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Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension

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Submitted 17 June 2008; accepted in final form 31 July 2008

Wang D, Strandgaard S, Iversen J, Wilcox CS. Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension. Am J Physiol Regul Integr Comp Physiol 296: R195–R200, 2009. First published August 6, 2008; doi:10.1152/ajpregu.90506.2008.—We reported impaired endothelium-derived relaxation factor/nitric oxide (EDRF/NO) responses and constitutive nitric oxide synthase (cNOS) activity in subcutaneous vessels dissected from patients with essential hypertension (n = 9) compared with normal controls (n = 10). We now test the hypothesis that the patients in this study have increased circulating levels of the cNOS inhibitor, asymmetric dimethylarginine (ADMA), or the lipid peroxidation product of linoleic acid, 13-hydroxyoctadecadienoic acid (HODE), which is a marker of reactive oxygen species. Patients had significantly (P < 0.001) elevated (means ± SD) plasma levels of ADMA (P ADMA: 766 ± 217 vs. 393 ± 57 nmol/l) and symmetric dimethylarginine (P SDMA: 644 ± 140 vs. 399 ± 70 nmol/l) but similar levels of L-arginine accompanied by significantly (P < 0.015) increased rates of renal ADMA excretion (21 ± 9 vs. 14 ± 5 nmol/μmol creatinine) and decreased rates of renal ADMA clearance (18 ± 3 vs. 28 ± 5 ml/min). They had significantly increased plasma levels of HODE (P HODE: 309 ± 30 vs. 226 ± 24 nmol/l) and renal HODE excretion (433 ± 93 vs. 299 ± 67 nmol/μmol creatinine). For the combined group of normal and hypertensive subjects, the individual values for plasma levels of ADMA and HODE were both significantly (P < 0.001) and inversely correlated with microvascular EDRF/NO and positively correlated with mean blood pressure. In conclusion, elevated levels of ADMA and oxidative stress in a group of hypertensive patients could contribute to the associated microvascular endothelial dysfunction and elevated blood pressure.

blood pressure; 13-hydroxyoctadecadienoic acid; endothelium-derived relaxation factor/nitric oxide; albuminuria

MOST OF THE ESTABLISHED AND NONTRADITIONAL CARDIOVASCULAR RISK FACTORS ARE ASSOCIATED WITH ENDOTHELIAL DYSFUNCTION (3, 5, 7, 18, 45). THIS INCLUDES THOSE WITH ESSENTIAL HYPERTENSION IN SOME (29, 33, 34, 35, 39, 42, 50), BUT NOT ALL, STUDIES (54). ENDOTHELIAL FUNCTION DEPENDS ON THE INTEGRITY OF CONSTITUTIVE NITRIC OXIDE SYNTHASE (cNOS) AND THE AVAILABILITY AND VASCULAR SIGNALING OF ITS PRODUCT, NITRIC OXIDE (NO). ENDOTHELIAL DYSFUNCTION IS OF CLINICAL IMPORTANCE SINCE IT PREDISPOSES TO THE DEVELOPMENT OF HYPERTENSION AND Atherosclerosis AND CONSEQUENTLY IS A PREDICTOR OF SUBSEQUENT ADVERSE CARDIOVASCULAR EVENTS (34).

Endothelial dysfunction has been ascribed to two principal factors. One is an enhanced vascular generation of superoxide anion (O2·−) that bioinactivates endothelial-derived NO (5), thereby reducing endothelial function and forming peroxynitrite (ONOO−). Peroxynitrite itself can nitrosate and inactivate prostacyclin synthase (46), which is an additional pathway mediating endothelium-dependent relaxation. A second factor is generation of asymmetric dimethylarginine (ADMA), which inhibits nitric oxide synthase (6) and inhibits the cellular uptake of L-arginine by the cationic amino acid transporters (CATs) (8).

