The heme oxygenase system attenuates pancreatic lesions and improves insulin sensitivity and glucose metabolism in deoxycorticosterone acetate hypertension

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Ndisang JF, Jadhav A. The heme oxygenase system attenuates pancreatic lesions and improves insulin sensitivity and glucose metabolism in deoxycorticosterone acetate hypertension. Am J Physiol Regul Integr Comp Physiol 298: R211–R223, 2010. First published October 28, 2009; doi:10.1152/ajpregu.91000.2008.—Recent clinical reports indicate that impaired glucose tolerance is a common phenomenon in primary aldosteronism. Aldosterone stimulates NF-κB and activating protein-1 (AP-1) to cause oxidative injury. Elevated oxidative stress impairs insulin signaling. We recently showed that the heme oxygenase (HO) system lowers blood pressure (BP) in deoxycorticosterone-acetate (DOCA)+salt hypertension, a model of primary aldosteronism. However, the effect of the HO system on insulin sensitivity in this model remains largely unclear. Here we report the effects of the HO-inducer hemin and the HO-blocker [chromium mesoporphyrin (CrMP)] on insulin sensitivity/glucose metabolism. Our experimental design included the following 10 groups: (A) controls [(i) surgery-free or normal Sprague-Dawley (SD), (ii) uninephrectomized (UnX)-sham, (iii) UnX+salt (0.9%NaCl+0.2%KCl)]; (B) DOCA+salt; (C) hemin+DOCA+salt; (D) hemin+CrMP+DOCA+salt; (E) CrMP+DOCA+salt; (F) vehicle-treated rats and (G) normal SD+hemin. Hemin therapy lowered BP and increased plasma insulin and the insulin-sensitizing protein adiponectin with slight but significant reduction of glycemia, while CrMP abolished the hemin effects. Furthermore, hemin improved intraperitoneal glucose and insulin tolerance, suggesting that although DOCA+salt-hypertensive rats were normoglycemic, insulin signaling may be impaired. In contrast, the HO-inhibitor CrMP aggravated insulin resistance and exacerbated glucose and insulin tolerance. Interestingly, the enhanced insulin sensitivity in hemin-treated animals was accompanied by reduced urinary/gastrocnemius muscle 8-iso-prostaglandin F₂α (8-isoprostane), inflammatory/oxidative transcription factors like NF-κB, AP-1, JNK, and heme content, whereas HO-1, HO-activity, cGMP, and plasma/gastrocnemius muscle antioxidants including bilirubin, ferritin, SOD, catalase, and the total antioxidant capacity were increased. Similarly, hemin enhanced pancreatic HO, cGMP, and cAMP but suppressed 8-isoprostane and attenuated pancreatic histopathological lesions including fibrosis, interstitial edema, acinar cell necrosis, vacuolization, and mononuclear cell infiltration, with corresponding improvement of insulin production. Our results suggest that impaired insulin signaling may be a forerunner to hyperglycemia in aldosteronism. By preserving pancreatic morphology, potentiating insulin signaling, and lowering BP, the HO system may prevent metabolic and cardiovascular complications in aldosteronism.

DOCA+salt hypertension; insulin sensitivity; oxidative stress; adiponectin.

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muscles (58). Besides its antioxidant effects, the HO system modulates other cellular activities, such as insulin release (40, 43). Given that HO-1 is activated by a wide variety of physical/chemical stimuli including glucose (23, 24), the HO system may be important for the regulation of glucose metabolism. Although recent evidence underscores the role of the HO system in diabetes, no study has investigated the effects of HO on insulin sensitivity in DOCA hypertension, an animal model of human primary aldosteronism (53). We hypothesize that an upregulated HO system will suppress NF-κB, AP-1, and JNK but enhance adiponectin and improve insulin signaling in DOCA-hypertension. Therefore, this study will delineate the role of the HO system on insulin signaling and glucose metabolism in mineralocorticoid-induced hypertension.

MATERIALS AND METHODS

Animals and treatment groups. Our experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics, which is in conformity with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats (SD) of 8 wk of age were purchased from Charles River Laboratories (Willington, MA). The animals were housed at 21°C with 12:12-h light-dark cycles, fed with standard laboratory chow, and had access to drinking water ad libitum. After a week of acclimatization, DOCA+salt hypertension was induced in some animals as previously described (28, 29, 47). Our experimental protocol included the following 10 groups (n = 6–29 per group): (A) controls [(i) surgery-free or normal SD rats], (ii) SD rats with one kidney removed or uninephrectomized (UnX)-sham, (iii) UnX rats that received water containing 0.9% NaCl + 0.2% KCl (UnX+salt), and (iv) UnX rats implanted subcutaneously at the midscapular region with a Silastic strip impregnated with DOCA (100 mg/kg body wt, Sigma-Aldrich, St. Louis, MO) (UnX+DOCA); (B) UnX rats implanted with DOCA-stripe that received water containing 0.9% NaCl + 0.2% KCl + 0.2% Na (UnX+DOCA+salt hypertensive rats), which for simplicity would be referred to as DOCA+salt; (C) DOCA+salt rats treated with (D) the HO inducer, hemin or (E) heme together with the HO blocker, chromium mesoporphyrin (CrMP), or (F) CrMP alone, or (G) the vehicle dissolving heme and CrMP. In addition, normal SD rats were given (H) heme (SD+hemin).

