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

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Abstract

Recent clinical reports indicate that impaired glucose tolerance is a common phenomenon in primary aldosteronism. Aldosterone stimulates nuclear-factor kappa-B (NF-κB) and activating-protein (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 groups: (A) controls [(i) surgery-free or normal Sprague-Dawley (SD), (ii) uninephrectomized (UnX)-Sham, (iii) UnX-salt (0.9%NaCl+0.2%KCl) and (iv) UnX-DOCA]; (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. DOCA-salt-hypertensive rats were normoglycemic. Hemin therapy lowered BP, 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 (IPGTT) and insulin tolerance (IPITT), 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 IPGTT and IPITT. Interestingly, the enhanced insulin-sensitization in hemin-treated animals was accompanied by reduced urinary/gastrocnemius muscle 8-isoprostane, inflammatory/oxidative transcription factors like NF-κB, AP-1, c-Jun-NH2-terminal kinase and heme content, whereas HO-1, HO-activity, cGMP and plasma/gastrocnemius muscle anti-oxidants including bilirubin, ferritin, superoxide dismutase, catalase and the total anti-oxidant 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.
INTRODUCTION

Clinical studies indicate that a high percentage of patients with primary aldosteronism suffer from glucose intolerance and insulin resistance (9, 17, 19). In the recent National Cholesterol Education Program Adult Treatment Panel III study, it was reported that the prevalence of metabolic syndrome and cardiovascular events was higher in patients with primary aldosteronism than those with essential hypertension (1, 17). Impaired glucose metabolism is linked to insulin resistance and considered the major contributor to the development of metabolic syndrome (21). The linkage could be explained by several mechanisms including abnormalities of insulin-signalling (54, 55), cation alterations, excessive activity of the sympathetic nervous system (52), enhanced renin-angiotensin-aldosterone activity (54, 55), and elevated inflammatory/oxidative events (54, 55). There is a general consensus that oxidative stress plays a major patho-physiological role in diabetes and its complications. However, at lower concentrations, reactive oxygen species also serve as messenger molecules that regulate cellular activities (20). On the other hand, cellular redox state may modulate the expression of stress proteins like heme oxygenase (HO) in an attempt to limit tissue insult (2, 57). Thus, the management of oxidative stress may constitute an important therapeutic approach to combat insulin-resistant diabetes and its complications.

We recently showed that upregulating the HO system abated oxidative stress and lowered blood pressure in different hypertensive models including spontaneously hypertensive rats (SHR) and deoxycorticosterone acetate (DOCA-salt) hypertension (29, 47). Like angiotensin, aldosterone causes oxidative stress (26, 27), inflammation and fibrosis by several mechanisms including the stimulation of nuclear factor kappa-B (NF-κB) and activating-protein (AP-1) (32, 39, 56). Besides NF-κB, c-Jun-N-terminal kinase (JNK) is activated by oxidative stress (10, 35). JNK regulates AP-1 (5) and blocks insulin biosynthesis (35). Therefore, in aldosteronism the activation of JNK by oxidative stress may contribute to the impairment of insulin-signaling. Moreover, aldosterone
triggers the formation of superoxide anion which in turn quenches nitric oxide by forming peroxynitrite (42), that subsequently oxidizes arachidonic acid to generate the potent vasoconstrictor anti-natriuretic substance, 8-iso-prostaglandin F$_{2\alpha}$ (8-isoprostane) (18). Accordingly, the high levels of aldosterone alongside 8-isoprostane, JNK, NF-κB and AP-1 may potentiate the oxidative destruction of tissues. Importantly, elevated oxidative stress has been shown to deplete the insulin-sensitizing protein, adiponectin (15, 33, 59). Therefore, strategies that concomitantly enhance adiponectin and suppress NF-κB, AP-1 and JNK may improve insulin-signaling and glucose metabolism in aldosteronism and metabolic syndrome.

