Heme Oxygenase Improves Renal Function by Potentiating Podocyte-Associated Proteins in $\omega$-Nitro-L-Arginine-Methyl Ester (L-NAME)-Induced Hypertension

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BACKGROUND
Although heme-oxygenase (HO) is cytoprotective, its effects on podocyte regulators like podocalyxin, podocin, CD2-associated protein (CD2AP) in renal dysfunction in $\omega$-nitro-L-arginine-methyl ester (L-NAME) hypertension are largely unclear.

METHODS
Hypertension was induced in normotensive Sprague Dawley rats by administering L-NAME for 4 weeks. Enzyme immunoassay, enzyme-linked immunosorbent, histology/morphology, spectrophotometry, and western immunoblotting were used. HO was enhanced with heme-arginate (HA) or inhibited with chromium mesoporphyrin (CrMP).

RESULTS
Treatment with heme-arginate reduced several renal histo-pathological lesions including renal arteriolar thickening, glomerular abnormalities, tubular cast, tubular atrophy/fibrosis, and mononuclear cell infiltration in L-NAME-hypertensive rats. Similarly, HA abated the elevated levels of renal extracellular matrix/profibrotic proteins like collagen and fibronectin that deplete nephrin, a fundamental transmembrane protein that forms the scaffoldings of the podocyte slit diaphragm permitting small ions to filter, but not massive excretion of proteins, hence proteinuria. Correspondingly, HA enhanced the aberrant expression of nephrin alongside other important regulators of podocyte like podocalyxin, podocin, and CD2AP, and improved renal function by reducing albuminuria/proteinuria, while increasing creatinine clearance. The renoprotection by HA were accompanied by significant reduction of inflammatory/oxidative mediators including nuclear factor-kappaB, macrophage inflammatory protein-1-alpha, macrophage chemotactant protein-1, tumor necrosis factor-alpha, interleukin (IL)-6, IL1β, 8-isoprostane, endothelin-1, and aldosterone. These were associated with increased levels of adiponectin, HO-1, HO activity, cyclic guanosine monophosphate, and atrial natriuretic peptide (ANP), whereas the HO inhibitor, CrMP annulled the renoprotection and exacerbated renal dysfunction.

CONCLUSIONS
HA improves renal function by attenuating histopathological lesions, suppressing inflammatory/oxidative mediators, abating profibrotic/extracellular matrix proteins, and reducing albuminuria/proteinuria, while concomitantly potentiating the HO-adiponectin-ANP axis, enhancing nephrin, podocin, podocalyxin, CD2AP and increasing creatinine clearance. Our study underscores the benefit of potentiating the HO-adiponectin-ANP against nephropathy.

Keywords: adiponectin; atrial natriuretic peptide; blood pressure; CD2-associated protein; extracellular matrix; heme oxygenase; hypertension; inflammation; nephrin; nephropathy; oxidative stress; podocalyxin; podocin.

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The growing incidence and prevalence of chronic diseases including kidney dysfunction remains a global health challenge.¹² Chronic kidney disease ultimately progress to end-stage renal disease. Many pathophysiological factors including excessive inflammation and oxidative stress are implicated in renal dysfunction.³⁴ Accordingly, elevated levels of macrophage chemotactant protein-1 (MCP-1)/CC chemokine ligand, macrophage inflammatory protein-1-alpha (MIP-1α)/chemokine ligand-3, nuclear factor-kappaB (NF-κB), tumor necrosis factor-alpha, (TNF-α), interleukin (IL)-6, IL1β, and endothelin-1 have been reported in nephropathy.³⁻⁵ During tissue insult, the profibrotic and damaging effects of these inflammatory/oxidative mediators are further accentuated by increased deposition of extracellular matrix such as transforming growth factor beta collagen and fibronectin.³⁴ Moreover, elevated levels of profibrotic/extracellular matrix proteins such as collagen and fibronectin are known to deplete nephrin,⁶ and thus compromise kidney function.⁷ Thus, substances that suppress collagen and fibronectin while enhancing nephrin may improve renal function.