The involvement of the HO system in the heme-induced insulin-sensitizing/antidiabetic effect was ascertained by giving 4 μmol/kg of the HO blocker CrMP (2.61 mg/kg ip; Porphyrin Products) with or without heme (30 mg/kg ip; Sigma-Aldrich) (50, 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 (28, 29, 47). Treatment with heme, CrMP, or the vehicle-dissolving heme and CrMP began after the animals were severely hypertensive with a blood pressure of 196.2 ± 2.4 mmHg recorded 4 wk after the surgical implantation of DOCA strips. Each injection was 0.5 ml and was given twice weekly for 4 wk. Fasting glucose was monitored weekly with a glucose meter (BD, Franklin Lakes, NJ) after overnight fasting in metabolic cages. Systolic blood pressure was determined by inculating at 65°C for 1 min followed by a 1°C per minute rise over 30 min.

Determination of plasma adiponectin. Adiponectin was measured by ELISA (Phoenix Pharmaceuticals) as we previously reported (45, 46, 48, 49). Briefly, blood samples were centrifuged, and the plasma aliquoted into wells of a microplate containing adiponectin antibody. After treatment with horseradish peroxidase-conjugated secondary antibody and streptavidin, the absorbance at 450 nm was read with a microplate (SpectraMax-340PC; Molecular Devices).

Histological and morphological analyses of pancreas. Pancreatic tissue obtained from duodenal and splenic lobes were fixed in 10% Tissues not used immediately were snap frozen in liquid nitrogen and stored at −80°C for biochemical assays.

Determination of HO activity, HO-1 concentration, and heme content. HO activity in the pancreas and gastrocnemius muscle was measured as bilirubin production using our established method (29, 47). In brief, tissues were homogenized on ice in 4 volumes of 5.1 K/Na and 100 mmol/l phosphate buffer with 2 mmol/l MgCl2 (HO-activity buffer) and 0.25 M sucrose solution and then centrifuged at 27,000 g for 15 min. Aliquots of 100 μl were collected from the supernatant and transferred into another beaker containing 500 μl of a mixture of 0.8 mmol/l nicotinamide disulfoxide phosphate, 20 μmol/l hemin, 2 mmol/l glucose-6-phosphate, 0.002 U/ml glucose-6-phosphate dehydrogenase, and 100 μl liver cytosol as the source of biliverdin reductase. The reaction was carried out in darkness for 1 h at 37°C and was stopped by adding 500 μl of chloroform. Subsequently, the tubes were agitated vigorously and centrifuged at 27,000 g for 5 min to extract bilirubin. The chloroform layer was collected and read on a spectrophotometer at 464 nm minus the background at 530 nm. The amount of bilirubin in each sample was determined spectrophotometrically (extinction coefficient for bilirubin 40 μM/ cm) and expressed as nanomoles per milligram protein per hour. The protein content was measured using the Bradford assay. Spleen tissue was used as a positive control.

Gastrocnemius muscle and pancreatic HO-1 levels were determined by ELISA (EKS-810A; Stressgen-Assay Design), while heme was assessed using QuantiChrom Heme Assay kit (model DIHM-25; BioAssay Systems) following the manufacturer’s instructions we have previously described (29).