The HO system is emerging as an important pathway for the regulation of glucose metabolism (8, 37, 45, 46, 48, 49). HO is a microsomal enzyme with inducible (HO-1) and constitutive (HO-2) isoforms (2). HO is ubiquitously distributed and expressed in different tissues including gastrocnemius skeletal muscles (58). Besides its anti-oxidant 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-signalling and glucose metabolism in mineralocorticoid-induced hypertension.
MATERIALS AND METHODS (see supplemental file for extended methodology)

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 weeks of age were purchased from Charles River Laboratories (Willington, MA). The animals were housed at 21°C with 12-hour 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 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 weight, Sigma-Aldrich, MO) (UnX-DOCA); (B) UnX rats implanted with DOCA-strip that received water containing 0.9 % NaCl + 0.2 % KCl (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) hemin together with the HO blocker, chromium mesoporphyrin (CrMP), or (F) CrMP alone, or (G) the vehicle dissolving hemin and CrMP. In addition normal SD rats were given (H) hemin.

The involvement of the HO system in the hemin-induced insulin-sensitizing/anti-diabetic effect was ascertained by giving the HO blocker, CrMP, 4 μmol/kg (2.61 mg/kg) i.p., Porphyrin Products, UT) with or without hemin (30 mg/kg, i.p., Sigma-Aldrich, MO) (50, 60). Hemin and CrMP were dissolved in 0.1M NaOH, titrated to pH 7.4 with 0.1M HCl and diluted 1:10 with
phosphate buffer as we previously reported (28, 29, 47). Treatment with hemin, CrMP or the vehicle dissolving hemin and CrMP began after the animals were severely hypertensive with a blood pressure of 196.2±2.4 mmHg, recorded 4 weeks after the surgical implantation of DOCA-strips. Each injection was 0.5 ml, and was given twice weekly for 4 weeks. Fasting glucose was monitored weekly with a glucose meter (BD, Franklin Lakes, NJ, USA) after overnight fasting in metabolic cages. Systolic blood pressure was determined weekly in conscious animals by standard tail-cuff non-invasive method (Model 29-SSP, Harvard Apparatus, Canada) during therapy. At the end of the 4-week treatment period, the study was terminated. The animals were weighed, anaesthetized with pentobarbital sodium (50 mg/kg i.p.), killed by decapitation and blood samples collected. The pancreas and gastrocnemius muscles were quickly excised, cleaned from adipose and connective tissues in ice-cold phosphate-buffered saline (PBS). The composition of PBS was as follows: 140 mM NaCl, 3mM KCl, 10 mM Na₂HPO₄, and 2mM KH₂PO₄. From the plasma, ferritin and bilirubin were routinely measured by the Royal University Hospital, Saskatoon. Tissues not used immediately were snap-frozen in liquid nitrogen and stored at -80ºC 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 100 mmol/L phosphate buffer with 2 mmol/L MgCl₂ (HO-activity buffer), 0.25 M sucrose solution and centrifuged at 27,000 g for 15 minutes. 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 dinucleotide phosphate, 20 μmol/L hemin, 2 mmol/L glucose-6-phosphate, 0.002 U/μl glucose-6-phosphate dehydrogenase and 100 μl liver cytosol as source of biliverdin reductase. The
reaction carried out in darkness for 1 hour 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 minutes 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 mM⁻¹cm⁻¹), and expressed as nmole/mg protein/hour. The protein content was measured using Bradford assay. As a positive control, spleen tissue was used.

Gastrocnemius muscle and pancreatic HO-1 levels were determined by Enzyme-linked immunosorbent assay (ELISA) (EKS-810A, Stressgen-Assay Design, MI), while heme was assessed using Quantichrom Heme Assay kit (DIHM-25- BioAssay Systems, CA) following the manufacturer’s instructions as we previously described (29).

**Total RNA Isolation and Quantitative Real-Time Reverse Transcription 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, CA) according to the manufacturer’s specifications. Reverse transcription was carried out using First Strand cDNA Synthesis Kit (Novagen, WI) 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 MMLV reverse transcriptase according to manufacturer’s instruction. Quantitative PCR was done with Applied Biosystems 7300 Real Time PCR system (Foster City, CA) and iQ SYBR Green Supermix (Bio-Rad, CA) containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM each free dNTP, hot start enzyme iQTaq DNA polymerase (25 U/ml), 3 mM MgCl₂, SYBR Green 1 and 10 nM fluorescein as passive 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'CATGCCTTCCGTACAAGTGCG
A-3' and reverse 5'TGGGTGCGTCTTAGTGGTATCTGT-3'); AP-1 (forward, 5' AGCAGATGCT
TGAGTTGAGAGCCA-3' and reverse, 5'TTCCATGGGTCCCTGCTTTGAGAT-3'); JNK,
forward 5' AAGCAGCAAGGCTACTCTCTTTCTCA-3' and reverse 5'-ATCGAGACTGCTGTCTG
TGTCTGA-3' and β-actin (forward, 5'TCATCAGATCGGAATGAGCGGT-3' and reverse,
5'ACAGCAGCTGTTGGCATAGAGGT-3') in a final volume of 25 µl. The National Research
Council of Canada, Saskatoon, confirmed the sequences of all primers used. The program for
thermal cycle was 10 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 56°C and 15
sec at 72°C. The melting points of PCR product were determined by incubating at 65°C for 1 min
followed by a 1°C per min rise over 30 min.

