Heme Arginate Therapy Enhanced Adiponectin and Atrial Natriuretic Peptide, but Abated Endothelin-1 with Attenuation of Kidney Histopathological Lesions in Mineralocorticoid-Induced Hypertension

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ABSTRACT
We investigated the role of heme oxygenase (HO), adiponectin, and atrial natriuretic peptide (ANP) in uninephrectomized (UnX) deoxycorticosterone-acetate (DOCA)-salt hypertensive rats, a volume-overload model characterized by elevated endothelin-1 (ET-1), mineralocorticoid-induced oxidative/inflammatory insults, fibrosis, hypertrophy, and severe renal histopathological lesions that closely mimic end-stage renal disease (ESRD). HO was enhanced with heme arginate (HA) or blocked with chromium mesoporphyrin (CrMP). Histological, morphological/morphometrical, quantitative reverse transcription-polymerase chain reaction, Western blot, enzyme immunoscassay, and spectrophotometric analysis were used. Our experimental design included the following groups of rats: A, controls (surgery-free Sprague-Dawley, UnX-sham, UnX-salt (0.9% NaCl + 0.2% KCl), and UnX-DOCA); B, UnX-DOCA-salt hypertensive; C, UnX-DOCA-salt + HA; D, UnX-DOCA-salt + HA + CrMP; E, UnX-DOCA-salt + CrMP; F, UnX-DOCA-salt + captopril; G, UnX-DOCA-salt + l-arginine; H, UnX-DOCA-salt + spironolactone; and I, UnX-DOCA-salt + vehicle. HA lowered blood pressure and abated kidney hypertrophy and renal lesions, including glomerulosclerosis, tubular dilation, tubular cast formation, interstitial mononuclear cell infiltration, glomerular hypertrophy, and renal-arteriolar thickening in UnX-DOCA hypertension. Correspondingly, HO activity, adiponectin, adenosine monophosphate-activated protein kinase (AMPK), ANP, cGMP, antioxidants such as bilirubin, ferritin, superoxide dismutase, and catalase, and total antioxidant capacity were increased, whereas ET-1, transformed growth factor β (TGF-β), fibronectin, and 8-isoprostane were abated. These were accompanied by reduced proteinuria/albuminuria, but increased creatinine clearance. Interestingly, HA was more renoprotective than spironolactone, l-arginine, and captopril, whereas the HO blocker CrMP exacerbated oxidative injury, aggravating renal lesions and function. Because 8-isoprostane stimulates ET-1 to potentiate oxidative stress and fibrosis, up-regulating HO-1 enhanced tissue antioxidant status alongside cellular targets such as adiponectin, AMPK, ANP, and cGMP to suppress ET-1, TGF-β, and fibronectin with a corresponding decline of renal lesions, proteinuria/albuminuria, and thus improved renal function. The potent renoprotection of HA could be explored to combat renal hypertension and histopathological lesions characteristic of ESRD.

The incidence of end-stage renal disease (ESRD) has almost doubled in the past 10 years (Rosamond et al., 2008). In a recent survey in the United States, after adjustment for age, sex, and race, patients with ESRD increased by 43% during the decade after 1991 (U.S. Renal Data Systems, 2007). Moreover, from 1994 to 2004, diabetes, hypertension, and glomerulonephritis accounted for 80% of all cases of ESRD (Rosamond et al., 2008). With an aging population and the high incidence of metabolic syndrome, this trend may prevail. Therefore, the growing incidence of ESRD is a great concern, and novel renoprotective strategies are needed. Uninephrectomized (UnX) deoxycorticosterone acetate (DOCA)-salt hypertension is a model of human primary aldosteronism, characterized by elevated endothelin-1 (ET-1), high levels of a synthetic mineralocorticoid (DOCA), and severe renal histopathological lesions that closely mimic ESRD (Larivière et al., 1993; Dworkin et al., 1996). Although aldosterone is a
mineralocorticoid that promotes sodium/water retention, recent evidence indicates that it triggers the formation of superoxide anion, which in turn quenches nitric oxide by forming peroxynitrite (Pryor and Squadrito, 1995) and subsequently oxidizes arachidonic acid, generating 8-isoprostane, a potent vasoconstrictor and stimulator of ET-1 (Fukunaga et al., 1995). In addition to its antidiuretic/sodium retention responses and regulation of systemic/renal hemodynamics (Piechota et al., 2007), ET-1 causes oxidative injury (Fukunaga et al., 1995; Fiore et al., 2005). Because ET-1 and aldosterone have mutual stimulatory effects (Khanna et al., 2005), the elevated levels of mineralocorticoid and 8-isoprostane in UnX-DOCA-salt hypertension may exacerbate the oxidative destruction of tissues (Pryor and Squadrito, 1995; Blasi et al., 2003; Ortmann et al., 2004; Artunc et al., 2006). Thus, the high levels of the synthetic mineralocorticoid DOCA and 8-isoprostane in UnX-DOCA-salt hypertension may constitute a powerful vasoconstrictor axis that amplifies the vasopressor action of ET-1, in addition to promoting remodeling and disrupting the architectural morphology and function of tissues (Pryor and Squadrito, 1995; Blasi et al., 2003; Ortmann et al., 2004; Artunc et al., 2006). Therefore, strategies capable of suppressing ET-1/aldosterone-induced insults would be useful for restoring tissue structure and function.

The activities of ET-1 and atrial natriuretic peptide (ANP) are closely related (Piechota et al., 2007). Generally, ANP and ET-1 have opposing effects (Pandey, 2005; Piechota et al., 2007). For example, ANP promotes natriuresis and vaso-dilatation (Pandey, 2005), whereas ET-1 stimulates sodium retention and vasoconstriction (Piechota et al., 2007). Likewise, ANP reduces fibrosis by inhibiting transforming growth factor β1 (TGFB-1) and fibronectin (Piechota et al., 2007), whereas ET-1 acts in concert with TGFB-1 to stimulate the synthesis of the matrix-associated protein fibronectin (Shi-Wen et al., 2007). On the other hand, ANP stimulates the production of adiponectin (Teukamoto et al., 2009), a cytoprotective adipokine with anti-inflammatory effects (Folco et al., 2009). Moreover, a putative link between ANP and adiponectin has been reported in patients with heart failure (Tanaka et al., 2008). Given that the effects of ANP are mediated by cGMP (Pandey, 2005), and adiponectin has recently been shown to enhance cGMP (Ribá et al., 2008), novel strategies that concomitantly enhance ANP, adiponectin, and cGMP, but suppress the effects of aldosterone, ET-1, TGFB-1, and fibronectin may attenuate tissue damage. Among the cytoprotective pathways that could be pharmacologically modulated to combat renal lesions is the heme oxygenase (HO) enzymatic pathway (Levere et al., 1990; Abraham and Kappas, 2008; Jadhav et al., 2009).