Total RNA isolation and quantitative real-time RT-PCR. This was performed as we previously reported (45, 46, 48, 49). The gastrocnemius muscle was homogenized in 0.5 ml Trizol reagent (Invitrogen Life Technologies) according to the manufacturer’s specifications. Reverse transcription was carried out using First Strand cDNA Synthesis Kit (Novagen) with 0.5 μg oligo(dT)6, 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 75 mM KCl, 3 mM MgCl2, 50 mM DTT, 10 mM each free dNTP and 100 U of Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instruction. Quantitative PCR was done with real-time PCR system (model 7300; Applied Biosystems, Foster City, CA) and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 50 mM KCl: 20 mM Tris-HCl (pH 8.4); 0.2 mM each of free dNTP, hot start enzyme iQTag DNA polymerase (25 U/ml), 3 mM MgCl2, SYBR Green 1; and 10 μM cDNA as reference. Triplicate samples containing 1 μl of cDNA were assayed using a template of 3.2 pmol of primers for p65-NF-κB (forward, 5’-CATGCTTTCCGGATCAAGTGCGCA-3’ and reverse, 5’-TGTTTGTGCTTTAGTGGTGATCTGT-3’); AP-1 (forward, 5’-ACGACATGCTG TAGTGTTAGAGCAGC-3’ and reverse, 5’-TCCATGCGTTCCTTGGTCTAGTGTGT-3’); JNK (forward, 5’-AAGCAGCAAGGCTTACTCCCTTCTCA-3’ and reverse, 5’-ATCGGAGAATCGTGTCGTGGTCTGTA-3’); and β-actin (forward, 5’-TCTTCATGGTTCCTCCTGTCTTGT-3’ and reverse, 5’-ACAGCAGCTGTGTTGCGATAGGTGCT-3’) in a final volume of 25 μl. The National Research Council of Canada, Saskatoon, confirmed the identification of all primers used. The program for the thermal cycle was 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 56°C, and 15 s at 72°C. The melting points of the PCR product were determined by incubating at 65°C for 1 min followed by a 1°C per minute rise over 30 min.

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formalin phosphate buffer for 48 h, processed, and paraffin embedded. Sections of 5-μm thicknesses were then cut and stained with hematoxylin and eosin for histological analysis as we previously reported (45). Whole pancreatic sections were examined for acinar cell necrosis, vacuolization, interstitial edema, fibrosis, and mononuclear cell infiltration by a pathologist. In addition, morphologic evaluation of acinar cell necrosis, vacuolization, interstitial edema, fibrosis, and mononuclear cell infiltration were blindly done in 20 randomly selected fields using light microscopy (Olympus BH-2; Olympus, Tokyo) semiquantiatively with 0 to 3 scales (0, normal or almost normal; 1, mild; 2, moderate; 3, severe) in each pancreatic section, and the mean score was calculated as we previously reported (45). The images were captured with a Nikon, Eclipse, 80i microscope equipped with a digital camera using the NIS-elements BR-Q imaging software.

**Determination of glucose and insulin tolerance.** Glucose tolerance was assessed by an intraperitoneal glucose tolerance test (IPGTT) after overnight fasting as we previously reported (45, 46, 48, 49). In brief, a bolus of glucose (2 g/kg ip) was injected and under anesthesia blood samples were collected from the tail vein at intervals of 0, 30, 60, 90, 120 min and tested for glucose and insulin. Plasma glucose was measured using an auto-analyzer (BD), while insulin was assayed by ELISA (Ultrasiensitive Rat Insulin kit, model 10-113-01; Mercodia, Uppsala, Sweden) according to the manufacturer’s instructions. Briefly, the assay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the samples react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to a microritration well. After removing the unbound antibody by washing, the bound conjugate is detected by reaction with 3,3’,5,5’-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm with a microplate (SpectraMax 340PC; Molecular Devices).

To evaluate insulin tolerance, an intraperitoneal insulin tolerance test (IPITT) was done. A bolus of insulin (2 U/kg ip) was administered to the animals, and blood samples were taken under anesthesia at intervals of 0, 30, 60, 90, and 120 min for glucose measurement as described above.

**Determination of 8-isoprostane.** Urinary 8-isoprostane is a noninvasive index of oxidative stress (14). This was determined by enzyme immunoassay (EIA; Cayman Chemical, MI) as we previously described (45, 46, 48, 49). Briefly, 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. Thereafter, the eluent was further purified on a silica column and eluted with 1:1 (vol/vol) ethylacetate-methanol and then aliquoted into plates precoated with monoclonal antibody. After 8-isoprostane tracer and isoprostane antiserum were added to each well and the wells incubated and washed, Ellman’s reagent containing the substrate of acetylcholinesterase was added. The absorbances were read at 412 nm in a plate reader (SpectraMax 340PC; Molecular Devices), and the values of 8-isoprostane were calculated from a standard curve.

Generally, urinary 8-isoprostane gives the overall status of oxidative stress in an organism, while pancreatic or gastrocnemius muscle 8-isoprostane reflects tissue-specific oxidative stress or damage (30). The tissues were homogenized in phosphate buffer containing 0.005% butylated hydroxy toluene in a ratio of 10 μl buffer/mg tissue. Subsequently, an equal volume of 15% KOH was added to the homogenate. Samples were incubated at 40°C for 1 h, followed by centrifugation, and the supernatant was neutralized with KH₂PO₄, and the absorbance read. Levels of pancreatic and gastrocnemius muscle 8-isoprostane were read in a plate reader as reported above and expressed as picograms per milligram of protein.

