Heme Arginate Suppresses Cardiac Lesions and Hypertrophy in Deoxycorticosterone Acetate-Salt Hypertension

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In hypertension, elevated levels of oxidative/inflammatory mediators including nuclear factor kappaB (NF-κB), activating protein (AP-1), c-Jun-NH2-terminal kinase (JNK), and cell-regulatory proteins such as transforming growth factor beta (TGF-β), trigger the mobilization of extracellular matrix (ECM) leading to fibrosis, hypertrophy and impairment of cardiac function. Although the heme oxygenase (HO) system is cytoprotective, its effects on cardiac fibrosis and hypertrophy in deoxycorticosterone acetate (DOCA-salt) hypertension are not completely elucidated. Here, we report cardioprotection by the HO inducer, heme arginate against histopathological lesions in DOCA-hypertension. Treatment with heme arginate restored physiological blood pressure, and abated cardiac hypertrophy (3.75 ± 0.12 vs. 3.19 ± 0.09 g/kg body wt; n =16, $P < 0.01$), left-to-right ventricular ratio (6.67 ± 0.62 vs. 4.39 ± 0.63; n = 16, $P < 0.01$), left ventricular mass (2.48 ± 0.14 vs. 2.01 ± 0.09 g/kg body wt; n = 16, $P < 0.01$) and left-ventricular wall thickness (2.82 ± 0.16 vs. 1.98 ± 0.14 mm; n = 16, $P < 0.01$), whereas the HO inhibitor, chromium mesoporphyrin, exacerbated hypertrophy and cardiac lesions. The suppression of cardiac hypertrophy was accompanied by a robust increase in HO-1, HO activity, cyclic guanosine monophosphate (cGMP), ferritin and the total antioxidant capacity, whereas 8-isoprostane, NF-κB, JNK, AP-1, TGF-β, fibronectin and collagen-I were significantly abated. Correspondingly, histopathological parameters that depict progressive cardiac damage, including fibrosis, interstitial/perivascular collagen deposition, scarring, muscle-fiber thickness, muscular hypertrophy and coronary-arteriolar thickening were abated. Our study suggests that upregulating the HO system lowers blood pressure, potentiates the antioxidant status in tissues, suppresses oxidative stress/mediators such as NF-κB, AP-1 and cJNK, and suppresses the mobilization of ECM proteins like TGF-β, collagen and fibronectin, with corresponding reduction of cardiac histopathological lesion and hypertrophy. Exp Biol Med 234:764–778, 2009

Key words: heme arginate; heme oxygenase-1; DOCA-salt hypertension; cardiac hypertrophy; fibrosis; collagen deposition; TGF-β; fibronectin; NF-κB

Introduction

Hypertension is a major factor for the development of cardiac hypertrophy. Among the different cardiovascular diseases associated with hypertension, hypertrophy and remodelling are characterized by increased deposition of extracellular matrix (ECM) proteins like collagen and fibronectin (1, 2). The accumulation of ECM proteins in the cardiovascular system increases wall stiffness and may ultimately lead to development of heart failure (3). Moreover, increased collagen deposition in the myocardium and around coronary arteries is a common pathophysiological characteristic that depicts progressive heart impairment in different hypertensive models, including deoxycorticosterone acetate (DOCA-salt) hypertension (4). In DOCA hypertensive model, the administration of synthetic mineralocorticoid, DOCA-salt, triggers severe hypertension that gradually leads to cardiac damage (4). Moreover, DOCA-salt hypertension mimics the pathophysiology of aldosterone overload (5).

The deleterious effects of aldosterone on the heart include the induction of inflammation, myocardial fibrosis and hypertrophy, ventricular arrhythmia, and cardiac dysfunction (6, 7). In addition, aldosterone stimulates ECM proteins such as fibronectin and collagen, and causes inflammation by activating nuclear factor kappa beta (NF-κB) and activating protein (AP-1) (1, 2, 8). This results in a net accumulation of interstitial collagen deposition which leads to fibrosis (9). Besides activating NF-κB, the NF-κB receptor has also been reported to stimulate AP-1 and c-Jun

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NH2-terminal kinase (cJNK), which in turn mediates aldosterone-induced oxidative insults (8, 10). In DOCA-salt hypertension, local production of aldosterone in cardiac tissue (11) stimulates transforming growth factor beta (TGF-β_1), fibronectin and collagen-1 leading to fibrosis and hypertrophy (12). Given that TGF-β_1 gene promoter harbours consensus binding sites for NF-κB and AP-1 (13), cross-talk among TGF-β_1, NF-κB and AP-1 is envisaged. Accordingly, the simultaneous activation of TGF-β_1, fibronectin, NF-κB, AP-1 and JNK constitute a potent deleterious force in tissues.

In physiological systems, many pathways are activated in an attempt to counteract tissue insult. These include the heme oxygenase (HO) system (14–20), a pathway that has been shown to counteract hypertension and hypertrophy (21, 22). HO is a microsomial enzyme with different isoforms (HO-1 and HO-2) that breaks down the pro-oxidant, heme to generate cytoprotective products such as carbon monoxide, bilirubin, biliverdin and ferritin with effects against apoptosis, inflammation and oxidative stress (14–16, 19, 20). HO-1 is inducible while HO-2 is constitutively expressed, so HO-1 can be pharmacologically modulated by an inducer of HO like heme arginate or a blocker such as chromium mesoporphyrin (CrMP) (23, 24). By breaking down the pro-oxidant, heme, HO depletes the levels of heme and thus reduces oxidative stress (18). Thus, upregulating the HO system may ameliorate tissue injury resulting from oxidative and inflammatory events. However, the effects of the HO system on mineralocorticoid-induced mobilization of ECM and cardiac histopathological lesions including collagen deposition, fibrosis, scarring, muscle-fiber thickness, muscular hypertrophy and coronary arteriolar thickening in DOCA-salt hypertension have not been fully characterized. Moreover, cardiovascular impairment in DOCA-salt hypertension mimics end-stage organ damage (4, 25). With the increasing incidence of end-stage-organ damage, especially in children and adolescents with hypertension (26–28), novel strategies to simultaneously suppress hypertension, hypertrophy and fibrosis are needed. Moreover, left ventricular hypertrophy is the most prominent clinical evidence of end-stage-organ damage in childhood hypertension, and recent data indicates that it occurs in as many as 41% of patients with childhood hypertension (26–28). Thus, there is need for new strategies to combat end-organ damage.

Therefore, this study was designed to investigate the effects of the HO inducer, heme arginate, on cardiac lesions in DOCA-salt hypertension. The chronic exposure to synthetic mineralocorticoid in the DOCA-salt model makes it ideal to study the effects of sustained aldosterone overload on the cardiac injury since chronic increase in aldosterone contributes to the pathophysiology of heart failure and end-stage-organ damage (4, 25, 29).

Materials and Methods

Our experimental protocol was approved by University of Saskatchewan Standing Committee on Animal Care and Research Ethics, and is in conformity with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male Sprague Dawley (SD) rats of 8 weeks were purchased from Charles River Laboratories (Willington, MA, USA). 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 one week acclimatization, some animals were anaesthetised, and their right kidney surgically removed. The animals were divided into the following experimental groups ($n = 6$–16 per group): (A) controls [surgery-free or normal SD, uninephrectomized (UnX)-sham, UnX-salt (0.9% NaCl + 0.2% KCl) and UnX-DOCA]; (B) DOCA-salt hypertensive rats; (C) heme arginate-treated DOCA-salt; (D) DOCA-salt rats treated together with heme arginate and the HO inhibitor, chromium mesoporphyrin (CrMP), and (E) vehicle-treated DOCA-salt. Heme arginate was given at a dose of 30 mg/kg i.p. daily, for 4 weeks, while CrMP was given at 4 μmol/kg i.p. daily for 4 weeks (23, 24, 30). Although many HO inhibitors are non-specific, and may affect other hemo enzymes or even increase HO-1 expression (31), however, CrMP has been shown to be selective against HO activity at a dose of 4 μmol/kg (32). Both heme arginate 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, 33). The volume of heme arginate, CrMP or vehicle injected was 0.5 ml. Systolic BP was determined by non-invasive tail-cuff method (Model 29 SSP, Harvard Apparatus, Montreal, Canada). BP was monitored weekly for the entire duration of therapy. At least six different readings were taken to calculate the mean systolic BP.