Nephrin is an important transmembrane zipper-like protein that is fundamental for the formation of the scaffolding of the podocyte slit diaphragm of the glomerular

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barrier that regulates the aperture size of the renal filtration barrier, selectively allowing small molecules like ions to pass through, but not larger molecules like proteins.\textsuperscript{8–10} A reduction in the expression of nephrin may cause massive excretion of proteins, hence proteinuria.\textsuperscript{8–11} Besides nephrin, other important regulators of the podocyte slit diaphragm include podocin, podocalyxin, and CD2-associated protein (CD2AP).\textsuperscript{11} Defects in these podocyte regulators cause proteinuria.\textsuperscript{11} Therefore, novel strategies that sustain nephrin, podocin, podocalyxin, and CD2AP would uphold renal function.

An important physiological enzyme with cytoprotective effects is heme-oxygenase (HO). HO is a microsomal enzyme with two main isoforms HO-1 (inducible) and HO-2 (constitutive) that generate cytoprotective products including biliverdin, bilirubin, ferritin, and carbon monoxide to suppress oxidative stress and inflammation.\textsuperscript{12} Although a third isoform HO-3 has been reported, it has little enzymatic activity and considered a pseudotranscript of HO-2, so HO activity is derived mainly from the inputs of HO-1 and HO-2.\textsuperscript{12} Generally, HO-1 can be activated by different agents including physical, chemical, and pathophysiological stimuli.\textsuperscript{12} However, the pathophysiological stimulation of HO-1 results only to a transient increase of HO activity that may fall below the threshold necessary to activate the downstream components of the HO system like the cyclic guanosine monophosphate (cGMP) secondary messenger system, so a robust enhancement of HO-1 and HO activity by pharmacological agents such as HO inducers is needed to surmount the threshold.\textsuperscript{12,13}

The role of the HO system on renal dysfunction in N\textsuperscript{ω}-nitro-l-arginine methyl ester (l-NAME) hypertension has not been completely elucidated. Importantly, the effects of the HO system on the master regulators of podocyte slit diaphragm such as podocin, podocalyxin, and CD2AP are largely unclear. Similarly, the effects of the HO system on renal expression of nephrin, MCP-1, MIP-1\textalpha, NF-\kappaB, fibronectin, and collagen in l-NAME-induced hypertension have not been reported. Therefore, a major objective of this study is to investigate the effects of the HO inducer, heme-arginate (HA) on podocin, podocalyxin, CD2AP, nephrin, MCP-1, MIP-1\textalpha, NF-\kappaB, fibronectin, collagen and correlate changes in these proteins to renal function in l-NAME-induced hypertension. Although l-NAME is traditionally known to cause hypertension by blocking nitric oxide synthase, emerging evidence indicate that the renin-angiotensin-aldrosterone system is implicated in many deleterious effects of the l-NAME hypertensive model including renal impairment.\textsuperscript{14} Accordingly, in l-NAME-induced hypertension, aldosterone has been shown to cause renal fibrosis and proteinuria.\textsuperscript{14} Whether an upregulated HO system with HA will suppress aldosterone and reduce proteinuria and albuminuria in l-NAME hypertension will be investigated. Thus the present study will assess the role of the HO system on renal function in l-NAME hypertension.

Since HO is cytoprotective, we hypothesize that HA would: (i) reduce the levels of aldosterone, (ii) abate proinflammatory/oxidative mediators, (iii) suppress the elevated expression of profibrotic/extracellular matrix proteins, (iv) attenuate renal histopathological lesions, and (v) improve renal function by enhancing the expression of important proteins of the podocyte slit diaphragm such as nephrin, podocin, podocalyxin, CD2AP in l-NAME hypertension.

**METHODS**

**Animals, treatment groups, and biochemical assays**

Our experimental protocol was approved by the University of Saskatchewan Animal Ethics Committee and is in conformity with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council of Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague Dawley (SD) rats (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed at 21 °C with 12-h light/dark cycles and fed with standard laboratory chow, and had access to drinking water ad libitum. The animals were allowed to acclimatize for 1 week, and at 7 weeks of age, they were randomly divided into the following groups: (i) normotensive control SD, (ii) SD + l-NAME (60 mg/kg/day, intraperitoneally) to induce hypertension, and (iii) SD + vehicle dissolving l-NAME. The administration of l-NAME to normotensive SD rats for 4 weeks induced severe hypertension (189.1±7.1 mmHg) as previously reported.\textsuperscript{15,16} The SD + l-NAME hypertensive group was further divided into different treatments as groups follow: (iv) SD + l-NAME + the HO inducer, HA (15 mg/kg, intraperitoneally), (v) SD + l-NAME + HA + the HO inhibitor, chromium mesoporphyrin (CrMP, 4 \mu mol/kg, intraperitoneally), (vi) SD + l-NAME + CrMP, and (vii) SD + l-NAME + vehicle dissolving HA + CrMP.

