Heme oxygenase-mediated endothelial dysfunction in DOCA-salt, but not in spontaneously hypertensive, rat arterioles

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Heme oxygenase-mediated endothelial dysfunction in DOCA-salt, but not in spontaneously hypertensive, rat arterioles. Am J Physiol Heart Circ Physiol 286: H1681–H1687, 2004. First published December 23, 2003; 10.1152/ajpheart.00409.2003.— Vascular heme oxygenase (HO) metabolizes heme to form carbon monoxide. Carbon monoxide inhibits nitric oxide synthase and promotes endothelium-dependent vasoconstriction. We reported HO-1-mediated endothelial dysfunction in Dahl salt-sensitive hypertension. Previous studies suggested that salt-sensitive hypertensive rats, but not spontaneously hypertensive rats (SHR), display endothelial dysfunction. This study examines the hypothesis that HO-1-mediated arteriolar endothelial dysfunction develops in deoxycorticosterone acetate (DOCA)-salt hypertensive (DOCA) rats, but not in SHR.

METHODS

Carbon monoxide can be generated in vivo mainly via the enzymatic degradation of heme by heme oxygenase (HO) (40). Numerous tissues (28), including vascular endothelial and smooth muscle cells, express HO (5, 9). The two major active isoforms of HO (28) are the inducible HO-1 and the constitutive HO-2. Pathological conditions (28), such as ANG II-induced (1, 14, 15) or Dahl/Rapp salt-sensitive (17) hypertension, can increase HO-1 expression. Although carbon monoxide relaxes vascular smooth muscle (6, 7, 20), it also interferes with the vasodilator effects of the nitric oxide (NO) system (29, 41, 46) and promotes endothelium-dependent vasoconstriction (18). Furthermore, induction of HO-1 has been shown to attenuate muscarinic agonist-induced NO release (41) and vasorelaxation (21) in isolated renal arteries.

Decreased endothelium-dependent vasodilation, associated with endothelial dysfunction, is an important characteristic of some forms of hypertension (33). Severely salt-sensitive models of hypertension, such as Dahl/Rapp salt-sensitive (11, 27) or deoxycorticosterone acetate (DOCA)-salt hypertension (26, 43), are generally thought to be associated with endothelial dysfunction. Some studies suggest that minimally salt-sensitive spontaneously hypertensive rats (SHR) display increased vascular NO production (11, 33), whereas others reported decreased NO bioavailability in these animals (12, 22, 23). We previously found increased blood carboxyhemoglobin (HbCO) and vascular HO-1 protein levels (17) in Dahl/Rapp salt-sensitive rats with salt-induced hypertension. Skeletal muscle arterioles isolated from these hypertensive Dahl/Rapp salt-sensitive rats showed impaired endothelium-dependent vasodilation in response to ACh, but acute in vitro pretreatment with an HO inhibitor abolished the difference between hypertensive high-salt and normotensive low-salt groups (17). These data suggest that increased endogenous HO function contributes to endothelial dysfunction during Dahl/Rapp salt-sensitive hypertension. However, it was unclear whether HO-mediated endothelial dysfunction extends to all forms of hypertension.

Previous studies by Hayakawa and Raij (10, 11) and Raij (33) suggested that, in contrast to SHR, severely salt-sensitive hypertension is associated with decreased NO production. Therefore, we hypothesized that HO-mediated arteriolar endothelial dysfunction develops in DOCA-salt hypertensive (DOCA) rats, but not in SHR. To test this hypothesis, we measured vascular HO-1 content and blood HbCO levels in DOCA rats and SHR. To examine endothelial function, we conducted experiments using skeletal muscle arterioles from these hypertensive rats and examined the responses to an endothelium-dependent vasodilator in the presence or absence of an inhibitor of endogenous carbon monoxide production.