**Measurement of SOD activity.** Pancreatic and gastrocnemius muscle SOD activity (total) were determined by EIA (Cayman Chemical), as we previously reported (29, 47). The Cayman assay kit detects activity of all three types of SOD (Cu/Zn⁺, Mn⁺⁺, and Fe⁺⁺). Briefly, the tissues were homogenized in a solution containing 20 mM HEPES at pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose/g tissue, and centrifuged at 1,500 g for 5 min at 4°C. The supernatant was recovered, and the EIA reaction performed and read at 450 nm using a plate reader (SpectraMax 340PC; Molecular Devices).

**Determination of catalase activity.** Gastrocnemius muscle catalase activity was evaluated by EIA (Cayman Chemical) as we previously described (29). The assay is based on the reaction of the enzyme with methanol in the presence of H₂O₂. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. The gastrocnemius muscle was homogenized in a solution containing 50 mM potassium phosphate and 1 mM EDTA at pH 7.4 in a ratio of 5 ml buffer to 1 g tissue. After being centrifuged at 10,000 g for 15 min, the supernatant was recovered for the quantification of catalase by means of a plate reader at 540-nm (Synergy Microplate Reader; BioTek Instruments).

**Total antioxidant capacity assay.** The total antioxidant capacity of the gastrocnemius muscle was evaluated using an EIA Kit (cat. no. 709001; Cayman Chemical) as we have previously reported (45, 46, 48, 49). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2’-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble tocopherol analog, and is quantified as molar Trolox equivalents. In brief, the tissues were homogenized in the presence of protease inhibitors, and we followed the manufacturer’s instructions step-by-step to quantify the total antioxidant capacity. The absorbance was read at 750 nm using Synergy Microplate Reader (BioTek Instruments) with Gen5 Data Analysis Software. The results were expressed as Trolox equivalent antioxidant capacity per milligram protein.

**Measurement of cAMP and cGMP.** The concentrations of cAMP and cGMP in the gastrocnemius muscle and pancreas were evaluated by EIA (Cayman Chemical), as previously described (29, 47). Briefly, left ventricular samples were homogenized in 6% trichloroacetic acid at 4°C in the presence of 3-[isobutyl-1-methyl]xanthine to inhibit phosphodiesterase activity and centrifuged at 2,000 g for 15 min. The supernatant was recovered, and after being washed with water-saturated diethyl ether, the upper ether layer was aspirated and discarded, while the aqueous layer was recovered and lyophilized. The dry extract was dissolved in assay buffer, and the cAMP or cGMP content was detected by EIA following the manufacturer’s instructions and expressed as picomol of cAMP or cGMP per milligram of protein. The protein content was detected by Bradford assay (7).

**Statistical analyses.** All data are expressed as means ± SE from at least six independent experiments unless otherwise stated. Statistical analyses were performed using unpaired Student’s t-test and ANOVA in conjunction with the Bonferroni test for repeated measures where appropriate. Group differences at the level of P < 0.05 were considered statistically significant.

**RESULTS**

**Hemin therapy enhanced HO-1, HO activity, and cGMP levels in the gastrocnemius muscles of DOCA-hypertensive rats.** To evaluate the role of the HO system in insulin signaling, we measured HO-1, HO activity, and cGMP. The basal HO-1 and HO-activity in the controls (surgery-free or normal SD, UnX-sham, UnX+salt, and UnX+DOCA) were comparable but lower than the levels in DOCA+salt-hypertensive rats. Hemin increased HO-1 and HO activity in DOCA+salt-hypertensive rats by 3.8- and 4.3-fold, respectively (Figs. 1, A and B), whereas cotreatment with the HO inhibitor CrMP nullified the effect of hemin. Similarly, treatment with CrMP alone depleted the
basal HO-1 and HO activity (Figs. 1, A and B). The enhanced HO activity would increase endogenous carbon monoxide production that would, in turn, stimulate cGMP synthesis (50). Both cGMP and carbon monoxide have been shown to increase insulin release (43, 44) and thus improve glucose metabolism. Accordingly, we detected a 3.7-fold increase of cGMP in hemin-treated animals (Fig. 1C). Contrarily, in CrMP-treated animals, the effect of hemin on cGMP was abolished. In animals treated with CrMP alone, a further decrease of cGMP that fell below the basal level was observed. Although the basal HO activity in DOCA+salt-hypertensive rats was higher than the controls, it did not evoke an increase in cGMP content (Fig. 1C). The reason for this is not fully understood and needs to be clarified by further studies. However, a possible explanation could be that the magnitude of basal HO in DOCA+salt-hypertensive rats might have fallen below the threshold necessary to trigger an increase in cGMP content. A similar observation has been previously reported (29, 50). Hemin therapy also enhanced HO-1, HO activity, and cGMP in SD, although a greater increment was observed in hemin-treated DOCA+salt animals (Fig. 1). The higher magnitude of HO-signaling may be responsible for the more intense insulin-sensitizing effect in DOCA+salt rats compared with SD. Alternatively, the less preponderant increase of HO activity in SD may suggest greater stability of the HO system in normal conditions.