We have studied endothelial function and cNOS activity directly in subcutaneous resistance vessels dissected from gluteal biopsies. We detected a profound reduction in endothelium-derived relaxation factor/nitric oxide (EDRF/NO) responses (assessed from ACh-induced, NOS-dependent relaxation of preconstricted vessels) and in cNOS activity (assessed from conversion of [14C]-arginine to [14C]-citrulline) in gluteal vessels from 9 patients with essential hypertension, compared with 10 age-matched normal controls. These patients formed an additional control group in a publication on endothelial function in patients with autosomal dominant polycystic kidney disease (50). The present study tests the hypothesis that these defects in EDRF/NO and the elevated blood pressure in this group of patients with uncomplicated essential hypertension are related to increases in plasma levels of ADMA (P ADMA) or lipid peroxidation markers of reactive oxygen species (ROS). To test the hypothesis, we measured these markers in plasma and urine samples from the patients whose microvascular function has already been reported.

METHODS

Patients. The protocol was approved by the Medical Ethics Committee, Copenhagen County, Denmark. All individuals gave informed, written consent before entering the study. Nine patients with essential hypertension and ten age-matched normal controls aged 23–60 yr were studied. All subjects were Caucasians. The diagnosis of essential hypertension has already been reported.
hypertension was established by a clinic record of systolic blood pressure >140 mmHg, a diastolic blood pressure >90 mmHg, the absence of clinical or laboratory evidence of secondary hypertension or diabetes mellitus, and normal values for serum electrolytes, creatinine, cholesterol, and urinalysis. Four patients and four control subjects were smokers. All were requested to refrain from smoking on the day of study. All patients were receiving antihypertensive treatment with two or more antihypertensive drugs (angiotensin-converting enzyme inhibitors (ACEs), angiotensin receptor blockers (ARBs), beta-blockers, alpha-beta-blockers, calcium antagonists, or diuretics). All antihypertensive therapy was withdrawn 24 h prior to testing. No patient or normal subject was taking any other medications, including vitamins, health supplements, or aspirin. To establish the true level of blood pressure in the hypertensive subjects, an ambulatory blood pressure monitor (ABPM) was undertaken over 24 h with a Takeda 2420 monitor, while the patients were off treatment. The glomerular filtration rate was estimated from the 4-h, one sample plasma clearance method using [51Cr]-ethylenediamine pentaacetic acid (EDTA) (24).

Urine collections and ABPM studies were completed within 1 wk of the collection of blood and urine samples and gluteal biopsies.

The details of the measurements of EDRF/NO and cNOS activity in gluteal vessels have been published, together with the mean values obtained (50). In brief, subcutaneous microvessels were mounted in a Mulpvany-Halpern myograph, preconstricted with 10^-5 M norepinephrine and relaxed with graded concentrations of ACh alone, or in the presence of L-nitroarginine methyl ester (L-NAME) to inhibit NO generation. The EDRF/NO response was calculated as the L-NAME-inhibitable maximal relaxation response to ACh. The constitutive nitric oxide synthase (cNOS) activity was assessed in other dissected subdermal vessels from the conversion of [14C]-arginine to [14C]-citrulline.

Measurement of ADMA, SDMA, and L-arginine in plasma and urine. ADMA, SDMA, and L-arginine were determined by a modification of an HPLC method (49) and validated according to U.S. Food and Drug Administration standards, as described in detail elsewhere (1). Within-assay and between-assay coefficients of variation for ADMA were 1.7% and 2.5%, respectively and did not differ in plasma or urine samples. The limits of detection were less than 50 nmol/l.

Measurement of plasma 13-hydroxyoctadecadienoic acid. Hydroxy polyunsaturated fatty acids were extracted and quantified by a modification of the HPLC method of Browne and Armstrong (4), as described in detail previously (1). Total lipid extracts were made from 500 µl of plasma collected in EDTA tubes using hexane: isopropanol (3:2) and saponified in 500 µl ethanolic NaOH to release the free acids (44).

Renal excretions of ADMA, SDMA, arginine, and 13-hydroxyoctadecadienoic acid (HODE) were factored by creatinine to correct for incomplete bladder emptying or urine collection.

Statistics. Data are expressed as means ± SD. The hypothesis requires testing of two primary variables: ADMA and HODE plasma levels. Therefore, a Bonferroni correction was applied requiring a P value of 0.05/2 = 0.025 for statistical significance. An independent-samples t-test was performed to compare means from control and patient groups using GraphPad software.

