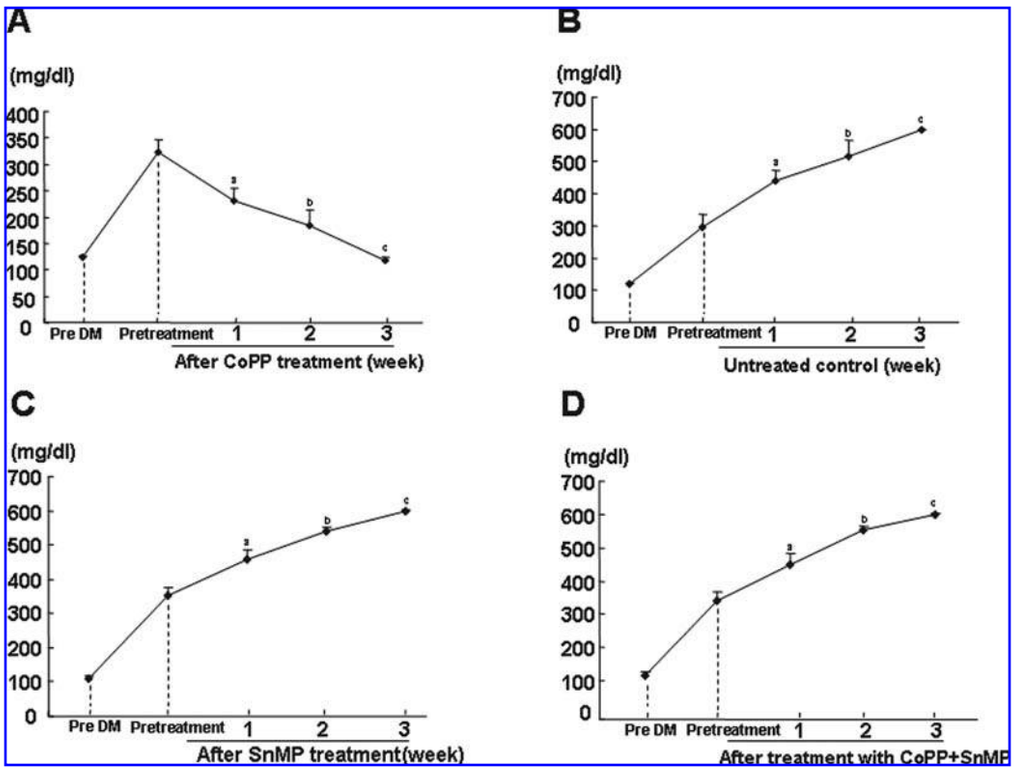

FIG. 1. Changes in blood glucose levels. (**A**) CoPP-treated NOD (a) $p = 0.0174$ *vs*. pretreatment, (b) $p = 0.0026$ vs. pretreatment, (c) $p < 0.0001$ *vs*. pretreatment. (**B**) Untreated NOD controls (a) $p = 0.0082$ *vs*. pretreatment, (b) $p = 0.0007$ *vs*. pretreatment, (c) $p < 0.0001$ *vs*. pretreatment. (**C**) SnMP-treated NOD (a) $p = 0.0042$ *vs*. pretreatment, (b) $p < 0.0001$ *vs*. pretreatment, (c) $p < 0.0001$ *vs*. pretreatment. (**D**) CoPP-treated NOD administered SnMP (a) $p = 0.0035$ *vs*. pretreatment, (b) $p < 0.0001$ *vs*. pretreatment, (c) $p < 0.0001$ *vs*. pretreatment. $N = 6$–7 for each graph.