DOCA+salt-hypertensive rats were normoglycemic (Table 1). However, hemin therapy slightly, but significantly reduced glucose levels, whereas CrMP abolished the modest effect of hemin on glycemia. Hemin and CrMP also affected body weight. The body weights of hemin- and CrMP-treated animals were lower than the control. A difference (≤10%) was observed (Table 1). In SD+hemin, DOCA+hemin, DOCA+hemin+CrMP, and DOCA+CrMP groups body weights were lower by 6.2, 6.8, 9.3, and 6.7%, respectively, compared with the controls. Although changes of body weight can affect blood glucose levels, it is unlikely, in this case, since the slightly reduced body weight in hemin- and CrMP-treated groups were accompanied by opposite glycemic effects (Table 1). Accordingly, we observed a slight decrease of glucose levels in hemin-treated animals but a small increase in CrMP-treated animals, suggesting that the HO system may be endowed with intrinsic antidiabetic effects. On the other hand, the expected reduction of blood pressure was observed in hemin-treated animals, and was more intense than the effect on blood glucose. A 32.3% drop of systolic blood pressure was observed in hemin-treated animals, whereas blood glucose was reduced only by 15.9% (Table 1). The vehicle-dissolving hemin and CrMP did not affect any of the parameters measured. The lower of body weights observed in hemin- and CrMP-treated animals may not be due to toxicity, as we ensured. The lower of body weights observed in hemin- and CrMP-treated animals, and was more intense than the effect on blood glucose. A 32.3% drop of systolic blood pressure was observed in hemin-treated animals, whereas blood glucose was reduced only by 15.9% (Table 1). The vehicle-dissolving hemin and CrMP did not affect any of the parameters measured. The lower of body weights observed in hemin- and CrMP-treated animals may not be due to toxicity, as we ensured. The lower body weights observed in hemin- and CrMP-treated animals were not reinstated. Contrarily, in CrMP-treated animals, the effect of hemin on 8-isoprostane was annulled, and interestingly, 8-isoprostane was reversed to similar levels as observed in untreated DOCA+salt rats. On the other hand, in CrMP-treated animals, the levels of 8-isoprostane were further
increased, suggesting that oxidative stress is further potentiated by blockade of basal HO activity. Besides using 8-isoprostane to evaluate oxidative stress, we also measured the concentration of heme, a prooxidant (31). The basal levels of heme in DOCA+salt rats were significantly elevated but were abated by hemin and enhanced by CrMP (Fig. 2C). Hemin therapy also reduced 8-isoprostane and heme levels in SD rats albeit to a lesser extent compared with DOCA+salt rats.

Since antioxidants like bilirubin and ferritin are generated by the HO system (4, 25, 31) and SOD and catalase are modulated by blockade of basal HO activity. Besides using 8-isoprostane to evaluate oxidative stress, we also measured the concentration of heme, a prooxidant (31). The basal levels of heme in DOCA+salt rats were significantly elevated but were abated by hemin and enhanced by CrMP (Fig. 2C). Hemin therapy also reduced 8-isoprostane and heme levels in SD rats albeit to a lesser extent compared with DOCA+salt rats.

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Fig. 2. Effects of hemin and CrMP on oxidative stress in DOCA+salt-hypertensive rats. Hemin therapy reduced urinary 8-isoprostane and gastrocnemius muscle 8-isoprostane (A), 8-isoprostane (B), and heme content (C), while CrMP nullified the effects of hemin. Hemin increased plasma ferritin (D) and bilirubin (E) as well as gastrocnemius muscle SOD activity (F), catalase activity (G), and the total antioxidant capacity (H), whereas CrMP abolished the effects of hemin. Bars are means ± SE; n = 6 rats per group (†P < 0.05 vs. all groups; *P < 0.01 vs. all groups; #P < 0.01 vs. DOCA+salt or DOCA+salt+Hem+CrMP).
would be accompanied by a parallel increase of insulin. Interestingly, the attenuation of JNK in hemin-treated DOCA-salt-hypertensive rats was associated with a significant increase of insulin levels (Fig. 4B), whereas CrMP abolished the hemin-mediated increase of insulin. Since cAMP mediates insulin release (44), we investigated the effects of hemin on pancreatic cAMP. Hemin therapy enhanced pancreatic cAMP by 1.9-fold, while CrMP abolished the hemin effect. Bars are means ± SE; n = 6 rats per group (*P < 0.01 vs. all groups; †P < 0.05 vs. all groups; #P < 0.05 vs. all groups).