MATERIALS

Materials. Chromium mesoporphyrin (CrMP) was purchased from Frontier Scientific (Logan, UT); thiobutabarbital sodium (Inactin), DOCA, sesame oil, ACh, SDS, Tween 20, hematoxylin solution (Gills no. 3), and 3,3′-diaminobenzidine from Sigma Aldrich (St. Louis, MO); and polyclonal HO-1 antibody from Stressgen Biotechnologies (San Diego, CA). All other chemicals were obtained from Fisher Scientific (Houston, TX). CrMP stock solution (15 mmol/l) was prepared in 50 mmol/l Na2CO3 solution and diluted in modified Krebs solution.
buffer (15 μmol/l) immediately before use. ACh stock solution (10 mmol/l) was prepared in saline and diluted in modified Krebs buffer immediately before use. The composition of the modified Krebs buffer was as follows (mmol/l): 118.5 NaCl, 4.7 KCl, 1.4 CaCl₂, 1.2 KH₂PO₄, 1.1 MgSO₄, 25.0 NaHCO₃, and 11.1 dextrose.

**Table 1. General characteristics of the DOCA-salt hypertensive model**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>DOCA</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>138±4(9)</td>
<td>197±7*(12)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>362±7(9)</td>
<td>327±8*(12)</td>
</tr>
<tr>
<td>HbCO, %</td>
<td>3.1±0.1(9)</td>
<td>4.1±0.1*(12)</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>321±5(9)</td>
<td>287±12*(12)</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>1.06±0.03(9)</td>
<td>2.66±0.10*(12)</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.09±0.03(8)</td>
<td>1.35±0.05*(6)</td>
</tr>
</tbody>
</table>

*Values are means ± SE of number of rats in parentheses. DOCA, deoxy-corticosterone acetate (DOCA)-salt hypertensive rats; sham, sham controls; MAP, mean arterial pressure; HR, heart rate; HbCO, carboxyhemoglobin. +P < 0.05 vs. sham.*

**RESULTS**

**Blood pressure and HbCO measurements.** In the salt-sensitive DOCA rats, mean arterial pressures were higher than in the sham control group and blood HbCO levels were elevated (Table 1). Although mean arterial pressure was also increased in SHR, blood HbCO levels were not elevated compared with WKY controls (Table 2). Body weights were lower, but kidney and heart weights were higher, in DOCA rats than in sham controls (Table 1). In contrast, body weights were higher in

**Table 2. General characteristics of SHR hypertensive model**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>111±2(10)</td>
<td>187±5*(8)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>317±6(10)</td>
<td>375±4*(8)</td>
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<tr>
<td>HbCO, %</td>
<td>2.9±0.1(8)</td>
<td>3.0±0.1(8)</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>278±10(10)</td>
<td>310±9*(8)</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>0.99±0.04(10)</td>
<td>0.98±0.03(8)</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.03±0.08(8)</td>
<td>1.01±0.04(3)</td>
</tr>
</tbody>
</table>

*Values are means ± SE of number of rats in parentheses. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. +P < 0.05 vs. WKY.*
SHR, but kidney and heart weights were not different between SHR and WKY groups.

**HO-1 protein measurements.** Abdominal aortic segments isolated from the severely salt-sensitive DOCA rats showed approximately sixfold higher HO-1 protein levels than did sham controls (Fig. 1). However, HO-1 protein levels were not increased in abdominal aortic segments of SHR rats compared with WKY controls (Fig. 2).

**HO immunohistochemistry.** First-order gracilis muscle arteries isolated from DOCA rats showed enhanced immunostaining for HO-1 in the vascular wall compared with the sham group (Fig. 3). In contrast, there was no difference in HO-1 staining between SHR and WKY arterioles (Fig. 3). Control sections, where the HO-1 antibody was omitted, did not show signs of immunostaining (data not shown).