**Determination of plasma adiponectin**

Adiponectin was measured by ELISA (Phoenix Pharmaceuticals, CA) 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 Device, CA).

**Histological and morphological analyses of pancreas**

Pancreatic tissue obtained from duodenal and splenic lobes were fixed in 10 % formalin
phosphate buffer for 48 hrs, processed and paraffin embedded, then sections of 5 µm thicknesses
were 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 was blindly done in 20 randomly selected fields using light microscopy (Olympus BH-2
Olympus Co, Tokyo) semi-quantitatively 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 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 i.p.)
was injected and blood samples were collected from the tail vein under anaesthesia at intervals of 0,
30, 60, 90, 120 min, and tested for glucose and insulin. Plasma glucose was measured using an auto-
alyzer (BD, Franklin Lakes, NJ) while insulin was assessed by ELISA [Mercodia Ultrasensitive
Rat Insulin kit (10-113-01), 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 microtitration 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 mn with a
microplate (SpectraMax 340PC, Molecular Device, CA).
To evaluate insulin tolerance, intraperitoneal insulin tolerance test (IPITT) was done. A bolus of insulin (2 U/kg, i.p.) was administered to the animals and blood samples taken under anaesthesia at intervals of 0, 30, 60, 90, 120 min for glucose measurement as described above.

**Determination of 8-isoprostane**

Urinary 8-isoprostane is a non-invasive index of oxidative stress (14). This was determined by enzyme immunoassay (EIA), (Cayman Chemical Company, MI) as we previously described (45, 46, 48, 49). Briefly, urine samples collected over a period 24 hrs were diluted 1:15 with ultra pure 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 aliquoted into plates precoated with monoclonal antibody. After, 8-isoprostane tracer and isoprostane antiserum were added to each well, incubated and after washing, Ellman’s reagent containing the substrate of acetylcholinesterase was added. The absorbance were read at 412 nm in a plate reader (SpectraMax 340PC, Molecular Device, CA), and the values of 8-isoprostane 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. The samples were incubated at 40°C for 1 h, followed by centrifugation, and the supernatant was neutralized with KH₂PO₄ and the absorbance read. The 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 superoxide dismutase activity

Pancreatic and gastrocnemius muscle superoxide dismutase (SOD) activity (total) were determined by EIA (Cayman Chemical Company, MI), as we previously reported (29, 47). The Cayman assay kit detects the activity of all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). Briefly, the tissues were homogenised in a solution containing 20 mM HEPES at pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose per gram tissue, and centrifuged at 1500 g for 5 minutes at 4°C. The supernatant was recovered and the EIA reaction performed and read at 450 nm using a plate reader (SpectraMax 340PC, Molecular Device, CA).

Determination of catalase activity

Gastrocnemius muscle catalase activity was evaluated by EIA (Cayman Chemical Company, MI) as we previously described (29). The assay is based on the reaction of the enzyme with methanol in the presence of H$_2$O$_2$. 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, 1 mM EDTA at pH 7.4 in a ratio of 5 ml buffer to 1 gram of tissue. After centrifuging 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, Vermont).

Total antioxidant capacity assay

The total antioxidant capacity of the gastrocnemius muscle was evaluated using EIA Kit (catalogue # 709001, Cayman Chemical Company, MI) as we 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 Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, 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, Vermont) with Gen5 Data Analysis Software. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) per mg protein.