HO is a microsomal enzyme with inducible (HO-1) and constitutive (HO-2) isofoms (Levere et al., 1990; Abraham and Kappas, 2008; Jadhav et al., 2009). The HO-catalyzed breakdown of the heme moiety generates biliverdin/bilirubin, ferritin, and carbon monoxide (CO) to suppress oxidative stress/inflammation and lower blood pressure (BP) (Abraham and Kappas, 2008). Because the role of the HO inducer HA in renal histopathological lesions has not been fully characterized, we investigated the effects of HA on renal morphology and function in UnX-DOCA-salt hypertensive rats, a model characterized by severe renal lesions that is widely accepted to mimic ESRD (Larivière et al., 1993; Dworkin et al., 1996). Moreover, with the high incidence of ESRD (Rondon et al., 2008), novel therapeutic modalities are needed. Given that ESRD frequently occurs in patients with chronic diseases such as hypertension and diabetes (Rosamond et al., 2008), two pathophysiological conditions characterized by elevated oxidative/inflammatory insults associated with progressive tissue destruction and gradual loss of function, we investigated the multifaceted interaction among HA, adiponectin, AMPK, ANP, cGMP, ET-1, TGFB-1, and fibronectin in UnX-DOCA-salt hypertensive rats. In addition, we compared renoprotection by HA with other drugs including; captopril, an angiotensin-converting enzyme inhibitor; spironolactone (SPL), a mineralocorticoid receptor blocker; l-arginine, a constituent of HA; and the HO blocker chromium mesoporphyrin (CrMP). Because captopril does not affect BP in UnX-DOCA-salt rats (Brown et al., 1999), whereas BP is lowered by SPL, HA, and l-arginine (Fenning et al., 2005; Abraham and Kappas, 2008; Iwazu et al., 2008), and CrMP increases BP (Jadhav and Ndisang, 2009; Ndisang and Jadhav, 2010a,b), the relative input/contribution of BP lowering of each drug to its antihypertrophic/fibrotic effect was assessed.

**Materials and Methods**

**Animals, Treatment Groups, and Biochemical Parameters.**

Our experimental protocol was approved by University of Saskatchewan Standing Committee on Animal Care and Research Ethics and conformed with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (National Institutes of Health Publication 85-23, revised 1996). Male Sprague-Dawley (SD) rats of age 8 weeks, purchased from Charles River Laboratories, Inc. (Willington, MA), were housed at 21°C with 12-h light/dark cycles, fed standard chow, and had access to drinking water ad libitum. After a week of acclimatization, UnX-DOCA-salt hypertension was induced as described previously (Ndisang et al., 2008; Jadhav and Ndisang, 2009). In brief, the right kidney was removed through a dorsal flank incision under isoflurane anesthesia, and a silastic strip with or without the synthetic mineralocorticoid DOCA was implanted subcutaneously in the midscapular region. Our experimental design included the following groups of rats (n = 6–20 per group): A, controls [surgery-free or normal SD, UnX-sham, UnX-salt (0.9% NaCl + 0.2% KCl), and UnX-DOCA]; B, UnX-DOCA-salt hypertensive; C, UnX-DOCA-salt + HA; D, UnX-DOCA-salt + HA + CrMP, the HO inhibitor; E, UnX-DOCA-salt + CrMP; F, UnX-DOCA-salt + captopril, an angiotensin-converting enzyme inhibitor; G, UnX-DOCA-salt + l-arginine; H, UnX-DOCA-salt + SPL, a mineralocorticoid receptor blocker; I, UnX-DOCA-salt + vehicle for HA and CrMP; and J, UnX-DOCA-salt + vehicle for SPL (sesame oil). HA and CrMP were prepared as reported previously (Jadhav and Ndisang, 2009). l-Arginine and captopril were dissolved in water (Gimeno et al., 1998; Brown et al., 1999), and SPL was dissolved in sesame oil (Klanke et al., 2008). Captopril and SPL were purchased from Sigma-Aldrich (St Louis, MO), and CrMP was purchased from Frontier Scientific (Logan, Utah). Treatment with HA (30 mg/kg i.p.), CrMP (4 μmol/kg i.p.), l-arginine (30 mg/kg i.p.) (Gimeno et al., 1998), captopril (100 mg/kg by oral gavage) (Brown et al., 1999), SPL (100 mg/kg i.p.) (Klanke et al., 2008), or the different vehicles began after the animals were severely hypertensive (192.7 ± 2.6 mm Hg), and treatment was given daily for 4 weeks. Many HO inhibitors are nonspecific and may affect other heme enzymes or even increase HO-1; however, CrMP is selective against HO at a dose of 4 μmol/kg (Jadhav and Ndisang, 2009).

Systolic BP was determined by the noninvasive tail-cuff method (model 29-SSP; Harvard Apparatus Inc., Montreal, ON, Canada). BP was monitored weekly for the entire duration of therapy. Six different readings were taken to calculate the mean systolic BP. At the end of the chronic treatment period, rats were anesthetized briefly, the right kidney was removed through a dorsal flank incision and had access to drinking water ad libitum. After a week of acclimatization, UnX-DOCA-salt hypertension was induced as described previously (Ndisang et al., 2008; Jadhav and Ndisang, 2009). In brief, the right kidney was removed through a dorsal flank incision under isoflurane anesthesia, and a silastic strip with or without the synthetic mineralocorticoid DOCA was implanted subcutaneously in the midscapular region. Our experimental design included the following groups of rats (n = 6–20 per group): A, controls [surgery-free or normal SD, UnX-sham, UnX-salt (0.9% NaCl + 0.2% KCl), and UnX-DOCA]; B, UnX-DOCA-salt hypertensive; C, UnX-DOCA-salt + HA; D, UnX-DOCA-salt + HA + CrMP, the HO inhibitor; E, UnX-DOCA-salt + CrMP; F, UnX-DOCA-salt + captopril, an angiotensin-converting enzyme inhibitor; G, UnX-DOCA-salt + l-arginine; H, UnX-DOCA-salt + SPL, a mineralocorticoid receptor blocker; I, UnX-DOCA-salt + vehicle for HA and CrMP; and J, UnX-DOCA-salt + vehicle for SPL (sesame oil). HA and CrMP were prepared as reported previously (Jadhav and Ndisang, 2009). l-Arginine and captopril were dissolved in water (Gimeno et al., 1998; Brown et al., 1999), and SPL was dissolved in sesame oil (Klanke et al., 2008). Captopril and SPL were purchased from Sigma-Aldrich (St Louis, MO), and CrMP was purchased from Frontier Scientific (Logan, Utah). Treatment with HA (30 mg/kg i.p.), CrMP (4 μmol/kg i.p.), l-arginine (30 mg/kg i.p.) (Gimeno et al., 1998), captopril (100 mg/kg by oral gavage) (Brown et al., 1999), SPL (100 mg/kg i.p.) (Klanke et al., 2008), or the different vehicles began after the animals were severely hypertensive (192.7 ± 2.6 mm Hg), and treatment was given daily for 4 weeks. Many HO inhibitors are nonspecific and may affect other heme enzymes or even increase HO-1; however, CrMP is selective against HO at a dose of 4 μmol/kg (Jadhav and Ndisang, 2009).
of therapy, the animals were weighed and anesthetized, and samples were collected. Kidney hypertrophy was determined by the kidney-to-body weight ratio, an index of kidney hypertrophy (Jadhav et al., 2009), and plasma (bilirubin, ferritin, and creatinine) and urine (proteinuria, albuminuria, and creatinine) were routinely analyzed at Saskatoon Royal University Hospital (Saskatoon, SK, Canada) as reported previously (Jadhav et al., 2009).