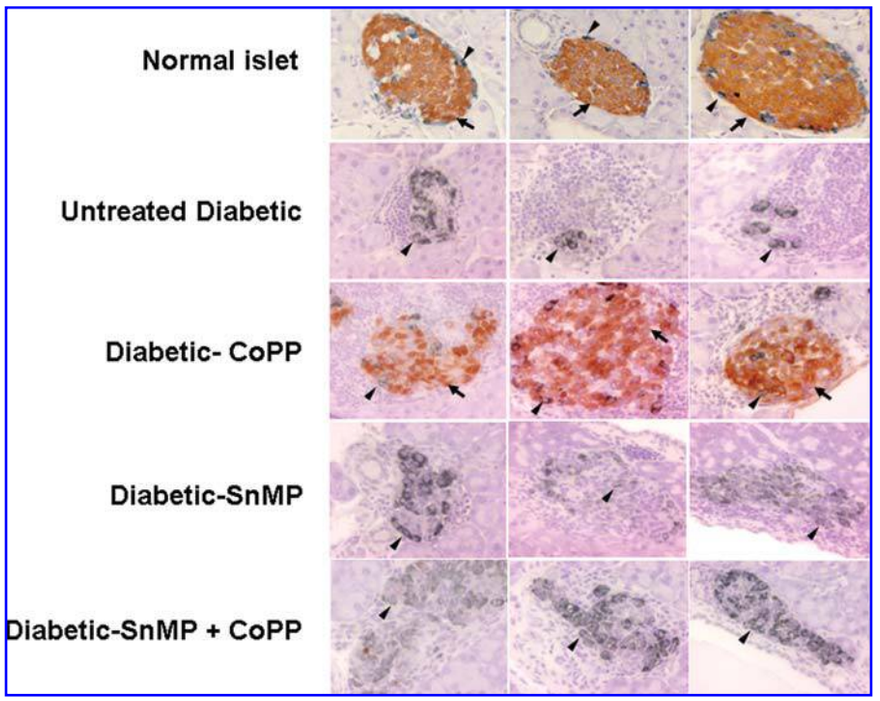

FIG. 2. Insulin and glucagon expression. Insulin-producing cells (brown) were observed in normal islets (*arrow*) in prediabetic (*arrows*) and in CoPP-treated NOD (*arrows*). No beta cells were present in untreated, SnMP-treated, or CoPP-treated NOD mice administered SnMP. Glucagon-producing cells (black) were observed in the islets of each group (*arrowheads*) (original magnification ×400). Representative slides are shown.

Effect of CoPP on HO-1 expression, HO activity, CO production, and heme

Treatment with CoPP caused a robust increase in HO-1 after 48 h (Fig. 3A). The inductive effect of CoPP was not limited to the pancreas but was also seen in the aorta (Fig. 3A, lower panel). Accordingly, we examined whether repeated administration of CoPP over the duration of the study would sustain HO-1 expression and whether the effect of CoPP on the restoration of beta cells was attributable to the pharmacologic effect of CoPP. CoPP treatment resulted in a significant increase in HO-1 protein levels as early as 6 h after the first administration ($p < 0.005$) compared with the levels in untreated animals. As shown in Fig. 3B, the repeated administration of CoPP resulted in a continuous, sustained increase in HO-1 protein ($p < 0.001$) compared with untreated NOD animals. HO-2, the constitutive form of HO, was unaffected by CoPP treatment (Fig. 3B).

Because Western blot analysis demonstrated a significant increase in HO-1 protein levels after CoPP administration, we assessed the effect of CoPP treatment on HO activity. As seen in Fig. 3C, a significant increase occurred in total HO activity in CoPP-treated mice as early as 6 h after the first administration. Because HO-2 activity remained unchanged with CoPP, the increase in HO activity contributed to the induction of HO-1 as a result of CoPP administration. In addition, CO production was measured in pancreatic islets incubated for 30 min in the presence of heme, as described in Methods. CO release increased as a function of time. CO levels in nondiabetic and untreated NOD mice were 1,785 ± 115 and 1,739 ± 131 nmol/mg/h, respectively. CoPP caused more than a twofold (3,977 ± 293) increase in CO generation, which was inhibited by coadministration the HO inhibitor, SnMP (572 ± 105 nmol/mg/h). Pancreatic heme levels averaged 152 ± 39 pmole heme/mg protein in nondiabetic animals and increased to 225 ± 47 pmole heme/mg protein in diabetic mice. The CoPP-mediated increase in HO-1 resulted in a significant decrease in heme to 130 ± 45 pmole heme/mg protein ($p < 0.05$). Because HO-1 and HO-2 degrade heme to equimolar amounts of biliverdin/bilirubin and CO, we measured bilirubin formation over the duration of the study. As seen in Fig. 3C, the continued administration of CoPP caused a sustained increase in bilirubin levels. The CoPP-mediated upregulation of HO-1 and the associated increase in HO activity were prevented by the administration of SnMP, which diminished HO activity by 75% ($p < 0.0001$).

