Heme oxygenase system enhances insulin sensitivity and glucose metabolism in streptozotocin-induced diabetes

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Ndisang JF, Jadhav A. Heme oxygenase system enhances insulin sensitivity and glucose metabolism in streptozotocin-induced diabetes. Am J Physiol Endocrinol Metab 296: E829–E841, 2009. First published February 3, 2009; doi:10.1152/ajpendo.90783.2008.—Hyperglycemia-induced oxidative stress is a common phenomenon in diabetes. Since oxidative stress depletes adiponectin and insulin levels, we investigated whether an upregulated heme oxygenase (HO) system would attenuate the oxidative destruction of adiponectin/insulin and improve insulin sensitivity and glucose metabolism in streptozotocin (STZ)-induced type 1 diabetes. HO was upregulated with hemin (15 mg/kg ip) or inhibited with chromium mesoporphyrin (CrMP, 4 μmol/kg ip). Administering hemin to STZ-diabetic rats reduced hyperglycemia and improved glucose metabolism, whereas the HO inhibitor CrMP annulled the antidiabetic effects and/or exacerbated fasting/postprandial hyperglycemia. Interestingly, the antidiabetic effects of hemin lasted for 2 mo after termination of therapy and were accompanied by enhanced HO-1 and HO activity of the soleus muscle, along with potentiation of plasma antioxidants like bilirubin, ferritin, and superoxide dismutase, with corresponding elevation of the total antioxidant capacity. Importantly, hemin abated c-Jun NH2-terminal kinase (JNK), a substance known to inhibit insulin biosynthesis, and suppressed markers/mediators of oxidative stress including 8-isoprostane, nuclear factor (NF)-κB, activating protein (AP)-1, and AP-2 of the soleus muscle. Furthermore, heme therapy significantly attenuated pancreatic histopathological lesions including acinar cell necrosis, interstitial edema, vacuolization, fibrosis, and mononuclear cell infiltration. Correspondingly, hemin increased plasma insulin and potentiated agents implicated in insulin sensitization and insulin signaling such as adiponectin, adenosine monophosphate-activated protein kinase (AMPK), cAMP, cGMP, and glucose transporter (GLUT)4, a protein required for glucose uptake. These were accompanied by improved glucose tolerance [intraperitoneal glucose tolerance test (IPITT)], decreased insulin intolerance [intraperitoneal insulin tolerance test (IPITT)], and reduced insulin resistance [homeostasis model assessment of insulin resistance (HOMA-IR) index], whereas CrMP nullified the heme-dependent antidiabetic and insulin-sensitizing effects. In conclusion, by concomitantly enhancing insulin and paradoxically potentiating insulin sensitivity, this study unveils a novel, unique, and long-lasting antidiabetic characteristic of upregulating HO with hemin that could be exploited against insulin-resistant and insulin-dependent diabetes.

type 1 diabetes; glucose transporter 4; c-Jun NH2-terminal kinase; adiponectin; insulin resistance; oxidative stress; nuclear factor κB; activating protein-1

Diabetes mellitus is a chronic syndrome of impaired carbohydrate, protein, and fat metabolism caused by insufficient secretion of insulin (type 1) or target tissue insulin resistance (type 2). Although they have differing etiologies, both type 1 and type 2 diabetes mellitus have the common pathophysiological feature of consistently elevated plasma glucose. In type 1 diabetes, hyperglycemia and alterations of lipid metabolism lead to increased oxidative stress (1). Moreover, hyperglycemia increases the formation of advanced glycation end products (AGE) and oxidative/inflammatory events (49, 54, 56), which in turn further enhance the production of AGE, thus creating a vicious cycle that potentiates the oxidative destruction of β-cells in both type 1 and type 2 diabetes (14, 27, 34, 49). Therefore, the progressive loss of β-cell function and the corresponding decline of insulin production observed in type 1 and type 2 diabetes could be attributed, at least in part, to oxidative stress (39, 50). Thus the maintenance of specialized islet architecture and the regulation of β-cell number by antioxidants and antiapoptotic agents may be important for the preservation of intact pancreatic structure to safeguard the insulin-producing capability of β-cells.

Besides depleting insulin, oxidative stress also reduces adiponectin, a cytoprotective and anti-inflammatory adipokine that regulates insulin sensitivity and glucose metabolism (26, 31, 53). Interestingly, adiponectin has been shown to attenuate the activation of the oxidative/inflammatory transcription factor nuclear factor (NF)-κB by a adenosine 3’,5’-cyclic monophosphate (cAMP)-dependent mechanism (48). Moreover, both cAMP and guanosine 3’,5’-cyclic monophosphate (cGMP) also stimulate insulin release (42). Besides NF-κB, c-Jun NH2-terminal kinase (JNK) is another mediator of oxidative injury that inhibits insulin biosynthesis (10, 28) and regulates activating protein (AP)-1 (8). Therefore, in the highly oxidative environment that characterizes diabetes, the combined effects of NF-κB, AP-1, and JNK would constitute a potent destructive force capable of depleting insulin and adiponectin (26, 28) and thus impairing glucose metabolism.

Recent evidence has underscored the emerging role of the heme oxygenase (HO) system in diabetes (9, 30, 32, 35, 41). HO is a microsomial enzyme with inducible (HO-1) and constitutive (HO-2) isoforms (2, 44). Interestingly, the HO-1 gene response element (3, 33), suggesting that the HO system may regulate oxidative stress (39, 50). Thus the maintenance of specialized islet architecture and the regulation of β-cell number by antioxidants and antiapoptotic agents may be important for the preservation of intact pancreatic structure to safeguard the insulin-producing capability of β-cells.

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muse was used to delineate the multifaceted interaction among the HO system, NF-κB, AP-1, AP-2, and JNK. In addition, the effect of HO on pancreatic morphology was examined. Since the HO system increases adiponectin (30, 32, 35), which enhances glucose metabolism by stimulating adenosine monophosphate-activated protein kinase (AMPK) (17, 62) with subsequent activation of glucose transporter (GLUT)4 (17, 22), we also studied the effects of HO on AMPK and GLUT4.