HO activity was evaluated spectrophotometrically by our established method, and HO-1 was evaluated by enzyme-linked immunosorbent assay (Assay Designs, Ann Arbor, MI) (Jadhav and Ndsang, 2009). Other biochemical parameters including 8-iso prostane, ANP, SOD activity (total), catalase activity, cGMP, total antioxidant capacity, and ET-1 were measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI), and adiponectin was measured by enzyme-linked immunosorbent assay (Phoenix Pharmaceuticals, Inc., Burlingame, CA) as reported previously (Ndsang et al., 2008, 2009; Jadhav and Ndsang, 2009).

Histological and Morphological Analyses of Kidney. Sections of 5 μm of paraffin-embedded tissue were cut and stained with hematoxylin and eosin, and the diameter of 30 glomeruli was measured randomly by using NIS-elements BR-Q imaging software (Nikon, Tokyo, Japan) (0.95 μm/pixel) as reported previously (Jadhav et al., 2009). Morphologic evaluation of glomerular hypertrophy, glomerulosclerosis, tubular dilation, tubular cast, and mononuclear cell infiltration were performed blindly with light microscopy and assessed semiquantitatively as described previously (Jadhav et al., 2009).

Western Immunoblotting. Tissues were homogenized and centrifuged, proteins were quantified by Bradford, and we proceeded as described previously (Ndsang et al., 2008; Jadhav et al., 2009). Primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for fibronectin, TGF-β1/2/3, and AMPK (Cell Signaling Technology, Danvers, MA) were used. Densitometric analysis was done with UN-SCAN-IT software (Silk Scientific Inc., Orem, Utah). β-Actin antibody (Sigma-Aldrich) was used as a control to ascertain equivalent loading.

Extended methodology is available as Supplemental Information.

Statistical Analyses. All data are expressed as means ± S.E.M. from at least six independent experiments unless stated otherwise. Statistical analyses were done by using unpaired Student’s t test and analysis of variance in conjunction with Bonferroni test for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Statistical analyses were done by using unpaired Student’s t test and analysis of variance in conjunction with Bonferroni test for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures. Statistical analyses were done by using unpaired Student’s t test and analysis of variance in conjunction with Bonferroni test for repeated measures. Group differences were determined using unpaired Student’s t test and analysis of variance for repeated measures.

Results

HA Lowered BP and Abated Kidney Hypertrophy. UnX-DOCA-salt rats were severely hypertensive (192.7 ± 2.6 mm Hg), whereas control animals received UnX-sham, UnX-salt, UnX-DOCA, and surgery-free SD were normotensive with BP of 118.7 ± 2.8, 123.6 ± 4.5, 122.5 ± 3.7, and 121.5 ± 3.1 mm Hg, respectively. The 4-week regimen of HA lowered BP in UnX-DOCA-salt hypertensive rats (192.7 ± 2.6 versus 136.6 ± 1.2 mm Hg; p < 0.01) (Table 1), whereas HA + CrMP or CrMP alone nullified the effects of HA and exacerbated hypertension, suggesting an important role for basal HO activity in BP regulation. After the first week of treatment, BP dropped from 192.7 ± 2.6 to 178.4 ± 3.4 mm Hg and decreased gradually to 163.3 ± 2.4, 143.4 ± 2.4, and 136.6 ± 1.2 mm Hg at the end of the second, third, and fourth week, respectively. Likewise, l-arginine and SLP lowered BP, whereas the animals treated with captopril and the vehicle dissolving HA and CrMP had sustained hypertension (Table 1). The vehicle dissolving SLP (sesame oil) did not affect BP (192.7 ± 2.6 versus 195.3 ± 5.8 mm Hg; n = 4). The

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<tr>
<th>Animal type</th>
<th>Controls</th>
<th>UnX-DOCA-salt + HA</th>
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<td>BP</td>
<td>178.4 ± 3.4</td>
<td>136.6 ± 1.2</td>
<td>143.4 ± 2.4</td>
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<td>Albuminuria (mg/24 h)</td>
<td>3.4 ± 0.4</td>
<td>3.1 ± 0.3</td>
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<td>Urinary albumin-to-creatinine ratio</td>
<td>0.4 ± 0.1</td>
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<td>Plasma Na (mEq/l)</td>
<td>72.6 ± 1.7</td>
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<td>Plasma K (mEq/l)</td>
<td>3.4 ± 0.2</td>
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<td>Plasma glucose (mg/dl)</td>
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<td>Plasma triglycerides (mg/dl)</td>
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<td>Plasma cholesterol (mg/dl)</td>
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<td>Plasma HDL (mg/dl)</td>
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<td>Plasma LDL (mg/dl)</td>
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antihypertensive effects of HA, SPL, and l-arginine were accompanied by significant reduction of kidney hypertrophy, albeit control levels were not reinstated. However, HA was more effective than l-arginine and SPL. In UnX-DOCA-salt + vehicle and UnX-DOCA-salt + HA + CrMP groups, hypertrophy remained elevated and was further exacerbated in the UnX-DOCA-salt + CrMP group, suggesting a role for basal HO against hypertrophy. Although captopril did not lower BP, it reduced kidney hypertrophy, although less effectively than did HA, SPL, and l-arginine (Table 1).

Because urinary proteins such as creatinine, proteinuria, and albuminuria are important indices of renal function (Artunc et al., 2006), we assayed and detected elevated levels of these proteins in UnX-DOCA-salt hypertensive rats. It is noteworthy that HA reduced urinary creatinine, proteinuria, and albuminuria by 33, 85, and 90% respectively, whereas cotreatment of HA and CrMP abolished the effects of HA, and treatment with CrMP alone exacerbated the excretion of these urinary proteins. On the other hand, SPL and l-arginine were less effective than HA but more effective than captopril against creatinine and proteinuria/albuminuria (Table 1).