l-NAME, HA, and CrMP were prepared as we previously reported.\textsuperscript{15–20} Treatment with HA, CrMP, or vehicle dissolving HA and CrMP began after the animals were hypertensive, and were given daily for 4 weeks as we previously reported.\textsuperscript{15–20} Although many HO inhibitors are nonspecific and may affect other hemoenzymes or even increase HO-1, however, CrMP is reportedly selective against HO-1 at a dose of 4 \mu mol/kg.\textsuperscript{21} Systolic blood pressure was measured weekly by noninvasive tail-cuff method (Model 29 SSP; Harvard Apparatus, Montreal, Québec, Canada). A day prior to killing, the animals were placed in metabolic cages for 24 h urine collection. Subsequently, the animals were weighed, anesthetized with pentobarbital sodium (50 mg/kg body weight) and the kidneys were isolated, cleaned, weighed, and collected for biochemical assays. Kidney hypertrophy was measured as kidney-to-body weight ratio, an index of kidney hypertrophy,\textsuperscript{19} while sodium and creatinine in plasma and urine creatinine, proteinuria, albuminuria, sodium and creatinine clearance were analyzed as we previously reported.\textsuperscript{19}

HO activity was determined by spectrophotometric assay as we previously reported,\textsuperscript{19} while enzyme-linked immunosorbent assay was used for the determination of HO-1 (Stressgen-Assay Design, Ann Arbor, MI), TNF-\beta, IL-6, and IL1\beta (Immu-no-Biological Laboratories Ltd, Takasaki-shi, Japan), MCP-1 and MIP-1\alpha (OmniKine, Assay Biotechnology Company, Sunnyvale, CA), and enzyme immunoassay for atrial natriuretic peptide (ANP), endothelin-1 (ET-1), cGMP, and...
aldosterone (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instruction as we previously reported.17,22,23

Histology and morphological analyses of kidney tissue

Histology and morphometric analyses of the kidney was done as we previously reported.18 Briefly, paraffin-embedded whole kidney sections of 5 μm were cut and treated with Masson’s trichrome stain to assess collagen deposition. Morphologic evaluation of collagen deposition was done by a blinded researcher using a virtual microscope (Aperio Scan Scope Model CS; Aperio Technology Inc, Vista, CA), and analyzed using Aperio Image Scope V11.2.0.780 software (Aperio, e-Pathology Solution, Vista, CA). Each kidney section was magnified at ≥x200, and 20 random snaps shots were taken per slide per group of 6 animals (totaling 120 images per group), and scored semiquantitatively as we previously reported.18,19,24

Western immunoblotting

Kidney samples 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 proteins extracted and quantified as we previously reported.18,19,24,25 Aliquots of 100 μg were loaded on a 10% SDS polyacrylamide gel for nephrin, podocalyxin, podocin, CD2AP, collagen IV, fibronectin, NF-κB, and phosphorylated NF-κB. The fractionated proteins were electrophoretically transferred into nitrocellulose paper and nonspecific bindings blocked with 3% non-fat milk.

The blots were incubated overnight with primary antibodies, targeting nephrin, podocalyxin, podocin, CD2AP, collagen IV, fibronectin, phosphorylated NF-κB, and phosphorylated peroxide/luminol chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA). Beta-actin antibody (Sigma, St Louis, MO) was used as a control. Densitometric analysis was done with UN-SCAN-IT software (Silk Scientific, Orem, UT).