**Isolated microvessel experiments.** During the stabilization period, internal diameter of isolated skeletal muscle arterioles decreased spontaneously in all four groups: from 214 ± 4 to 138 ± 6 μm in sham rats (n = 18), from 174 ± 7 to 132 ± 6 μm in DOCA rats (n = 24), from 219 ± 6 to 126 ± 6 μm in WKY rats (n = 15), and from 199 ± 5 to 127 ± 7 μm in SHR (n = 14). Pretreatment with the HO inhibitor CrMP (15 μmol/l) promoted arteriolar vasoconstriction in all four groups [from 139 ± 6 to 102 ± 6 μm in sham rats (n = 9), from 131 ± 8 to 104 ± 6 μm in DOCA rats (n = 12), from 121 ± 6 to 73 ± 7 μm in WKY rats (n = 7), and from 130 ± 12 to 90 ± 11 μm in SHR (n = 7)] whereas vehicle pretreatment had no significant effect on internal diameter [from 136 ± 10 to 136 ± 8 μm in sham rats (n = 9) from 133 ± 8 to 129 ± 8 μm in DOCA rats (n = 12), from 130 ± 10 to 125 ± 11 μm in WKY rats (n = 8), and from 124 ± 8 to 124 ± 7 μm in SHR (n = 7)].

In skeletal muscle arterioles isolated from DOCA rats, responses to an endothelium-dependent vasodilator, ACh (1 nmol/l–3 μmol/l), were attenuated compared with responses from sham controls [maximum difference (Δmax) = 79 ± 6 μm (n = 9) and 44 ± 5 μm (n = 12) in vehicle-pretreated sham-operated and DOCA rats, respectively, P < 0.05; Fig. 4A]. Acute in vitro pretreatment with an inhibitor of HO, CrMP (15 μmol/l), enhanced ACh-induced vasodilatory responses in the DOCA arterioles and abolished the difference between DOCA and sham groups [Δmax = 83 ± 12 μm (n = 9) and 76 ± 7 μm (n = 12) in CrMP-pretreated sham-operated and DOCA rats, respectively, P > 0.05; Fig. 4B]. In contrast, ACh-induced vasodilator responses (Fig. 5) were not different between arterioles isolated from the SHR and control WKY groups with or without CrMP pretreatment [Δmax = 82 ± 5 μm (n = 8) and 84 ± 3 μm (n = 7) in vehicle-pretreated WKY rats and SHR, respectively, and 99 ± 10 μm (n = 7) and

![Fig. 1. Heme oxygenase-1 (HO-1) protein levels in abdominal aortic segments isolated from sham control rats and deoxycorticosterone acetate (DOCA)-salt hypertensive (DOCA) rats. Top: quantification of HO-1 protein expression by laser densitometry in sham and DOCA aortic tissues. Bottom: Western blot of HO-1 in abdominal aortic segments isolated from WKY rats and SHR. Values are means ± SE; n = 6. *P < 0.05 vs. sham.](image1)

![Fig. 2. HO-1 protein levels in abdominal aortic segments isolated from 12-wk-old Wistar-Kyoto (WKY) control rats (n = 5) and spontaneously hypertensive rats (SHR, n = 4). Top: quantification of HO-1 protein expression by laser densitometry in WKY and SHR aortic tissues. Bottom: Western blot of HO-1 in abdominal aortic segments isolated from WKY rats and SHR. Values are means ± SE.](image2)

![Fig. 3. Immunohistochemical staining (ABC peroxidase method) for HO-1 in 1st-order gracilis muscle arteries isolated from sham rats (A), DOCA rats (B), WKY rats (C), and SHR (D). Blue area is hematoxylin background staining. Chromogen is 3,3′-diaminobenzidine; brown color indicates immunoreactivity. Magnification ×60.](image3)
102 ± 5 μm (n = 7) in CrMP-pretreated WKY rats and SHR, respectively, P > 0.05 for both.

DISCUSSION

In this study, we found that vascular HO-1 expression and endogenous carbon monoxide production were increased in DOCA rats, but not in SHR. Vessels from DOCA animals displayed attenuated endothelium-dependent vasodilator responses, and acute in vitro treatment with an inhibitor of endogenous carbon monoxide production abolished the differences between DOCA and sham arterioles. In contrast, endothelium-dependent vasodilatory responses were not different between SHR and WKY arterioles with or without the HO inhibitor.