Because mineralocorticoids favor sodium/water retention and facilitate potassium excretion, we examined the effects of HA and the other drugs on these parameters. UnX-DOCA hypertension was characterized by elevated plasma sodium and increased water intake (Table 1). It is noteworthy that HA and SPL were equally effective in reducing plasma sodium and water intake, whereas l-arginine and captopril were less effective. The reduction of plasma sodium by HA was accompanied by increased excretion of urinary sodium, creatinine, and urine volume with increased creatinine clearance, but reduced potassium excretion. l-Arginine was less effective than HA, but more effective than SPL and captopril. In contrast, CrMP abolished the effect of HA, aggravated potassium excretion, and reduced creatinine clearance (Table 1). HA and SPL also increased plasma potassium. On the other hand, HA greatly reduced the urinary albumin-to-creatinine index, suggesting less glomerular hyperpermeability (Artunc et al., 2006) and improved renal function after up-regulation of HO. In contrast, blocking the HO system with CrMP increased and/or exacerbated the urinary albumin-to-creatinine index, suggesting aggravation of glomerular function.

The administration of HA, CrMP, SPL, captopril, and l-arginine did not affect body weight (Table 1). However, the coapplication of HA and CrMP reduced body weight. The reason for this remains unclear, although food intake was reduced.

**HA Increased HO-1, HO Activity, and cGMP in UnX-DOCA Hypertensive Rats.** The basal HO-1 concentration and HO activity levels in sham-operated and SD controls were comparable but lower than the levels in UnX-DOCA hypertensive rats. HA robustly increased HO-1 and HO activity by 3.9- and 5.3-fold, respectively, whereas the coapplication of the HO inhibitor CrMP with HA abolished the increase, and CrMP alone depleted basal HO levels (Fig. 1, A and B). The HA-dependent enhancement of HO activity increased endogenous CO, which in turn stimulated the cGMP. Correspondingly, a robust increase of cGMP (3.1-fold) was observed in HA-treated animals (Fig. 1C). Likewise, l-arginine also increased cGMP, although to a lesser magnitude than HA, whereas captopril and SPL had no effect. Although basal HO activity in UnX-DOCA hypertensive rats was higher than the controls, it did not evoke an increase in cGMP content (Fig. 1C), suggesting that the magnitude might have been below the threshold necessary that triggers an increase in cGMP content. A similar observation has been reported previously (Jadhav et al., 2008; Ndisang et al., 2008).

**HA Increased ANP but Abated ET-1 in UnX-DOCA Hypertensive Rats.** UnX-DOCA-salt hypertension is characterized by elevated ET-1 (Lariviere et al., 1993). The administration of HA significantly reduced plasma and kidney ET-1 levels (Fig. 2, A and B). l-Arginine, captopril, and SPL also reduced ET-1 levels, although to a lesser extent than did HA. The coadministration of CrMP and HA reversed the
Fig. 2. Effect of HA on ET-1, 8-isoprostane, and ANP in UnX-DOCA-salt hypertensive rats. A, treatment with HA reduced plasma ET-1. B–D, HA reduced kidney ET-1 (B), abated urinary 8-isoprostane (C), and attenuated kidney 8-isoprostane (D) in UnX-DOCA-salt hypertensive rats. E–G, HA increased plasma ANP (E), enhanced kidney ANP (F), and increased urinary cGMP, a surrogate marker of ANP (G) in UnX-DOCA-salt hypertensive rats. ++, $p < 0.01$ versus UnX-DOCA-salt, UnX-DOCA-salt + CrMP, or UnX-DOCA-salt + CrMP; †, $p < 0.01$ versus all groups; *, $p < 0.05$ versus UnX-DOCA-salt + captopril or UnX-DOCA-salt + spironolactone; §, $p < 0.01$ versus UnX-DOCA-salt + l-arginine, UnX-DOCA-salt + captopril, or UnX-DOCA-salt + spironolactone; †, $p < 0.05$ versus all groups; @, $p < 0.05$ versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 6 rats per group).
effect of HA on ET-1, whereas treatment with CrMP alone further increased ET-1 levels, suggesting a role of HA in the regulation of ET-1. Because ET-1 is stimulated by 8-isoprostane (Fukunaga et al., 1995), and both ET-1 and 8-isoprostane are involved in the oxidative destruction of tissue, we also assessed 8-isoprostane. It is noteworthy that HA markedly reduced the elevated levels of urinary and kidney 8-isoprostane in UnX-DOCA hypertensive rats, whereas CrMP abolished the effect of HA (Fig. 2, C and D). Generally, urinary 8-isoprostane is indicative of the overall status of oxidative stress in an organism, whereas kidney 8-isoprostane reflects tissue-specific oxidative stress (Janssen, 2001). On the other hand, l-arginine, captopril, and SPL also reduced 8-isoprostane, although the magnitude of reduction was lesser than with HA.

Given that ET-1 and ANP interact reciprocally (Piechota et al., 2007), we assayed and observed that the reduction of ET-1 by HA was accompanied by the concomitant potentiation of ANP levels (Fig. 2, E and F). Consistently, urinary cGMP, a surrogate marker of ANP (Benigni et al., 2004), was increased by 1.9-fold by HA, whereas CrMP abated the HA-induced increase of urinary cGMP (Fig. 2G). Increased levels of urinary cGMP were also observed in l-arginine- and captopril-treated animals, whereas SPL had no effect. Although the basal ANP in UnX-DOCA hypertensive rats was higher than controls, it might not have been high enough to trigger an increase in cGMP, a secondary messenger through which ANP elicits its effect (Pandey, 2005). The reduced levels of urinary cGMP in UnX-DOCA hypertensive rats may be supportive of this notion (Fig. 2G). According to Spearman’s analyses, there is a significant positive correlation between urinary and kidney cGMP ($r = 0.748, p = 0.005$).

**HA Enhanced the Antioxidant Status in UnX-DOCA Hypertensive Rats.** Because antioxidants are modulated by the HO system (Abraham and Kappas, 2008; Ndisang and Jadhav, 2010a,b; Ndisang et al., 2010), we investigated the effects of HA on different antioxidants, including bilirubin, ferritin, SOD, and catalase. We detected significantly lower basal levels of bilirubin, ferritin, SOD, and catalase in UnX-DOCA-salt hypertensive rats (Fig. 3, A–D), which were increased by HA by 5.9-, 5.2-, 2.8-, and 3.2-fold, respectively. In contrast, the coadministration of CrMP and HA reversed the effect of HA on these antioxidants, whereas treatment with CrMP alone further depleted the antioxidants. HA seemed to have greater impact on bilirubin because it was enhanced to levels beyond the controls, whereas ferritin, SOD, and catalase were reinstated to control levels. Although l-arginine, captopril, and SPL had no effect on bilirubin and ferritin, l-arginine and SPL increased SOD, whereas captopril enhanced catalase. However, the magnitudes of these increments were lower than those triggered by HA. It is noteworthy that the HA-mediated increase of bilirubin, ferritin, SOD, and catalase was accompanied by significant elevation of the total antioxidant capacity (Fig. 3E). l-Arginine, captopril, and SPL increased the total antioxidant capacity to a lesser extent than did HA.