A day prior to killing, the animals were placed in metabolic cages overnight, weighed, anaesthetized with pentobarbital sodium (50 mg/kg body wt), blood samples were collected and the hearts were isolated. The hearts were cleaned in ice-cold phosphate-buffered saline (PBS), weighed and left ventricular hypertrophy established. The composition of PBS was as follows: (mM) 140 NaCl, 3 KCl, 10 Na_2HPO_4, and 2 KH_2PO_4. Tissues not used immediately were snap-frozen in liquid nitrogen and stored at –80°C for biochemical assays. From the plasma ferritin was routinely measured by Saskatoon Royal University Hospital, Canada. Immediately on sacrifice, whole blood was collected in chilled 50 ml beakers containing heparin as anticoagulant. The whole blood was then centrifuged at 2000 *g* at 4°C for 10 minutes. The resulting plasma was immediately removed by Pasteur pipette, and dispensed into sample tubes for ferritin measurement.

Histological and Morphological Analyses of Left Ventricular Tissue. The heart was isolated and

fixed in 10% formalin, processed and paraffin embedded as we previously reported (33). Sections of 5 μm thickness were cut from the left ventricle and stained with hematoxylin and eosin. By means of light microscopy, semi-quantitative assessment to evaluate muscular hypertrophy and the degree of scarring in the different experimental groups was performed in a blinded manner. A scale of 0 to 2 was used, with 0 signifying normal or almost normal; 1 for mild and 2 for severe lesion. The mean value for each experimental group was obtained and compared statistically. In addition, thickening of the coronary arteriolar wall was determined randomly using NIS-Elements BR-Q imaging software (Nikon) [0.95 μm/Px], while other morphometrical parameters of the coronary arterioles such as media-to-lumen ratio, media cross-sectional area and media thickness were recorded and compared between the different experimental groups (34). Masson trichrome staining was used to detect interstitial/perivascular collagen deposition. The total area of vessels and perivascular fibrosis was determined, and assessment of perivascular fibrosis was done by calculating the ratio of the fibrosis area surrounding the vessel to the total vessel area and averaged per group (34).

We also quantified longitudinal and cross-sectional muscle fiber thickness. The morphometric evaluation of left ventricular myocyte width (longitudinal/cross-sectional) was done randomly in at least 20 muscle fibers from each tissue using NIS-Elements BR-Q imaging software [0.95 μm/Px].

Total RNA Isolation and Quantitative Real-Time Reverse Transcription PCR. This was done by our established method (30, 33). In brief, left ventricular tissues were homogenized in 0.5 ml Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's specifications. Reverse transcription was done with First Strand cDNA Synthesis Kit (Novagen, Madison, WI, USA) with 0.5 μg Oligo (dT)6, 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$, 50 mM DTT, 10 mM each free dNTP and 100 U of MMLV reverse transcriptase following the manufacturer's instruction. Quantitative PCR was done with Applied Biosystems 7300 Real Time PCR system (Foster City, CA, USA) and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) 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 $MgCl_2$, SYBR Green and 10 nM fluorescein as passive reference. Triplicate samples of 1 μl of cDNA were run using a template of 3.2 pmol of primers for AP-1 (forward, 5′AGCAGATGCTTGAGTTGAGAGCCA-3′ and reverse, 5′TTCCATGGGTCCCTGCTTTGAGAT-3′), p65-NF-κB (forward, 5′CATGCGTTTCCGTTACAAGTGCGA-3′ and reverse 5′TGGGTCGTCTTAGTGGTATCTGT-3′), JNK (forward 5′AAGCAGCAAGGCTACTCCTTCTCA-3′ and reverse 5′ATCGAGACTGCTGTCTGTGTCTGA-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5′AGCAAGGATACTGAGAGCAAGA-3′ and reverse 5′TCTGGGATGGAATTGTGAGGGAGA-3′) in a final volume of 25 μl.

The sequences of all primers used were confirmed by the National Research Institute of Canada, Saskatoon. The program for thermal cycle was 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 56°C and 15 seconds at 72°C. PCR product melting points were determined by incubation at 65°C for 1 minute followed by a 1°C per minute rise over 30 minutes.

Determination of Left Ventricular HO Activity and HO-1 Concentration. HO activity in the left ventricle was evaluated as bilirubin production using our established method (23, 30, 33). Briefly, left ventricular tissue was homogenized on ice in 4 volumes of 5:1 K/Na 100 mmol/L phosphate buffer with 2 mmol/L $MgCl_2$ (HO-activity buffer), centrifuged at 13,000 rpm for 15 minutes, and 100 μl of supernatant used for HO enzyme activity assay. This was added to 500 μl, containing 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 was done in darkness at 37°C for 1 hour, and stopped by adding 500 μl of chloroform. To extract bilirubin, the tubes were vigorously agitated and centrifuged at 13,000 rpm for 5 minutes. 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 by spectrophotometric assay and expressed as nmole/mg protein/hour. The protein content was measured using Bradford assay.

Left ventricular HO-1 concentration was quantified by ELISA (Stressgen, StressXpress, Ann Arbor, MI, USA) as we previously reported (33). This is a quantitative assay in which mouse monoclonal antibody specific for HO-1 was pre-coated on wells of a plate to capture and detect HO-1 when the secondary rabbit polyclonal antibody conjugated to horseradish was added. The assay uses tetramethylbenidine substrate which develops a blue colour in proportion of the amount of HO-1. The addition of an acid stop solution converts the endpoint colour to yellow, and the intensity of the yellow colouration was read in a microplate at 540 nm (SpectraMax 340PC, Molecular Devices, CA, USA). HO-1 concentrations in the samples were calculated from a standard curve generated with calibrated HO-1 standards (0.195–12.5 ng/ml) that were provided by the manufacturer.

Measurement of Left Ventricular cGMP Content. The concentration of cGMP was assessed as we previously described (30, 33). Briefly, left ventricular tissues were homogenized in 6% trichloroacetic acid at 4°C in the presence of 3'-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity. After centrifuging at 2000 *g* for 15 minutes, the supernatant was recovered and washed with water-saturated diethyl ether. Thereafter, the upper ether layer was aspirated and discarded while the

aqueous layer containing cGMP was recovered and lyophilised for the assay. The dry extract was dissolved in assay buffer and the cGMP content was quantified following the manufacturer's protocol. The results were expressed as picomol of cGMP per mg of protein.

Western Immunoblotting. Left ventricular tissue was homogenized in 10 mM Tris-buffered saline (20 mM Tris-HCl of pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of freshly prepared cocktail of protease inhibitors, as we previously described (23, 30, 33). Aliquots of 50 μg of proteins were loaded on a 10% SDS-polyacrylamide gel for fibronectin and collagen-α1 type-1, while 12.5% for TGF-β. The fractionated proteins were electrophoretically transferred to nitrocellulose paper. Non-specific bindings sites were blocked with 3% non-fat milk dissolved in PBS for 2 hours at room temperature. Thereafter, the membranes were incubated overnight with primary antibodies against fibronectin (sc-18825, 1:5000 dilution, Santa Cruz Biotechnology, CA, USA), collagen-α1 type-1 (sc-25974, 1:1000 dilution, Santa Cruz Biotechnology, CA, USA) and TGF-β1/2/3 [(1:1000 dilution; H-112 sc-7892 (with molecular weights for TGF-β_1, TGF-β_2 and TGF-β_3 being 12.5, 13 and 12.5 kD, respectively), Santa Cruz Biotechnology Inc., CA, USA)]. After several washes, the nitrocellulose blot was incubated with secondary antibody conjugated to horseradish peroxide (Bio-Rad, CA, USA) for 2 hours at room temperature, and the immuno-reactivity visualized with enhanced horseradish peroxide/luminol chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA, USA). Relative densitometric scanning and analysis of respective bands of blot were carried out using UN-SCAN-IT software (Silk Scientific, Utah, USA). A monoclonal antibody raised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to ascertain equivalent loading.