DOCA is an aldosterone mimetic that similarly promotes sodium and water retention. Uninephrectomized rats that are treated with DOCA and receive saline drinking solution (DOCA group) develop severe hypertension (34, 35). DOCA-salt hypertension is a model of severely salt-sensitive high blood pressure that is associated with end-organ damage, such as cardiac hypertrophy and severe renal injury (34). We also found higher blood pressure and higher heart and kidney weights, consistent with cardiac and renal injury, in DOCA than in sham control rats.

The SHR is a genetic model of minimally salt-sensitive hypertension, and, with age, this strain progressively develops hypertension in the absence of exacerbating dietary factors (8). Hayakawa and Raij reported that, at comparable levels of systemic hypertension, Dahl/Rapp salt-sensitive rats develop renal injury (10) and cardiac hypertrophy (11), whereas SHR do not (10, 11). We similarly found that although mean arterial pressures in SHR were comparable with those in the DOCA group, SHR did not show increased heart and kidney weights, potentially arguing against cardiac and renal injury.

Carbon monoxide is a vasoactive by-product of HO-catalyzed breakdown of heme (6, 20). Carbon monoxide generated in vivo is highly stable but, eventually, diffuses into the bloodstream, where it binds to hemoglobin to form HbCO (20). HbCO can be measured from a small blood sample (100–150 μl) with the use of a clinical-grade machine (47). We previously found that blood HbCO levels may be used as an index to evaluate the status of the endogenous carbon monoxide system (47) and reported that HbCO levels are increased in Dahl/Rapp salt-sensitive rats with salt-induced hypertension (17). Our present study found higher blood HbCO levels in DOCA rats than in sham animals. However, blood HbCO levels did not differ between SHR and control WKY groups. These data serve as a cursory index that salt-sensitive hypertension in DOCA rats is accompanied by increased endogenous carbon monoxide production. Furthermore, this increase in endogenous carbon monoxide production may not be a consequence of high blood pressure per se but may be, rather, associated with the salt-sensitive trait.

Fig. 4. Concentration-dependent effects of an endothelium-dependent vasodilator, ACh, on changes in internal diameter of 1st-order gracilis muscle arterioles isolated from sham control (sham) and DOCA rats. Arterioles were acutely pretreated in vitro with vehicle (A) or an inhibitor of HO, chromium mesoporphyrin (CrMP, 15 μmol/l; B), for 20 min before experiments. Values are means ± SE. *P < 0.05 vs. sham.

Fig. 5. Concentration-dependent effects of ACh on changes in internal diameter of 1st-order gracilis muscle arterioles isolated from 12-wk-old WKY rats or SHR. Arterioles were acutely pretreated in vitro with vehicle (A) or CrMP (15 μmol/l; B) for 20 min before experiments. Values are means ± SE.
The major endogenous source of carbon monoxide production is the HO-catalyzed enzymatic degradation of heme (6, 20, 40). Numerous tissues (28), including vascular endothelial and smooth muscle cells, express HO (5, 9). Three HO isoforms have been described (28, 30). HO-1 (heat shock protein 32) is the inducible isoform because its gene expression can be increased severalfold by various stimuli (28). HO-2 is regarded as a constitutive isoform, because its expression is relatively constant (28). Little is known about HO-3, except for a single report indicating that it has negligible catalytic activity with respect to the other two isoforms (30). ANG II-induced hypertension was reported to increase cardiac (14), aortic (15), and renal (1) expression of HO-1. We previously found that vascular HO-1 expression is increased in Dahl/Rapp salt-sensitive rats with salt-induced hypertension (17). In contrast, a recent report suggested that HO-1 expression is decreased in 8-wk-old, but not in adult, SHR (31). Our present data show higher HO-1 protein levels in abdominal aortic segments and first-order gracilis muscle arterioles isolated from DOCA rats than from the sham group. However, HO-1 levels were not different between SHR and WKY groups. These data suggest that DOCA-salt hypertension is accompanied by increased vascular HO-1 protein content, which may contribute to the increased endogenous carbon monoxide production.

