Impaired Endothelial Function and Microvascular Asymmetrical Dimethylarginine in Angiotensin II–Infused Rats
Effects of Tempol

Dan Wang, Zaiming Luo, Xiaoyan Wang, Pedro A. Jose, John R. Falck, William J. Welch, Shakil Aslam, Tom Teerlink, Christopher S. Wilcox

Abstract—Angiotensin (Ang) II causes endothelial dysfunction, which is associated with cardiovascular risk. We investigated the hypothesis that Ang II increases microvascular reactive oxygen species and asymmetrical dimethylarginine and switches endothelial function from vasodilator to vasoconstrictor pathways. Acetylcholine-induced endothelium-dependent responses of mesenteric resistance arterioles were assessed in a myograph and vascular NO and reactive oxygen species by fluorescent probes in groups (n=6) of male rats infused for 14 days with Ang II (200 ng/kg per minute) or given a sham infusion. Additional groups of Ang or sham-infused rats were given oral Tempol (2 mmol·L⁻¹). Ang II infusion increased mean blood pressure (119±5 versus 89±7 mm Hg; P<0.005) and plasma malondialdehyde (0.57±0.02 versus 0.37±0.05 μmol·L⁻¹; P<0.035) and decreased maximal endothelium-dependent relaxation (18±5% versus 54±6%; P<0.005) and hyperpolarizing (19±3% versus 29±3%; P<0.05) responses and NO activity (0.9±0.1 versus 1.6±0.2 U; P<0.01) yet enhanced endothelium-dependent contraction responses (23±5% versus 5±5%; P<0.05) and reactive oxygen species production (0.82±0.05 versus 0.15±0.03 U; P<0.01). Ang II decreased the expression of dimethylarginine dimethylaminohydrolase 2 and increased asymmetrical dimethylarginine in vessels (450±50 versus 260±35 pmol/mg of protein; P<0.01) but not plasma. Tempol prevented any significant changes with Ang II. In conclusion, Ang redirected endothelial responses from relaxation to contraction, reduced vascular NO, and increased asymmetrical dimethylarginine. These effects were dependent on reactive oxygen species and could, therefore, be targeted with effective antioxidant therapy. (Hypertension. 2010;56:950-955.)

Key Words: reactive oxygen species • NO • hypertension • endothelial dysfunction • dimethylarginine dimethylaminohydrolase

Normal vessels display endothelium-dependent relaxation responses to acetylcholine (Ach). The endothelium-dependent relaxation factor (EDRF) is mediated by endothelial NO synthase (NOS). The mediators of the endothelium-dependent hyperpolarizing factor (EDHF) vary by species and vascular bed but can include hydrogen peroxide (H₂O₂),¹ epoxyeicosatrienoic acids (EETs),² and electromechanical coupling.³ These cause activation of calcium-dependent potassium channels on vascular smooth muscle cells (VSMCs) that can be blocked by a combination of apamin and charybdothoxin.² Some vessels also display a cyclooxygenase (COX)-dependent relaxation response attributable to prostacyclin, although this was not apparent in rat afferent arterioles.² An endothelium-dependent contracting factor (EDCF) occurs in models with increased reactive oxygen species (ROS). The EDCF in renal afferent arterioles from rabbits infused with angiotensin (Ang) II entailed a vasoconstrictor prostaglandin generated by COX that activated thromboxane prostanoid receptors (TP-Rs) on VSMCs.⁴

Ang II generates ROS in blood vessels that can bioactivate NO.⁴ However, NOS-dependent EDRF responses of resistance arterioles are not consistently reduced⁵ by Ang infusion. Moreover, ROS can generate an EDCF.⁴,⁶ However, it is not clear whether prolonged inhibition of ROS can prevent the defects in endothelium-dependent relaxations and prevent the EDCF responses of resistance vessels from animals infused with Ang II.