Despite the higher kidney HO activity (Figs. 1, A and B, and 3, A and B), it was puzzling that the levels of bilirubin and ferritin in UnX-DOCA hypertensive rats were lower. To address this problem, we measured HO activity in the liver because the liver and spleen are almost entirely responsible for bilirubin/iron production (Levere et al., 1990). It is noteworthy that the basal levels of hepatic HO activity in UnX-DOCA-salt hypertensive rats were lower than the controls (Fig. 3F). This may account for the diminished levels of plasma bilirubin and ferritin.

**HA Enhanced Adiponectin and the Expression of AMPK in UnX-DOCA Hypertensive Rats.** Because adiponectin is an anti-inflammatory protein (Folco et al., 2009) with renoprotective effects (Sharma et al., 2008), we investigated the effects of HA on adiponectin in UnX-DOCA-salt hypertension. In UnX-DOCA-salt rats, the depressed levels of plasma adiponectin (Fig. 4A) were markedly enhanced by HA by 4.6-fold, whereas the HO blocker CrMP nullified the HA-induced increase. On the other hand, l-arginine and captopril also increased adiponectin, although to a lesser magnitude compared with HA. In contrast, SPL had no effect on adiponectin levels.

Given that the effects of adiponectin are mediated via AMPK (Yamauchi et al., 2002), we investigated whether the HA-induced increase of adiponectin was accompanied by a parallel increase of AMPK. It is noteworthy that HA robustly enhanced AMPK expression in UnX-DOCA-salt rats (Fig. 4B), although control levels were not reinstated. CrMP abolished the effect of HA on AMPK.

Because a putative link between adiponectin and ANP has been reported in patients with heart failure (Tanaka et al., 2008), we used Spearman’s analyses to assess whether this association is applicable in our study. It is noteworthy that a significant positive correlation between circulating plasma adiponectin and kidney ANP was observed ($r = 0.853, p = 0.001$).

**HA Reduced Histopathological Lesions of the Kidney.** The suppression of hypertrophy and renal histopathological lesions by HA was accompanied by increased cGMP. Given that the elevation of cGMP attenuates kidney lesions (Wang et al., 2005), we used Spearman’s analyses to establish a link between cGMP, renal lesions, and HA. In UnX-DOCA-salt hypertensive rats, Spearman’s analyses revealed a significant negative correlation between kidney cGMP, the formation of tubular cast ($r = -0.642, p = 0.045$), glomerulosclerosis ($r = -0.633, p = 0.037$), interstitial mononuclear cell infiltration ($r = -0.693, p = 0.018$), glomerular diameter ($r = -0.818, p = 0.002$), tubular cast per section ($r = -0.687, p = 0.028$), and renal-arteriolar thickening ($r = -0.79, p = 0.004$). It is noteworthy that the elevated cGMP in HA-treated animals coincided with a marked reduction of glomerulosclerosis, tubular cast, and tubular dilation (Fig. 5A). Correspondingly, HA greatly attenuated glomerular diameter, reduced sclerotic/damaged glomeruli, and abolished tubular cast formation (Fig. 5B). The reduction of glomerular diameter and tubular cast was more effective because they were restored to control levels, whereas the attenuation of sclerotic/damaged glomeruli was partial. The reason for this selective effect remains unclear and needs further investigation.

Our results also revealed that the elevated levels of ET-1, 8-isoprostane, TGF-$\beta$-1, and fibronectin in UnX-DOCA hypertension positively correlate to renal lesions, and the reduction of these substances coincided with deceased glomerular necrosis, glomerular atrophy, interstitial mononuclear cell infiltration, and glomerular hypertrophy (Fig. 6A). Correspondingly, Western immunoblotting revealed that the reduction of renal lesions was associated with a significant
reduction of TGF-β1 and fibronectin protein expressions (Fig. 6, B and C).

Remodeling of renal arterioles is a pathophysiological phenomenon that compromises renal function. TGF-β1, fibronectin, ET-1, and 8-isoprostane stimulate remodeling and fibrogenesis (Lee et al., 2000; Shi-Wen et al., 2007). It is noteworthy that our data also indicate that HA abolished remodeling of renal arterioles, reinstating the dimensions of media-to-lumen ratio, media thickness, and cross-sectional area to control levels (Fig. 7).

Our histological observations were further confirmed by semiquantitative morphological analyses executed blindly (Table 2). A significant reduction of glomerular hypertrophy, renal-arteriolar thickening, glomerulosclerosis, tubular dilation, tubular cast formation, and interstitial mononuclear cell infiltration was observed in HA-treated animals.

**Discussion**

This study demonstrates that HA is an effective renoprotective agent against renal injury in UnX-DOCA hypertension. In this model, mineralocorticoid overload and the high levels of 8-isoprostane and ET-1 act in concert to potentiate the oxidative/fibrotic destruction of tissue (Pryor and Squadrito, 1995; Blasi et al., 2003; Ortmann et al., 2004; Artunc et al., 2006). Accordingly, severe histopathological lesions such as glomerulosclerosis, tubular atrophy, glomerular hypertrophy, tubular cast, interstitial mononuclear cell infiltration, and renal-arteriolar thickening were accompanied by functional damage, evidenced by elevated albuminuria, proteinuria, and urinary creatinine (Blasi et al., 2003; Ortmann et al., 2004; Artunc et al., 2006). Moreover, elevated albuminuria has been linked to adiponectin deficiency (Sharma et al., 2007).
AMPK may blunt albuminuria/proteinuria and prevent the possibility that increasing adiponectin levels or stimulating and kidney AMPK is a further confirmation of the intriguing correlation. Likewise, the HA-mediated increase of plasma adiponectin proves renal function by decreasing glomerular hyperpermeability, a defect associated with ESRD (Artunc et al., 2006). Interestingly, HA concomitantly enhanced suppressive Western immunoblot and relative densitometry of expressed protein normalized by β-actin reveals that HA significantly enhanced the protein expression of AMPK in UnX-DOCA-salt hypertensive rats, whereas CrMP blocked the HA-mediated increase. *p < 0.05 versus all groups; **, p < 0.01 versus UnX-DOCA-salt or UnX-DOCA-salt + HA + CrMP; †, p < 0.01 versus UnX-DOCA-salt + l-arginine, UnX-DOCA-salt + captopril, or UnX-DOCA-salt + spiran lactone; @, p < 0.05 versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 4–6 rats per group).

al., 2008). Interestingly, HA concomitantly enhanced suppression of albuminuria-to-creatinine index, whereas the HO inhibitor CrMP nullified the effect of HA. B, representative Western immunoblot and relative densitometry of expressed protein normalized by β-actin reveals that HA significantly enhanced the protein expression of AMPK in UnX-DOCA-salt hypertensive rats, whereas CrMP blocked the HA-mediated increase. *p < 0.05 versus all groups; **, p < 0.01 versus all groups; †, p < 0.01 versus UnX-DOCA-salt or UnX-DOCA-salt + HA + CrMP; †, p < 0.01 versus UnX-DOCA-salt + l-arginine, UnX-DOCA-salt + captopril, or UnX-DOCA-salt + spiran lactone; @, p < 0.05 versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 4–6 rats per group).