RESULTS

Clinical characteristics of the subjects. The two groups were well matched for age, gender, blood hemoglobin, fasting plasma glucose, plasma cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, plasma total homocysteine, blood urea nitrogen, serum creatinine, creatinine clearance and [51Cr]-EDTA plasma clearance (Table 1). The systolic, diastolic, and mean blood pressures and the excretion of albumin were significantly higher in the patients with hypertension.

Plasma levels, renal excretion, and renal clearance of ADMA, SDMA, L-arginine, and HODE. Plasma levels of L-arginine were similar in the two groups (Table 2). However, patients with hypertension had significantly higher plasma concentrations of ADMA and SDMA, a reduced plasma ratio of L-arginine/ADMA, but a similar plasma ratio of ADMA/SDMA. The 24-h renal excretion of ADMA was significantly increased, but the renal clearance of ADMA was reduced in the patients with hypertension. The excretion and clearance of SDMA and L-arginine were not significantly different between the groups.

Patients with hypertension had increased plasma levels and renal excretion of HODE, but the renal clearance of HODE was not significantly different between groups.

Correlations of EDRF/NO and MBP with plasma ADMA and HODE. Among the composite group of normal subjects and patients with essential hypertension, individual values for EDRF/NO from L-NAME inhibitable, ACh-induced relax-

Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls</th>
<th>Essential Hypertension</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Number studied</td>
<td>10</td>
<td>9</td>
<td>ns</td>
</tr>
<tr>
<td>Age, yr</td>
<td>39 ± 4</td>
<td>47 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>5/5</td>
<td>3/6</td>
<td>ns</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>113 ± 11</td>
<td>139 ± 14</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>72 ± 9</td>
<td>95 ± 9</td>
<td>0.01</td>
</tr>
<tr>
<td>24 h mean blood pressure, mmHg</td>
<td>92 ± 6</td>
<td>107 ± 9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Blood hemoglobin, g/dl</td>
<td>13.5 ± 1.1</td>
<td>13.4 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>170 ± 27</td>
<td>185 ± 146</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mg/dl</td>
<td>55 ± 11</td>
<td>59 ± 18</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma LDL cholesterol, mg/dl</td>
<td>101 ± 32</td>
<td>107 ± 37</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>82 ± 9</td>
<td>89 ± 8</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma total homocysteine, mg/dl</td>
<td>12.4 ± 0.4</td>
<td>11.6 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>12.5 ± 0.4</td>
<td>15.2 ± 4.5</td>
<td>ns</td>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td>1.05 ± 0.1</td>
<td>1.03 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>[51Cr]-EDTA clearance, ml/min•1.73 m²⁻¹</td>
<td>100 ± 9</td>
<td>92 ± 10</td>
<td>ns</td>
</tr>
</tbody>
</table>

Conversion factors for SI units: 1 g/dl of hemoglobin = 100 g/l; 1 mg/dl of cholesterol = 0.0259 mmol/l; 1 mg/dl glucose = 0.0555 mmol/l; 1 mg/dl of homocysteine = 7.397 µmol/l; 1 mg/dl of urea nitrogen = 0.357 mmol/l; 1 mg/dl of creatinine = 88.4 µmol/l; 1 µg/mg creatine of albumin = 1.712 pmol/µmol creatinine. HDL, high-density lipoprotein; LDL, low-density lipoprotein; ns, not significant.
ations of norepinephrine preconstricted subdermal resistance vessels (50) correlated negatively with plasma levels of ADMA and HODE (Fig. 1, A and B). Mean blood pressure correlated positively with plasma ADMA and HODE (Fig. 1, C and D). These two plasma markers were significantly interrelated (Fig. 2). Inspection of data in Fig. 1 shows that these were complete separations of values for EDRF/NO and PHODE between the two groups, and only a single patient had values for PADMA that overlapped the values in normal subjects.