Increasing evidence suggests that an endogenous inhibitor of NOS, asymmetrical dimethylarginine (ADMA), and its metabolism by dimethylarginine dimethylaminohydrolase...
(DDAH)\(^7\) may regulate vascular NO. Ang II infusion can increase circulating levels of ADMA.\(^8,9\) However, it is unclear whether increased ADMA levels in plasma or tissues can be prevented by an effective antioxidant. We reported recently that culture of VSMCs with Ang II increased cellular, but not medium, levels of ADMA.\(^10\) To resolve these issues, we assessed the 3 major endothelium-dependent pathways and related these responses to vascular NO and ROS activities and plasma and tissue ADMA concentrations in mesenteric resistance arteries in rats during a slow pressor infusion of Ang II. Other groups of sham and Ang II–infused rats received the antioxidant drug Tempol throughout.\(^11\)

These experiments were designed to test the hypothesis that ROS, generated in microvascular resistance vessels during prolonged Ang II–induced hypertension, impairs EDRF, EDHF, and NO activity and increases microvascular ADMA and EDCF. We selected Tempol, which is a redox-cycling nitroxide, to reduce ROS and enhance vascular NO.\(^11\) These experiments are significant because restoration of endothelial function and NO and abrogation of EDCF, ROS, and ADMA would reverse some of the earliest manifestations of hypertension and vascular disease.\(^12\)

**Methods**

Male Sprague-Dawley rats (200 to 220 g; Taconics Laboratory) were maintained on tap water and standard chow (Na\(^+\) 0.4 g · 100 g \(^{-1}\)) under constant humidity and temperature and 12-hour light-dark cycles. The protocols were approved by Georgetown University Institutional Animal Care and Use Committee. Details of methods appear in the online Data Supplement (please see http://hyper.ahajournals.org).

**Animal Model**

Three sets each of 4 groups of Sprague-Dawley rats were prepared (6 per group were used). Rats were anesthetized with sodium pentobarbital (50 mg · kg \(^{-1}\)) for subcutaneous insertion of a sham minipump (sham) or an osmotic minipump (model 202, Alzate) at the dorsum of the neck. Group 1 had a sham infusion. Group 2 received Ang II (Peninsula Laboratory) at 200 ng · kg \(^{-1}\) · min \(^{-1}\) for 14 days. Group 3 received a sham minipump and oral Tempol (2 mmol · L \(^{-1}\) of water). Group 4 received an Ang II minipump and Tempol. On the experimental day, rats were anesthetized with inhaled isoflurane (3% balance with oxygen). The carotid artery was cannulated for measurement of mean arterial pressure (model DPM-1B, Biotek). Blood was withdrawn, and the plasma was stored at \(-80^\circ\)C. Thereafter, rats were euthanized by exsanguination, the abdomen was opened, and mesenteric arteries were isolated and studied in a myograph or snap frozen and stored at \(-80^\circ\)C for analysis of ADMA or protein.

**EDRF/NO, EDHF, and EDCF Responses of Mesenteric Resistance Arterioles**

The mesenteric vessels were isolated, mounted in a Mulvany-Halpern myograph (JP Trading, Science Park), studied as described in detail previously,\(^13\) described in the online Data Supplement, and summarized in Figure S1 (see online Data Supplement).

**Fluorescence Detection of Ach-Induced NO and ROS in Mesenteric Resistance Arterioles**

These followed methods\(^13\) described in details in the online Data Supplement.

**Protein Expression Studies**

The protein expression in lysates of isolated mesenteric resistance arterioles was studied as described previously.\(^13\)

**Table 1.** Effects of Ang II Infusion Alone or With Tempol on Mean Arterial Pressure and Plasma Malondialdehyde, L-Arginine, ADMA, and SDMA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Ang II</th>
<th>Tempol</th>
<th>Tempol+Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Arterial Pressure, mm Hg</td>
<td>89±7</td>
<td>119±5†</td>
<td>90.0±3</td>
<td>98.2±6</td>
</tr>
<tr>
<td>Malondialdehyde, µmol · L (^{-1})</td>
<td>0.37±0.05</td>
<td>0.57±0.02*</td>
<td>0.46±0.05</td>
<td>0.45±0.05</td>
</tr>
<tr>
<td>L-Arginine, µmol · L (^{-1})</td>
<td>173±13</td>
<td>179±55</td>
<td>137±48</td>
<td>141±66</td>
</tr>
<tr>
<td>ADMA, nmol · L (^{-1})</td>
<td>510±46</td>
<td>594±127</td>
<td>610±46</td>
<td>700±20</td>
</tr>
<tr>
<td>SDMA, nmol · L (^{-1})</td>
<td>280±30</td>
<td>309±31</td>
<td>300±12</td>
<td>300±9</td>
</tr>
</tbody>
</table>

Data show mean±SEM values (n=6 per group).
*Effect of Ang II is P<0.05.
†Effect of Ang II is P<0.005.

