Altered Nitric Oxide Synthase 3 Distribution in Mesenteric Arteries of Hypertensive Rats

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Abstract—A high-salt (HS) diet and angiotensin II (Ang II) are both associated with the development of hypertension and impaired endothelial function. We hypothesize that alterations in nitric oxide synthase (NOS) activity or subcellular localization of NOS 3 protein may contribute to endothelial dysfunction in salt-dependent hypertension. To test this hypothesis, two models of salt-dependent hypertension were studied: DOCA-salt and Ang II. For Ang II hypertension, rats were divided into 4 groups: control on normal or HS diet, Ang II-infused on normal or HS diet. The mesenteric arterial bed was isolated and frozen in liquid nitrogen. Frozen arteries were homogenized and separated into cytosolic and particulate fractions. NOS activity was assayed by determining the conversion of 3H-arginine to 3H-citrulline in the absence and presence of the NOS inhibitor, Nω-nitro-L-arginine. NOS 3 protein expression was significantly increased in the cytosol of arteries from DOCA-salt compared with placebo rats and in Ang II-infused and Ang HS rats compared with control. NOS 3 expression in the particulate fraction was comparable among all groups. NOS activity (pmol/30 min/total protein) was significantly increased in the cytosolic fraction of arteries from DOCA-salt rats compared with placebo and in Ang HS rats compared with control. NOS activity was comparable in the particulate fraction in all rat groups. In conclusion, there is an altered subcellular distribution of NOS 3 in salt-dependent hypertension that may contribute to the development of hypertension and endothelial dysfunction. (Hypertension. 2002;39[part 2]:597-602.)

Key Words: nitric oxide synthase ■ deoxycorticosterone ■ mesenteric arteries ■ arginine ■ hypertension, arterial ■ angiotensin II

The vascular endothelium is a dynamic structure that plays a central role in cardiovascular health, in part via the release of vasoactive substances.1 The balance between the production of endothelium-derived constrictors and dilators is an important determinant of vascular tone. Dysfunction of the endothelium is associated with the development and progression of a number of cardiovascular diseases, including hypertension. Studies involving both humans2 and experimental animals3 have demonstrated a decline in endothelium-dependent vasodilation in hypertensive subjects compared with healthy subjects, resulting in an overall increase in vascular tone.

Human hypertension is often caused or exacerbated by high salt. Epidemiological studies have reported that endothelium-dependent vasodilator responses are significantly reduced in patients with salt-dependent hypertension.4 In experimental forms of hypertension, including those that require a high-salt intake, endothelium-dependent relaxation has been reported to decrease in large conduit arteries such as the aorta5-9 and carotid.10 While these findings are informative, it is smaller caliber arteries that regulate and determine vascular resistance and blood pressure.11 There is not a consensus in the literature as to the effects of salt-induced hypertension on vascular responses in resistance arteries. Endothelium-dependent vasodilation has been reported to decrease in isolated pressurized mesenteric resistance arteries from Dahl salt-sensitive rats8,12 and is unchanged in arteries from deoxycorticosterone acetate (DOCA)-salt rats.10 DOCA-salt treatment and chronic infusions of angiotensin II (Ang II) both result in experimental forms of salt-dependent hypertension. In salt-dependent hypertension, a high-salt diet intensifies increases in blood pressure. A decrease in endothelial-derived NO has been suggested to contribute to the development of salt-dependent hypertension. DOCA-salt and Ang II hypertension are both characterized by high superoxide (O2·−) production.2,9 O2·− is a free radical that scavenges nitric oxide (NO) thereby decreasing NO bioavailability. Previous studies have not specifically examined the effects of salt-dependent hypertension on endothelial nitric oxide synthase (NOS 3) in resistance arteries. Additionally, while in healthy arteries NOS 3 is predominately membrane associated, subcellular localization of NOS 3 in hypertension has not been examined. We hypothesize that alterations in compartmentalization of NOS 3 may contribute to endothelial dysfunction. Therefore, the purpose of this study was to examine the effects of hypertension, a

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high-salt diet, and their interaction on NOS 3 protein expression, subcellular localization, and activity in the mesenteric arterial bed.