One of the major concerns of the current treatment of diabetes is based on the maintenance of glycemic levels within a fairly narrow range, reflecting the recommendations of the Diabetes Control and Complications Trial (4, 16). However, such tight glycemic control may not be feasible, and even patients who diligently regulate their blood glucose are still vulnerable to the many complications that characterize diabetes. In these circumstances, novel antidiabetic strategies capable of improving insulin levels and/or insulin sensitivity would be of obvious therapeutic benefit.

MATERIALS AND METHODS

Animal treatment and plasma measurements. The experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics. Seven-week-old male Sprague-Dawley (SD) rats were purchased from Charles River (Wilmington, MA). The animals were housed at 2°C with 12:12-h light-dark cycles, were fed with standard laboratory chow, and had access to drinking water ad libitum. After a week of acclimatization, diabetes was induced with STZ (Sigma, St. Louis, MO) (150 mg·kg⁻¹·day⁻¹ ip in 0.1 mol/l citrate buffer, pH 4.5) (38). As previously reported, a single large (>140 mg/kg) dose, but not smaller single or multiple STZ doses, reliably induces insulin-deficient diabetes that is similar to human type 1 diabetes and is associated with similar secondary complications (57). One week after STZ injection, the occurrence of polyuria, polydipsia, polyphagia, and elevated blood glucose (>20 mmol/l) confirmed the diabetic state, and so we subjected the animals to different treatments. Hemin (15 mg/kg ip, Sigma) was administered to induce HO-1. The involvement of HO-1 in the hemin-induced antidiabetic effect was ascertained by giving the HO blocker chromium mesoporphyrin (CrMP, 4 μmol/kg ip; Porphyrin Products, Logan, UT) with or without hemin. Although many HO inhibitors are nonspecific, and affect other hemeoxygenases or even increase HO-1 expression (59), CrMP given at a dose of 4 μmol/kg is reportedly selective against HO activity (60). Hemin and CrMP were dissolved in 0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl, and diluted 1:10 with phosphate buffer as we previously reported (23, 43, 46). As an additional control, some animals received the vehicle used to dissolve hemin and CrMP. Each injection was 0.5 ml and was given twice weekly for 4 wk. The experimental design included the following groups (n = 6–12 per group): 1) control SD, 2) SD+vehicle (citrate buffer) for STZ, 3) STZ alone, 4) STZ+hemin, 5) STZ+hemin+CrMP, 6) STZ+CrMP, and 7) STZ+vehicle dissolving hemin and CrMP. Fasting and postprandial glucose were monitored weekly with a glucose meter (BD, Franklin Lakes, NJ), during the treatment period and biweekly after therapy. Glucose was measured after 12 h of fasting in metabolic cages, while postprandial levels were taken at the same time (4:00 PM) each day throughout the study. Before measurement of postprandial glucose, we ensured that the animals had eaten. Since postprandial glycemic levels are highest 1 h after a meal (49), each animal was given 1.2 g of crushed chow by gavage 1 h before measurement. The mean daily food intake by SD rats is 16.4 ± 6.1 g (47). The study was terminated after 2 mo of posttreatment observations. A day before killing, the animals were fasted for 12 h overnight in metabolic cages and weighed, urine samples were collected, and after anesthesia (pentobarbital sodium, 50 mg/kg body wt) the animals were decapitated and plasma was collected for the routine determination of bilirubin and ferritin by Saskatoon Royal University Hospital, while the tissues harvested were used immediately or frozen in liquid nitrogen and kept at −80°C for biochemical assays. Histological and morphological analyses of pancreas. Pancreatic tissue obtained from duodenal and splenic lobes were fixed in 10% formalin, processed, and paraffin embedded; then sections of 5-μm thickness were cut and stained with hematoxylin and eosin for histological analysis. Whole pancreatic sections were examined by a pathologist for acinar cell necrosis, vacuolization, interstitial edema, fibrosis, and mononuclear cell infiltration. In addition, morphological evaluation of acinar cell necrosis, vacuolization, interstitial edema, fibrosis, and mononuclear cell infiltration was done in a blinded manner in 20 randomly selected fields by light microscopy (Olympus BH-2, Olympus, Tokyo, Japan) semiquantitatively with a scale of 0 to 3 (0, normal or almost normal; 1, mild; 2, moderate; 3, severe) in each pancreatic section, and the mean score was calculated (15). Determination of HO activity and HO-1 levels in soleus muscle. HO activity was measured as bilirubin production by our established method (23, 43, 45), while the concentration of HO-1 was determined by ELISA (EKS-810A, Stressgen-Assay Design, Ann Arbor, MI) according to the instructions from the manufacturer.

Total RNA isolation and quantitative RT-PCR for NF-κB, AP-1, GLUT4, and JNK in soleus muscle. The soleus muscle was homogenized in 0.5 ml of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) as we previously reported (23, 43). Reverse transcription was carried out with a First Strand cDNA Synthesis Kit (Novagen, Madison, WI) with 0.5 μg of oligo(dT)15, 50 nM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM MgCl2, 50 mM DTT, each free dNTP at 10 mM, and 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase, according to the manufacturer’s instructions. Quantitative PCR was done with the Applied Biosystems 7300 Real Time PCR system (Foster City, CA) and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), each free dNTP at 0.2 mM, hot start enzyme iTaq DNA polymerase (25 U/μl), 3 mM MgCl2, and SYBR Green 1 (1:2000 in water as passive reference). Triplicate samples of 1 μl of cDNA each were run with a template of 3.2 pmol of primers for NF-κB (forward 5′-CATGGCGTTTCCGTTAACAAGTGGCA-3′ and reverse 5′-TGGGTGTGGTTCTTGTTATCTGGTG-3′), AP-1 (forward 5′-ACGACAGTCTGGATTGAGACCAC-3′ and reverse 5′-TTCTATGGGTCCTGCTTTGATG-3′), GLUT4 (forward 5′-AATGAGCGCCCTTGTTCAGGAGCGGACA-3′ and reverse 5′-ATGTTATTTGTTTACGAGGTACGTC-3′), and JNK (forward 5′-AGCGCTACAAAGATCCTAAGAGGGA-3′, and reverse 5′-TGTACTACATCGCACTGGGCTC-3′, and reverse 5′-AGACGAGCCCTTGTTCAGGAGCGGACA-3′ and reverse 5′-TGTACTACATCGCACTGGGCTC-3′, and reverse 5′-AGACGAGCCCTTGTTCAGGAGCGGACA-3′ and reverse 5′-TGTACTACATCGCACTGGGCTC-3′, and reverse 5′-AGACGAGCCCTTGTTCAGGAGCGGACA-3′ and reverse 5′-TGTACTACATCGCACTGGGCTC-3′). The sequences of all primers were confirmed by the National Research Institute of Canada, Saskatchewan.