Severely salt-sensitive hypertension (33), such as Dahl/Rapp salt-sensitive (11, 27) or DOCA-salt hypertension (26, 43), is reported to be associated with endothelial dysfunction. Some studies suggest that minimally salt-sensitive SHR display increased vascular NO production (11, 33), whereas others reported decreased NO bioavailability in these animals (12, 22, 23). Carbon monoxide has been shown to inhibit NO synthase (29, 41, 46) and promote endothelium-dependent vasoconstriction (18). Furthermore, induction of HO-1 has been shown to attenuate muscarinic agonist-induced NO release (41) and vasorelaxation (21) in isolated renal arteries. We previously reported impaired ACh-induced vasodilation in skeletal muscle arterioles isolated from hypertensive Dahl/Rapp salt-sensitive rats, but acute in vitro pretreatment with an HO inhibitor abolished the difference between hypertensive and normotensive groups (17). These data suggested that increased endogenous HO function may contribute to endothelial dysfunction during Dahl/Rapp salt-sensitive hypertension.

Our present study shows that skeletal muscle arterioles isolated from DOCA rats show attenuated vasodilator responses to an endothelium-dependent vasodilator, ACh, compared with sham controls. Furthermore, acute in vitro pretreatment with an inhibitor of endogenous carbon monoxide production, CrMP, enhanced arteriolar responses to ACh in the DOCA arterioles and diminished the differences between the DOCA and sham groups. CrMP is a photostable (44), specific (2), competitive inhibitor of HO that has been shown to decrease carbon monoxide formation in isolated gracilis muscle arterioles (49). In contrast, ACh-induced responses were not different between SHR and WKY arterioles with or without pretreatment with the HO inhibitor. Our HbCO and HO-1 protein measurements indicate that endogenous carbon monoxide production is increased in DOCA rats, but not in SHR. These data suggest that DOCA-salt hypertension is accompanied by increased endogenous carbon monoxide production, which may contribute to arteriolar endothelial dysfunction. Furthermore, this increase in endogenous carbon monoxide production and the subsequent endothelial dysfunction may not be a consequence of high blood pressure per se but may be, rather, associated with salt retention and/or high salt intake.

Numerous studies suggest an important role for the renin-angiotensin-aldosterone system in the development of endothelial dysfunction during hypertension (16). Recent studies emphasize the role of increased mineralocorticoid activity in hypertensive vascular injury (34). However, other studies suggest that high salt intake alone can promote endothelial dysfunction (4, 25, 39). Given the series of interventions required to establish DOCA-salt hypertension (unilateral nephrectomy, DOCA, and high salt intake), one can generate multiple combinations of “controls” to examine the respective contributions of each of these factors to arteriolar endothelial dysfunction in this model. Although such studies are required to explore the detailed mechanism of HO-mediated endothelial dysfunction in DOCA-salt hypertension, they are beyond the scope of the present study.

In the present study, we compared arteriolar responses between the hypertensive and normotensive animals at matched constant intraluminal pressure of 80 mmHg. Because the arterioles from hypertensive (DOCA and SHR) animals are likely adapted to the higher intraluminal pressures, our experiments may not accurately reflect their responsiveness in vivo. However, because the mean arterial pressures in DOCA rats and SHR are comparable, we have no reason to believe that the lower pressure per se would affect their responsiveness differently. Furthermore, other studies also used matched normal pressures to compare isolated arteriolar responses between hypertensive SHR and normotensive WKY animals (12, 22, 23) and found altered flow-induced dilator responses (22, 23) but similar ACh-induced vasodilator responses (12). Therefore, we believe that the intraluminal pressure used in our in vitro study is not enough to explain the observed differences between DOCA and SHR arteriolar responses.