Methods

Animals

Male Sprague-Dawley rats (200 to 225 g) (Harlan Laboratories, Indianapolis, Ind) were used in all studies. All animal protocols were in accordance with National Institutes of Health guidelines and approved by the Medical College of Georgia Committee for Animal Use in Research and Education. Rats were housed in temperature and humidity controlled, light-cycled quarters. Rats were allowed to adjust to these conditions for several days before surgery. DOCA-salt and placebo animals were prepared as previously described. Uninephrectomized rats received either a DOCA pellet (200 mg, 60-day time release) or placebo pellet implanted subcutaneously. DOCA-salt rats were given 0.9% NaCl to drink and placebo rats were given tap water. Blood pressures were measured using tail-cuff plethysmography. To produce Ang II hypertension, rats were first anesthetized using methohexital sodium (50 mg/kg). Control rats were implanted with a subcutaneous mini-osmotic pump (ALZET) filled with saline and given either a normal-salt diet (0.8% NaCl) or a high-salt (HS) diet (10.15% NaCl). Ang II-treated rats were implanted with a mini-osmotic pump filled with Ang II (65 ng/min, 14 days) and either put on a normal (Ang) or high-salt (HS) diet. In a subset of animals, telemetry transmitters were implanted for continuous measurement of blood pressure as previously described. Animals were euthanized using sodium pentobarbital (50 mg/kg IP) followed by a thoracotomy. The mesenteric bed, including arteries and veins, was cut away from the intestinal wall. The superior mesenteric artery was separated from connective tissue and cut. The mesenteric bed, including the superior mesenteric artery, was placed in a dissecting dish containing ice cold homogenization buffer (50 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L EDTA, and 0.1 mmol/L EGTA, 250 mmol/L sucrose, 10% glycerol) in the presence of protease inhibitors (1 mmol/L PMSF, 1 μmol/L pepstatin A, 2 μmol/L leupeptin, and 0.1% aprotinin). The fat was carefully pulled off from the vessels, and then the vessels were removed, using an Olympus dissecting microscope. The arteries were placed in a tube containing 1 mL homogenization buffer then snap-frozen in liquid nitrogen. Slightly thawed arteries were homogenized on ice in the presence of fresh protease inhibitors with a glass-glass homogenizer containing 1 mL homogenization buffer then snap-frozen in liquid nitrogen. Fifty grams of mesenteric tissue was homogenized for 10 strokes. The homogenate was centrifuged at 4°C at 100 000g for 30 minutes to separate into cytosolic and particulate fractions. The particulate fraction was then resuspended in 0.5 mL homogenization buffer. Protein concentrations were determined by standard Bradford assay (Bio-Rad) using BSA as the standard.

Western Blotting of NOS 3

Western blotting protocol was performed as previously described. The primary antibody was a monoclonal anti-NOS 3 (1:500; Transduction Laboratories) and the secondary antibody was horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; Amersham). The primary antibody was stripped using ReBlot Plus Mild Antibody Stripping Solution (Chemicon International) and a second primary antibody to β-actin (1:5000, Sigma) was applied followed by a secondary antibody to rabbit (1:10 000, Amersham). Equal protein loading was calculated based on protein concentrations from the Bradford assay and verified by actin. Specific bands were detected with enhanced chemiluminescence (Supersignal Chemiluminescent Substrate, Pierce). Densitometry was performed using a digital imaging system (Alpha Innotech Corporation).