Statistical analysis

All data are expressed as means ± SEM from at least 4 independent experiments unless otherwise stated. Statistical analyses were done using analysis of variance (ANOVA) to evaluate the null hypothesis of no difference in mean levels of the various biomarkers between 2 or more treatment groups respectively. Given the small number of subjects within each group and the resultant inability to effectively evaluate the assumptions of parametric testing (ANOVA), confirmatory nonparametric comparison was also undertaken (Kruskal-Wallis test). Where the above overall tests results suggested that mean values were not the same across all the groups, multiple pairwise comparisons between groups were undertaken with Bonferroni corrected P values to ascertain which groups differed from each other. P values<0.05 were considered statistically significant in all testing. Where samples sizes were <5 per group, the chi-square distribution is assumed for Kruskal-Wallis testing.

All analyses were done using Statistical Analysis System, software Version 9.3 (SAS Institute, Cary, NC).

RESULTS

Treatment with HA potentiated the HO system, ANP and its surrogate marker cGMP and adiponectin in the kidneys of l-NAME-hypertensive rats

The administration of HA to l-NAME-hypertensive rats restored physiological blood pressure (187.4 ± 4.3 vs. 125.1 ± 2.9 mmHg, P < 0.01) and attenuated kidney hypertrophy (5.6 ± 0.4 vs. 3.9 ± 0.2 g/kg body weight, P = 0.07) (Table 1), whereas the coadministration of the HO inducer HA and the HO blocker, CrMP abolished the effect of HA on kidney hypertrophy (5.4 ± 0.3 g/kg body weight) and systolic blood pressure (194 ± 5.2 mmHg). The antihypertensive,

### Table 1. Effects of heme-arginate (HA) and chromium mesoporphyrin (CrMP) on physiological variables in N^6-nitro-l-arginine methyl ester (l-NAME)-induced hypertension in normotensive Sprague-Dawley (SD) rats

<table>
<thead>
<tr>
<th>Physiological variables</th>
<th>Control SD</th>
<th>SD + l-NAME + HA</th>
<th>SD + l-NAME + HA + CrMP</th>
<th>SD + l-NAME + CrMP</th>
<th>SD + vehicle for l-NAME + vehicle for HA and CrMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>366.4 ± 7.5</td>
<td>357.8 ± 6.4</td>
<td>341.5 ± 4.9</td>
<td>349.5 ± 8.6</td>
<td>361.5 ± 5.7</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>123 ± 3.1</td>
<td>187 ± 4.3*</td>
<td>125 ± 2.9*</td>
<td>194 ± 5.2*</td>
<td>214 ± 6.2*</td>
</tr>
<tr>
<td>Kidney hypertrophy (g/kg body weight)</td>
<td>3.4 ± 0.2</td>
<td>5.6 ± 0.4*</td>
<td>3.9 ± 0.2*</td>
<td>5.4 ± 0.3*</td>
<td>6.3 ± 0.5*</td>
</tr>
<tr>
<td>Albuminuria (mg/24 h)</td>
<td>2.5 ± 0.4</td>
<td>18.3 ± 2.6*</td>
<td>10.2 ± 0.7*</td>
<td>21.5 ± 1.4*</td>
<td>31.4 ± 2.2*</td>
</tr>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>7.3 ± 0.6</td>
<td>69.5 ± 4.1*</td>
<td>18.4 ± 1.6*</td>
<td>74.9 ± 2.7*</td>
<td>86.6 ± 3.5*</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min/g kidney)</td>
<td>4.4 ± 0.6</td>
<td>2.7 ± 0.3</td>
<td>3.6 ± 0.5</td>
<td>2.5 ± 0.6^</td>
<td>2.1 ± 0.2^</td>
</tr>
</tbody>
</table>

n = 6 per group.

*P < 0.05 vs. Control-SD or SD + vehicle for l-NAME; †P < 0.05 vs. SD + vehicle for l-NAME; ‡P < 0.05 vs. SD + l-NAME + HA + CrMP or SD + l-NAME + CrMP or SD + l-NAME + vehicle for HA and CrMP; ^P < 0.05 vs. SD + l-NAME + CrMP.
antihypertrophic, and renoprotective effects of HA were accompanied by significant enhancement of HO-1 and HO activity (Figure 1A,B). Similarly, treatment with HA significantly enhanced ANP and the depressed basal cGMP, a surrogate marker of ANP,26 and secondary messenger through which endogenously produced carbon monoxide by the HO system acts27 (Figure 1C,D). Both ANP and cGMP are renoprotective.19,28 Furthermore, HA treatment significantly
increased the levels of renal adiponectin (Figure 1E), another protein with renoprotective effects.29

Although the basal HO-1 and ANP levels in l-NAME-hypertensive rats were higher as compared to the normotensive SD controls (Figure 1A,C), they did not evoke any changes in cGMP, suggesting that the basal levels HO-1 and ANP might have been below the threshold that activates the cGMP secondary messenger system. On the other hand, the coadministration of the HO inducer HA and the HO blocker, CrMP abolished the effects of HA on HO-1, HO activity, cGMP, ANP, and adiponectin, whereas treatment with CrMP alone resulted in further depletion of HO-1, HO activity, cGMP, ANP, and adiponectin.