Studies regarding the functional status of the vascular NO system in SHR appear to be contradictory. Some investigators suggest that vascular NO production is increased in SHR (11, 33). They suggest that severely salt-sensitive models of hypertension, such as Dahl salt-sensitive rats, are more susceptible to end-organ damage than are SHR because they are unable to increase vascular NO formation in response to the enhanced hemodynamic forces associated with high blood pressure (11, 33). In contrast, other studies suggest that vascular NO formation and/or bioavailability is decreased in SHR (12, 22, 23). Because decreased vascular NO function is a key feature associated with endothelial dysfunction, understandably the issue of endothelial function in SHR is similarly controversial. The reason for this diversity in the scientific literature is unknown. The SHR is a genetic model of hypertension, and it is possible that the last two decades may have yielded some genetic drifts among different laboratories and breeders. In fact, an established stroke-prone substrain of SHR (SHRSP) exhibits severe hypertension, enhanced salt sensitivity, and endothelial dysfunction (decreased vasodilator responses to ACh) and develops malignant nephrosclerosis and stroke compared with the parent SHR strain (8). Nonetheless, there are studies reporting endothelial dysfunction in regular SHR also (13, 22, 23, 42). Some of those investigations used large blood vessels, such as aortic rings (48), which are not resistance vessels. However, there are reports of decreased vascular NO production in SHR.
function also in resistance vessels isolated from SHR (13, 22, 23, 42).

Our present study does not find evidence of endothelial dysfunction in skeletal muscle arterioles isolated from SHR, although we only used an endothelium-dependent vasodilator, ACh, to examine arteriolar endothelial function. Although agonists such as ACh are frequently used to examine endothelial function (17, 21, 27, 38, 39), it is important to recognize that such manipulations only test certain aspects of endothelial function. Therefore, the possibility exists that different methods of endothelial stimulation, e.g., shear stress-mediated flow-induced dilation vs. agonist stimulation, may yield different results. Investigators who reported impaired shear force-induced dilation (22, 23) and enhanced myogenic tone (13, 42) in SHR did not see attenuated ACh-induced vasodilation in skeletal muscle arterioles isolated from SHR (12). Perhaps by using different, perhaps larger, blood vessels or alternative methods of endothelial stimulation we might be able to demonstrate altered endothelial function in SHR. However, our aim was not to resolve the apparent controversy in the scientific literature regarding endothelial function in SHR. Our present study simply demonstrates a phenomenon of HO-mediated attenuation of ACh-induced vasodilation that is clearly present in DOCA rats, but absent in SHR. Our results show that such phenomena are not equally present in all forms of hypertension and, therefore, suggest that factors other than high blood pressure per se contribute to the development of HO-mediated altered endothelial function in some forms of hypertension.

The issue of salt sensitivity in SHR requires clarification. Some reports suggest that, on increased dietary salt intake, SHR display higher blood pressure; therefore, SHR should be classified as salt sensitive (3, 32). Others compare the salt sensitivity of blood pressure in SHR and SHRSP and suggest that SHR are only minimally salt sensitive (8). Blood pressure responses to salt intake are continuously distributed; therefore, the definition of salt sensitivity is arbitrary: it simply means that the blood pressure changes more than the currently defined increment (45). Salt sensitivity is defined as a ≥10% increase in blood pressure in response to high sodium intake (1 wk at 249 meq/day) (45). Therefore, SHR should be regarded as salt sensitive. Nonetheless, the degree of salt sensitivity is much less in SHR than in SHRSP, DOCA rats, or Dahl salt-sensitive rats.

In summary, our data show that, at similar levels of hypertension, vascular HO-1 protein levels and blood HbCO levels are increased in DOCA rats, but not in SHR. Arterioles isolated from the DOCA animals displayed attenuated endothelium-dependent vasodilator responses, and acute in vitro treatment with an inhibitor of endogenous carbon monoxide production abolished the differences between the DOCA and sham arterioles. In contrast, endothelium-dependent vasodilator responses were not different between SHR and WKY arterioles with or without the HO inhibitor. These data suggest that DOCA-salt hypertension is accompanied by increased endogenous carbon monoxide production, which may contribute to arteriolar endothelial dysfunction. Furthermore, this increase in endogenous carbon monoxide production and the subsequent endothelial dysfunction may not be a consequence of high blood pressure per se but may be, rather, associated with salt retention and/or high salt intake.

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**References**