Determination of Plasma 8-Isoprostane. 8-iso-prostane is a non-invasive index of oxidative stress (35). This was measured by EIA (Cayman Chemical, Ann Arbor, MI) as we previously reported (33). In brief, plasma samples 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 was added to each well, incubated and after washing, Ellaman's reagent containing the substrate of acetylcholinesterase was added. The absorbance were read at 412 nm in a plate reader (SpectraMax 340PC, Molecular Devices, CA, USA), and the values of 8-isoprostane calculated from a standard curve.

Determination of Plasma Total Antioxidant Capacity Assay. Plasma total antioxidant capacity was evaluated using EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA) as we previously reported (30, 33). The antioxidant system of living organisms is made up of an array of different enzymes including superoxide dismutase, catalase, and glutathione peroxidise as well as substances like ferritin, ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, biliverdin, bilirubin, etc. (14–16, 18–20, 36). The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system. The synergistic effect of all the different antioxidants provides greater protection against oxidative stress than any single compound alone. The Cayman Chemical Antioxidant Assay Kit measures the total antioxidant capacity (37). This assay is based on the ability of the antioxidants in samples to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS plus metmyoglobin. In brief, plasma was treated with Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), metmyoglobin and chromogen. The reaction was initiated by adding H_2O_2 and after 5 minutes, the absorbance was read at 750 nm using Synergy Microplate Reader (BioTek Instruments, Inc., Vermont, USA) with Gen5 Data Analysis Software. The results were expressed as Trolox equivalent antioxidant capacity (TEA C) per mg protein (37).

Statistical Analysis. All data were expressed as means ± SEM from at least six independent experiments unless otherwise stated. Statistical analyses were done using Student's *t* test, ANOVA in conjunction with Bonferroni's test for multiple variables, and ANOVA for repeated measures. Group differences at the level of $P < 0.05$ were considered statistically significant.

Results

Heme Arginate Reduces Blood Pressure and Abates Cardiac Hypertrophy in DOCA-Salt Rats. The implantation of DOCA strips to unilaterally nephrectomized rats caused severe hypertension (198.7 ± 3.6 mmHg), while the controls (surgery-free SD, UnX-sham, UnX-salt and UnX-DOCA) were within physiological range with readings 121.4 ± 1.6, 120.6 ± 2.4, 119.5 ± 1.5, 120.4 ± 3.1 and 116.4 ± 2.3 mmHg, respectively (Table 1). The application of heme arginate restored physiological blood pressure to DOCA-salt hypertensive (134.8 ± 1.6 mmHg). During the 4-week therapy, blood pressure progressively dropped. After the first week blood pressure dropped to 183.5 ± 1.8 mmHg, and subsequently to 165.4 ± 3.6, 143.8 ± 1.7 and 134.8 ± 1.2 mmHg, respectively, at the the second, third and fourth week of therapy. In contrast, the animals treated with the HO inhibitor, CrMP, had sustained hypertension (215.6 ± 4.2 mmHg) throughout the study, whereas the vehicle had no effect on blood pressure (198.7 ± 3.6 vs. 199.5 ± 3.7 mmHg). Interestingly, the blood pressure lowering effect of heme arginate was accompanied by significant reduction of several indices of hypertrophy (33, 38–40) including, the left-to-right ventricular ratio (6.67 ± 0.62 vs. 4.39 ± 0.63, $P < 0.01$), the left ventricle-to-body weight index (2.48 ±

0.14 vs. 2.01 ± 0.09 g/kg body wt, $P < 0.01$) and the left ventricular wall thickness (2.82 ± 0.16 vs. 1.97 ± 0.14 mm, $P < 0.01$) (Table 1). Correspondingly, cardiac hypertrophy (entire heart) was significantly abated (3.75 ± 0.12 vs. 3.19 ± 0.09 g/kg body wt, $P < 0.01$) in DOCA-salt hypertensive rats (Table 1). On the contrary, CrMP annulled the protective effect of heme arginate and/or exacerbated cardiac hypertrophy while the vehicle had no effect.

The application of heme arginate alone did not affect body weight; however, co-application with CrMP caused a slight reduction of body weight. The reason for this remains unclear and should be further investigated.

Heme Arginate Upregulates HO-1, HO Activity and cGMP Levels. To elucidate the mechanisms for the cardioprotective effects of heme arginate, HO-1 and HO activity were assayed. The basal levels of HO-1 in the controls (normal SD and UnX-sham) were similar, but slightly lower than in DOCA-salt hypertensive. Heme arginate greatly enhanced HO-1 concentration by 3.1-fold, while the HO inhibitor, CrMP, nullified the increase by heme arginate (Fig. 1A). A similar trend was observed for HO activity. The basal HO activity in the controls was similar, but lower than in DOCA-hypertensive rats. Treatment with heme arginate enhanced HO activity by 4.6-fold, whereas CrMP abolished the heme arginate effect (Fig. 1B). The enhanced HO activity will increase the production of endogenous carbon monoxide that will in turn stimulate the soluble guanylate cyclase-cGMP second messenger system, an important component through which the HO system abates cardiac injury and hypertrophy (41, 42). Accordingly, a robust increase in cGMP was observed in heme arginate-treated animals (Fig. 1C). On the other hand, CrMP abolished the heme arginate effect on cGMP. Although the basal HO activity in DOCA-salt hypertensive rats was higher than in the control, the magnitude might have been below the threshold that triggers an increase in cGMP content. A similar observation has been previously reported in spontaneously hypertensive rats (23, 30, 33).

Heme Arginate Potentiates the Antioxidant Status and Abates Oxidative Stress. Hypertrophy is generally characterized by intense inflammatory and oxidative insults, and in many cases mediators like JNK, NF-κB and AP-1 are implicated (8, 10). In DOCA-salt hypertensive rats, the basal JNK levels were significantly elevated as compared to the normotensive controls (normal SD and UnX-sham). However, heme arginate significantly attenuated JNK by 2.5-fold, whereas the HO blocker, CrMP, annulled the effect of heme arginate (Fig. 2A). Similarly, elevated levels of the oxidative/inflammatory transcription factors NF-κB and AP-1 were observed in DOCA-salt hypertensive rats (Fig. 2B and 2C). Interestingly, treatment with heme arginate abrogated the levels of NF-κB and AP-1 by 2.8- and 3.7–fold, respectively. Although heme arginate reduced the levels of JNK, NF-κB and AP-1, it was more effective against NF-κB and AP-1 as their levels were restored to those of the controls (normal SD and UnX-

Table 1. Physiological Profile of Animals

Parameter	Normal SD (surgery-free) ($n = 6$)	UnX-sham ($n = 6$)	UnX-salt ($n = 6$)	UnX-DOCA ($n = 6$)	DOCA-salt ($n = 16$)	DOCA-salt + HA ($n = 16$)	DOCA-salt + HA+CrMP ($n = 6$)	DOCA-salt + vehicle ($n = 6$)
Body weight (grams)	444 ± 19.4	476 ± 13.3	467 ± 12.6	449 ± 15.4	441 ± 17.6	420 ± 10.5	411 ± 13.4[#]	446.5 ± 15
Systolic blood pressure (mmHg)	121.4 ± 1.6	120.6 ± 2.4	119.5 ± 1.5	120.4 ± 3.1	198.7 ± 3.6*	134.8 ± 1.6[†]	215.6 ± 4.2*	199.5 ± 3.7*
Cardiac hypertrophy (g/kg B. wt.)	2.9 ± 0.09	3.1 ± 0.17	2.8 ± 0.2	3.1 ± 0.3	3.75 ± 0.11*	3.19 ± 0.09[†]	4.16 ± 0.21*	3.95 ± 0.24*
Left-to-right ventricular ratio	4.11 ± 0.19	4.33 ± 0.67	4.16 ± 0.48	4.25 ± 0.42	6.67 ± 0.62*	4.39 ± 0.63[†]	7.46 ± 0.88*	7.31 ± 0.42*
Left ventricle-to-body weight index (g/kg B. wt.)	2.01 ± 0.08	1.91 ± 0.17	1.94 ± 0.22	1.97 ± 0.05	2.48 ± 0.14*	2.01 ± 0.09[†]	2.97 ± 0.19*	2.63 ± 0.1*
Left ventricle wall thickness (mm)	1.68 ± 0.11	2.0 ± 0.05	1.75 ± 0.24	1.81 ± 0.16	2.82 ± 0.16*	1.97 ± 0.14[†]	2.97 ± 0.19*	2.95 ± 0.14*

[#] $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups; [†] $P < 0.01$ vs. all groups.