Measurement of NOS Activity

Aliquots of cytosolic and particulate fractions were incubated with [3H]arginine (10 μmol/L final arginine, 71 Ci/mmol) in the presence of 1 mmol/L NADPH, 30 nmol/L calmodulin, 3 μmol/L tetrahydrobiopterin, 2 mmol/L CaCl2, 1 μmol/L FAD, and 1 μmol/L FMN in a final volume of 50 μL for 30 minutes at room temperature. The remainder of the assay was performed as previously described. The NOS 1-specific inhibitor N’-(1-imino-3-butenyl)-l-ornithine (VNIO; 1 μmol/L) was used to assess NOS 1-specific activity and the NOS 2-specific inhibitor 1400W (1400W; 1400W; 100 mmol/L) was used to assess NOS 2-specific activity. The inhibitory constants of VNIO for NOS 1, NOS 2, and NOS 3 are 0.1, 60, and 12 μmol/L, respectively. The inhibitory constants of 1400W for NOS 1, NOS 2, and NOS 3 are 2 μmol/L, 7 nmol/L, and 50 μmol/L. NOS activity was defined as [3H] arginine to [3H] citrulline conversion that was inhibited by the nonselective NOS inhibitor No-nitro-l-arginine (LNNa, 1 mmol/L). Therefore, NOS activity was calculated using the following formula: Total NOS activity = (pmol NOS activity in the absence of LNNa) – (pmol NOS activity in the presence of LNNa). NOS 1- and 2-isofrom activity was calculated using the following formula: NOS isoform activity = Total NOS activity – (pmol of NOS activity in the presence of NOS isoform-specific inhibitor). NOS 3-specific activity was estimated using the following formula: NOS 3 activity = Total NOS activity – (NOS 1-specific activity + NOS 2-specific activity). NOS activity was either normalized to mg of protein (pmol/30 min/total protein) or expressed as pmol of NOS activity per 30 minutes of total protein in mesenteric arterial bed.

Materials

DOCA and control pellets were purchased from Innovative Research of America. Normal and high-salt rat chow was purchased from Harlan Teklad. Ang II, LNNa, leupeptin, pepstatin A, aprotinin, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company. VNIO and 1400W were purchased from Cayman Chemicals. [3H]arginine was obtained from Amersham.

Data Analysis

All values are expressed as mean±SEM. All comparisons of DOCA-salt/placebo rats were performed using a Student t test for independent samples. All comparisons among Ang-II rats were performed using a one-way ANOVA followed by a Newman-Keuls post-hoc test. A probability value <0.05 was considered significant. Densitometry values for Ang II rats were compared using a ranked ANOVA followed by a Fisher’s protected least significant difference.

Results

Blood Pressure Response

DOCA-salt treatment resulted in a significant increase in mean 24-hour blood pressure when compared with placebo rats; blood pressures were 185±5 and 135±8 mm Hg, respectively (P<0.001). Chronic infusions of Ang II also resulted in a significant increase in blood pressure compared with infusions of saline (control); blood pressures were 146±7 and 108±3 mm Hg, respectively (P<0.005). The addition of a high-salt diet had no effect on blood pressure in control rats (117±3 mm Hg) but resulted in an increase in blood pressure in Ang II-infused rats (174±8 mm Hg; P<0.001 compared with Ang on a normal-salt diet).

NOS 3 Expression in Mesenteric Arteries

Western blot analysis was performed to examine NOS 3 distribution in cytosolic and particulate subcellular fractions. There was a significant increase in NOS 3 expression by the cytosolic fraction of arteries from DOCA-salt–treated rats.
compared with placebo rats (Figure 1) \((P<0.001)\). Particulate NOS 3 expression was not different between these groups. NOS 3 expression in mesenteric arteries from Ang rats is shown in Figure 2. Ang II-infused rats and Ang rats on a high-salt diet had a significant increase in cytosolic expression of NOS 3 compared with both control rats (control vs Ang, \(P=0.00001\); control vs Ang HS, \(P=0.0001\)) and rats on a high-salt diet alone (HS vs Ang, \(P=0.002\); HS vs Ang HS, \(P=0.003\)). There were no differences in particulate NOS 3 expression.

### NOS Activity

NOS activity was calculated by measuring the conversion of \(^{[3}\text{H}] \text{arginine}\) to \(^{[3}\text{H}] \text{citrulline}\). There was a significant increase in both cytosolic and particulate protein extracted from the mesenteric arterial bed in DOCA-salt rats (protein concentrations, cytosolic, and particulate, respectively: placebo= 857±50 \(\mu\)g, 1093±54 \(\mu\)g; DOCA= 2966±307 \(\mu\)g, 2273±166 \(\mu\)g; \(P<0.001\)) and Ang HS rats (cytosolic and particulate, respectively: control= 881±68 \(\mu\)g, 1492±62 \(\mu\)g; HS=794±125 \(\mu\)g, 1300±201 \(\mu\)g; Ang=1254±116 \(\mu\)g, 1726±223 \(\mu\)g; Ang HS=2737±269 \(\mu\)g, 2569±309 \(\mu\)g; \(P<0.005\)) when compared with the other treatment groups. Because of this increase in protein concentration, NOS activity is expressed as pmol of activity normalized to mg of protein and pmol of NOS activity in total protein from the arterial bed.