DISCUSSION

There are four principal new findings in this study. First, the plasma levels and rates of renal excretion of ADMA and HODE were increased in a group of nine patients with essential hypertension who had severely reduced EDRF/NO responses and cNOS activities of their subdermal resistance vessels. These patients had no other risk factors for cardiovascular disease, except for microalbuminuria, which itself is considered a marker of endothelial dysfunction (17). This is the first time that both ADMA and oxidative stress have been quantitated in a group of hypertensive subjects with severely impaired microvascular EDRF/NO responses and cNOS activities measured directly in isolated resistance vessels. A second novel finding was the almost complete separation of the individual values of EDRF/NO responses and PADMA and PHODE between the two groups. This suggests that defective endothelial function and elevated ADMA and oxidative stress are rather uniform in patients with essential hypertension. A third

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls</th>
<th>Essential Hypertension</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Plasma l-arginine, µmol/l</td>
<td>78±7.0</td>
<td>64±17</td>
<td>ns</td>
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<tr>
<td>Plasma ADMA, nmol/l</td>
<td>38.3±5.6</td>
<td>76.7±138</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Plasma SDMA, nmol/l</td>
<td>39.2±7.0</td>
<td>64.4±140</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Plasma l-arginine/ADMA</td>
<td>206±55</td>
<td>90±31</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Plasma ADMA/SDMA</td>
<td>1.0±0.2</td>
<td>1.2±0.2</td>
<td>ns</td>
</tr>
<tr>
<td>Renal excretion of ADMA, nmol/µmol creatinine</td>
<td>13.9±5.6</td>
<td>21.2±2</td>
<td>=0.016*</td>
</tr>
<tr>
<td>Renal excretion of SDMA, nmol/µmol creatinine</td>
<td>20.9±9.4</td>
<td>23.5±8</td>
<td>ns</td>
</tr>
<tr>
<td>Renal clearance of ADMA, ml/min</td>
<td>28.5±5.5</td>
<td>26.4±5</td>
<td>ns</td>
</tr>
<tr>
<td>Renal clearance of SDMA, ml/min</td>
<td>36.1±13</td>
<td>32.8±7</td>
<td>ns</td>
</tr>
<tr>
<td>Renal clearance of l-arginine, ml/min</td>
<td>0.08±0.007</td>
<td>0.09±0.004</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma HODE, nmol/l</td>
<td>236±18</td>
<td>326±68</td>
<td>=0.001*</td>
</tr>
<tr>
<td>Renal excretion of HODE, nmol/µmol creatinine</td>
<td>299±67</td>
<td>433±93</td>
<td>=0.002*</td>
</tr>
<tr>
<td>Renal clearance of HODE, ml/min</td>
<td>11.2±2</td>
<td>10.2±2</td>
<td>ns</td>
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ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; HODE, 13-hydroxyoctadecadienoic acid. *Statistically significant difference after adjustment for two comparisons by the Bonferroni correction.

Fig. 1. Correlations between individual values for the composite group of normal control subjects (■) and patients with essential hypertension (○). Data are for L-NAME inhibitable, ACh-induced relaxations of phenylephrine preconstricted subdermal microvessels reported previously (50) or mean blood pressure (from 24 h ambulatory blood pressure recording) and plasma levels of asymmetric dimethylarginine (ADMA) and 13-hydroxyoctadecadienoic acid (HODE).
new finding was the strongly negative correlations between directly measured EDRF/NO responses and plasma levels of ADMA and HODE and strongly positive correlations between mean blood pressure and these plasma markers. A fourth new finding was the close correlation between plasma levels of ADMA and HODE in this group of subjects.

Although ADMA can inhibit cNOS activity, the role of circulating ADMA in impairing NO generation and endothelial dysfunction remains controversial (48). Our previous study of patients with autosomal dominant polycystic kidney disease (50) included patients with essential hypertension as an additional control group to assess the impact of hypertension on the microvascular defects in this patient group. We were struck with the profound inhibition of EDRF/NO response, NOS activity, and plasma NO$_3$ concentrations in this hypertensive group. Therefore, we undertook subsequent measures of ADMA and lipid peroxidation to investigate potential mechanisms. An intravenous infusion of ADMA in healthy volunteers augments peripheral and renal vascular resistances and arterial pressure (6). However, the plasma levels of ADMA required for these acute effects are well above those measured in this study. Nevertheless, increased p$_{\text{ADMA}}$ correlates inversely with indirect measures of endothelial function in patients with dyslipidemia (2) and hypertension (35). The present study extends previous reports of elevated plasma levels of ADMA in most (15, 16, 20, 28, 31, 35, 41, 52) but not all (39) studies of hypertensive humans to a group with directly measured defects in EDRF/NO and cNOS activity in their blood vessels.