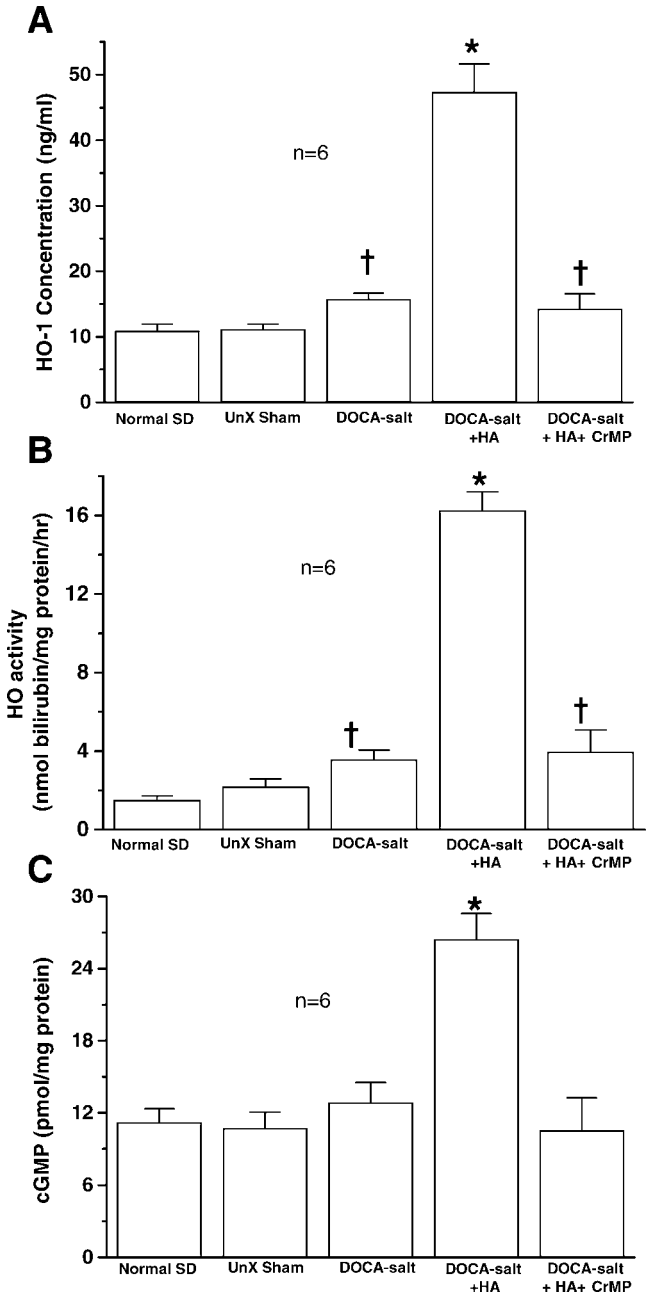

Figure 1. Effect of heme arginate (HA) and chromium meoporphyrin (CrMP) on HO-1, HO activity and cGMP content in the left ventricle of DOCA-salt hypertensive rats. (**A**) Heme arginate increased cardiac HO-1 concentration, but CrMP reversed the effect of heme arginate († $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups, $n = 6$ for each group). Bars represent means ± SE. (**B**) Treatment with heme arginate markedly increased HO activity while CrMP blocked the increase († $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups, $n = 6$ for each group). Bars represent means ± SE. (**C**) Heme arginate markedly enhanced cGMP content, but CrMP blocked the increase by heme arginate (* $P < 0.01$ vs. all groups, $n = 6$ for each group). Bars represent means ± SE. The acronym "UnX" signifies nephrectomised sham control.

sham), whereas JNK was only partially reduced. The reason for this selective effect is unclear and should be further investigated. On the other hand, the HO inhibitor, CrMP, reinstated comparable levels of JNK, NF-κB and AP-1 as observed in DOCA-salt hypertensive animals. Interestingly, the reduction of JNK, NF-κB and AP-1 in heme arginate-treated animals was accompanied by a parallel reduction of plasma-8-isoprostane, an index of oxidative stress (Fig. 2D). The levels of 8-isoprostane in heme arginate-treated animals were reduced to comparable levels of the normotensive controls.

Our results also indicate that heme arginate greatly enhanced the levels of antioxidants. In DOCA-salt hypertensive rats the basal levels of the antioxidant, ferritin, were significantly lower than in the normotensive controls. However, heme arginate increased ferritin levels by 7-fold (Fig. 2E). Correspondingly, the total antioxidant capacity in heme arginate-treated animals was robustly increased by 3.8-fold (Fig. 2F). Importantly, heme arginate potentiated the total antioxidant status to levels even beyond those observed in the normotensive controls, while the HO inhibitor, CrMP suppressed the effects of heme arginate.

Heme Arginate Attenuates Scarring Muscle-Fiber Thickness and Fibrosis. The application of heme arginate to DOCA-salt hypertensive rats greatly reduced histopathological lesions and evidence of cardiac hypertrophy. In DOCA-salt hypertensive animals, scarring was intense and diffused (Fig. 3A). The scarred areas contained muscle fibers with variations in cross-sectional area, focal crowding and grouping of capillaries, which indicated muscle loss. In addition, there was an evident increase in interstitial fibrosis, which was of patchy distribution in cardiac tissue of DOCA-salt hypertensive rat. The histopathological lesions in DOCA-salt hypertensive rats were indicative of intense fibrosis and hypertrophy (43). However, heme arginate greatly reduced the lesions and this coincided with the attenuation of cardiac hypertrophy (Fig. 3A).

We also examined the cross-sectional/longitudinal muscle fiber thickness, an index of cardiac fibrosis and hypertrophy (44). The cardiac muscle fibers from untreated DOCA-salt hypertensive rats were associated with high variations, marked interstitial fibrosis and greater thickness (Fig. 3A). Interestingly, in heme arginate-treated animals, the muscles fibers were more uniform with minimal interstitial connective tissues, reduced interstitial fibrosis and thickness. Interestingly, our histological observations were further confirmed by quantitative and semi-quantitative analyses. Accordingly, quantitative analyses indicated that heme arginate reduced longitudinal muscle fiber thickness (Fig. 3B), cross-sectional muscle fiber thickness (Fig. 3C) and perivascular fibrosis (Fig. 3D). Similarly, semi-quantitative analyses revealed that heme arginate attenuated muscular hypertrophy (Fig. 3E) and scarring (Fig. 3F) in DOCA-salt hypertensive rats. According to Spearman's analyses, there was a positive correlation