**Measurement of cAMP and cGMP**

The concentrations of cAMP and cGMP in the gastrocnemius muscle and pancreas were evaluated by EIA (Cayman Chemical Company, MI), 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-methylxanthine to inhibit phosphodiesterase activity and centrifuged at 2000g for 15 minutes. The supernatant was recovered and after washing 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 mg of protein. The protein content was detected by Bradford assay (7).

**Statistical analyses**

All data were expressed as means ± SEM from at least six independent experiments unless otherwise stated. Statistical analyses were done using unpaired Student’s *t*-test and analyses of
variance in conjunction with 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. 1A and 1B), whereas co-treatment with the HO inhibitor, CrMP nullified the
effect of hemin. Similarly, treatment with CrMP alone depleted the basal HO-1 and HO activity
(Figs. 1A and 1B). 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+hemin-treated animals, the effect of hemin on cGMP was abolished. In
animals treated with CrMP alone, a further decrease of cGMP which 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-signalling may be responsible for the more intense insulin-sensitizing effect in DOCA-salt rats as compared to 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 body weights were lower by 6.2, 6.8, 9.3 and 6.7% respectively as compared to 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 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 anti-diabetic 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 DOCA-salt+hemin group whereas blood glucose was reduced only by 15.9% (Table-1). The vehicle dissolving hemin and CrMP did not affect any of the parameters measure. The lower of body weights observed in hemin- and CrMP-treated animals may not be due to toxicity as we recently showed that important indices of toxicity including plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (γGGT) were within normal range (47, 48).
**Hemin enhanced the anti-oxidant status and reduced oxidative stress in DOCA-salt rats**

Given that oxidative stress is among the causes of insulin resistance, we measured 8-isoprostane, an important marker of oxidative stress (14). In DOCA-salt hypertensive rats, the basal levels of urinary and gastrocnemius 8-isoprostane were markedly elevated, suggesting enhanced oxidative stress (Figs. 2A and 2B). However, hemin therapy significantly reduced 8-isoprostane, although control levels were not reinstated. Contrarily, in hemin+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 pro-oxidant (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 as compared to DOCA-salt rats.

Since anti-oxidants like bilirubin and ferritin are generated by the HO system (4, 25, 31), and superoxide dismutase and catalase are modulated HO-1 (57), we investigated the effect of hemin on these anti-oxidants. In DOCA-salt-rats, the basal levels of ferritin, bilirubin, SOD and catalase were markedly reduced (Figs. 2D to 2G), and were increased by hemin by 6.4, 2.2, 2.1 and 2.2 fold respectively, whereas CrMP abolished the effect of hemin on these anti-oxidants. Interestingly, the hemin-induced increment of ferritin, bilirubin, SOD and catalase was accompanied by significant elevation of the total anti-oxidant capacity (Fig. 2H). In DOCA-hypertensive rats, the basal level of the total anti-oxidant capacity was 3.3 fold lower than the controls, but was enhanced by hemin to control levels. In hemin+CrMP-treated animals, the total anti-oxidant capacity was reversed to the levels of DOCA-salt hypertensive rats, and further
reduced in CrMP-treated animals to lower levels, suggesting that inhibiting the HO system may lead to the suppression of anti-oxidant defence mechanisms.

Hemin therapy abated transcription factors that reduce insulin-signaling

Oxidative and inflammatory transcriptions factors such as NF-κB, AP-1 and JNK are implicated in tissue damage and insulin resistance (5, 35). In DOCA-salt hypertensive rats, quantitative real-time RT-PCR analyses indicated that the levels of NF-κB, AP-1 and JNK in the gastrocnemius muscle were markedly elevated (Fig. 3). Hemin therapy abated NF-κB and AP-1 by 3.1 and 2.3 fold respectively, while the HO inhibitor, CrMP, nullified the effects of hemin (Fig. 3A and 3B). Importantly, CrMP alone further enhanced NF-κB and AP-1 in DOCA-salt rats, suggesting the involvement of basal HO activity in the regulation of these oxidative/inflammatory mediators. Furthermore, in DOCA-salt rats the basal expression of gastronecmius muscle JNK, a substance that suppresses insulin biosynthesis (35), was significantly elevated, but was abated by hemin (Fig. 3C). Although hemin therapy abated the levels of NF-κB, AP-1 and JNK in DOCA-salt rats, control values were not reinstated. Hemin therapy also reduced NF-κB, AP-1 and JNK in SD rat, although the effect was less intense as compared to DOCA-salt rats (Figs. 3A-3C).