**Plasma Malondialdehyde**

Plasma malondialdehyde was measured by high-performance capillary electrophoresis-micellar electrokinetic chromatography (see the online Data Supplement).

**l-Arginine, Asymmetrical, and Symmetrical Dimethylarginine Concentrations in Plasma and Tissue**

For details, see the online Data Supplement.

**Statistics**

Values are expressed as mean±SEM. The concentration-response curves to Ach were analyzed using nonlinear regression of sigmoidal concentration-response curves (GraphPad Prism), which were used to calculate the EC\(_{50}\). Other values were analyzed by ANOVA followed by a post hoc test for multiple comparisons (GraphPad Prism). A value of P<0.05 was considered statistically significant.

**Results**

Mean arterial pressure under anesthesia and plasma malondialdehyde were increased by Ang II, but this was prevented in rats receiving Tempol. There were no changes in plasma arginine, ADMA, or symmetrical dimethylarginine (SDMA; Table 1).

Figure S1 depicts how endothelium-dependent responses to Ach were assessed (see Figure S1). Responses to Ach are shown in the Figure and EC\(_{50}\) and maximal values in Table 2. Compared with sham-infused rats, those infused with Ang II had a 57% reduction in maximal relaxation with Ach and a 67% reduction in EDRF but a more modest 34% reduction in EDCF. Ang II enhanced EDCF responses by 4-fold. Ang II infusion also reduced the sensitivity of the Ach and EDCF responses but increased the sensitivity of the EDCF responses and did not change the sensitivity of EDHF responses. There were no significant effects of Ang II infusion on these responses in rats administered Tempol (Table 2).

Further data for mesenteric resistance arterioles are shown in Table 3. Ach-induced DAFFM (4-amino-5-methylamino-2,7'-difluorescein) fluorescence was reduced by 41% in vessels from Ang-infused rats, whereas Ach-induced Tempol-9AC-ROS fluorescence was increased 5-fold (Table 3). Both of these changes were prevented in rats given Tempol. Tissue concentrations of l-arginine and SDMA in mesenteric vessels were unaffected by Ang II infusion, but ADMA was increased significantly by 73%. This was prevented by Tempol, which led to a significant increase in tissue l-arginine in rats.
infused with Ang II. The expression of DDAH-2 protein was reduced by Ang II infusion but was prevented in rats given Tempol.

We evaluated whether a reduced response to NO, H₂O₂, or EETs could account for the reduced EDRF or EDHF responses of vessels from Ang-infused rats. The maximal relaxation to the NO donor, sodium nitroprusside, in vessels from sham-infused rats (10⁻⁴ M; 97 ± 8%) was not significantly different (94 ± 6%) in vessels from Ang II–infused rats. H₂O₂ elicited a modest relaxation of 9 ± 3% at 10⁻⁵ mol/L and a marked relaxation at 10⁻⁴ mol/L. However, these responses were similar in vessels from sham- and Ang-infused rats (Figure S2A). EETs did not elicit a significant relaxation even at 10⁻⁵ mol/L (Figure S2B). Therefore, differences in responsiveness to NO, H₂O₂, or EETs could not explain the reduced relaxation responses from Ang-infused rats.

To assess the role of vasodilator prostaglandins in EDRF responses, vessels from sham-infused rats were incubated with 5 × 10⁻⁵ mol/L of indomethacin (to block COX-1 and -2). Relaxation responses to 10⁻⁴ M Ach after vehicle

![Graph A](imageA.png)

**Figure.** Mean ± SEM values for relaxation responses to Ach in norepinephrine-preconstricted mesenteric arterioles (A) and calculated EDRF (B), EDHF (C), and EDCF responses in vessels under spontaneous tone (D). Comparing sham- and Ang-infused responses: *P < 0.05 and **P < 0.01. There were no significant differences between sham- and Ang-infused responses in rats given Tempol. B indicates before.