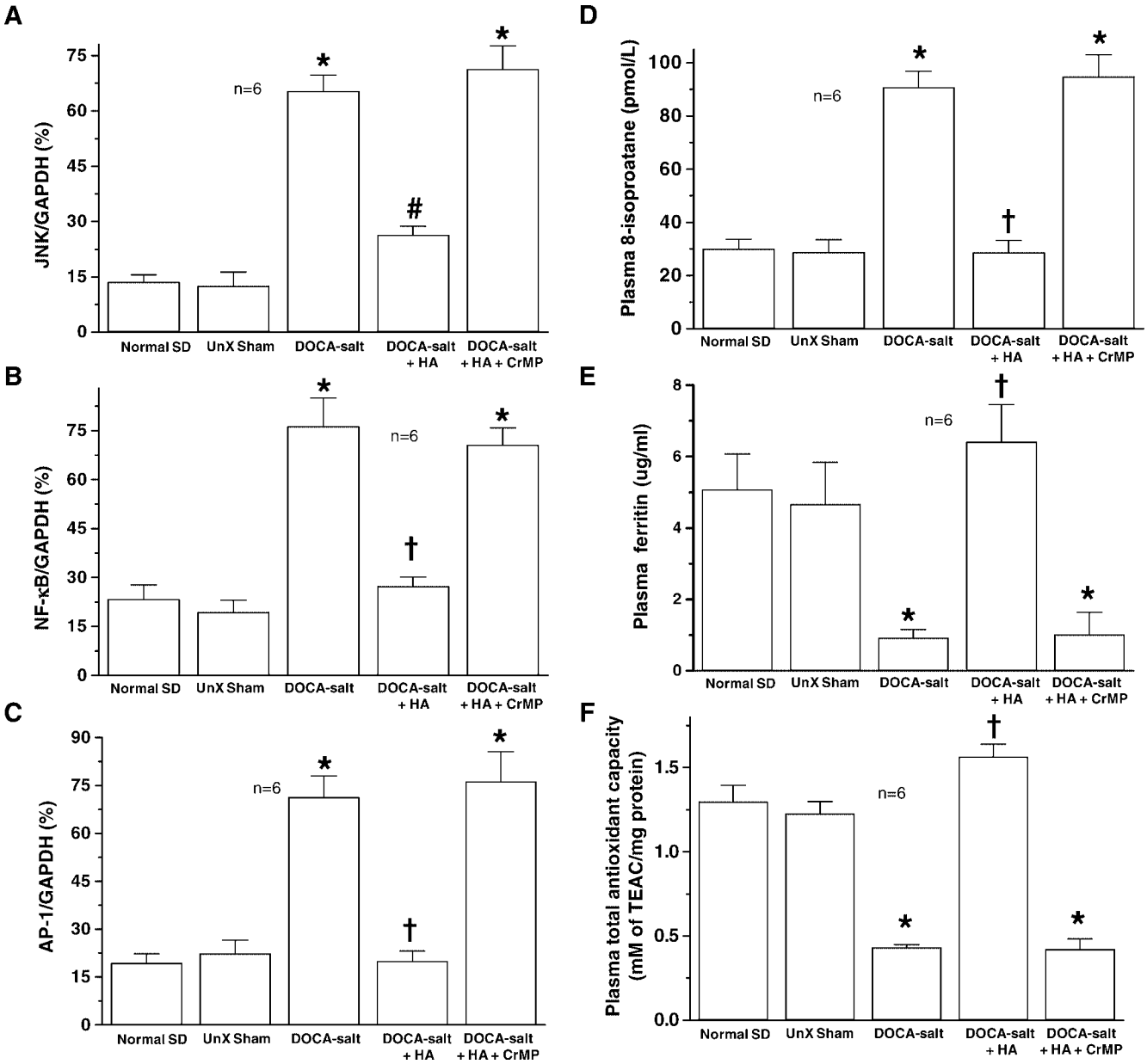

Figure 2. Effect of heme arginate (HA) and chromium meoporphyrin (CrMP) on oxidative in DOCA-salt hypertensive rats. (**A**) HA attenuated left ventricular JNK while CrMP increased it ($^{\#}$ $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups, $n = 6$ for each group). Bars represent means ± SE. (**B**) HA abated left ventricular NF-κB while CrMP increased it (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA+HA+CrMP, $n = 6$ for each group). Bars represent means ± SE. (**C**) HA suppressed left ventricular AP-1 while CrMP increased it (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA+HA+CrMP, $n = 6$ for each group). Bars represent means ± SE. (**D**) HA reduced plasma 8-isoprostane while CrMP increased it (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA+HA+CrMP, $n = 6$ for each group). Bars represent means ± SE. (**E**) HA enhanced plasma ferritin while CrMP decreased it (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA+HA+CrMP, $n = 6$ for each group). Bars represent means ± SE. (**F**) HA increased plasma total antioxidant capacity while CrMP reduced it (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA+HA+CrMP, $n = 6$ for each group). Bars represent means ± SE. The acronym "UnX" signifies nephrectomised sham control.

between scaring and the low HO activity ($r = 0.775$; $P = 0.005$) in DOCA-salt hypertensive rats. Interestingly, upregulation of HO activity by heme arginate significantly abated scarring. Further assessment of our result by Spearman's analyses revealed a positive association between the degree of longitudinal muscle fiber thickness and perivascular fibrosis ($r = 0.867$, $P = 0.001$), JNK ($r = 0.821$, $P = 0.005$), NF-κB ($r = 0.867$, $P = 0.002$), AP-1 ($r = 0.872$, $P = 0.002$), and 8-isoprostane ($r = 0.804$, $P = 0.006$) in DOCA-salt hypertensive rats.

Heme Arginate Abates Collagen Deposition and Remodelling or Coronary Arterioles. Coronary arteriolar thickening and collagen deposition are important pathophysiological evidence of cardiac damage. In DOCA-