Western immunoblotting. The soleus muscle was homogenized (1:10 wt/vol) in 10 mM Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of a cocktail of protease inhibitors and centrifuged, and proteins were extracted as we previously described (23, 43, 45). Briefly, proteins were extracted and quantified by Bradford assay, and aliquots of 50 μg were loaded on SDS-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to nitrocellulose paper, and nonspecific binding blocked with 3% nonfat milk, and incubated overnight with primary antibodies against AMPK (Cell Signaling Technology, Danvers, MA), AP-1 (Abcam, Cambridge, MA), NF-κB (p100/p52) (Applied Biological Materials, Richmond, BC, Canada), GLUT4 (Abcam), and JNK (Assay Designs, Ann Arbor, MI). Anti-mouse β-actin (Sigma) was used as control. Densitometric
scanning and analyses of blots were done with UN-SCAN-IT software (Silk Scientific).

**Determination of plasma adiponectin.** The concentration of adiponectin was determined by ELISA (Phoenix Pharmaceuticals, Burlingame, CA) according to the manufacturer’s instruction. Briefly, blood samples were collected and centrifuged, and the plasma was aliquoted into wells of a microplate containing adiponectin antibody. After treatment with horseradish peroxidase-conjugated secondary antibody and streptavidin, the samples were read at 450 nm with a microplate (SpectraMax 340PC, Molecular Devices).

**Determination of glucose tolerance, insulin tolerance, and insulin resistance by homeostasis model assessment of insulin resistance.** Glucose tolerance was assessed by intraperitoneal glucose tolerance test (IPGTT) after fasting for 12 h. A bolus of glucose (2 g/kg ip) was injected, and blood samples were collected sequentially from the tail vein at intervals of 0, 30, 60, 90, and 120 min and tested for glucose and insulin. Plasma glucose was measured with a glucose meter (BD), while insulin was assessed by ELISA [Mercodia Ultrasensitive Rat Insulin Kit (10-113-01), Uppsala, Sweden] according to the manufacturer’s instructions.

To evaluate insulin tolerance, an intraperitoneal insulin tolerance test (IPITT) was performed. A bolus of insulin (2 U/kg ip) was administered to the animals, and blood samples were taken sequentially at various time points from 0 to 120 min for glucose measurement as described above. In addition, homeostasis model assessment of insulin resistance (HOMA-IR) analysis was used to assess insulin resistance. Values for HOMA-IR were calculated from the product of fasting plasma glucose (mg/dl) and insulin (μU/ml) divided by 22.5 (40).

**Determination of cGMP and cAMP in soleus muscle.** The concentration of cGMP and cAMP was assessed by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI) as previously described (43, 45). Briefly, the soleus muscle was homogenized in 6% trichloroacetic acid at 4°C in the presence of 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity and centrifuged at 2000 g for 15 min. The supernatant was recovered and washed with water-saturated diethyl ether, and the upper ether layer was aspirated and discarded while the aqueous layer containing cGMP or cAMP was recovered and lyophilized. The dry extract was dissolved in 1 ml of assay buffer, and the cGMP or cAMP content was measured with the manufacturer’s protocol and expressed as picomoles per milligram of protein.

**Determination of 8-isoprostane, an index of oxidative stress.** Urinary 8-isoprostane is a noninvasive index of oxidative stress. This was determined by EIA (Cayman Chemical) as we previously reported (23). In brief, urine samples collected over a 24-h period were diluted 1:15 with ultrapure water, applied to a reverse-phase C-18 column at pH 3, and eluted with 1:1 (vol/vol) ethyl acetate-heptane. The eluent was further purified on silica column, eluted with 1:1 (vol/vol) ethyl acetate-methanol, and aliquoted into a 96-well plate precoated with monoclonal antibody. Next, 8-isoprostane tracer and isoprostane antigen were added to each well and incubated and, after washing, Ellaman’s reagent containing the substrate of acetylcholinesterase was added. The absorbance was read at 412 nm in a plate reader (SpectraMax 340PC, Molecular Devices), and the values of 8-isoprostane were calculated from a standard curve.

**Measurement of superoxide dismutase activity.** Plasma superoxide dismutase (SOD) activity was measured by EIA (Cayman Chemical) as we previously reported (23, 43). This assay detects both cytosolic and mitochondrial SOD activity and thus measures total SOD activity. The absorbance was read at 450 nm in a plate reader (SpectraMax 340PC, Molecular Devices), and the values of SOD were calculated from a standard curve.

**Total antioxidant capacity assay.** The total antioxidant capacity assay was done by EIA (Cayman Chemical) as we previously reported (23, 43). This assay is based on antioxidants inhibiting the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline] sulfonate (ABTS) to ABTS plus met-myoglobin. In brief, plasma samples were treated with
Tropol (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), met-myoglobin, and chromogen, and the absorbance was read at 750 nm with Synergy Microplate Reader (BioTek Instruments). The results were expressed as Tropol equivalent antioxidant capacity (TEAC) per milligram of protein.