Other mechanisms responsible for the renoprotection of HA include up-regulation of HO and its cellular targets such as ANP and cGMP and the reduction of BP, ET-1, oxidative stress, TGF-β, and fibronectin. The interaction between ET-1 and ANP is widely acknowledged (Piechota et al., 2007). For example, ANP inhibits ET-1 (Piechota et al., 2007), and interestingly, the HA-dependent increase of ANP is accompanied by a parallel reduction of ET-1. However, ET-1 may also stimulate ANP synthesis (Dietz, 2005). Therefore, ET-1 may stimulate ANP to promote natriuresis, and beyond a certain threshold, ANP is inhibited by ET-1 through a feedback loop. Alternatively, CO, a product generated by the HO system, may reduce ET-1 (Stanford et al., 2004). Interestingly, the elevation of ANP by HA was accompanied by increased natriuresis with a corresponding increase in urine volume. During the elevation of BP, ANP is released in response to increased atrial distension, leading to natriuresis, diuresis, vasorelaxation, and reduction of BP (Pandey, 2005). Thus, the concomitant increase of ANP and natriuresis may also account for the antihypertensive effects of HA. The stimulation of cGMP is an important mechanism by which ANP elicits its effects (Pandey, 2005). Because natriuretic peptide receptors A and B contain guanylate-cyclase catalytic activity, cGMP largely mediates the effects of ANP (Pandey, 2005). Moreover, cGMP increases natriuresis and diuresis via diuretic-sensitive Na⁺ and Cl⁻ transport in renal-tubular epithelial cells (Pandey, 2005). Therefore, the synergistic augmentation of ANP and cGMP by HA would potentiate natriuresis.

Another important observation from our study is the marked elevation of plasma sodium in the UnX-DOCA-salt, UnX-DOCA-salt + HA + CrMP, and UnX-DOCA-salt + vehicle groups. Notably, the levels of plasma sodium in these groups were in the realm of toxicity. The sodium increase was accompanied by severe hypertension, marked renal hypertrophy, and fibrosis with renal insufficiency, a defect that was evidenced by reduced creatinine clearance and elevated proteinuria/albuminuria. Moreover, the pathophysiological profile of these animals was further aggravated by the striking reduction of plasma potassium. Generally, the clinical hallmarks of mineralocorticoid excess include potassium depletion (hypokalemia), systemic hypertension, and metabolic alkalosis. Interestingly, HA reversed these pathophysiological events by concomitantly enhancing ANP and cGMP, thus increasing natriuresis. In HA-treated animals plasma sodium consistently was significantly reduced, whereas plasma potassium increased. In addition to their natriuretic effects, ANP and cGMP may counter-regulate the effects of elevated mineralocorticoid and ET-1 in UnX-DOCA hypertension via natriuresis, vasodilation, and reduction of inflammatory/oxidative insults.

Many studies have shown that up-regulating the HO system reduces oxidative stress, annuls inflammation, and lowers BP (Abraham and Kappas, 2008; Ndisang and Jadhav, 2010a,b). By breaking down the pro-oxidant heme, HO reduces oxidative stress. In addition, the HA-mediated increase of ferritin, bilirubin, SOD, and catalase would contribute in augmenting the total antioxidant capacity and suppressing 8-isoprostane, with a corresponding decline of histopathological lesions, an effect that would improve...
renal function. Moreover, in the first human case of HO-1 deficiency, renal damage characterized by tubulointerstitial injury, tubular dilatation, interstitial fibrosis, and inflammatory cell infiltration were among the pathophysiological profile of the patient (Ohta et al., 2000), suggesting a role of the HO system in combating ESRD.

The antihypertensive effects of the HO system have been widely acknowledged, and many mechanisms, including suppression of vasoconstrictors and/or potentiation of vasorelaxants, have been proposed (Levere et al., 1990; Abraham and Kappas, 2008; Ndisang et al., 2008). Likewise, we recently showed that up-regulating the HO system abates the phospholipase C/inositol-triphosphate prohypertensive axis to lower BP in spontaneously hypertensive rats and UnX-DOCA-salt hypertensive rats (Ndisang et al., 2008; Ndisang and Jadhav, 2010a,b). It is therefore possible that up-regulating the HO system with HA will lower BP through these different mechanisms. Because elevated BP is among the causes of hypertrophy, it could be envisaged that the antihypertensive effects of HA would be accompanied by the reduction of hypertrophy. Likewise, L-arginine and SPL lowered BP, abated kidney hypertrophy, and improved renal function, whereas captopril was ineffective against BP (Brown et al., 1999) and had only a modest, although significant, effect against hypertrophy/renal function compared with the other drugs. Therefore, in the present study, the antihypertensive effects of HA, although undoubtedly important, may not be exclusively responsible for renoprotection. Although the reduction of BP per se leads to renoprotection, HA may possess intrinsic renoprotective characteristics in addition to its antihypertensive properties. For example, HA may have antifibrogenic effects. Because a mutual profibrotic effect between ET-1 and TGF-β1 has been reported (Shi-Wen et al., 2007) the concomitant suppression of ET-1, TGF-β1, and fibronectin by HA would contribute to renoprotection.

Although we recently reported the renoprotective effects of another HO inducer, hemin (Jadhav et al., 2009), HA more effectively suppressed renal lesions and improved renal function. This may be due to the synergistic contribution of hemin and L-arginine because HA is made from hemin and L-arginine. Therefore, the antihypertensive effects of HA, although undoubtedly important, may not be exclusively responsible for renoprotection.

Fig. 5. Effect of HA on renal lesions of UnX-DOCA-salt hypertensive rats. A, histological sections showing glomerulosclerosis, tubular cast, and tubular dilation of kidney from normal SD (n = 6), UnX-sham (n = 6), UnX-DOCA-salt (n = 6), and HA-treated UnX-DOCA-salt (n = 6) rats. The arrows indicate areas of pronounced damage. B, quantitative evaluation reveals that HA reduced glomerular diameter, sclerotic/damaged glomeruli, and tubular cast formation in UnX-DOCA-salt rats. *, p < 0.01 versus all groups; †, p < 0.01 versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 6 rats per group).
Fig. 6. Effect of HA on renal histological, TGF-β, and fibronectin. A, representative images showing glomerular necrosis, glomerular atrophy, interstitial mononuclear cell infiltration, and glomerular hypertrophy of normal SD (n = 6), UnX-Sham (n = 6), UnX-DOCA-salt (n = 6), and HA-treated UnX-DOCA-salt (n = 6) rats. The arrows indicate areas of intense damage. B and C, representative Western blot and densitometry of kidney TGF-β (B) and fibronectin (C). *p < 0.01 versus all groups; #p < 0.01 versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 6 rats per group).
nine. Because the histopathological lesions and reduced renal function may eventually progress to ESRD, the HA-induced renoprotection by HA may set the stage for more intense research on other HO inducers as a new paradigm against ESRD. Nonetheless, the suppression of renal lesions unveiled by this study may represent only the tip of an iceberg and does not profoundly address many unsettling questions. Further investigations examining the pharmacokinetics/dynamics of HA are needed to understand half-life, metabolites, distribution, and interaction with active proteins in biological milieu. These are among the many challenges that have to be addressed to increase the translational

Fig. 7. HA and remodeling of renal arterioles. A, histological sections of kidney from normal SD (n = 6), UnX-sham (n = 6), UnX-DOCA-salt (n = 6), and HA-treated UnX-DOCA-salt (n = 6) rats. The boxes show thickening of renal arterioles. B, HA abated morphometrical indices in UnX-DOCA-salt rats. *p < 0.01 versus all groups; †p < 0.01 versus UnX-DOCA-salt. Bars represent means ± S.E. (n = 6 rats per group).