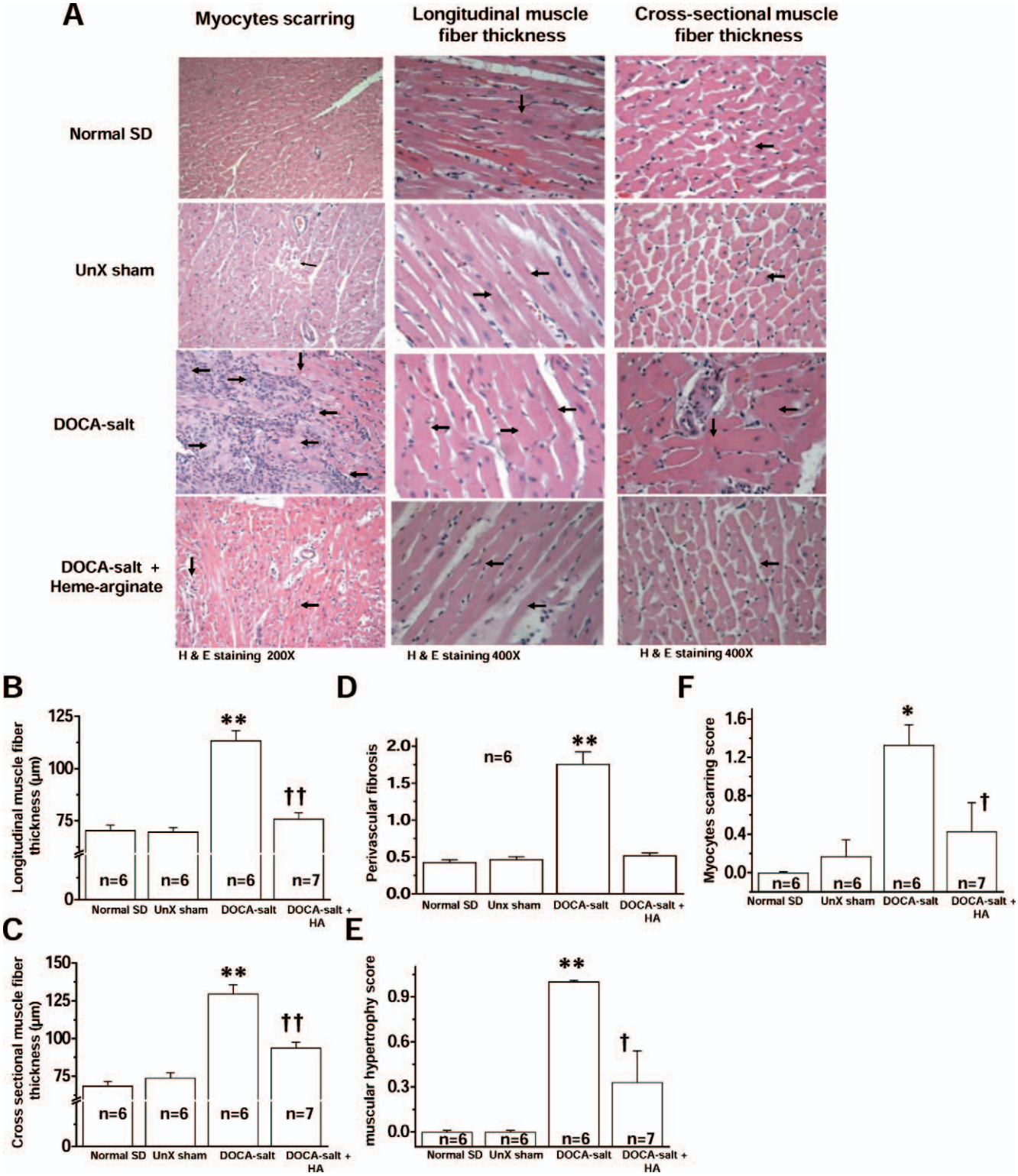

Figure 3. Effect of heme arginate (HA) on cardiac injury. (**A**) Representative images of histological sections indicate that HA reduced scarring and longitudinal/cross-sectional muscle fiber thickness in the left ventricle of DOCA-salt hypertensive rats ($n = 6$–7 for each group). The arrows indicate areas of intense damage. Quantitative evaluation revealed that HA reduced (**B**) longitudinal muscle fiber thickness (** $P < 0.01$ vs. all groups; †† $P < 0.01$ vs. DOCA-salt, $n = 6$–7 per group), (**C**) cross-sectional muscle fiber thickness (** $P < 0.01$ vs. all groups; †† $P < 0.01$ vs. DOCA-salt, $n = 6$–7 per group), and (**D**) perivascular fibrosis (** $P < 0.01$ vs. all groups, $n = 6$ per group). Bars represent means ± SE. Semi-quantitative analyses revealed that HA attenuated (**E**) muscular hypertrophy († $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups, $n = 6$–7 per group), and (**F**) scarring († $P < 0.05$ vs. all groups; * $P < 0.01$ vs. all groups, $n = 6$–7 per group). Bars represent means ± SE. The acronym "UnX" signifies nephrectomised sham control. A color version of this figure is available in the online journal.

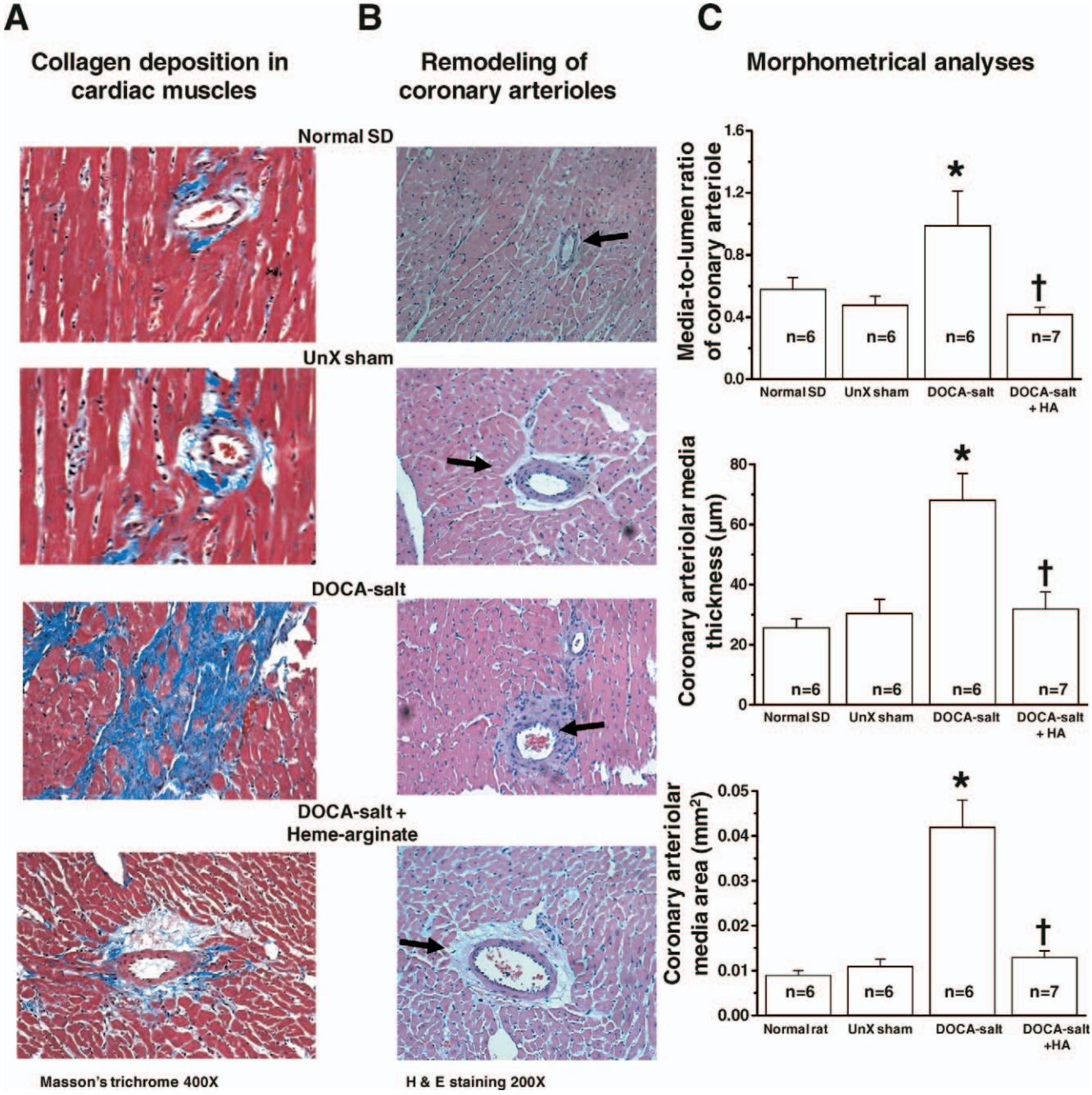

Figure 4. Effect of heme arginate (HA) on collagen deposition in the left ventricle and remodelling of coronary arterioles of DOCA-salt hypertensive rats. (**A**) Representative images of histological sections revealed that HA attenuated collagen deposition ($n = 6$–7 for each group). (**B**) Representative images of histological sections revealed that HA attenuated coronary arteriolar thickening ($n = 6$–7 for each group). (**C**) Morphometrical analyses indicated that HA reduced media-to-lumen ratio, media area, and media thickness of the coronary arterioles (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt, $n = 6$–7 per group). Bars represent means ± SE. The acronym "UnX" signifies nephrectomised sham control. A color version of this figure is available in the online journal.

salt hypertensive rats, collagen deposition was very intense and highly diffused in the interstitium and perivascular space (Fig. 4A). However, heme arginate greatly reduced collagen deposition. Our results also revealed increased coronary arteriolar thickness (remodelling) in DOCA-salt hypertensive rats (Fig. 4B). Accordingly, we detected elevated media-to-lumen ratio, media thickness and media area of coronary arterioles in DOCA-salt hypertensive rats (Fig. 4C). Interestingly, heme arginate significantly abated these morphometrical indices that characterize remodelling/hypertrophy of the coronary arterioles.

Heme Arginate Attenuates the Expression of TGF-β, Fibronectin, and Collagen. TGF-β mobilizes the ECM by stimulating fibronectin and collagen-1 to cause