Table 2. Maximal Relaxation Responses and EC₅₀ Values of Vessels to Ach of Mesenteric Arteries From Vehicle, Ang II, Tempol, and Tempol With Ang II Infusion Rats

<table>
<thead>
<tr>
<th>Response</th>
<th>Maximum, %</th>
<th>Maximum, %</th>
<th>P</th>
<th>Log EC₅₀, mol/L</th>
<th>Log EC₅₀, mol/L</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td>83.9 ± 7.9*</td>
<td>36.0 ± 7.0‡</td>
<td>&lt; 0.01</td>
<td>–6.17 ± 0.06*</td>
<td>–5.47 ± 0.04‡</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>EDRF</td>
<td>54.4 ± 4.6*</td>
<td>18.0 ± 4.7‡</td>
<td>&lt; 0.01</td>
<td>–6.55 ± 0.06*</td>
<td>–5.78 ± 0.05‡</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>EDHF</td>
<td>29.4 ± 2.7*</td>
<td>18.7 ± 2.7‡</td>
<td>&lt; 0.05</td>
<td>–5.18 ± 0.07*</td>
<td>–5.46 ± 0.16‡</td>
<td>NS</td>
</tr>
<tr>
<td>EDCF</td>
<td>5.2 ± 4.6*</td>
<td>23.0 ± 5.6‡</td>
<td>&lt; 0.05</td>
<td>–4.11 ± 0.35*</td>
<td>–4.49 ± 0.03‡</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Ach</td>
<td>88.9 ± 7.9†</td>
<td>69.9 ± 8.9§</td>
<td>NS</td>
<td>–6.21 ± 0.05†</td>
<td>–6.16 ± 0.08§</td>
<td>NS</td>
</tr>
<tr>
<td>EDRF</td>
<td>53.4 ± 5.7†</td>
<td>41.4 ± 4.7§</td>
<td>NS</td>
<td>–6.76 ± 0.09†</td>
<td>–6.67 ± 0.01§</td>
<td>NS</td>
</tr>
<tr>
<td>EDHF</td>
<td>35.4 ± 3.7†</td>
<td>27.4 ± 4.7§</td>
<td>NS</td>
<td>–5.38 ± 0.15†</td>
<td>–5.15 ± 0.14§</td>
<td>NS</td>
</tr>
<tr>
<td>EDCF</td>
<td>5.1 ± 6.2†</td>
<td>10.4 ± 4.6§</td>
<td>NS</td>
<td>–4.16 ± 0.13†</td>
<td>–4.44 ± 0.07§</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data were obtained from experiments described in Figure 1. NS indicates not significant.

*Data are for vehicle.
†Data are for Tempol.
‡Data are for Ang II.
§Data are for Tempol-Ang II.
(84±8%) were unchanged after inhibition of COX with 5×10⁻⁵ mol·L⁻¹ of indomethacin (79±6%) but were abolished by endothelium removal (1±3%). Therefore, indomethacin was not used in subsequent relaxation studies.

The mediation of EDHF was studied in vessels from sham-infused rats with the use of specific inhibitors (Table 4). EDHF response to 10⁻⁴ M Ach with vehicle was 29±3%. This was not significantly blunted by catalase to metabolize H₂O₂ or by 14,15-epoxyeicosa-5-(Z)-enoic acid to block EETs in concentrations shown to be effective in isolated vessels. This was not significantly blunted by catalase to metabolize H₂O₂ or by 14,15-epoxyeicosa-5-(Z)-enoic acid to block EETs.

The EDCF response of vessels from Ang-infused rats (Table 5) was not reduced significantly by incubation with parecoxib to block COX-2 or with OKY-046 to block thromboxane synthase. However, the response was inhibited similarly by SC-560 to block COX-1 or SQ-29 548 to block TP-Rs and was abolished by endothelium removal. Thus, the EDHF responses depended on the endothelium but were not mediated significantly by H₂O₂ or EETs.

Concentrations of L-arginine, ADMA, and SDMA in homogenates of aorta, kidney, and liver are shown in Table S1. Tissue levels of L-arginine and SDMA were unaffected by Ang II infusion, but levels of ADMA in all 3 of the tissues were increased. These effects were prevented in rats given Tempol.

### Discussion

The main new findings are that a prolonged slow pressor infusion of Ang II into rats changed endothelial function of mesenteric resistance vessels by diminishing vasodilator and increasing vasoconstrictor pathways. This was accompanied by a reduced endothelial release of microvascular NO and increased ROS and ADMA. Ang infusion downregulated the vascular expression of DDAH-2. These effects of Ang II were prevented by 2 weeks of oral Tempol administration. Plasma ADMA did not predict vascular, renal, or hepatic tissue levels of ADMA.