Hemin therapy enhanced plasma adiponectin and insulin in DOCA-hypertension

Since elevated oxidative stress has been shown to reduce adiponectin (33, 59), and adiponectin deficiency is among the causes of insulin resistance (22, 37), we investigated whether the suppression of oxidative stress by hemin would affect plasma adiponectin. In DOCA-salt hypertensive rats, the levels of plasma adiponectin were significantly lower than the controls (Fig. 4A). Interestingly, hemin therapy greatly enhanced adiponectin by 2.2 fold, whereas the HO blocker, CrMP, abolished the hemin-induced increase of adiponectin.
Given that JNK inhibits insulin biosynthesis (35), and our results indicated that hemin therapy suppressed JNK, we investigated whether the hemin-mediated reduction of JNK 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 increase (Fig. 4C). Importantly, CrMP alone further depleted the levels of cAMP, insulin and adiponectin in DOCA-salt hypertensive rats, suggesting the involvement of basal HO activity in glucose metabolism. On the other hand, hemin therapy also increased insulin, adiponectin and cAMP in normoglycemic control SD rats albeit to a lesser magnitude than in DOCA-salt hypertensive rats (Fig. 4C).

**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 anti-oxidant 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, IPGTT revealed that the levels of plasma glucose were significantly elevated than in hemin-treated animals at all time points tested from 30-120 min (Fig. 5A), suggesting improved glucose tolerance in hemin-treated animals. Interestingly, challenge with a bolus injection of glucose markedly stimulated insulin release in DOCA-salt+hemin, whereas only a modest effect was observed in DOCA-salt and DOCA-salt+hemin+CrMP groups (Fig. 5B). Interestingly, in hemin-treated animals, two distinct phases of insulin release were noted: an acute-phase (first-phase response) 0-30 minutes and a second-phase response of gradual decrease of
insulin that returned to fasting levels by 90 minutes and remained constant at that level. Contrarily, in DOCA-salt and DOCA-salt+hemin+CrMP, the two phases of glucose-stimulated insulin release were less-distinct. Therefore, hemin therapy reduces insulin intolerance, whereas the HO inhibitor, CrMP aggravates it. Correspondingly, IPITT analyses indicated enhanced insulin sensitivity in hemin-treated animals (Fig. 5C). Importantly, a rapid reduction of glucose levels was observed in hemin-treated animals after insulin challenge, suggesting improved insulin sensitivity, whereas only a modest reduction of glucose was observed in DOCA-salt and DOCA-salt+hemin+CrMP groups (Fig. 5C).

**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. 6A and 6B), whereas co-treatment 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 the 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 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. 6A-6C). 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 anti-oxidant 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 anti-oxidant 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. 6D-6E).

**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 beta-cells.

**DISCUSSION (see supplemental file for extended 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 diabetic complications in aldosteronism. Accordingly, the high levels of 8-isoprostone
alongside increased NF-κB, 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).

Besides its effects on insulin, cGMP counteract tissue insults by inhibiting NF-κB- and AP-1-induced inflammatory/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 anti-oxidants including superoxide dismutase, calatase, bilirubin and ferritin with potentiation of the total anti-oxidant 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). The levels of adiponectin are low in patients with obesity, atherosclerosis, insulin resistance (22) and primary aldosteronism (15). 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 enhance insulin sensitivity and improve glucose metabolism.
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 (1992) (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 (1992) (12), the animals were not nephrectomized and had blood pressure of 171.9±3 mmHg, and thus lower by ~24.3 mmHg when compared to 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 uninephrectomized animals receiving DOCA manifest insulin/glucose tolerance differently than animals receiving only DOCA should be investigated in future studies.

Although many HO inhibitors are non-specific and may affect other heme-dependent enzymes such as nitric oxide synthase, cytochrome P-450 and soluble guanylyl cyclase or even increase HO-1 (3, 57), however, 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 lethal (41).