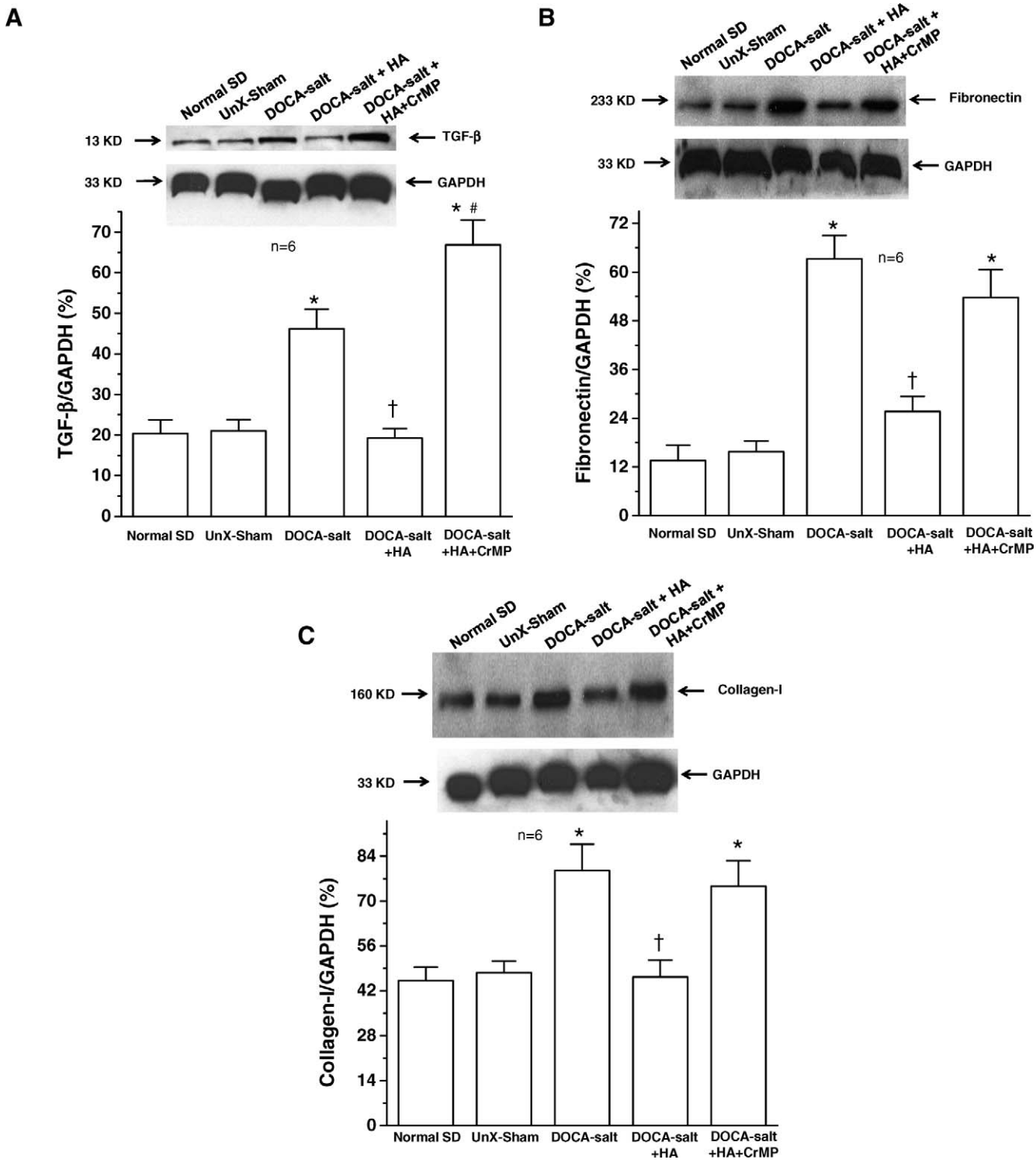

Figure 5. Effect of heme arginate (HA) on extracellular matrix proteins in the left ventricle of DOCA-salt hypertensive rats. (**A**) Representative Western immunoblot of TGF-β and the quantitative result of relative TGF-β levels normalized by GAPDH (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA-salt + HA+CrMP, # $P < 0.05$ vs. all groups, $n = 6$ per group). Bars represent means ± SE. (**B**) Representative Western blot of fibronectin and the quantitative result of relative fibronectin levels, normalized by GAPDH (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA-salt + HA+CrMP, $n = 6$ per group). Bars represent means ± SE. (**C**) Representative Western blot of collagen-I and the quantitative result of relative collagen-I levels, normalized by GAPDH (* $P < 0.01$ vs. all groups; † $P < 0.01$ vs. DOCA-salt or DOCA-salt + HA+CrMP, $n = 6$ per group). Bars represent means ± SE. The acronym "UnX" signifies sham-operated control.

fibrosis and hypertrophy (1, 2, 12). In DOCA-salt hypertensive rats, severe cardiac lesions accompanied by elevated levels of TGF-β were observed (Fig. 5A). Interestingly, heme arginate significantly suppressed TGF-β in DOCA-salt hypertensive rats, reinstating similar levels as observed in the normotensive controls, whereas the HO inhibitor, CrMP, completely cancelled the heme arginate effect and even slightly increased the levels of TGF-β. Similarly, heme arginate suppressed the expressions of fibronectin (Fig. 5B) and collagen-1 (Fig. 5C) in DOCA-salt hypertensive rats. However, heme arginate restored the expression of collagen-1 to control levels but only partially reduced fibronectin expression. The reason for this selective effect is unclear and should be addressed in future studies.

Discussion

The present study demonstrates the cardioprotective effects of heme arginate against hypertension, fibrosis and cardiac hypertrophy. In DOCA-salt hypertensive rats, myocardial damage was associated with severe lesions including collagen deposition, interstitial/perivascular fibrosis, scarring, muscle-fiber thickness, muscular hypertrophy and coronary arteriolar thickening. Interestingly the application of heme arginate greatly attenuated these lesions. The mechanisms underlying the cardioprotection of heme arginate include upregulation of HO activity and the guanylate cyclase-cGMP pathway. The potentiated HO-signaling was accompanied by the suppression of 8-isoprostane and mediators of oxidative stress like JNK, NF-κB, AP-1, alongside the reduction of TGF-β and corresponding decline of the deposition of ECM proteins like fibronectin and collagen. In contrast, the HO blocker, CrMP, abolished HO activity, reversed the protective effect of heme arginate and/or exacerbated cardiac insults. Although many HO inhibitors are non-specific, CrMP given at a dose of 4 μmol/kg is selective against HO activity (32, 45). Therefore, the exacerbation of cardiac lesions in CrMP-treated animals could be ascribed to the suppression HO-1.

Increased deposition of collagen and fibronectin in the myocardium and coronary arteries is a common pathophysiological event that impairs cardiac function (1, 2, 4). Interestingly, heme arginate abated cardiac fibronectin and collagen-1, and reduced thickening of small arteriolar coronaries. Since the accumulation of fibronectin and collagen in cardiac tissue frequently occurs in hypertrophic remodelling (1, 2), the restoration of histological lesions and attenuation of cardiac hypertrophy could also be attributed to the reduction of ECM proteins in heme arginate-treated animals. ECM matrix proteins are activated by many factors including TGF-β1 (12). Both TGF-β1 and fibronectin are involved in fibrosis and tissue damage (46). Thus, the abrogation of TGF-β1 may lead to reduced mobilization of fibronectin and collagen. This notion is consistent with previous studies that reported the reduction of TGF-β1-mediated increase of fibronectin in mice (47). Therefore the anti-fibrogenic effect of the HO system is not strain-dependent and may be considered a more diffused phenomenon that could be explored in the design of remedies against hypertrophy. Since TGF-β1 activates fibronectin and collagen-1 (44, 46, 47), the HO system may be critical for the regulation of fibrotic events in hypertrophy. Interestingly, TGF-β1 is also regulated by NF-κB and AP-1 (13). Therefore, the attenuation of TGF-β1 in heme arginate-treated animals may be linked to the parallel reduction of NF-κB and AP-1. However, this notion does not minimize the seemingly complex interaction among TGF-β1, NF-κB, AP-1 and the HO system, which remains largely unclear. Nevertheless, the simultaneous reduction of TGF-β1, NF-κB and AP-1 suggests a multifaceted interaction among these factors and the HO system. The characterization of this interaction remains challenging and would be the subject of future investigations. On the other hand, the HO system may directly regulate NF-κB, AP-1. The presence of consensus binding sites for NF-κB and AP-1 on the HO-1 gene promoter suggests greater interaction between these transcription factors and the HO system. Accordingly, it has been reported that blockade of HO system leads to increased levels of NF-κB and AP-1, with subsequent aggravation of inflammatory insults (48). This report is in agreement with our observation since the application of the HO blocker, CrMP, abolished the protective effects of heme arginate, with subsequent elevation of NF-κB, AP-1 and oxidative insults.