Hemin therapy improved glucose and insulin tolerance in DOCA-salt hypertension. Since elevated oxidative stress contributes to insulin resistance and glucose intolerance, we investigated whether the potentiation of the overall antioxidant status in hemin-treated animals would be accompanied by a concomitant improvement of glucose/insulin tolerance. In DOCA-salt and DOCA-salt+hemin+CrMP groups, the

in normoglycemic control SD rats, albeit to a lesser magnitude than in DOCA-salt-hypertensive rats (Fig. 4C).

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Hemin therapy upregulated pancreatic HO and suppressed oxidative stress. The basal levels of HO-1 and HO-activity in UnX-sham control rats were lower than the levels of DOCA+salt-hypertensive rats. Hemin greatly increased pancreatic HO-1 and HO activity (Figs. 6, A and B), whereas cotreatment with CrMP nullified the effect of hemin. On the other hand, treatment with CrMP alone depleted the basal levels of HO-1 and HO activity. The enhanced HO activity in DOCA+salt rats was paralleled by increased cGMP (Fig. 6C), a cyclic nucleotide that stimulates insulin release (44). Contrarily, in DOCA+salt+CrMP+hemin-treated group, the effect of hemin on cGMP was abolished. Similarly, the levels of cGMP were further decreased in the DOCA+salt+CrMP group (Fig. 6C). Although the basal HO activity in DOCA+salt-hypertensive rats was higher than the controls, it did not evoke an increase in cGMP content (Figs. 6, A–C). A similar observation has been previously reported (29, 50).

Interestingly, upregulation of pancreatic HO was accompanied by the enhancement of SOD (Fig. 6D) and the potentiation of the total antioxidant capacity (Fig. 6E), with the corresponding reduction of 8-isoprostane (Fig. 6F), a marker of oxidative stress. In DOCA+salt rats, the depressed levels of SOD and total antioxidant capacity were enhanced by 2.2- and 2.6-fold, whereas 8-isoprostane was reduced by 2.1-fold. In contrast, CrMP abolished the effects of hemin and exacerbated oxidative stress (Figs. 6, D–E).

Hemin therapy reduced pancreatic histopathological lesions in DOCA+salt-hypertensive rats. Severe inflammation was evident in pancreatic tissue from DOCA+salt-hypertensive rats (Fig. 7). This was characterized by significant elevation of mononuclear cell infiltration, increased formation of vacuoles, enhanced interstitial edema with elevated acinar cell necrosis, and fibrosis (Table 2). The massive infiltration of mononuclear cells in pancreatic tissue from DOCA+salt rats was associated with increased acinar cell necrosis and elevated fibrotic tissue, suggesting that the disappearance of acinar cells may be due to fibrosis. Interestingly, hemin therapy markedly reduced these lesions, although control levels were not attained. In contrast, the HO blocker CrMP abolished the protective effects of hemin and exacerbated histopathological lesions in DOCA+salt-hypertensive rats, suggesting an important role of the HO system in the maintenance and preservation of intact pancreatic architecture/morphology to safeguard the insulin-producing capability of β-cells.

**DISCUSSION**

The present study highlights the insulin-sensitizing effects of the HO system in DOCA+salt hypertension, a model of primary aldosteronism. It is now widely accepted that primary aldosteronism represents the most common form of endocrine hypertension (6). Hyperaldosteronism is associated with detrimental consequences on the cardiovascular system, renal function, insulin signaling, and glucose metabolism. Oxidative stress plays a crucial role in the development of diabetic

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**Fig. 5.** Effects of hemin and CrMP on glucose tolerance and insulin tolerance in DOCA+salt-hypertensive rats. Hemin therapy improved glucose intolerance (A), increased glucose-stimulated insulin release (B), and reduced insulin tolerance (C), whereas, CrMP abolished the effects of hemin. Bars are means ± SE; n = 6 rats per group (*P < 0.01 vs. all groups; †P < 0.05 vs. all groups).
complications in aldosteronism. Accordingly, the high levels of 8-isoprostane alongside increased NF-$\kappa$B, AP-1, and JNK observed in DOCA-hypertensive rats may accentuate the oxidative destruction of tissues (5, 10, 18, 34, 42). Importantly, the depressed insulin levels in DOCA-salt-hypertensive rats may be due to elevated oxidative stress (35), since the activation of JNK, a mediator of oxidative events, has been shown to suppress insulin biosynthesis (35). Alternatively, the preservation of pancreatic architecture/morphology to safeguard the insulin-producing capability of $\beta$-cells alongside upregulation of cAMP and cGMP may account for increased insulin levels since these cyclic nucleotides also promote insulin release (44).