Effect of HO on antiapoptotic signaling molecules pAKT, RSK, and BcL-xL

Because upregulation of HO-1 expression is shown to increase antiapoptotic proteins in various tissues (21, 37, 38), we measured the levels of the antiapoptotic signaling proteins under increased and decreased HO activity. As seen in Fig. 4, the administration of SnMP for 3 weeks resulted in a decrease in pAKT, RSK, and BcL-xL. Densitometry analysis showed

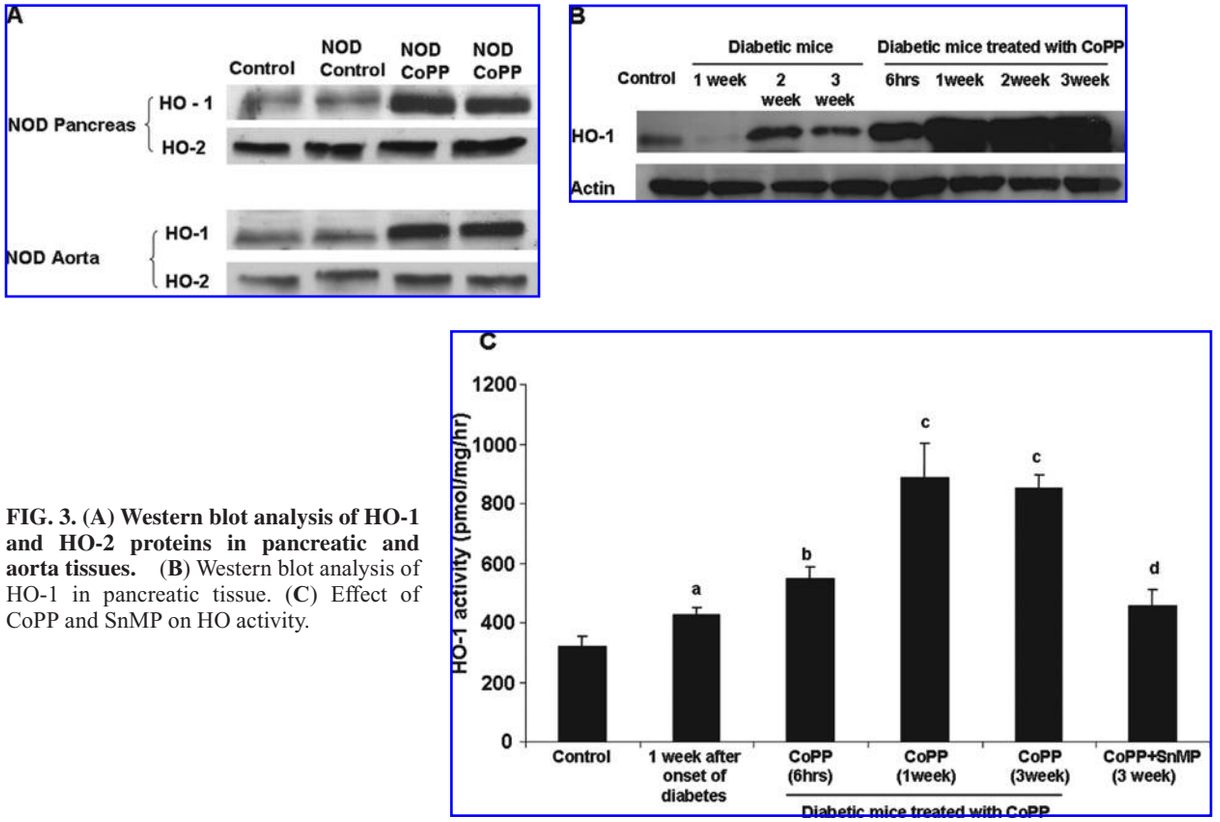

FIG. 3. (A) Western blot analysis of HO-1 and HO-2 proteins in pancreatic and aorta tissues. (**B**) Western blot analysis of HO-1 in pancreatic tissue. (**C**) Effect of CoPP and SnMP on HO activity.