Hemin therapy also enhanced the HO system and its related signaling pathways in SD, although the magnitude was smaller as compared to 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 physiological a certain 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 IPGTT, IPITT 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 (see supplemental file for extended perspectives)

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 studies. However, it appears that the metabolic complication arising from aldosteronism aggravates with time, and at its initial phase when glycemia 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 hyperglycaemia in aldosteronim 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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**Figure legends**

**Figure 1:** Effects of hemin and the HO inhibitor, CrMP, on HO-1, HO activity and cGMP in the gastrocnemius muscle of DOCA-salt hypertensive rats. The basal (A) HO-1 concentration, and (B) HO activity in DOCA-salt hypertensive rats was higher than in the controls. Hemin therapy greatly increased HO activity in DOCA-salt hypertensive rats, but the HO blocker, CrMP nullified the hemin effect. (C) Treatment with hemin markedly increased cGMP content of DOCA-salt hypertensive, while CrMP abolished the effects of hemin. Bars represent means ± SE; n=6 rats per group (*p<0.01 vs all groups; †p<0.05 vs all groups; #p<0.01 vs DOCA-salt+Hem).  

**Figure 2:** The effects of hemin and CrMP on oxidative stress in DOCA-salt hypertensive rats. Hemin therapy reduced (A) urinary 8-isoprostane, and gastrocnemius muscle (B) 8-isoprostane and (C) heme content, while CrMP nullified the effects of hemin. Hemin increased plasma (D) ferritin and (E) bilirubin as well as gastrocnemius muscle (F) superoxide dismutase activity, (G) catalase activity, and (H) the total anti-oxidant capacity, whereas, CrMP abolished the effects of hemin. Bars represent 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).  

**Figure 3:** The effects of hemin and CrMP on gastrocnemius muscle NF-κB, AP-1 and JNK. Quantitative real-time RT-PCR indicated that hemin therapy suppressed the elevated basal mRNA expression of (A) NF-κB, (B) AP-1 and (C) JNK in DOCA-salt hypertensive rats, but CrMP annulled the hemin effect. Bars represent 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).
**Figure 4:** Effects of hemin and CrMP on adiponectin, insulin and cAMP in DOCA-salt hypertensive rats. Hemin therapy enhanced plasma (A) adiponectin and (B) insulin in DOCA-salt rats, but CrMP blocked the effect of hemin. (C) Treatment with hemin increased pancreatic cAMP content. Bars represent means ± SE; n=6 rats per group (\(\#p<0.01\) vs Normal SD or UnX-Sham or Normal SD+Hem or DOCA-salt+Hem; \(\$p<0.05\) vs all groups; \(*p<0.05\) vs all groups).

**Figure 5:** Effects of hemin and CrMP on glucose tolerance (IPGTT) and insulin tolerance (IPITT) in DOCA-salt hypertensive rats. Hemin therapy (A) improved glucose intolerance (IPGTT), (B) increased glucose-stimulated insulin release, and (C) reduced insulin tolerance (IPITT), whereas, CrMP abolished the effects of hemin. Bars represent means ± SE; n=6 rats per group (*p<0.01 vs all groups; †p<0.05 vs all groups).

**Figure 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 (A) HO-1 concentration, and (B) HO activity 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 (D) pancreatic superoxide dismutase activity (E) and the total antioxidant capacity, with corresponding reduction of (F) 8-isoprostance. Bars represent 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).
Figure 7: Effect of hemin on pancreatic histopathological lesions in DOCA-salt hypertension. Representative images depicting lesions such as acinar-cell necrosis, vacuolization, interstitial edema, fibrosis and mononuclear cell-infiltration of UnX-sham control (n=6), DOCA-salt (n=6), DOCA-salt+hemin (n=6), DOCA-salt+CrMP+hemin (n=6), and DOCA-salt+CrMP (n=6). The arrows indicate areas of intense damage. Samples AA from UnX-sham control animals appeared healthy with no apparent lesions. These samples were scored 0 for vacuolisation, interstitial edema, acinar-cell necrosis, fibrosis and 1 for mononuclear cell-infiltration. In samples BB from DOCA-salt hypertensive rats, severe histological lesions characterized by increased vacuolisation, mononuclear cell-infiltration, acinar-cell necrosis and fibrosis were evident. These samples were scored 3 for vacuolisation, mononuclear cell-infiltration, acinar-cell necrosis and fibrosis, and 2 for interstitial edema. In samples CC of hemin+DOCA-salt group, the histological lesions were greatly attenuated. These samples were scored 1 for vacuolisation, mononuclear cell-infiltration, acinar-cell necrosis, fibrosis, and interstitial edema. In contrast, samples DD of DOCA-salt+CrMP+hemin showed the reversal of the hemin-induced protection. In these samples, increased vacuolisation and severe mononuclear cell-infiltration with intense fibrosis and elevated acinar-cell necrosis were observed. These samples were scored 3 for vacuolisation, mononuclear cell-infiltration, fibrosis, acinar-cell necrosis, and 2 for interstitial edema. Finally, samples EE of DOCA-salt+CrMP showed exacerbation of the histological lesions. These samples were scored 3 for vacuolisation, mononuclear cell-infiltration, fibrosis, acinar-cell necrosis, and interstitial edema.
Figure 1