Statistical analysis. All data are expressed as means ± SE from at least four independent experiments unless otherwise stated. Statistical analyses were done with unpaired Student’s t-test, analyses of variance in conjunction with Bonferroni test, and analyses of variance for repeated measures. Group differences at the level of P < 0.05 were considered statistically significant.

RESULTS

Hemin therapy reduced fasting and postprandial hyperglycemia in STZ-diabetic rats. The 4-wk regimen of hemin reduced fasting and postprandial hyperglycemia in STZ-diabetic rats (Fig. 1). STZ evoked 3.1- and 3.4-fold increments in fasting/postprandial glycemic levels. Interestingly, hemin therapy markedly reduced both fasting and postprandial hyperglycemia, although control levels were not reinstated. Importantly, the effect of hemin on fasting/postprandial glucose prevailed for 2 mo after stoppage of therapy (Fig. 1, A and C). To ascertain the implication of the HO system in the antidiabetic effects, some STZ-diabetic animals were treated together with the HO blocker CrMP or with CrMP alone. Interestingly, the coadministration of hemin and CrMP reversed the antidiabetic effect of hemin, whereas CrMP alone further exacerbated hyperglycemia (Fig. 1, A and C), suggesting an important role for basal HO activity in glucose homeostasis and metabolism. The vehicle had no effect on blood glycemia. Hemin therapy was more effective against fasting hyperglycemia. The reason for this selective effect needs to be further investigated.

The effect of the hemin regimen against both fasting and postprandial hyperglycemia was gradual and consistent throughout the 4-wk treatment period (Fig. 1, B and D). The fasting and postprandial levels were 21.2 ± 2.5 and 32.9 ± 2.7 mmol/l, respectively, before therapy but progressively decreased to 8.5 ± 0.6 and 11.9 ± 1.2 mmol/l, respectively, by the fourth week of treatment (Fig. 1, B and D). The application of hemin therapy to SD rats also lowered fasting (7.0 ± 0.6 vs. 6.05 ± 0.4 mmol/l; P < 0.05, n = 8) and postprandial (9.3 ± 0.4 vs. 7.1 ± 0.3 mmol/l; P < 0.01, n = 8) glucose; however, the antidiabetic effect was less intense than in STZ-diabetic rats.

Table 1. Effect of hemin therapy on body weight

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD (9 wk)</td>
<td>375±8</td>
</tr>
<tr>
<td>STZ (9 wk)</td>
<td>357.9±5</td>
</tr>
<tr>
<td>SD (13 wk)</td>
<td>416±4</td>
</tr>
<tr>
<td>STZ (13 wk)</td>
<td>390±7</td>
</tr>
<tr>
<td>STZ + hemin (13 wk)</td>
<td>379±5±</td>
</tr>
<tr>
<td>STZ + hemin + CrMP (13 wk)</td>
<td>374±6</td>
</tr>
<tr>
<td>STZ + CrMP (13 wk)</td>
<td>381±5±</td>
</tr>
<tr>
<td>SD + hemin (13 wk)</td>
<td>402±4</td>
</tr>
<tr>
<td>SD (21 wk)</td>
<td>493±5</td>
</tr>
<tr>
<td>STZ (21 wk)</td>
<td>481±7</td>
</tr>
<tr>
<td>STZ posthemin (21 wk)</td>
<td>479±6</td>
</tr>
<tr>
<td>STZ + hemin (21 wk)</td>
<td>484±8</td>
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</tbody>
</table>

Values are means ± SE for n = 6-12 rats per group. SD, Sprague-Dawley (control); STZ, streptozotocin-induced diabetic; CrMP, chromium mesoporphyrin. *P < 0.05 vs. age-matched SD rats; †P < 0.05 vs. STZ rats.

The induction of diabetes by STZ caused loss of body weight (Table 1). Similarly, the application of hemin alone or together with CrMP further reduced body weight slightly compared with STZ (Table 1). However, by 1 mo after therapy the hemin- and CrMP + hemin-treated STZ rats had body weights comparable to those of untreated STZ rats, although the weights were still lower than those of age-matched control SD rats. STZ is well known to cause loss of body weight and/or retard growth (11, 52). It is unlikely that the body weight loss is responsible for the antidiabetic effect since fasting/postprandial glucose levels were reduced only in hemin-treated STZ rats (Table 1). Similarly, comparable body weights were observed at 2 mo after therapy, but the antidiabetic effect was evident only in the hemin-treated STZ rats. The fasting/postprandial glycemic levels were reduced only in hemin-treated STZ rats, while CrMP abolished the effect of hemin. Values are means ± SE; n = 8 rats/group.

Fig. 2. Effects of hemin and the HO inhibitor CrMP on HO-1, HO activity, and cGMP in soleus muscle of STZ-diabetic rats. A: basal HO-1 concentration in STZ rats was lower than in euglycemic Sprague-Dawley (SD) control rats. Hemin therapy greatly increased HO activity in STZ rats, but the HO blocker CrMP nullified the hemin effect. B: HO activity in STZ rats was lower than in control rats and was increased by hemin, whereas CrMP blocked the effect of hemin. C: treatment with hemin markedly increased the depressed cGMP content of STZ rats, while CrMP abolished the effect of hemin. Values are means ± SE; n = 8 rats/group.
prandial glucose levels in the animals at 2 mo after therapy and in age-matched untreated STZ rats were (in mmol/l) 10.8 ± 0.7 vs. 19.8 ± 0.7 (n = 6, P < 0.01) and 17.6 ± 1.8 vs. 28.4 ± 2.2 (n = 6, P < 0.01), respectively.

Hemin therapy potentiated HO-1, HO activity, and cGMP in STZ-diabetic rats. To understand the mechanisms underlying the antidiabetic effects of hemin, we measured the concentration of HO-1 and HO activity. In hyperglycemic STZ rats, basal HO-1 and HO activity were depressed compared with euglycemic SD control rats. Interestingly, hemin increased HO-1 concentration and HO activity in STZ rats by 8.3- and 8.4-fold, respectively (Fig. 2, A and B). The enhanced HO activity would increase endogenous carbon monoxide (CO) that would in turn enhance cGMP (46), a substance implicated in the regulation of insulin (42) and GLUT4 signaling (36), and thus glucose metabolism. Hemin also enhanced HO-1 and HO activity in SD rats by 5.3- and 4.7-fold, respectively. The HO-induced increase of HO-1 and HO activity was significantly greater in STZ rats than in SD rats.