TABLE 2

Effect of heme arginate therapy on morphological parameters in UnX-DOCA-salt hypertensive rats

Heme arginate significantly reduced glomerular hypertrophy, thickening or renal arterioles, glomerulosclerosis, tubular dilation, tubular cast formation, and interstitial mononuclear cell infiltration in the kidneys of UnX-DOCA-salt hypertensive rats. Assessment was done in a blinded manner, using a scale from 0 to 3, with 0 = normal; 1 = mild, 2 = moderate, and 3 = severe. Data are expressed as means ± S.E.M. Statistical analyses were done by using unpaired Student’s t test and analysis of variance in conjunction with Bonferroni test for repeated measures. Group differences at the level of P < 0.05 are considered statistically significant.

<table>
<thead>
<tr>
<th>Morphological Analyses</th>
<th>Rat Type</th>
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<tbody>
<tr>
<td></td>
<td>Normal SD</td>
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<tr>
<td>Glomerular hypertrophy</td>
<td>0.2 ± 0.17</td>
</tr>
<tr>
<td>Remodeling and thickening of renal arterioles</td>
<td>0.2 ± 0.17</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>0.2 ± 0.17</td>
</tr>
<tr>
<td>Glomerular necrosis</td>
<td>0.2 ± 0.17</td>
</tr>
<tr>
<td>Glomerular atrophy</td>
<td>0.3 ± 0.21</td>
</tr>
<tr>
<td>Tubular dilation</td>
<td>0.3 ± 0.21</td>
</tr>
<tr>
<td>Tubular cast formation</td>
<td>0.2 ± 0.17</td>
</tr>
<tr>
<td>Interstitial mononuclear cell infiltration</td>
<td>0.2 ± 0.17</td>
</tr>
</tbody>
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*p < 0.05, **p < 0.01 vs. UnX-DOCA-salt;
1p < 0.05, †p < 0.01 vs. UnX-sham, n = 6–7 per group.
potential of HO inducers. Although UnX-DOCA-salt hypertensive rats are a model characterized by severe renal lesions, they may not ideally represent the progression of ESRD in subjects with two kidneys because uninephrectomy would create a condition that predisposes the remaining kidney to hypertension, and this would constitute an additional damaging factor that in combination with hypertension-induced hypertrophy, fibrosis, and oxidative/inflammatory insults would result in more accentuated damage of the remaining kidney. However, because the present study demonstrates that HA is protective against more severe renal lesions in UnX-DOCA hypertension, its effectiveness against renal injury of lesser severity could be envisaged.

Collectively, our results indicate that HA is a more effective renoprotective agent than SPL, L-arginine, and captopril in UnX-DOCA hypertension, suggesting that the HA-mediated induction of HO-1 and the resulting increase of bilirubin, ferritin, SOD, catalase, antioxidant status, and the concomitant potentiation of adiponectin/AMPK signaling and ANP/βcAMP signaling, plus the corresponding decline of ET-1, 8-isoprostane, TGF-β, and fibronectin may account for the reduction of renal hypertrophy and histopathological lesions with restoration of renal function.

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References


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Heme-arginate therapy enhanced adiponectin and atrial natriuretic peptide, but abated endothelin-1 with attenuation of kidney histopathological lesions in mineralocorticoid-induced hypertension

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MATERIALS AND METHODS

Animals, treatment groups and plasma/urinary measurements

Our experimental protocol was approved by University of Saskatchewan Standing Committee on Animal Care and Research Ethics, and was 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 (SD) rats of 8 weeks were purchased from Charles River Laboratories (Willington, MA, USA), housed at 21°C with 12-hour light/dark cycles, fed with standard chow and had access to drinking water ad libitum. After a week of acclimatization, DOCA-salt hypertension was induced as previously described (Ndisang et al., 2008; Jadhav et al., 2009). Briefly, the right kidney of the animals were removed through a dorsal flank incision under isoflurane anaesthesia, and a silastic strip with or without the synthetic mineralocorticoid, DOCA, was implanted subcutaneously in the mid-scapular region of the animals. Our experimental design included the following groups (n=6-20 per group): (A) controls [(i) surgery-free or normal SD, (ii) UnX-sham, (iii) UnX-salt (0.9% NaCl + 0.2% KCl) and (iv) UnX-DOCA]; (B) UnX-DOCA-salt hypertensive rats; (C) UnX-DOCA-salt+heme arginate (HA); (D) UnX-DOCA-salt+HA+chromium mesoporphyrin (CrMP), the HO inhibitor; (E) UnX-DOCA-salt+CrMP; (F) UnX-DOCA-salt+Captopril (angiotensin converting enzyme [angiotensin converting enzyme (ACE) inhibitor]); (G) UnX-DOCA-salt+L-arginine, (H) UnX-DOCA-salt+Spironolactone (a mineralocorticoid receptor blocker) (I), UnX-DOCA-salt+Vehicle for HA and CrMP, and (J) UnX-DOCA-salt+Vehicle for spironolactone (sesame oil). HA was prepared from hemin and L-arginine as we previously reported (Jadhav and Ndisang, 2009). 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 (Levere et al., 1990; Jadhav et al., 2008; Ndisang et al., 2008).
L-arginine and captopril were dissolved in water (Gimeno et al., 1998; Brown et al., 1999) while spironolactone in sesame oil as reported by Klande et al., (2008) (Klanke et al., 2008) and Fujisawa et al., (2003) (Fujisawa et al., 2003). Captopril, spironolactone, hemin and L-arginine were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) while CrMP from Porphyrin Products (Logan, UT, USA). Treatment with HA (30 mg/kg i.p.), CrMP (4 μmol/kg i.p), L-arginine (30 mg/kg i.p.) (Gimeno et al., 1998), captopril (100 mg/kg by oral-gavage) (Brown et al., 1999), spironolactone (100 mg/kg i.p.) (Klanke et al., 2008) or the different vehicles began after the animals were severely hypertensive (193±3 mmHg), and was given daily for 4 weeks. Although many HO inhibitors are non specific and affects other hemo-enzymes or even increase HO-1, however, CrMP is selective against HO at a dose of 4 μmol/kg (Vreman et al., 1993; Ndisang et al., 2002). The volume of each injection 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. At the end of therapy, the animals were weighed, anaesthetized, and samples collected. Kidney hypertrophy was determined by the kidney-to-body weight ratio, an index of kidney hypertrophy (Guo et al., 2006), while plasma (bilirubin, ferritin, creatinine) and urine (proteinuria, albuminuria, creatinine) were routinely analyzed by Saskatoon Royal University Hospital, Canada. Creatinine clearance was calculated using the formula:

\[
\text{Urine creatinine concentration} \times \text{Urine volume in 24 hrs} \div \text{Plasma creatinine concentration}
\]