Given that kidney hypertrophy may lead to renal insufficiency, we measured important indices of renal function including albuminuria, proteinuria, and creatinine clearance to assess kidney function. In l-NAME-hypertensive rats, renal hypertrophy (Table 1) was associated with significant elevation of albuminuria/proteinuria and reduced creatinine clearance, suggesting renal dysfunction (Table 1). These renal deficiencies were reversed by the HO inducer HA, whereas the coadministration with the HO inhibitor CrMP or treatment with CrMP alone annulled the renoprotection by HA and exacerbated renal dysfunction by aggravating albuminuria, proteinuria and further reducing creatinine clearance (Table 1).

Treatment with l-NAME, HA, and CrMP slightly reduced body weight. However, the total weight loss was less than 8% and was not significant. The vehicle dissolving l-NAME and the vehicle for HA and CrMP had no effect on any of the measured parameters (Table 1).

Treatment with HA reduce the levels of kidney endothelin-1, 8-isoprostane, and aldosterone in the kidneys of l-NAME-hypertensive rats

Given that aldosterone, ET-1, and 8-isoprostane are implicated in renal fibrosis,14,19 we measured ET-1 and 8-isoprostane in the kidneys. In l-NAME-hypertensive rats, the basal levels of aldosterone, ET-1, and 8-isoprostane were markedly elevated as compared to SD controls (Figure 2A–C). Interestingly, treatment with HA significantly abated the elevated levels of aldosterone, ET-1, and 8-isoprostane. On the other hand, the cotreatment of HA and the HO inhibitor CrMP abolished the effects of HA, while treatment with CrMP alone exacerbated the levels of aldosterone, ET-1, and 8-isoprostane.

Since aldosterone favors sodium/water retention we measured water intake and sodium excretion. In l-NAME-hypertensive rats, the elevated levels of aldosterone was accompanied by significant water intake as compared to SD controls (263.9 ± 10.4 vs. 42.9 ± 2.7 ml/24h, P < 0.01, n = 6). However, treatment with HA greatly reduced water intake (263.9 ± 10.4 vs. 162.5 ± 5.4 ml/24h, P < 0.01, n = 6), whereas cotreatment with CrMP abolished the effect of HA (271.4 ± 14.5 ml/24h), while treatment with CrMP alone further increased water intake (292.7 ± 13.4 ml/24h). Given that HA therapy abated aldosterone and correspondingly reduced water intake, we assessed the effect of HA on plasma sodium...
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since aldosterone regulates sodium excretion. We observed that in l-NAME-hypertensive rats, plasma sodium retention was markedly increased as compared to SD controls (156.7 ± 2.6 vs. 138.4 ± 2.5 mmol/l, P < 0.01, n = 6), but was reduced by HA to 145.3 ± 1.8 mmol/l, while the coadministration with the HO inhibitor, CrMP, abolished the effects of HA (162.5 ± 2.1 mmol/l), whereas treatment with CrMP alone exacerbated the levels of plasma sodium (170.5 ± 2.8 mmol/l).