To further evaluate the importance of the basal HO input to glucose metabolism, we gave the HO inhibitor CrMP alone or with hemin to some STZ-diabetic animals. Treatment with CrMP+hemin abolished the hemin-dependent increase of HO-1, HO activity, and cGMP, with corresponding reversal of the antidiabetic effects, while CrMP alone further increased hyperglycemia. Importantly, the CrMP-induced exacerbation of hyperglycemia was accompanied by further reduction of basal HO-1, HO activity, and cGMP in STZ rats (Fig. 2), suggesting an important contribution of basal HO-cGMP signaling in glucose metabolism and homeostasis. Interestingly, after termination of therapy for 2 mo, HO activity and cGMP remained significantly elevated. Since cGMP reg-

![A](https://www.ajpendo.org/ajpendo/figures/A.png)  ![B](https://www.ajpendo.org/ajpendo/figures/B.png)  ![C](https://www.ajpendo.org/ajpendo/figures/C.png)  ![D](https://www.ajpendo.org/ajpendo/figures/D.png)  ![E](https://www.ajpendo.org/ajpendo/figures/E.png)  ![F](https://www.ajpendo.org/ajpendo/figures/F.png)

**Fig. 3.** Effects of hemin and CrMP on oxidative stress in STZ-diabetic rats. Hemin therapy increased plasma levels of bilirubin (A), ferritin (B), and superoxide dismutase (C) and total antioxidant capacity (D), whereas CrMP abolished the effects of hemin. Treatment with hemin abated soleus muscle (E) and urinary (F) 8-isoprostane in STZ-diabetic rats. Values are means ± SE; n = 6–8 rats per group. TEA C, Trolox equivalent antioxidant capacity.
ulates insulin release (42) and enhances skeletal muscle GLUT4 (36), it is possible that the improved glucose metabolism and insulin sensitivity in hemin-treated STZ rats may be attributed, at least in part, to the sustained elevation of cGMP. The higher magnitude of HO activity and cGMP in STZ after hemin therapy (Fig. 2) may be responsible for the more intense antidiabetic effect in STZ rats. Alternatively, the less preponderant increase in HO activity in SD may suggest greater stability of the HO system in euglycemic conditions.

**Upregulating HO system suppressed oxidative stress in STZ-diabetic rats.** Given that the HO system enhances antioxidants (59), we measured bilirubin, ferritin, and SOD. The basal levels of plasma bilirubin, ferritin, and SOD in STZ-diabetic rats were significantly depressed (Fig. 3, A–C). Interestingly, hemin therapy increased bilirubin, ferritin, and SOD by 2.4-, 2.6-, and 2.2-fold, respectively, with a corresponding increase of the total antioxidant capacity (Fig. 3, A–D). A similar increment was also observed in hemin-treated SD, although the percentage of increase observed in hemin-treated STZ was greater. In contrast, the HO inhibitor CrMP abolished the potentiation of antioxidants. It is important to note that the hemin-induced increments of antioxidants were sustained for 2 mo after termination of therapy (Fig. 3).

Since hyperglycemia is a major trigger of oxidative stress, we investigated whether the hemin-dependent antidiabetic effect would be accompanied by reduced levels of the oxidative stress marker 8-isoprostane. While levels of urinary 8-isoprostane reflect systemic oxidative stress, soleus muscle 8-isoprostane indicates tissue-specific oxidative stress. The levels of urinary and soleus muscle 8-isoprostane were markedly elevated in STZ-diabetic rats (Fig. 3, E and F). However, hemin therapy reduced urinary and soleus muscle 8-isoprostane to control levels. Hemin also reduced 8-isoprostane in SD rats, although to a lesser extent compared with STZ rats. In contrast, the HO inhibitor CrMP nullified the antioxidant effect of hemin by enhancing 8-isoprostane. The hemin-dependent reduction of 8-isoprostane remained evident for 2 mo after termination of therapy.

**Hemin therapy abated soleus muscle NF-κB, AP-1, AP-2, and JNK but increased plasma adiponectin and insulin.** Many transcription factors reduce insulin signaling in diabetes (8, 28). In hyperglycemic STZ rats, quantitative real-time RT-PCR

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**Fig. 4.** Effects of hemin and CrMP on soleus muscle nuclear factor (NF)-κB, activating (AP)-1, AP-2, and c-Jun NH₂-terminal kinase (JNK) and plasma insulin and adiponectin of STZ-diabetic rats. Quantitative real-time RT-PCR indicated that hemin therapy abated the elevated basal mRNA expression of NF-κB (A), AP-1 (B) AP-2 (C), and JNK (D) in the soleus muscle, but CrMP annulled the hemin effect. Treatment with hemin enhanced plasma insulin (E) and adiponectin (F), while CrMP abolished the effect of hemin. Values are means ± SE; *n = 6 rats/group.
analyses indicated that the levels of NF-κB, AP-1, and AP-2 in the soleus muscle were significantly elevated (Fig. 4, A–C). Interestingly, hemin therapy reduced NF-κB, AP-1, and AP-2 by 3.9-, 3.1-, and 2.6-fold, respectively, while the HO inhibitor CrMP abolished the effects of hemin. It is worthwhile to note that hemin effectively restored NF-κB to control levels, while AP-1 and AP-2 were partially reduced, suggesting greater sensitivity for NF-κB. The reasons for this selective effect remain unclear and should be further investigated.