We confirmed previous findings that oral Tempol prevented the increased blood pressure with a slow pressor infusion of Ang II. Vascular ROS with Ang II are derived from NADPH oxidase activity and a reduction in extracellular superoxide dismutase expression. Tempol prevented superoxide anion generation by Ang II in VSMCs and inhibited lipid peroxidation and microvascular ROS in response to Ang II in the present study. We selected a slow pressor rate of Ang II infusion in this study because high rates of Ang II infusion caused renal damage that reduced DDAH activity.

A reduction in EDRF in microvessels of rabbits infused with Ang II was improved by bath addition of Tempol. A reduced EDRF has been related to bioinactivation of NO by ROS. This is consistent with our findings that Tempol administration prevented the reduction in microvascular EDRF and NO and the increase in ROS in rats infused with Ang II.

The maximal EDHF response was also reduced by prolonged Ang II infusion, but the effect was smaller than on EDRF, and the EC₅₀ response was not changed. Mediation of EDHF varies

### Table 3. Effects of Ang II Infusion Alone or During Tempol Administration on Mesenteric Resistance Arterioles

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Ang II</th>
<th>Tempol</th>
<th>Tempol + Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO activity with Ach, F/Fo DAF</td>
<td>1.6±0.2</td>
<td>1.0±0.1†</td>
<td>1.7±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>ROS activity with Ach, F/Fo tempo 9AC</td>
<td>0.15±0.03</td>
<td>0.82±0.05‡</td>
<td>0.22±0.05</td>
<td>0.42±0.11</td>
</tr>
<tr>
<td>L-Arginine, nmol·mg of protein⁻¹</td>
<td>71±25</td>
<td>95±23</td>
<td>33±4</td>
<td>57±8*</td>
</tr>
<tr>
<td>ADMA, pmol·mg protein⁻¹</td>
<td>260±35</td>
<td>450±50†</td>
<td>228±36</td>
<td>312±36</td>
</tr>
<tr>
<td>SDMA, pmol·mg protein⁻¹</td>
<td>80±50</td>
<td>127±76</td>
<td>56±9</td>
<td>103±28</td>
</tr>
<tr>
<td>DDAH-2 protein, relative to β-actin</td>
<td>1.4±0.2</td>
<td>0.7±0.1‡</td>
<td>1.2±0.3</td>
<td>1.1±0.2</td>
</tr>
</tbody>
</table>

Data show mean±SEM values (n=6 per group) for mesenteric resistance arterioles.

*Effect of Ang II is P<0.05.
†Effect of Ang II is P<0.01.
‡Effect of Ang II is P<0.005.

F/Fo indicates fluorescence relative to basal; DAF, 4-amino-5-methylamino-2',7'-difluorofluorescein.

### Table 4. Maximal Endothelium-Dependent Hyperpolarization Responses in Mesenteric Resistance Arterioles From Sham-Infused Rats

<table>
<thead>
<tr>
<th>Added to the Bath</th>
<th>Relaxation, %</th>
<th>P vs Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>29±3</td>
<td></td>
</tr>
<tr>
<td>Catalase, 125 U·ml⁻¹</td>
<td>26±6</td>
<td>NS</td>
</tr>
<tr>
<td>14,15-epoxyeicosa-5-(Z)-enoic acid, 10⁻⁶ M</td>
<td>32±5</td>
<td>NS</td>
</tr>
<tr>
<td>Endothelium removal</td>
<td>1±5</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>

Data show mean±SEM values (n=6 per group). All of the vessels were preconstricted with 10⁻⁵ mol·L⁻¹ of norepinephrine, pretreated with 10⁻⁴ mol·L⁻¹ of L-nitroarginine to inhibit NOS and relaxed with 10⁻⁴ mol·L⁻¹ of Ach. NS indicates not significant.