The reduction of plasma sodium by HA was accompanied by increased excretion of urinary sodium in HA-treated animals as compared to untreated l-NAME-hypertensive rats (139.2 ± 1.4 vs. 126.5 ± 1.5 mmol/l, P < 0.05, n = 6). On the other hand, treatment with the HO inhibitor nullified the effects of HA reinstating comparable levels as observed in l-NAME-hypertensive animals (128.4 ± 1.9 mmol/l). Interestingly, the increased urinary sodium excretion in

Figure 3. Effects of the heme oxygenase (HO) inducer heme-arginate (HA) and the HO inhibitor CrMP on macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP1-α), tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β in the kidneys of Nω-nitro-l-arginine methyl ester (l-NAME) hypertension. Treatment with HA markedly abated (A) MCP-1, (B) MIP1-α, (C) TNF-α, (D) IL-6, and (E) IL-1β in l-NAME hypertension, while the HO inhibitor CrMP nullified the effects. Bars represent means ± SEM; n = 7 rats per group. ANOVA/Kruskal-Wallis testing for the null hypothesis of no difference in means across all group had P values <0.01 for each of A–E; follow-up pairwise comparisons between each possible pairing of groups, again testing the null hypothesis of no difference in mean values, were undertaken with Bonferroni correction. Differences are indicated by the following superscripts: *P < 0.05 vs. all groups; ¥P < 0.05 vs. SD + l-NAME or SD + l-NAME + HA + CrMP or SD + l-NAME + CrMP; &P < 0.05 vs. SD + l-NAME or SD + l-NAME + CrMP. Abbreviations: ANOVA, analysis of variance; CrMP, chromium mesoporphyrin; SD, Sprague Dawley.
HA-treated animals was accompanied by increased urinary volume. Accordingly, in HA-treated animals urinary volume was $231.4 \pm 11.5$ ml/24 h as opposed to $154.6 \pm 8.2$ ml/24 h in l-NAME-hypertensive animals. Treatment with HA attenuated the levels of MCP-1, MIP-1α, TNF-α, IL-6, and IL-1β in the kidneys of l-NAME-hypertensive rats. We also assessed the effects of HA on MIP-1α and MCP-1 because these chemokines are implicated in tissue injury. In l-NAME-hypertensive rats, the basal levels of MCP-1 and MIP-1α were markedly elevated (Figure 3A,B), but were significantly reduced by HA. In contrast, the coadministration of HA with CrMP nullified the effects of HA, while treatment with CrMP alone further increased the levels of MCP-1 and MIP-1α.

Given that TNF-α, IL-6, and IL-1β are implicated in inflammatory insults and tissue damage, we investigated the effects of HA on these inflammatory cytokines. The basal levels of TNF-α, IL-6, and IL-1β in l-NAME-hypertensive rats significantly elevated, but were reduced by HA (Figure 3C–E), whereas cotreatment with CrMP abolished the effect of HA.

Figure 4. Effects of the heme oxygenase (HO) inducer heme-arginate (HA) on the expression of nuclear factor-kappaB (NF-κB), collagen IV, and fibronectin in the kidneys of $N^\omega$-nitro-l-arginine methyl ester (l-NAME) hypertension. Western immunoblotting indicates that treatment with HA markedly reduced the expressions of (A) NF-κB, (B) p-NF-κB, (C) collagen IV, and (D) fibronectin in l-NAME hypertension. Bars represent means ± SEM; n = 4 rats per group. ANOVA/Kruskal-Wallis testing for the null hypothesis of no difference in means across all group had P values <0.01 for each of A–D; follow-up pairwise comparisons between each possible pairing of groups, again testing the null hypothesis of no difference in mean values, were undertaken with Bonferroni correction. Differences are indicated by the following superscripts: *P < 0.05 vs. SD control, †P < 0.05 vs. SD + l-NAME. Normality/chi-square distribution assumed for respective ANOVA/Kruskal-Wallis testing in this small sample context. Abbreviations: ANOVA, analysis of variance; SD, Sprague Dawley.

Treatment with HA suppressed NF-κB, p-NF-κB, and extracellular matrix/profibrionic proteins in the kidneys of l-NAME-hypertensive rats

To further investigate the renoprotective effects of HA, we measured the expression of NF-κB, a transcription factor that together with extracellular matrix proteins such as collagen IV and fibronectin are known to cause fibrosis and renal injury. In l-NAME-hypertensive rats, the basal expression of renal NF-κB, p-NF-κB, collagen IV, and fibronectin were significantly elevated as compared to the SD control (Figure 4A–D). Interestingly, treatment with HA...
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Treatment with HA potentiates proteins associated with podocyte and glomerular function in the kidneys of L-NAME-hypertensive rats

Given that nephrin, podocalyxin, podocin, and CD2AP are important for the formation of the podocyte slit diaphragm of the glomerular barrier that regulates the renal filtration barrier, we evaluated the effects of HA on these proteins.