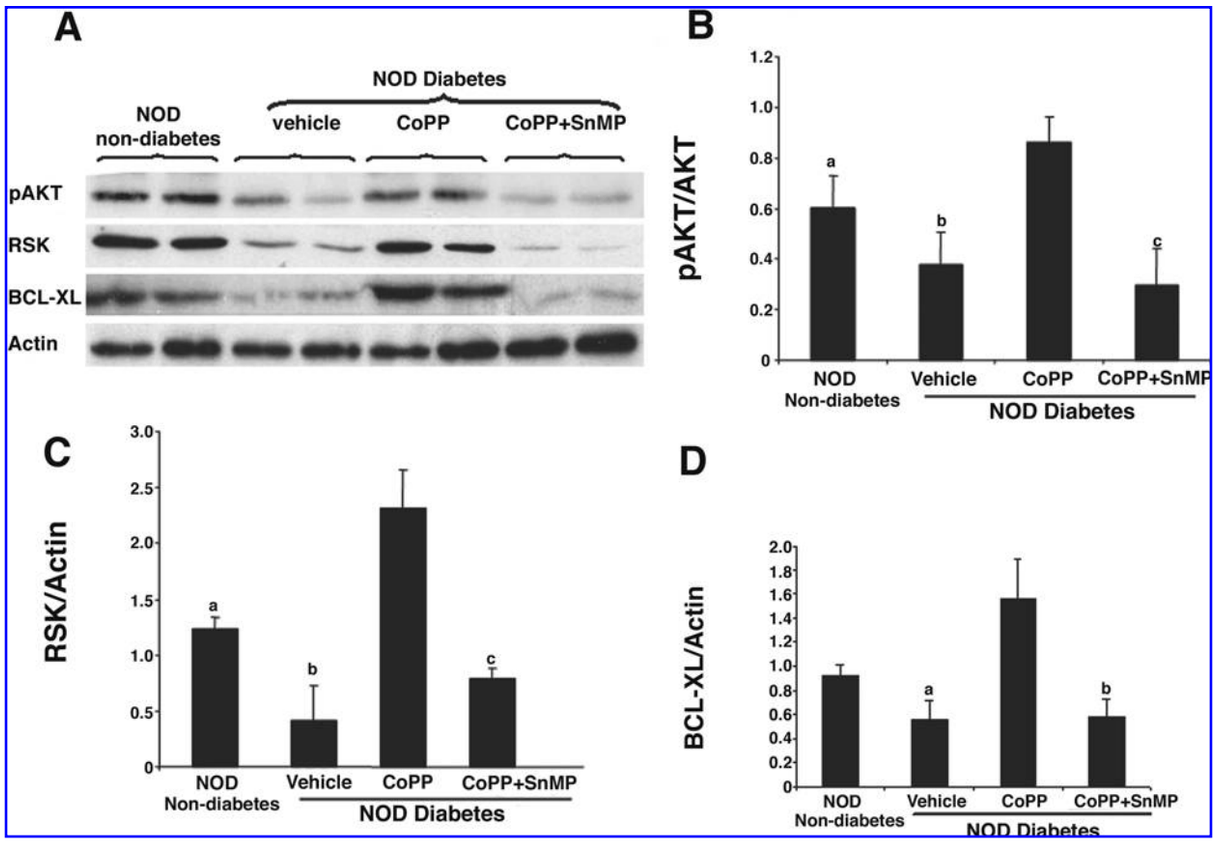

FIG. 4. (A) Western blot and densitometry analyses of pAKT, RSK, and BcL-xL in pancreatic tissue. Mean band density for (**B**) pAKT, (**C**), RSK, and (**D**) BcL-xL normalized to α-actin, except pAKT normalized to total AKT ($n = 3$ for each point). Each bar represents mean ± SEM of the ratio of each antiapoptotic protein. For HO-1, $p < 0.01$ *vs*. untreated NOD. For pAKT, $p < 0.01$ *vs*. untreated NOD and nondiabetic NOD. For RSK, $p < 0.001$ *vs*. untreated NOD and nondiabetic NOD. For BcL-xL, $p < 0.05$ *vs*. untreated NOD and nondiabetic NOD. $N = 4$.

that pAKT, which was not increased in untreated animals, was significantly elevated in CoPP-treated mice overexpressing HO-1 (Fig. 4B, $p < 0.022$, vs. untreated diabetic NOD). The HO-1–mediated increase in pAKT was presumably dependent, in part, on the generation of bilirubin and CO because the administration of SnMP inhibited HO activity and prevented a CoPP-mediated increase in RSK and BcL-xL (Fig. 4C and D; $p < 0.007$ vs. untreated NOD and $p < 0.025$ vs. untreated NOD, respectively), suggesting a link between HO-1 expression and antiapoptotic molecule levels.

Effect of CoPP on p47 phox, 3-NT, and O_2^-

With the onset of diabetes, the levels of p47 phox protein, an essential component for NADPH oxidase activity, increased severalfold compared with levels in prediabetic animals ($p < 0.05$). As shown in Fig. 5A (lower panel), CoPP administration decreased p47 phox