Given that JNK inhibits insulin biosynthesis (28), we evaluated the effects of hemin on JNK and insulin levels. Importantly, we detected increased levels of JNK in the soleus muscle of STZ, which were abated by hemin although control levels were not reinstated (Fig. 4D). Interestingly, the attenuation of JNK in hemin-treated STZ was accompanied by a parallel increase of plasma insulin (Fig. 4E), whereas CrMP reversed the effects of hemin.

Since oxidative stress reduces adiponectin levels (26, 53), and adiponectin deficiency is among the causes of insulin resistance (18, 31), we investigated whether the suppression of oxidative stress by hemin would affect plasma adiponectin. In STZ-diabetic rats, the levels of plasma adiponectin were significantly reduced compared with control rats (Fig. 4F). However, hemin therapy greatly enhanced adiponectin by 3.2-fold, whereas the HO blocker CrMP abolished the hemin-induced increase of adiponectin. Importantly, the concomitant reduction of NF-κB, AP-1, AP-2, and JNK and the enhancement of adiponectin/insulin in hemin-treated STZ rats were sustained for the 2-mo stoppage of therapy, suggesting a role of these pathways in the long-lasting antidiabetic effect of hemin.

Hemin therapy reduced protein expression of NF-κB, AP-1, and JNK in soleus muscle. To further clarify whether the hemin-induced reduction of NF-κB, AP-1, and JNK mRNA expressions was accompanied by a parallel reduction of the corresponding proteins, we used Western immunoblotting to detect the protein expressions of NF-κB, AP-1, and JNK (Fig. 5). Interestingly, Western immunoblot and quantitative analysis of the expressed proteins normalized by β-actin revealed that hemin therapy significantly attenuated the protein expressions of NF-κB, AP-1, and JNK in the soleus muscle of STZ rats. It is important to note that the effect of hemin on the mRNA and protein levels of NF-κB and AP-1 were consistent, whereas hemin had a greater effect on JNK protein compared with JNK mRNA. The reasons for this selective efficacy remain unclear and should be further investigated.

Hemin therapy enhanced cAMP, AMPK, and GLUT4 in soleus muscle of STZ rats. AMPK, GLUT4, and cAMP are important agents that enhance insulin signaling and glucose metabolism (17, 22, 42). In hyperglycemic STZ rats, the levels of cAMP, AMPK, and GLUT4 were significantly depressed (Fig. 6). However, hemin therapy robustly increased cAMP, AMPK, and GLUT4 by 4.2-, 2.8-, and 3.8-fold, respectively, whereas CrMP abrogated the antidiabetic effects of hemin.

Since adiponectin enhances insulin sensitization and improves glucose metabolism (31) by activating AMPK (17, 62), which increases glucose transport by stimulating GLUT4 (17, 22), the sustained levels of AMPK and GLUT4 in hemin-treated STZ rats (Fig. 6, B–D) that lasted 2 mo after stoppage of therapy would, at least in part, account for the enduring antidiabetic effect. Similarly, sustained enhancement of cAMP in hemin-treated STZ rats (Fig. 6A) would improve glucose metabolism and thus counteract fasting and postprandial hyperglycemia given that cAMP mediates insulin release (42). Importantly, hemin therapy concomitantly enhanced GLUT4 mRNA and protein levels (Fig. 6, C and D). Since the stimulation of glucose uptake by insulin in muscle requires the translocation of GLUT4 from intracellular storage sites to the cell surface (64), the hemin-mediated increase in GLUT4 is an important revelation from our study.
Hemin therapy improves glucose and insulin tolerance but enhances insulin sensitivity in STZ rats. Since elevated oxidative stress contributes to insulin resistance and glucose tolerance, we investigated whether the potentiation of the overall antioxidant status and the reduction of hyperglycemia in hemin-treated STZ rats would be accompanied by improved glucose/insulin tolerance. Interestingly, in STZ and STZ+hemin+CrMP groups, IPGTT analyses indicated that the levels of plasma glucose were significantly elevated at all time points tested over those in the STZ+hemin and STZ-posthemin groups (Fig. 7A), suggesting that hemin therapy improved glucose intolerance in STZ. Interestingly, a bolus injection of glucose markedly stimulated insulin release in STZ+hemin and STZ-posthemin (Fig. 7B), whereas the levels of glucose-stimulated insulin in the STZ+hemin and STZ-posthemin groups remained significantly low at all time points during the IPGTT analyses. Importantly, two distinct phases of insulin release were observed in hemin-treated animals: an acute-phase or first-phase response (0–30 min) and a second-phase response characterized by gradual decrease and subsequent return to fasting levels by 90 min, and remaining constant at that level for the entire duration of the experiment. In contrast, in the STZ and STZ+hemin+CrMP group the two phases of glucose-stimulated insulin release were less distinguishable. Thus upregulating the HO system with hemin attenuates insulin intolerance. Correspondingly, IPITT analyses indicated enhanced insulin sensitivity in hemin-treated animals (Fig. 7C). Importantly, a rapid reduction of glucose levels was observed in the STZ+hemin and STZ-posthemin groups after insulin challenge, suggesting the enhancement of insulin sensitivity (Fig. 7C), whereas only a slight reduction of glucose was observed in the STZ and STZ+hemin+CrMP groups.

To further investigate the effect of hemin therapy on insulin sensitivity, HOMA-IR analysis was done. Interestingly, HOMA-IR analysis revealed that insulin resistance in the STZ+hemin and STZ-posthemin groups was markedly reduced compared with the elevated levels of the STZ and STZ+hemin+CrMP groups (Fig. 7D), suggesting greater insulin sensitivity in hemin-treated animals.

Hemin therapy reduced histopathological lesions of pancreas. In STZ-diabetic rats, severe inflammation was evident (Fig. 8). This was characterized by significant elevation of mononuclear cell infiltration, increased interstitial edema, and more pronounced acinar cell necrosis and fibrosis (Table 2). The massive infiltration of mononuclear cells in pancreatic tissues from STZ rats was accompanied by the disappearance of acinar cells, which were replaced with fibrous tissue. Interestingly, hemin therapy markedly reduced these lesions, albeit control levels were not attained. Similarly, hemin therapy greatly reduced vacuolization in STZ-diabetic rats. In contrast, the HO blocker CrMP abolished the protective effects of hemin and exacerbated the histopathological damage in STZ-diabetic rats.