In L-NAME-hypertensive rats, western immunoblotting revealed that the basal expressions of nephrin, podocalyxin, podocin, and CD2AP were significantly reduced as compared to the SD control (Figure 5A–D). However, treatment with HA greatly enhanced the expressions of nephrin, podocalyxin, podocin, and CD2AP to comparable levels as in the SD controls.

Treatment with HA reduce renal histopathological lesions in L-NAME-hypertensive rats

Our results indicate that images of kidney sections from L-NAME-hypertensive animals were characterized by lesions in the cortex and medulla. Distinct areas of tubular atrophy and fibrosis with associated mononuclear inflammatory infiltrate were evident, particularly around the tubules and blood vessels (Figures 6, 7 and 8). These areas involved approximately 5 to up to 20% of cortex. There

Figure 5. Effects of the heme oxygenase (HO) inducer heme-arginate (HA) on the expression of nephrin, podocalyxin, podocin, and CD2-associated protein (CD2AP) in the kidneys of Nω-nitro-L-arginine methyl ester (L-NAME) hypertension. Western immunoblotting reveals that treatment with HA greatly enhanced the expressions of (A) nephrin, (B) podocalyxin, (C) podocin, and (D) CD2AP in L-NAME hypertension. Bars represent means ± SEM; n = 4 rats per group. ANOVA/Kruskal-Wallis testing for the null hypothesis of no difference in means across all group had P values <0.01 for each of A–D; follow-up pairwise comparisons between each possible pairing of groups, again testing the null hypothesis of no difference in mean values, were undertaken with Bonferroni correction. Differences are indicated by the superscript: *P < 0.05 vs. all groups. Normality/chi-square distribution assumed for respective ANOVA/Kruskal-Wallis testing in this small sample context. Abbreviation: ANOVA, analysis of variance.
were tubular casts within the collecting ducts in medulla (Figure 7). In contrast, tubular atrophy, fibrosis inflammation, and tubular casts were almost absent in control and HA-treated kidneys.

The most significant changes in the kidneys of \(\text{L-NAME}\)-hypertensive rats involved small arteries and arterioles which were characterized by mucoid intimal thickening, moderate fibroelastic intimal thickening, and thickening of media (Figures 6, 7 and 8). The average medial thickening of small artery medial wall was 31.5, 39.0, and 24.5 nm respectively in SD control, \(\text{L-NAME}\)-hypertensive rats, and HA-treated hypertensive rats. In addition, rare blood vessels in the hypertensive kidney revealed fibrinoid necrosis of vessel wall. Furthermore, the arterioles showed moderate to marked narrowing of vascular lumen due to mucoid intimal thickening. This was noted in approximately 25–30% of arterioles in hypertensive rat kidneys. These vascular changes were not present in SD controls or HA-treated animals. The small arteries also appeared dilated in HA-treated rats compared to SD controls.

**Figure 6.** Effects of the heme oxygenase (HO) inducer heme-arginate (HA) on vascular lesions in the kidneys of \(N^\omega\)-nitro-L-arginine methyl ester (\(\text{L-NAME}\)) hypertension. Representative images demonstrating mucoid intimal thickening with almost complete obliteration of the lumen in arterioles and medial-intimal thickening of small arteries were more prominent in \(\text{L-NAME}\) hypertensive rats as compared to the Sprague Dawley (SD) controls and HA-treated animals. Mason trichrome staining. Magnification ×200.
Glomerular changes in the L-NAME-hypertensive kidneys were characterized by ischemic wrinkling and collapse of glomerular capillary loops, focal area of segmental sclerosis, and focal hypertrophy. Ischemic insults were predominantly noted in glomeruli with marked arteriolar narrowing (Figure 8). No glomerular abnormalities were seen in kidneys of normal and HA-treated rats.