protein to near-normal levels. When SnMP was administered to CoPP-treated animals, p47 phox protein levels increased significantly ($p < 0.05$) compared with both prediabetic animals and those treated with CoPP only (Fig. 5A, lower panel).

Because peroxynitrite is increased in diabetes and results in increased protein oxidation, we measured 3-NT levels. Relative to prediabetic NOD, diabetic mice showed significantly higher levels of 3-NT (Fig. 5). The increased levels of HO-1–derived bilirubin and CO were associated with a significant decrease in the level of 3-NT immunoreactivity in CoPP-treated mice. CoPP in combination with SnMP significantly ($p < 0.02$) increased pancreatic 3-NT levels (Fig. 5B).

Because CoPP was shown to increase HO-1 protein levels and activity with a resultant decrease in pancreatic heme, an increase in CO and bilirubin production, and a decrease in NADPH oxidase activity, we examined O_2^- formation in the pancreas. O_2^- levels were increased in diabetic compared with nondiabetic animals. However, O_2^- levels significantly decreased from 11,080 ± 3,295 CPM/mg protein/min chemiluminescence count in untreated NOD to 1,895 ± 380 in CoPP-treated NOD ($p < 0.002$), reaching levels below those seen in nondiabetic controls (4,256 ± 1,032 CPM/mg protein/min).

DISCUSSION

This study demonstrates, for the first time, that upregulation of HO-1 gene expression in the early development of diabetes in NOD mice results in the acquisition of a new pancreatic