NOS activity/mg protein values for DOCA-salt rats are shown in Figure 3A. DOCA-salt treatment had no effect on cytosolic NOS activity compared with placebo (\(P=0.10\), NS). NOS activity in the particulate fraction was significantly decreased by DOCA-salt treatment (\(P<0.001\)).

NOS activity/mg protein values for Ang rats are shown in Figure 3B. There were no differences in NOS activity in the cytosolic fraction with either Ang II-infusions alone or with the addition of a high-salt diet. In the particulate fraction, neither Ang, a high-salt diet, or the combination of Ang and a high-salt diet altered NOS activity relative to control; however, there was a significant decrease in NOS activity in Ang-HS–treated rats compared with HS-alone–treated rats (\(P<0.01\)).

### Relative Contributions of NOS-Isoforms to Total NOS Activity

The relative contributions were assessed using isoform-specific inhibitors, and NOS activity was expressed as pmol/30 min/total protein in arterial bed. As shown in Figure 4A, there were no differences in either the NOS 1 or NOS 2 activity in the cytosolic fraction of arteries from DOCA-salt rats compared with placebo rats. NOS 3-specific activity, however, was significantly increased in DOCA-salt–treated rats (\(P=0.001\)). There were no differences in the activity of the three NOS isoforms in the particulate fraction (Figure 4B).

**Figure 1.** NOS 3 protein expression in mesenteric arteries from DOCA-salt and placebo rats. A, Shows representative Western blots. Lanes 1 and 2 are the cytosolic (C) and particulate (P) fractions from a DOCA-salt rat. Lanes 3 and 4 are the cytosolic and particulate fractions from a placebo rat. A total of 65 \(\mu\)g of protein was loaded per lane. Relative densitometry is shown in B, \(n=4\). * Indicates significant difference from placebo.

**Figure 2.** NOS 3 protein expression in mesenteric arteries from Ang II rats. A, Shows a representative Western blot. Lanes 1 and 2 are the cytosolic (C) and particulate (P) fractions from a control rat. Lanes 3 and 4 are the cytosolic and particulate fractions from an Ang II rat. Lanes 5 and 6 are the cytosolic and particulate fractions from a HS rat, and lanes 7 and 8 are from an Ang HS rat. Relative densitometry is shown in B: \(n=5\) for control and Ang, \(n=6\) for HS, and \(n=8\) for Ang HS. A total of 55 \(\mu\)g of protein was loaded per lane. * Indicates significant difference from control. † Indicates significant difference from HS.
The relative contribution of the three NOS isoforms to total NOS activity in Ang rats is shown in Figure 5. There were no differences in either NOS 1- or NOS 2-specific activity in the cytosolic fraction of Ang II-treated rats (Figure 5A). There was, however, a significant increase in NOS 3-specific activity in Ang HS rats compared with the other three treatment groups (P<0.001 for all comparisons). There were no differences in the relative contribution of any of the NOS isoforms to NOS activity in the particulate fraction (Figure 5B).

Discussion
In this study we found an altered distribution of NOS 3 protein in mesenteric arteries in two distinct forms of experimental salt-dependent hypertension, with a significant increase in NOS 3 protein expression in the cytosol. When total NOS activity/total protein in the arterial bed was calculated, we found that NOS 3 activity was increased in the cytosolic fraction of mesenteric arteries from DOCA-salt and Ang plus high-salt hypertensive rats. Therefore, increased NOS 3 expression and activity was demonstrated in mesenteric arteries from hypertensive rats with vascular remodeling. Increased activity did not coincide with the increased NOS 3 expression in mesenteric arteries in Ang hypertension.