### Table 5. Maximum Endothelium-Dependent Contraction Factor Responses in Mesenteric Resistance Arterioles From Ang II-Infused Rats

<table>
<thead>
<tr>
<th>Added to Bath</th>
<th>Contraction, %</th>
<th>P vs Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>23±5</td>
<td></td>
</tr>
<tr>
<td>SC-560, 10⁻⁷ M</td>
<td>7±5</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Parecoxib, 10⁻⁵ M</td>
<td>19±4</td>
<td>NS</td>
</tr>
<tr>
<td>OKY-046 NA, 10⁻⁵ M</td>
<td>13±3</td>
<td>NS</td>
</tr>
<tr>
<td>SQ-29,584, 10⁻⁵ M</td>
<td>6±4</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Endothelium removal</td>
<td>1±4</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>

Data show mean±SEM values (n=6 per group). Vessels were studied under spontaneous tone. They were pretreated with 10⁻⁶ mol·L⁻¹ of L-nitroarginine, 10⁻⁶ mol·L⁻¹ of apamin and 10⁻⁴ mol·L⁻¹ of charybdotoxin to block relaxation responses and constricted with 10⁻⁴ mol·L⁻¹ of Ach. NS indicates not significant.
by vessel and species but can include \( \text{H}_2\text{O}_2 \) and EETs. However, the impaired EDHF responses in this study in mesenteric arterioles of rats were independent of \( \text{H}_2\text{O}_2 \) or EET generation or action. Thus, neither \( \text{H}_2\text{O}_2 \) nor EETs caused significant vasorelaxation except at very high \( \text{H}_2\text{O}_2 \) concentrations, and the addition of catalase to metabolize \( \text{H}_2\text{O}_2 \) or the EET antagonist, 14,15- epoxyeicosa-5-(Z)-enoic acid, to the bath did not alter EDHF responses. The mediator of EDHF was not identified in this study.

EDHF is upregulated in blood vessels of endothelial NOS knockout mice. We confirm that Ang II–infused rats have impaired EDRF responses in mesenteric arterioles. Kang et al reported that Ang II reduced the sensitivity of mesenteric vessels to Ach, as in the present study, but reduced the maximal response only after blockade of NOS. They proposed that NOS was upregulated in Ang II–infused rats to compensate for a defect in \( K^+ \)-channel–mediated relaxation. However, in our study, the maximal EDRF response and the Ach-induced NO activity were reduced in vessels from Ang II–infused rats. The difference may relate to the lower rate of Ang II infusion.

An endothelium-dependent contraction is apparent in many models of hypertension. EDCF responses entailed an endothelial COX-dependent production of a factor under the influence of ROS that activates TP-Rs on VSMCs. The contractile responses of vessels of Ang II–infused rats in the present study were prevented by blockade of COX-1, TP-Rs, or endothelium removal, but blockade of TXA\(_2\) (thromboxane A\(_2\)) synthase had no significant effect. The present study did not identify the COX-1 product responsible, but prostaglandin endoperoxides have been implicated. The vascular signaling of Ang II contractile response via TP-Rs in this study may underlie the finding that TP-R knockout mice have an impaired pressor and renal vasoconstrictor response to infused Ang II.

The kidney and the liver clear plasma ADMA, and the liver clears L-arginine. Avid uptake by these organs may account for the many-fold higher arginine concentrations in the liver and ADMA concentrations in the kidney, relative to the other organs sampled. DDAH-2 is the principal isoform expressed in the rat mesenteric resistance arterioles and DDAH-1 in the renal proximal tubules. The downregulation by Ang II of DDAH-2 in cultured endothelial cells and in mesenteric resistance arterioles in this study and of DDAH-1 in the kidney cortex in a previous study could, therefore, have reduced the metabolism of ADMA in these organs and contributed to the increased tissue levels of ADMA. Indeed, Tempol prevented the Ang-induced reduction in DDAH-2 expression in the mesenteric vessels, which could have contributed to the prevention of an Ang II–induced increase in vascular ADMA in Ang-infused rats receiving Tempol. DDAH activity was not assessed in these studies. However, Tain and Baylis reported that directly increasing ROS in the kidney cortex decreased DDAH activity. Because Tempol prevented the Ang II–induced increased plasma malondialdehyde and vascular ROS activity, this relates the decreased tissue DDAH-2 expression and increased tissue ADMA to oxidative stress. However, tissue ADMA is also increased by upregulation of protein arginine methyltransferases and by downregulation of cationic amino acid transporters, which could have contributed to the increased tissue ADMA in this study. We reported recently that incubation of VSMCs with Ang II increased NADPH oxidase and reduced DDAH and cationic amino acid transporter activity and increased cellular, but not medium, levels of ADMA. The increase in cellular ADMA was prevented by Tempol or blockade of Ang type 1 receptors. Further studies in VSMCs transfected with \( p2^{\text{NOS}} \) to increase NADPH oxidase directly demonstrated reduced DDAH-1 and -2 expression and activity, increased protein arginine methyltransferase 3 expression and activity, decreased cationic amino acid transporter 2A expression and activity, and increased cellular ADMA concentrations. Thus, ROS can direct cellular metabolic pathways to increase cellular ADMA concentrations, but a reduction in cationic amino acid transporter activity may restrain ADMA export and limit accumulation of ADMA in the extracellular fluid or plasma.