Fig. 6. Effects of hemin and CrMP on cAMP, adenosine monophosphate-activated protein kinase (AMPK), and glucose transporter (GLUT)4 in the soleus muscle of STZ-diabetic rats. A: hemin therapy robustly enhanced cAMP in the soleus muscle, while CrMP blocked the effect of hemin. B: Western immunoblot of soleus muscle AMPK and quantitative analyses of relative AMPK levels normalized by β-actin reveal that hemin therapy significantly enhanced AMPK levels in STZ rats. C: quantitative real-time RT-PCR indicated that hemin therapy greatly enhanced GLUT4 expression in the soleus muscle of STZ rats, while CrMP reversed the hemin effect. D: Western immunoblot of soleus muscle GLUT4 and quantitative analyses of relative GLUT4 levels normalized by β-actin reveal that hemin therapy significantly enhanced GLUT4 levels in STZ rats. Values are means ± SE; n = 4–6 rats/group.
The present study demonstrates the enduring antidiabetic effect of hemin against fasting and postprandial hyperglycemia in STZ-induced diabetes. The mechanisms involved include upregulation of the HO system, cAMP, and cGMP, along with enhancement of antioxidants such as SOD, bilirubin, and ferritin with potentiation of the total antioxidant capacity. Correspondingly, marker/mediators of oxidative injury like 8-isoprostane, NF-κB, AP-1, AP-2, and JNK were reduced and pancreatic histopathological lesions including acinar cell necrosis, interstitial edema, vacuolization, fibrosis, and mononuclear cell infiltration were significantly attenuated. The concomitant improvement of pancreatic morphology along with the reduction of JNK and the enhancement of cAMP and cGMP contributed to increasing insulin production (28, 42). Interestingly, the increased insulin production in hemin-treated animals was accompanied by a concomitant and paradoxical enhancement of insulin sensitization. Accordingly, in hemin-treated animals, glucose tolerance (IPGTT) was improved, insulin intolerance (IPITT) reduced, and insulin resistance (HOMA-IR index) decreased. Importantly, the improvement of insulin sensitivity was associated with enhanced expression of GLUT4, an essential protein for glucose uptake. The effect of hemin on GLUT4 is a novel and intriguing observation. Reduced GLUT4 is implicated in insulin resistance and impaired glucose metabolism (13, 64). Therefore, increased GLUT4 along with the reduction of glucose/insulin intolerance are important mechanisms responsible for the sustained antidiabetic effect of hemin. Moreover, hemin also enhanced adiponectin, an insulin-sensitizing protein that stimulates AMPK to enhance glucose transport via GLUT4 (17, 22, 62). Given that a cGMP-dependent mechanism has been shown to increase GLUT4 (36), the increased levels of cGMP observed in our study may account for the enhancement of GLUT4. Alternatively, the hemin-induced reduction of NF-κB may improve GLUT4 signaling since the suppression of NF-κB is associated with insulin-stimulated phosphorylation of Akt and the translocation of GLUT4 (61). Thus our study unveils the multifaceted interaction between the HO system and insulin-sensitizing and insulin-inhibitory pathways. Therefore, upregulating the HO system with hemin potentiates insulin-sensitizing pathways like adiponectin, AMPK, and GLUT4 but abates insulin-inhibitory pathways like NF-κB and JNK (28) in the soleus muscle. In contrast, the HO inhibitor CrMP suppressed HO activity and reduced adiponectin, AMPK, and GLUT4 to annul the antidiabetic effects of hemin. Although many HO inhibitors are nonspecific and affect other hemoenzymes or even increase HO-1 (59), CrMP given at a dose of 4 μmol/kg is reportedly selective for HO (60).

Hemin therapy also increased HO-1, HO activity, and cGMP in SD rats, although to a lesser magnitude than in STZ rats. Since SD rats are healthy animals with normal glycemia, the HO system may be acting in conjunction with other pathways to control blood glucose. Therefore, the less intense effect of the HO system in SD rats might not have altered other healthy/functional pathways that act in concert with HO to regulate glucose metabolism. Whether this is an intrinsic homeostatic and/or defensive mechanism to maintain healthy conditions in SD rats within a certain physiological range remains unclear and needs to be clarified in future studies. On
the other hand, the greater potentiation of HO signaling in STZ rats was accompanied by more intense antidiabetic effect, suggesting that the HO system in STZ rats may be more susceptible to pharmacological manipulations. Therefore, hemin therapy may have greater selectivity for diabetic conditions. Moreover, because STZ rats are diabetic/unhealthy, the greater increment of HO signaling may be necessary to attain the threshold that initiates the restoration of glucose metabolism. This notion is consistent with previous studies in which upregulation of the HO system improved glucose metabolism (41).

Table 2. Effect of hemin therapy on morphological parameters of pancreas

<table>
<thead>
<tr>
<th>Morphological Analyses</th>
<th>SD (n = 6)</th>
<th>STZ (n = 6)</th>
<th>STZ + Hemin (n = 6)</th>
<th>STZ + Hemin + CrMP (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial edema</td>
<td>0 ± 0</td>
<td>2.67 ± 0.21*</td>
<td>1.0 ± 0.22‡</td>
<td>2.83 ± 0.17*</td>
</tr>
<tr>
<td>Vacular formation</td>
<td>0.17 ± 0.17</td>
<td>3.0 ± 0.00*</td>
<td>0.57 ± 0.43‡</td>
<td>3.0 ± 0.00*</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>0 ± 0</td>
<td>3.0 ± 0.00*</td>
<td>1.14 ± 0.14†</td>
<td>2.83 ± 0.17*</td>
</tr>
<tr>
<td>Acinar cell necrosis</td>
<td>0 ± 0</td>
<td>2.67 ± 0.21*</td>
<td>0.57 ± 0.43‡</td>
<td>2.67 ± 0.21*</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.17 ± 0.17</td>
<td>3.00 ± 0.00*</td>
<td>1.0 ± 0.22‡</td>
<td>3.00 ± 0.00*</td>
</tr>
</tbody>
</table>