**Histological and morphological analyses of kidney**

Sections of 5 μm of paraffin embedded tissue were cut, stained with hematoxylin and eosin, and the diameter of 30 glomeruli measured randomly using NIS-elements BR-Q- imaging software.
Morphologic evaluation of glomerular hypertrophy, glomerulosclerosis, tubular dilation, tubular cast and mononuclear cell-infiltration were performed blindly with light-microscopy, and assessed semi-quantitatively using 0 to 3 scale (0, normal or almost normal; 1, mild; 2, moderate; 3, severe) in each kidney section and the mean calculated. Further evaluation of renal damage was done by counting the damaged and sclerotic glomeruli, and tubular protein casts in each section as we previously reported (Jadhav et al., 2009).

**Determination of HO activity and HO-1 concentration**

HO activity was evaluated as bilirubin production by our established method (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009). The 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), centrifuged at 13,000 rpm for 15 minutes. From the supernatant, aliquots of 100 µl was to measure HO enzyme activity in a reaction volume of 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 carried out in dark at 37ºC 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 spectrophotometrically (extinction coefficient for bilirubin 40 mM⁻¹cm⁻¹), and expressed as nmole/mg protein/hour. As a positive control, spleen tissue was used. HO-1 concentration was determined by ELISA (EKS-810A, Stressgen-Assay Design, Ann Arbor, MI, USA) as previously reported (Jadhav et al., 2008).
**Western Immunoblotting**

Kidney tissues were homogenized (1:10, w:v) in 10 mM Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of a cocktail of protease inhibitors, and centrifuged as we previously reported (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009). Briefly, proteins were extracted and quantified by Bradford assay, and aliquots of 50 µg were loaded on a 10% SDS-polyacrylamide gel for fibronectin and 12.5% for ANP and TGF-β. The fractionated proteins were electrophoretically transferred to nitrocellulose paper, non-specific bindings blocked with 3% non-fat milk, and incubated overnight with primary antibodies (Santa Crutz Biotechnology Inc, CA, USA) against fibronectin (1: 1000 dilution, sc-18825) ANP (1: 1000 dilution, sc-20158), and TGF-β1/2/3 [(1:1000 dilution; H-112 sc-7892 (with molecular weights for TGF-β1, TGF-β2 and TGF-β3 being 12.5, 13 and 12.5 kD respectively)], and AMPK (1: 1000 dilution, Cell Signaling Technology Inc, Danvers, MA, USA). Beta-actin (Sigma St Louis, MO, USA) (1: 5000 dilution) was used as a control to ascertain equivalent loading. After washing, blots were incubated with anti-rabbit IgG conjugated to horseradish peroxide (Bio-Rad, CA, USA), and the immuno-reactivity visualized using enhanced horseradish peroxide/luminol chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA).

**Determination of superoxide dismutase activity**

Superoxide dismutase (SOD) activity was determined by EIA (Cayman Chemical Company, Ann Arbor, MI, USA), as we reported previously (Jadhav et al., 2008; Ndisang et al., 2008). This kit detects all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). Briefly, kidney tissues were homogenised in a solution of 20 mM HEPES at pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose per gram tissue, and centrifuged. The supernatant was recovered and the EIA reaction
performed and read at 450 nm using a plate reader (SpectraMax 340PC, Molecular Device, CA, USA).

**Determination of catalase activity**

Kidney catalase activity was evaluated with an EIA (Cayman Chemical, Ann Arbor, MI, USA) as previously reported (Jadhav et al., 2008). The assay is based on the reaction of the enzyme with methanol in the presence of $\text{H}_2\text{O}_2$. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. The kidney 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, Inc Vermont, USA).

**Determination of 8-isoprostane**

Urinary 8-isoprostane is a non-invasive index of oxidative stress. This was determined by EIA (Cayman Chemical, Ann Arbor, MI) as we previously reported (Jadhav et al., 2008). Briefly, urine samples collected over a 24 hr period were diluted 1:15 with ultra pure water, and applied to a reverse-phase C-18 column at pH 3. Thereafter, the samples were eluted with 1:1 (vol/vol) ethyl acetate/heptane. Subsequently, 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, Ellaman’s reagent containing the substrate of acetylcholinesterase was added. The
absorbance were read at a wavelength of 412 nm in a microplate reader (SpectraMax 340PC, Molecular Device, CA, USA), and the values of 8-isoprostane calculated from a standard curve.

**Measurement of cGMP content**

The concentration of cGMP was determined as previously reported (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009). Briefly, homogenized tissues or urine samples were treated with 6% trichloroacetic acid at 4°C and centrifuged. The supernatant was treated with water-saturated diethyl-ether, and the upper ether layer discarded while the aqueous layer containing cGMP recovered and lyophilized. The dry extract was dissolved in 1 ml assay buffer and cGMP quantified by EIA (Cayman Chemical, Ann Arbor, MI, USA) as following the manufacturer’s instructions.

**Determination of Endothelin-1**

ET-1 was quantified by EIA (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. This immunometric assay is based on a double-antibody 'sandwich' technique that detects ET-1 within the range of 0-250 pg/ml. Briefly, supernatants from homogenized tissues and previously obtained plasma were purified by cold spike extraction, concentrated and the absorbance read at 405 nm in a plate reader (SpectraMax 340PC, Molecular Device, CA, USA).

**Total antioxidant capacity assay**

The total antioxidant capacity was evaluated by EIA (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s instruction, as we previously reported (Ndisang et al., 2008; Jadhav et al., 2009). This assay relies on the ability of the anti-oxidants in samples to
inhibit the oxidation of 2,2’-azino-di-[3-ethylbenzthiazoline sulphonate (ABTS) to ABTS plus metmyoglobin. In brief, tissues were homogenized in the presence of protease inhibitors and treated with Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), metmyoglobin and chromogen. The reaction was initiated by adding H₂O₂ and after 5 min, 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 (TEAC) per mg protein.

**Determination of plasma adipopnectin**

Adipopnectin was measured by ELISA (Phenix Pharmaceuticals, Inc, Burlingame, CA, USA) as we previously reported (Ndisanga and Jadhav, 2009a; Ndisang and Jadhav, 2009b; Ndisang et al., 2009a; Ndisang et al., 2009b). 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, USA).

**REFERENCES**