It has been reported that both hypertension and a high-salt diet alone are associated with decreases in endothelium-dependent vasodilation. Additionally, in both humans and experimental animals, decreases in vascular relaxation have been reported to be exacerbated in the presence of both hypertension and a high-salt diet. Our study corroborates these reports, in that the combination of Ang and a high-salt diet produced the largest alteration in NOS 3 protein distribution. NOS 3 is a membrane-associated enzyme that catalyzes the formation of NO from L-arginine. NOS 3 is normally targeted to caveolar domains and Golgi membranes by posttranslational N-myristoylation and palmitoylation. We found that there were no changes in particulate NOS 3 expression among any of the treatment groups studied. Particulate NOS activity/mg protein in DOCA-salt rats decreased, most likely because there was significantly more protein in the particulate fraction of arteries from this group. Therefore, when particulate NOS activity was expressed as pmol/total protein in the mesenteric arterial bed, no differences were observed in NOS activity. This finding suggests that membrane-bound NOS 3 is not contributing to the endothelial dysfunction in these models of hypertension.
in NOS 3 activity most likely reflects an increase in NOS 3 protein in DOCA-salt and Ang HS models of hypertension. Increased cytosolic NOS 3 protein expression was also demonstrated in mesenteric arteries from Ang hypertensive rats; however, no increase in NOS activity was observed in either the cytosolic or particulate fractions. Additionally, we did not find an increase in protein in arteries from Ang hypertensive animals, indicating less vascular remodeling than in arteries from DOCA-salt– and Ang-HS–treated rats. At the current time, it is unknown why we observed this difference. From all of our evidence and the results of Sessa et al, we hypothesize that cytosolic NOS 3 produces less bioavailable NO than particulate NOS 3. However, we cannot rule out the possibility that cytosolic NOS 3 is actively making NO and potentially working as a compensatory protective mechanism to antagonize increases in vascular tone.

One of the novel findings of this study was the altered compartmentalization of NOS 3 protein expression in salt-dependent hypertension; the cause of this altered localization, however, is unknown. Both DOCA-salt and Ang hypertension have high O$_2^-$ production. O$_2^-$ avidly binds to NO thereby reducing NO bioavailability and forming the free radical peroxynitrite (ONOO$^-$). Therefore, while it might be expected that an increase in NOS 3 protein expression would maintain endothelium-dependent vasodilation, a corresponding increase in O$_2^-$ may antagonize this effect and contribute to endothelial dysfunction. Using a porphyrinic sensor to directly measure NO production, Tschudi et al reported an increase in O$_2^-$ scavenging of NO in mesenteric arteries of hypertensive rats. NAD(P)H oxidase in the medial layer has been identified as the primary source of O$_2^-$ in the vasculature. One might hypothesize that the increase in O$_2^-$ production in salt-dependent hypertension may drive the altered compartmentalization of NOS 3. Additionally, cytosolic NOS 3 may be uncoupled and produce O$_2^-$ in cases of tetrahydrobiopterin (BH$_4$) deficiency or inhibition of Hsp90. In DOCA-salt and Ang HS hypertension there may be a deficiency of BH$_4$, since ONOO$^-$ has been shown to oxidize BH$_4$ resulting in NOS 3 uncoupling and increased cytosolic NOS 3 expression. Because in our NOS activity assay all cofactors are present at maximal concentrations, a decrease in cofactor availability would not be reflected in our assay as a decrease in activity. Thus, we are actually measuring the maximal potential for NOS to produce NO. Alternatively, a recent report by Dedio et al found that NOSIP (NOS 3 interaction protein) can interfere with NOS 3 binding to caveolin-1, resulting in altered NOS 3 compartmentalization and activity. Dedio et al also observed that NOSIP was associated with cytoplasmic filamentous structures. Since there is an abundance of vascular remodeling in DOCA and Ang hypertension and a resultant increase in cytoskeletal proteins, there may also be an increase in NOSIP, which facilitates altered NOS 3 localization.

In conclusion, there is an altered distribution of NOS 3 in the mesenteric arterial bed of DOCA-salt and Ang-HS hypertension with an increase in cytosolic protein expression. These findings are consistent with our hypothesis that an altered subcellular localization of NOS 3 protein may con-
tribute to endothelial dysfunction in salt-dependent hypertension.

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