In DOCA-salt hypertension, many factors contribute to increase oxidative stress. These include the high levels of superoxide, 8-isoprostane and pro-inflammatory/oxidative mediators such as NF-κB, AP-1 and JNK (11–13, 49). However, upregulating the HO system with heme arginate abated oxidative stress and its mediators, whereas the HO inhibitor, CrMP, abolished heme arginate protection and exacerbated oxidative injury. The cytoprotective effects of upregulating HO system have been widely acknowledged (14–17). Heme arginate unregulated HO-1, HO activity and cGMP levels. Both HO activity and cGMP are closely related components of the HO system as the carbon monoxide generated from this system stimulates the soluble guanylyl cyclase/cGMP signalling (23) to reduce tissue injury (14–17, 50, 51). Moreover, HO breaks down the pro-oxidant, heme, and thus suppresses oxidative stress (18). In addition, other products from the HO system such as bilirubin and biliverdin possess anti-inflammation and antioxidant properties (14–16), while the iron formed enhances ferritin synthesis and thus confers additional protection against oxidative stress (19, 20). Interestingly, the increased levels of ferritin observed in heme arginate-treated animals alongside the corresponding increase of total antioxidant capacity would attenuate cardiac injury. Our results are in accordance with recent findings that demonstrated the potentiation of antioxidant status by upregulating the HO system (31) and the suppression of oxidant-induced activation of JNK and other mitogen-

activated protein kinases (52). Since another HO inducer, cobalt protoporphyrin, has been shown to attenuate oxidative injury in diabetes by increasing catalase and superoxide dismutase (SOD) activities (31), it is possible that the HO system is capable of regulating other antioxidant enzymes like catalase and SOD during tissue insult as an attempt to mount greater protection. Accordingly, the enhancement of the total antioxidant status in tissues after administering HO inducers may represent an intrinsic characteristic of HO in upregulating not only the traditional antioxidant which are directly linked to the HO system like bilirubin, biliverdin, ferritin, but also other antioxidant with different origins like SOD and catalase. However, this notion is complex and should be further investigated, to clarify how the HO system elicits such a central regulatory role for diverse antioxidants. Nevertheless, the potentiation of antioxidant status by the HO system is an important mechanism that acts synergistically with the reduction of TGF-β1 to attenuate histological lesions, nullify the mobilization ECM proteins like fibronectin and collagen-1 to ameliorate fibrosis and left ventricular hypertrophy.

Although the HO system is cytoprotective (16, 18, 20), some studies have shown that that carbon monoxide may interfere with nitric oxide-induced vasodilation (53) to cause endothelium-dependent vasoconstriction (54). These studies suggest that carbon monoxide may have a biphasic effect, and therefore the specific conditions under which different experiments are done may be critical to the observed effect. In our experimental setting, pharmacologically administered heme arginate upregulated the HO system and increased endogenous carbon monoxide production, which alongside bilirubin, biliverdin and ferritin would act in concert to abate oxidative stress and inflammation not only by enhancing cGMP, but also by suppressing JNK, NF-κB, and AP-1. Although the endothelium-dependent vasoconstriction (54) should be further investigated, our study highlights cardioprotection by heme arginate, suggesting that the concomitant suppression of 8-isoprostane JNK, NF-κB, AP-1 and TGF-β1, alongside the suppression of ECM proteins resulted in the attenuation of cardiac lesions including, scarring muscular hypertrophy, collagen deposition, and muscle fiber thickness, all of which are important indices of fibrosis and hypertrophy (43, 44). Our study also indicated that heme arginate and CrMP affected body weight. The reduction of body weight in the animals that received CrMP and heme arginate should be further investigated. It is difficult to account for the slight weight loss. However, food intake was slightly reduced. Given that a similar protocol has been used in other studies without toxicity (23, 30), further investigations will be carried out in future.

The anti-hypertensive effect of upregulating the HO system is a well known phenomenon, and many mechanisms have been proposed. These include the reduction of 20-hydroxyeicosatetraenoic acid, stimulation of calcium-activated potassium channels, and improved vascular relaxation, etc. (23, 24, 30, 55). Given that elevated blood pressure is among the causes of hypertrophy, it is possible that the anti-hypertensive effect of heme arginate is accompanied by attenuation of hypertrophy. However, hypertrophy may occur without hypertension, and the HO system has been shown to be effective against hypertrophy in normotensive conditions (22). Accordingly, HO-1 was reported to abate angiotensin-induced hypertrophy *in vitro* and *in vivo* (22), suggesting that the HO system may be endowed with intrinsic anti-hypertrophic properties which, together or independent to its anti-hypertensive effect, would abate cardiac lesions. Furthermore, HO-induced cardioprotection in normotensive conditions have been reported in rat cardiac myocytes (56) and H(mox-1)-knockout mice (57). Consistently, these reports and the present findings suggest that the HO system may combat hypertrophy in both normotensive and hypertensive conditions. The cardioprotection by heme arginate may arise from upregulating the HO system and the modulation of pro-inflammatory/oxidative transcription factors like JNK, NF-κB and AP-1 that cause tissue damage. As such, the presence of consensus binding sites for NF-κB and AP-1 on HO-1 gene (58, 59) may be indicative of an important role of the HO system in cellular defense, since blockade of the HO system caused increased NF-κB and AP-1, with corresponding increase in inflammatory (48) damage. Although the reduction of blood pressure *per se* is cardioprotective, heme arginate may possess other protective characteristics besides its anti-hypertensive properties. For example, the heme arginate-mediated reduction of left ventricular TGF-β1 and fibronectin observed in our studies has also been reported in normotensive mice (47). Importantly, the anti-fibrogenic effect of the HO system has been reported in normotensive conditions. In a related study it was shown that the HO system regulates fibronectin levels (47). Accordingly, knocking-out the HO-1 gene led to increased levels of fibronectin (47). Since the HO-1 knockout mice has higher levels of fibronectin and TGF-β1 is known to activate fibronectin (47), the concomitant reduction of TGF-β1 and fibronectin in heme arginate-treated animals may attenuate left ventricular fibrosis, even in hypertensive conditions. Although we have recently reported the effects of hemin on cardiac hypertrophy (33), examining the effects of another HO inducer provides additional confirmation of the potent cardioprotective effects of HO inducers. Therefore the suppression of NF-κB and AP-1, TGF-β1 and fibronectin by heme arginate further supports the protective effects of the HO system against oxidative and fibrotic mediators in mineralocorticoid-induced damage.

Although the current study provides important insights of the HO system against mineralocorticoid-induced cardiac damage, further studies are needed to fully characterize and distinguish the cardioprotective and antihypertensive effects of heme arginate. Some limitations of this study include heme arginate toxicity, which may cause hepatic iron

overload with adverse effects (60). Similarly, the pharmacological administration of heme arginate may cause vasculotoxicity (61). However, toxicity occurs primarily when the therapeutic dose of heme arginate is exceeded (60, 61). Another limitation of our study is the lack of functional data to demonstrate that the suppression of cardiac histopathological lesions in DOCA-salt hypertensive rats was accompanied by improved cardiac function. Although systolic blood pressure was the only hemodynamic parameter measured in the present study, we had previously shown that upregulating the HO system with hemin, another HO inducer, led to improved cardiac function in a model of cardiac anaphylaxis (50). We showed that during cardiac anaphylaxis, the HO system attenuates the exaggerated increase in inotropism, chronotropism and heart rate after antigen challenge (50). Similarly, the HO system has been shown to improve cardiac output, stroke volume and ejection fraction during exercise (62), whereas blockade of the HO system impairs cardiac hemodynamics (63). Accordingly, it could be envisaged that the attenuation of cardiac histopathological lesions in DOCA-salt hypertensive rats would be accompanied by improved cardiac function.

Collectively, our study suggests that the cardioprotective effects of heme arginate could be attributed to upregulating the HO system and cGMP-signalling, alongside the reduction of oxidative stress and its mediators with consequential reduction of collagen and fibronectin deposition in the myocardium and small arteriolar coronaries. Correspondingly, the attenuation of histopathological lesions resulted in the reduction of fibrosis and cardiac hypertrophy. Although our study attempts to delineate the mechanisms underlying the cardioprotective effects of the HO system in DOCA-salt hypertension, many challenging questions needs to be resolved. The multifaceted interaction among the HO system and mediators of fibrogenic/oxidative events like JNK, NF-κB, AP-1 and TGF-β1 should be further investigated at molecular/gene levels for a more comprehensive understanding. Nevertheless, the translational potential of our study remains enormous given that HO inducers like heme arginate are already used in clinical settings against porphyria (64).

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