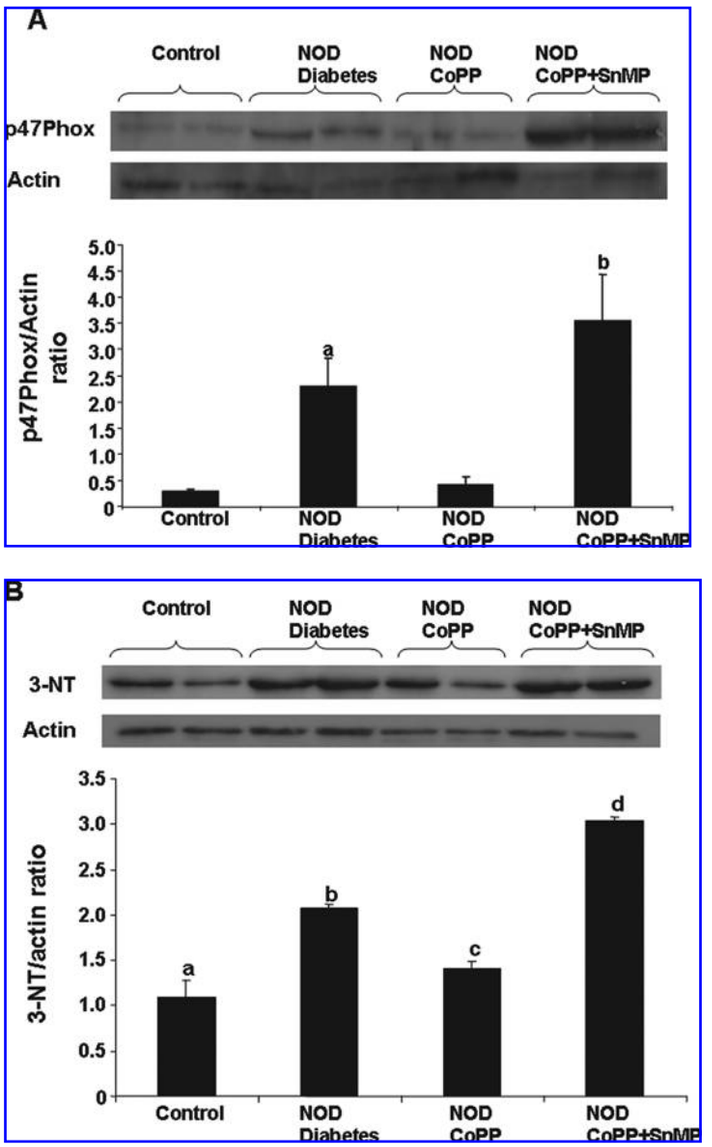

FIG. 5. Western blot and densitometry analysis showing (A) p47 phox and (B) 3-NT expression in pancreatic tissue. For p47 phox, (a) $p < 0.05$ *vs*. CoPP-treated and nondiabetic NOD and (b) $p < 0.05$ *vs.* CoPP-treated NOD. For 3-NT, (a) $p < 0.02$ *vs*. untreated NOD, (b) $p < 0.005$ *vs*. CoPP-treated NOD administered SnMP, (c) $p < 0.01$ *vs.* untreated NOD, (d) $p < 0.05$ *vs.* CoPP-treated NOD. Results are expressed as mean ± SEM; $N = 3$.

phenotype, as reflected by decreased p47 phox and O_2^- generation and increases in antiapoptotic signaling proteins, thus preventing beta-cell destruction and delaying the development of diabetes over the study period. First, the increase in HO-1, after CoPP administration, resulted in a decrease in pancreatic heme and increases in CO and bilirubin production, which paralleled the prevention of beta-cell destruction and the normalization of glucose levels. CO and bilirubin have been shown to be important regulators of vascular and pancreatic function (1, 32). Bilirubin is an important antioxidant, and its increase appears to decrease the risk of cardiovascular disease (40). Interestingly, a deficiency in HO, such as that seen in HO-1, HO-2 knockout animals and in animals with genetically suppressed HO-1, has been shown to exacerbate vascular dysfunction (10, 26, 43). The decrease in HO-1 in diabetic mice resulted in an increase in pancreatic heme, which may contribute to pancreatic dysfunction, because heme is a prooxidant and is involved in the generation of ROS and lipid peroxidation (24).

CoPP-mediated increases in HO-1 protein levels and HO activity were associated with the prevention of $CD11c^+$ dendritic cell infiltration into pancreatic tissues and an improvement in insulin secretion. More important, the euglycemic effect of HO-1 induction and prevention of $CD11c^+$ dendritic cell infiltration were reversed when NOD mice were treated with the HO inhibitor, SnMP. These results demonstrate that HO-1 upregulation and the associated increase in the heme degradation products, CO and bilirubin, are essential to cytoprotection.