Fig. 6. Effects of hemin and the HO inhibitor CrMP on HO-1, HO activity, cGMP, and oxidative stress in the pancreas of DOCA-salt-hypertensive rats. The basal levels of HO-1 concentration (A) and HO activity (B) in DOCA-salt-hypertensive rats were higher than those of the control. Hemin therapy greatly increased HO activity, whereas CrMP annulled the hemin effect. C: hemin increased cGMP content of DOCA-salt hypertensive, while CrMP abolished the effect of hemin. Hemin therapy enhanced pancreatic SOD activity (D) and the total antioxidant capacity (E), with corresponding reduction of 8-isoprostane (F). Bars are means ± SE; n = 6 rats per group (*P < 0.01 vs. all groups; †P < 0.05 vs. all groups; #P < 0.05 vs. DOCA-salt+Hem+CrMP).
oxidative stress (36). Thus, the cGMP/cAMP-secondary messenger system constitutes an important mechanism through which the HO system elicits cytoprotection and improves glucose metabolism. On the other hand, hemin therapy concomitantly enhanced several antioxidants including SOD, catalase, bilirubin, and ferritin with potentiation of the total antioxidant status in DOCA/salt-hypertensive rats. Correspondingly, markers/mediators of oxidative stress, including 8-isoprostane, NF-κB, AP-1, and JNK, were greatly attenuated, whereas the HO inhibitor, CrMP, nullified the hemin-induced protection and exacerbated oxidative insults with aggravation of insulin signaling/glucose metabolism. Since oxidative stress depletes adiponectin (15, 33, 59), the suppression of oxidative stress may account for the increased levels of adiponectin in hemin-treated animals. The insulin-sensitizing effect of adiponectin has been widely acknowledged (22, 37). Levels of adiponectin are low in patients with obesity, atherosclerosis, insulin resistance (22), and primary aldosteronism...
Moreover, adiponectin knockout leads to insulin-resistant type 2 diabetes in mice (37). On the other hand, adiponectin reduces tissue injury by suppressing the activation of NF-κB via a cAMP-dependent mechanism (51). Therefore, the reduction of oxidative stress coupled to increased levels of adiponectin, cAMP, and cGMP are among the mechanisms by which the HO system enhances insulin sensitivity and improves glucose metabolism in DOCA+salt hypertension. This concept is further strengthened by the finding that hemin therapy reduced glucose intolerance (IPGTT) and improved insulin tolerance (IPGTT). Therefore, it could be postulated that, although DOCA-hypertensive rats are normoglycemic, insulin signaling may be impaired. Consistent with this notion are reports indicating that insulin production in DOCA+salt hypertension is reduced (12, 13). However, Dai et al. (12) reported normal glucose tolerance in DOCA rats. This discrepancy may be due to the different protocols used. In the present study, DOCA+salt-hypertensive rats were uninephrectomized and were severely hypertensive (196.2 ± 2.4 mmHg), whereas in the study of Dai et al. (12) the animals were not nephrectomized and had blood pressure of 171.9 ± 3 mmHg and, thus, lower by ~24.3 mmHg compared with the blood pressure registered in the present study. Arguably, the severity of hypertension in our protocol may aggravate oxidative insults leading to greater adversity in tissues with more accentuated damage that may compromise different cellular functions including the response of glucose to insulin. However, further studies are needed to clarify this discrepancy. Whether UnX animals receiving DOCA manifest insulin/glucose tolerance differently than animals receiving only DOCA should be investigated in future studies.

Although many HO inhibitors are nonspecific and may affect other heme-dependent enzymes, such as nitric oxide synthase, cytochrome P-450, and soluble guanylyl cyclase or even increased HO-1 (3, 57), CrMP given at a dose of 2.61 mg/kg is reportedly selective for HO (60). Nevertheless, CrMP should be used cautiously because, besides the aforementioned phototoxic collateral effects, CrMP may have other harmful effects, and higher doses of intravenous administration could even be lethal (41).

Hemin therapy also enhanced the HO system and its related signaling pathways in SD, although the magnitude was smaller compared with DOCA+salt-hypertensive rats. The reasons for this selective effect of HO are not fully understood. However, it is possible that since SD rats are healthy animals with normal/functional insulin signaling, the HO system may be acting in conjunction with other functional pathways to maintain insulin sensitivity. Therefore, the less intense effect of the HO system would not alter other healthy pathways that act in concert with the HO system to preserve insulin sensitivity. Whether this is an intrinsic homeostatic and/or defensive mechanism to maintain healthy conditions in SD within a certain physiological range remains unclear and needs to be clarified in future studies. Nevertheless, the selectivity of the HO system in diseased conditions could be explored in the design of novel therapeutic agents against insulin resistance and diabetes.