Values are means ± SE for n rats per group. Hemin significantly reduced interstitial edema, vacular formation, inflammatory cell infiltration, acinar cell necrosis, and fibrosis in the pancreas of STZ-diabetic rats. Assessment was done in a blinded manner according to a scale from 0 to 3, with 0 = normal, 1 = mild, 2 = moderate, and 3 = severe. *P < 0.01 vs. all groups; †P < 0.05, ‡P < 0.01 vs. all groups.
and type 2 diabetes (39, 50). The suppression of NF-κB, AP-1, and AP-2 was accompanied by corresponding reduction of markers of oxidative stress like 8-isoprostane. Importantly, the hemin-dependent attenuation of oxidative stress could be linked to concomitant enhancement of antioxidants, including SOD, bilirubin, and ferritin, with subsequent potentiation of the total antioxidant status in STZ-diabetic rats. In tissues, the antioxidant system comprises different antioxidants such as SOD, catalase, glutathione peroxidase, ferritin, ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, biliverdin, bilirubin, etc (5, 6, 21, 24, 55), and the sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system (5). The additive effect of all the different antioxidants provides greater protection against oxidative stress than any single compound alone. Therefore, in hemin-treated STZ rats, reduced oxidative stress coupled to enhanced adiponectin, AMPK, and GLUT4 may lead to improved insulin sensitivity. Consistent with this, HO-1 gene has been associated with insulin sensitization and cellular defense against oxidative stress (9). Moreover, in diabetic conditions, the oxidative destruction of adiponectin and insulin (26, 28) may compromise glucose metabolism. Oxidative stress depletes adiponectin mRNA and thus reduces adiponectin levels (26, 53), suggesting the susceptibility of adipocytes to oxidative destruction. However, the potentiation of the HO system by hemin suppressed oxidative stress to prevent the oxidative destruction of adiponectin and insulin. The hemin-mediated increase in adiponectin is consistent with recent studies showing the enhancement of adiponectin by another HO inducer, cobalt protoporphyrin (CoPP), in Zucker fatty rats and obese diabetic mice (30, 32, 35).

The role of the HO system in the regulation of insulin release has been widely acknowledged (19, 20, 41). Depressed HO status was detected in pancreatic islets of Goto-Kakizaki rats (41). The defective HO system was corrected by hemin or CO, suggesting an important role of these substances in glucose metabolism (19, 38). Moreover, pancreatic β-cells produce CO to regulate insulin and glucagon secretion and thus glucose metabolism (19). Similarly, glucose was shown to stimulate the production of CO by pancreatic islets, which in turn triggered insulin release (19), and to protect/preserve β-cell vitality (63). Thus CO generated by the HO system along with the hemin-dependent reduction of JNK are important mechanisms that may account for the increase in plasma insulin and the corresponding reduction of fasting/postprandial hyperglycemia. The abrogation of JNK by hemin is consistent with reports indicating that the HO system abates JNK activity (12). Although several studies indicate that the HO system increases insulin levels (19, 38, 41), some conflicting observations have been reported. A recent study showed that the HO inducer CoPP did not increase insulin levels in obese diabetic mice (35). Although the reason for this discrepancy remains unclear, further investigations are needed to verify whether the different effects of hemin and CoPP on insulin are strain dependent and/or specific to the type of HO inducers. Our study also indicates loss of body weight in hemin-treated STZ rats. The 4-wk hemin regimen slightly decreased body weight in STZ rats. However, it is unlikely that the loss of body weight may account for the sustained antidiabetic effect. At 2 mo after therapy there was no significant difference in body weight between hemin-treated and untreated STZ rats, but the antidiabetic effect prevailed only in the hemin-treated animals. Moreover, STZ is a compound that causes loss of body weight and/or retards growth (11, 52). It is unlikely that hemin therapy was toxic because we recently showed (43) that indexes of toxicity including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyltransferase (γGT) remained unchanged after hemin therapy.

The suppression of markers/mediators of oxidative stress including 8-isoprostane, NF-κB, JNK, AP-1, and AP-2 has provided additional testimony for the hemin-dependent normalization of the oxidative environment associated with diabetes and insulin resistance. Moreover, the presence of binding sites for NF-κB, AP-1, AP-2, and glucocorticoid-responsive elements (33) in HO-1 gene promoter may be indicative of important roles in many cellular events (51) including metabolism. Given that glucocorticoids regulate glucose metabolism and insulin resistance (37), the HO system may not only suppress inflammatory/oxidative transcription factors to limit tissue insults (51) but also regulate other glucose metabolism (9, 19) via the glucocorticoid-responsive elements (33) of the HO-1 gene. Interestingly, high glucose levels are among the different stimuli that induce HO-1 (25, 29). Accordingly, the diversity of HO inducers may be indicative of multiple regulatory elements for the HO-1 gene with binding sites for different transcription factors or genes. These arrays of genes may account for the pleiotropic role of HO-1 in different cellular processes including glucose metabolism and defense. However, further investigations are needed to clarify how HO-1 and the genes modified by it modulate insulin secretion and insulin sensitization and improve glucose metabolism in type 1 and type 2 diabetes.

Collectively, our study unveils the potent effect of hemin against fasting/postprandial hyperglycemia in STZ-induced diabetes and suggests that the potentiation of antioxidants and subsequent decline of JNK, NF-κB, AP-1, AP-2, and 8-isoprostane resulted in the attenuation of histopathological lesions, and thus underscores the important role of the HO system in the maintenance and preservation of intact pancreatic architecture to safeguard the insulin-producing capability of β-cells. Correspondingly, increased insulin production was accompanied by the paradoxical enhancement of insulin sensitization and thus improved glucose metabolism.

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GRANTS

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REFERENCES

Heme oxygenase enhances insulin sensitivity