O_2^- generation was prevented by increased HO-1 expression. A reduction in O_2^-, through the induction of HO, has been shown to provide vascular protection in previous studies (1). It is thus possible that the observed small increase in $CD11c^+$ dendritic cell infiltration contributes to early stage beta-cell injury and an increase in blood glucose, resulting in a later

increase in O_2^- formation. Although the destruction of beta cells, which underlies type 1 diabetes, is probably due to an autoimmune response, the particular susceptibility of beta cells to oxidative damage from ROS produced during inflammation may be a predisposing factor (18, 20). In our study, the upregulation of HO-1 decreased O_2^- generation, and, as expected, increased CO release and bilirubin formation. Conversely, the administration of SnMP blocked the effect of CoPP, preventing the increased generation of CO and bilirubin, and was associated with a reversal of the beneficial effects observed with the induction of HO-1. Inducers of HO-1 have been shown to decrease O_2^-, presumably via a decrease in NADPH oxidase, a heme-dependent protein (34). The preservation of beta cells could be accomplished, at least in part, by avoiding mechanisms that involve decreased O_2^- generation.

Fourth, the upregulation of HO-1 expression changed the pancreas from a naïve to a defensive phenotype by producing a robust increase in pAKT, RSK, and BcL-xL. The antiapoptotic Bcl-2 family proteins, such as Bcl-XL, prevent the release of apoptotic proteins from mitochondria (6a, 41). One of the important substances released from mitochondria during apoptosis is cytochrome *c* (22). Released cytosolic cytochrome *c* binds to Apaf-1, inducing a conformational change in Apaf-1 (13). The binding to the Apaf-1–cytochrome *c* complex triggers its oligomerization to form the apoptosome, which recruits procaspase-9 (44). Further, HO-1–mediated increase in RSK is an important for prevention of cell death. RSK phosphorylates Bad at Ser 112 (35) and prevents the proapoptotic effect of Bad.

The HO-1–mediated increase in pAKT, RSK, and BcL-xL was abolished when NOD mice were treated with SnMP, thus affirming the critical role of HO-1 induction in these processes. In a recent study, a decrease in activated AKT was observed after the inhibition of HO activity using SnMP; however, pAKT was restored by the upregulation of HO-1 (7). More recently, AKT activation in human islets was shown to be associated with increased beta-cell proliferation and survival (31). These results indicate that HO-1 overexpression can mediate the preservation of beta cells and/or prevent beta-cell destruction in the early development of diabetes *via* a mechanism that involves a decrease in $CD11c^+$ dendritic cell infiltration and an increase in AKT activation.

Recently, Chen *et al.* (4) reported that IL-10 regulates inflammatory and immunosuppressive responses in a HO-1–dependent manner. Thus, the anti-inflammatory and immunosuppressive effects of CoPP on dendritic cells seen in NOD mice could be achieved by the upregulation of HO-1 *via* IL-10–mediated suppression. Multiple lines of evidence suggest that $CD4^+CD25^+T_{regs}$ are important in preventing an immunoresponse to oxidants in which IL-10 and/or other cytokines play a critical role (11).

Our results suggest that beta cells may escape autoreactive T cell–mediated destruction as a result of the CoPP-mediated induction of HO-1 and the associated increase in production of CO and bilirubin. This could be explained, in part, by the significantly decreased numbers of $CD8^+$ T cells and $CD11c^+$ dendritic cells in CoPP-treated NOD mice compared with untreated animals. It is also evident that CO and bilirubin prevent pancreatic cell death *via* a mechanism(s) that involves increased activation of phosphorylated AKT and increases in antiapoptotic signaling molecules. Therapeutic interventions aimed at inducing a sustained increase in HO-1 activity, with its associated increase in production of CO and bilirubin, may help to delay and/or moderate the development or the severity of diabetes.

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