Gastrocnemius muscle cGMP (pmol/mg protein)

Gastrocnemius muscle HO activity (nmolbilirubin/mg protein/hr)

Gastrocnemius muscle HO-1 (concentration (ng/ml))

Normal SD
UnX-Sham
UnX-Salt
UnX-DOCA
DOCA-salt
Normal SD+Hem
DOCA-salt+Hem
DOCA-salt+CRMP

n=6

0 5 10 15 20 25

0 9 18 27 36 45 54

0 40 80 120 160

* †† * # *
Figure 4

A

Plasma Adiponectin (μg/ml)

B

Plasma Insulin (μg/L)

C

Pancreatic cAMP content (pmol/mg protein)
Figure 5

A

**IPGTT**

Plasma glucose (mmol/L)

- **DOCA-salt**
- **DOCA-salt + Hemin+CrMP**
- **UnX-sham**

Time (min)

n=6

B

Plasma insulin (μg/L)

- **UnX-sham**
- **DOCA-salt+Hemin**
- **DOCA-salt**
- **DOCA-salt + Hemin+CrMP**

Time (min)

n=6

C

**IPITT**

Glucose (mmol/L)

- **DOCA-salt + Hemin+CrMP**
- **DOCA-salt**
- **UnX-sham**

Time (min)
Hemin attenuates histopathological damages in the pancreas

A: UnX-Sham

B: DOCA-salt

C: DOCA-salt + Hemin

D: DOCA-salt + CrMP + Hemin

E: DOCA-salt + CrMP

H&E x200
Table 1: Effect of the HO inducer, hemin and the HO blocker, CrMP on physiological variables

<table>
<thead>
<tr>
<th>Physiological variable</th>
<th>Controls (grams)</th>
<th>DOCA-Salt</th>
<th>DOCA-salt +Hemin</th>
<th>Normal SD +Hemin</th>
<th>DOCA-salt + Hemin +CrMP</th>
<th>DOCA-salt +CrMP</th>
<th>DOCA-salt +Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>451 ± 19.4</td>
<td>437 ± 17.3</td>
<td>419 ± 8.5†</td>
<td>409 ± 10.5†</td>
<td>421 ± 9.8†</td>
<td>446.5 ± 15</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>6.8 ± 0.6</td>
<td>6.9 ± 0.3</td>
<td>5.8 ± 0.4$</td>
<td>6.9 ± 0.4</td>
<td>7.8 ± 0.3#</td>
<td>6.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>116.0 ± 0.96</td>
<td>196.2 ± 2.4*</td>
<td>132.8 ± 1.6§</td>
<td>110.5 ± 2.4§</td>
<td>218.5 ± 3.7*</td>
<td>228.5 ± 5.4*</td>
<td>202.4 ± 4.3*</td>
</tr>
</tbody>
</table>

†p<0.05 vs controls; $p<0.05 vs DOCA-salt; #p<0.05 vs DOCA-salt +Hemin; *p<0.01 vs all groups; §p<0.01 vs DOCA-salt, n=6-24 rats per group
Table-2: Effect of hemin and CrMP on morphological parameters in DOCA-salt rats

<table>
<thead>
<tr>
<th>Morphological analyses</th>
<th>UnX-Sham (n=6)</th>
<th>DOCA-salt (n=6)</th>
<th>DOCA-salt+Hem (n=6)</th>
<th>DOCA-salt+CrMP+Hem (n=6)</th>
<th>DOCA-salt+CrMP (n=6)</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>

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. Assessment was blindly done using 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 vs all groups