Our finding of elevated plasma levels and renal excretion of HODE in patients with essential hypertension extends prior reports in essential hypertension of increased plasma levels of the ROS markers of hydrogen peroxide (22), plasma protein carbonyl derivatives (19, 40), and lipid peroxidation products (9, 10, 19, 28, 36, 37, 38, 43), oxidized to reduced ratio of glutathione (26, 27, 38) and O$_2^{-}$ in activated polymorphonuclear leukocytes (21, 25, 37, 53). However, other studies have failed to detect an increase in renal excretion of lipid peroxidation products (11) or ROS in lymphocytes (30) in hypertensive subjects.

The finding that EDRF/NO is negatively related to plasma ADMA and to plasma HODE is consistent with, but does not itself prove, that elevated ADMA or oxidative stress may be a cause of impaired endothelial function in this group.

Limitations of this study include the relatively small number of patients. The relatively invasive method to assess directly and unequivocally the EDRF/NO responses and cNOS activity of resistance vessels restricted enrollment. The numbers were too small to detect significant correlations of the data within the group of hypertensive subjects. A second limitation is that patients with essential hypertension were receiving antihypertensive therapy until 24 h before study, as required by our institutional Human Subjects Review Group. However, prior reports have shown that the treatment of hypertension with an angiotensin receptor blocker (12, 14, 16, 26, 52), an angiotensin-converting enzyme inhibitor (12, 16, 19, 28, 52), or a calcium channel blocker (13, 26, 37, 53) all reduce plasma levels of oxidative stress markers (13, 14, 19, 26, 28, 37, 52, 53) or ADMA (12, 16, 52). Thus, the elevated plasma levels of ADMA and HODE are not likely a consequence of prior treatment of hypertension. A third limitation is the associative method of study. Thus, for example, the inverse correlation between EDRF/NO and p$_{\text{HODE}}$ is consistent with both a role for oxidative stress in causing endothelial dysfunction and with a role for endothelial dysfunction in causing oxidative stress, perhaps by an uncoupling of the eNOS enzyme (5).

A new finding in this study is the very close correlation between the plasma levels of ADMA with the marker of oxidative stress in the group of normotensive and hypertensive patients. Recent experimental evidence reviewed in Palm et al. (32) suggests a tight interrelationship between the production and metabolism of ROS and ADMA. Thus oxidation of a reactive thiol in the active site of DDAH inactivates DDAH and could thereby increase plasma and tissue levels of ADMA, and reduce renal ADMA clearance as found in the hypertensive patients in this study with oxidative stress. The protein arginine methyl transferase isofoms that catalyze the methylation of arginine epitopes on proteins (47) also are redox sensitive (32, 47). This might account for our finding of elevated plasma levels of SDMA in the hypertensive subject in our study. SDMA is not a substrate for DDAH (23).

**Perspectives and Significance**

The positive correlations detected in the composite group of subjects between blood pressure and plasma levels of ADMA and HODE, and the negative correlations with EDRF/NO are consistent with a role for ADMA and oxidative stress in elevated blood pressure and endothelial dysfunction in human subjects. A test of this hypothesis must await the development of methods to reduce ADMA or oxidative stress in human subjects. This could have clinical impact since endothelial dysfunction and hypertension are powerful predictors of cardiovascular events and loss of renal function (51).

**ACKNOWLEDGMENTS**

The authors thank Pia Linne Olsen, Bodil Hellström, Inger Marie Leth, and Britta Vilhelmsen for their meticulous laboratory work during the study. We thank Anna Leone at Oxonon BioAnalysis (oxononus@aol.com) for developing, validating, and undertaking the analysis for ADMA, SDMA, l-arginine, and 13-HODE. We thank Emily Wing Kam Chan for editing and preparing the manuscript.

**GRANTS**

This work was supported by grants from the George E. Schreiner Chair of Nephrology and from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-49870 and DK-36079) and National Heart, Lung, and Blood Institute (HL-68686).
DISCLOSURE

Data for ACh-induced vascular relaxation and cNOS activity for normal controls and patients with essential hypertension were published previously as control groups for a study on patients with autosomal-dominant polycystic kidney disease (50).

REFERENCES