Collectively, our results suggest that impaired insulin signaling may be a forerunner to hyperglycemia and other metabolic/cardiovascular complications associated with aldosteronism. The improved intraperitoneal glucose tolerance and intraperitoneal insulin tolerance and increased insulin sensitivity would enhance glucose metabolism in hemin-treated animals. Moreover, the observed reduction of insulin in DOCA+salt rats is consistent with impaired pancreatic insulin release in humans with primary aldosteronism (11). Given that impaired glucose metabolism appears to be the major contributor of metabolic dysfunction in primary aldosteronism (16), hemin therapy would be beneficial not only in lowering blood pressure and abating oxidative/inflammatory events, but also in potentiating insulin signaling and thus improving glucose metabolism. The metabolic and immune/inflammatory systems have been evolutionarily conserved throughout species and are among the most fundamental requirements for survival. By modulating these systems, HO-1 may be an important chaperon molecule with pleitropic effects against metabolic, immune/inflammatory, and cardiovascular disorders including hypertension, diabetes, and obesity (29, 38). Seen in this light, HO inducers may be considered as novel strategies to prevent aldosteronism from evolving into metabolic syndrome and other related cardiovascular complications.

**Perspectives and Significance**

Although this study suggests that impaired insulin signaling in aldosteronism may lead to hyperglycemia, further investigations are needed to fully clarify this notion. In recent studies, we showed that the paradoxical increase of insulin secretion and insulin sensitivity was associated with marked reduction of fasting glucose levels in Zucker diabetic fatty rats, streptozotocin-induced diabetes, and Goto-Kakizaki rats (45, 46, 48, 49). Therefore, it remains puzzling why the robust increase of insulin and enhanced insulin sensitivity in hemin-treated DOCA+salt rats were only accompanied by a modest, though significant, reduction of glucose levels. These are some of the challenging questions that should be addressed in future stud-

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**Table 2. Effect of hemin and CrMP on morphological parameters in DOCA-salt rats**

<table>
<thead>
<tr>
<th>Morphological Analyses</th>
<th>UnX-Sham</th>
<th>DOCA+Salt</th>
<th>DOCA+Salt+Hemin</th>
<th>DOCA+Salt+CrMP+Hemin</th>
<th>DOCA+Salt+CrMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial edema</td>
<td>0.67±0.21</td>
<td>2.33±0.21†</td>
<td>1.17±0.17†</td>
<td>2.33±0.21†</td>
<td>2.83±0.17*</td>
</tr>
<tr>
<td>Vacuole formation</td>
<td>0.17±0.17</td>
<td>2.83±0.17*</td>
<td>0.57±0.43†</td>
<td>2.83±0.17†</td>
<td>3.0±0.0*</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>0±0.0</td>
<td>2.67±0.21†</td>
<td>1.00±0.22†</td>
<td>2.83±0.17†</td>
<td>3.00±0.00*</td>
</tr>
<tr>
<td>Acinar cell necrosis</td>
<td>0.67±0.21</td>
<td>3.00±0.0*</td>
<td>0.57±0.43†</td>
<td>3.00±0.00*</td>
<td>3.00±0.00*</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0±0.0</td>
<td>2.67±0.21†</td>
<td>1.14±0.14†</td>
<td>2.67±0.21†</td>
<td>2.83±0.17*</td>
</tr>
</tbody>
</table>

*Values are mean scores ± SE; n = 6 rats per group. Assessment was blind using a scale from 0 to 3, with 0 = normal; 1 = mild, 2 = moderate, and 3 = severe. Hemin significantly reduced interstitial edema, vacuole formation, inflammatory cell infiltration, acinar cell necrosis, and fibrosis in the pancreas of DOCA+salt hypertensive rats, while CrMP reversed the effects of hemin with aggravation of pancreatic lesions. *P < 0.01 vs. all groups; †P < 0.05 vs. all groups.
ies. However, it appears that the metabolic complication arising from aldosteronism aggravates with time, and, at its initial phase when glyceremia is still normal, the input from other functional pathways may be sufficient for glucose homeostasis. Nevertheless, future studies should investigate whether prior to the manifestation of hyperglycemia in aldosteronism other factors besides impaired insulin signaling are involved. The identification and characterization of these factors would advance our knowledge on aldosteronism and its progression to metabolic/cardiovascular complications.

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DISCLOSURE

No conflicts of interest are declared by the author(s).

REFERENCES