DISCUSSION

The present study demonstrates that HA is a potent renoprotective agent against L-NAME-induced renal abnormalities. In L-NAME hypertension, excessive oxidative stress, increased inflammation, and elevated extracellular matrix deposition are cardinal pathophysiological features that were evidenced by the high levels of 8-isoprostane, ET-1, MIP-1α, MCP-1, NF-κB, IL-6, IL-1β, and aldosterone.3–6,14 These pathophysiological agents are among the complex molecular processes that characterize the intricate relationship between inflammation, renal fibrosis, and the development and progression of nephropathy. Importantly, our study unveils for the first time that HA therapy potentiates the expression of several transmembrane proteins including podocin, podocalyxin, and CD2AP8–11 which are critical for the formation of the podocyte slit diaphragm that regulates the aperture size of the glomerular filtration barrier, selectively allowing passage of small molecules like ions, but not larger molecules like proteins. Similarly, nephrin, another transmembrane protein necessary for the morphology of podocyte slit diaphragm8–11 was also enhanced by HA therapy.

Interestingly, the potentiation of podocyte-related proteins in HA-treated animals was associated with the reduction of proteinuria and albuminuria, while creatinine clearance increased suggesting improved renal function. The HA-dependent suppression of oxidative/inflammatory mediators and extracellular matrix/profibrotic proteins in L-NAME-hypertensive animals was accompanied by the parallel reduction of several histopathological lesions including, glomerular abnormalities, tubular casts, tubular atrophy/fibrosis, mononuclear cell infiltration, renal arteriolar narrowing, and medial thickening. These lesions were greatly attenuated by HA therapy. Thus the improved renal function in HA-treated animals may be due to the concomitant restoration of renal lesions, the suppression of oxidative/inflammatory mediators, the reduction of renal hypertrophy, the attenuation of extracellular matrix/profibrotic proteins alongside the potentiation of podocyte-related proteins such as nephrin, podocin, podocalyxin, and CD2AP.

The mechanisms underlying the renoprotection of HA include the potentiation of ANP and adiponectin by an
upregulated HO system in the kidney. The activities of HO, ANP, and adiponectin are closely related. For example, ANP can stimulate adiponectin.\textsuperscript{30} Similarly, ANP is also capable of enhancing the HO system.\textsuperscript{31,32} On the other hand, the HO system can potentiate ANP.\textsuperscript{19} Therefore, it is possible that the mutual stimulatory effect between the HO system and ANP and could synergistically potentiate renoprotection because both ANP and the HO system are renoprotective.\textsuperscript{19,28} Interestingly, the HO system, adiponectin, and ANP have all been shown to increase cGMP.\textsuperscript{19,33} Moreover, the potentiation of ANP, a substance that promote natriuresis and vasodilatation and alongside the parallel reduction of ET-1, a substance that stimulates sodium retention and vasoconstriction,\textsuperscript{34} together with the suppression of aldosterone may account for the increased sodium excretion and enhanced urinary volume. Alternatively, the increased sodium excretion and enhanced urinary volume observed in HA-treated animals may result from the concomitant enhancement of ANP, and its surrogate marker urinary cGMP. Thus, the potentiation of HO-adiponectin-ANP by HA therapy may

\textbf{Figure 8.} Effects of the heme oxygenase (HO) inducer heme-arginate (HA) on renal lesions in N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) hypertension. Representative images showing the magnitude of glomerular and vascular abnormalities in L-NAME hypertensive rats as compared to the Sprague Dawley (SD) controls and HA-treated animals. Mason trichrome staining. Magnification \times200.
account for improved renal function in l-NAME-hypertensive rats. On the other hand, the administration of the HO inhibitor, CrMP with HA or alone abolished the renoprotection by HA, abrogated the basal levels of ANP, adiponectin, and HO, with robust elevation of NF-κB, TNF-α, IL-6, IL1β, aldosterone, MCP-1, MIP-1α, proteinuria, albuminuria, and exacerbated renal dysfunction, suggesting an important role of the HO-adiponectin-ANP axis in nephropathy.

The renoprotective effects of ANP may be hindered if cGMP response to ANP stimulation is aberrant. In a related study, elevated ANP levels were reportedly accompanied by reduced response to ANP stimulation is aberrant. In a related study, elevated ANP levels were reportedly accompanied by reduced response to ANP stimulation is aberrant. In a related study, elevated ANP levels were reportedly accompanied by reduced response to ANP stimulation is aberrant. In a related study, elevated ANP levels were reportedly accompanied by reduced response to ANP stimulation was prevented by deletion of protein kinase Cα signaling in vivo. Kidney Int 2006; 70:1456–1462.


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