The 50% reduction in DDAH-2 expression in mesenteric vessels with Ang II in this study was similar to that produced by in vivo gene silencing, which impaired EDRF responses and NO bioactivity in mesenteric arterioles substantially. Moreover, Torondel et al reported that overexpression of DDAH-1 or -2 in vascular endothelial cells reduced ADMA concentrations, enhanced in vitro vascular NO production, and conferred resistance to administration of ADMA in DDAH heterozygote mice. The 70% increase in ADMA and 33% reduction in the arginine:ADMA ratio in mesenteric vessels of Ang-infused rats should contribute to the increased tissue ADMA, because this would reduce the substrate:inhibitor ratio for NOS. Ang II increased the mesenteric vascular arginine levels in rats given Tempol, which also could have contributed to improved EDRF responses in Tempol-treated rats, but the reason for this was unclear. Moreover, the increase in superoxide in the blood vessels during Ang II infusion should have enhanced NO bioinactivation. The finding that the Ang II–induced functional defects, reduced NO and DDAH-2 expressions, and increased tissue levels of ADMA and ROS were all prevented by Tempol implies that an increase in ROS was an upstream event that impaired endothelial responses by coordinating these pathways for endothelial dysfunction.

Perspectives
Infusions of Ang II have variable effects on plasma ADMA. Thus, prolonged Ang II infusions into mice doubled plasma ADMA in one study, whereas in others Ang II did not change plasma ADMA, as in the present study, except at high rates of Ang II infusion that caused renal damage and reduced renal DDAH expression. We find that tissue ADMA can increase during a slow pressor Ang II infusion despite unchanged plasma levels.

Endothelial dysfunction or plasma markers of ROS or ADMA predict future cardiovascular events or death in high-risk patients. The present study shows that tissue ADMA may not always be reflected by plasma levels but that plasma malondialdehyde was a valid predictor of vascular ROS. A recent review concluded that the mechanisms that regulate the balances between NO and EDCF and the processes that transform the endothelium from a protective organ to a source of vasoconstriction and proaggregatory and promitogenic responses in human hypertension are important but remain to be determined. The present findings that...
Tempol given to Ang II–infused rats prevented the defective endothelial relaxation responses and NO bioactivity and the enhanced ROS, endothelial contractions, and ADMA suggest that vascular ROS is an important component of this transformation of endothelial function. Thus, maneuvers to reduce ROS could have therapeutic potential.\(^\text{11}\)

**Acknowledgments**

We thank Sigrid de Jong for expert technical assistance and Emily Wing Kam Chan for preparing and editing the article.

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**Disclosures**

None.

**References**


20. Wang et al. Endothelium, ADMA, and ROS.
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IMPAIRED ENDOTHELIAL FUNCTION AND MICROVASCULAR ADMA IN ANGIOTENSIN II INFUSED RATS: EFFECTS OF TEMPOL

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Methods

EDRF/NO, EDHF and EDCF responses of mesenteric resistance arterioles: After equilibration, the vessels were preconstricted with $10^{-5} \text{ mol} \cdot \text{l}^{-1}$ norepinephrine and relaxed with acetylcholine (Ach: $10^{-4}$ to $10^{-3} \text{ mol} \cdot \text{l}^{-1}$). Thereafter, preconstricted vessels were relaxed with the endothelium-independent agent, sodium nitroprusside ($10^{-8}$ to $10^{-3} \text{ mol} \cdot \text{l}^{-1}$). The EDRF/NO response was taken as the relaxation induced by $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ Ach (maximally effective dose) in physiological salt solution (vehicle) minus the same response in the presence of $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ N$^\text{G}$-nitro-l-arginine (L-NA) to inhibit nitric oxide synthase. The EDHF response was taken as the inhibition of relaxation by $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ Ach and blockade of calcium-activated potassium channels with $10^{-6} \text{ mol} \cdot \text{l}^{-1}$ apamin plus $10^{-5} \text{ mol} \cdot \text{l}^{-1}$ charybdotoxin in the presence of $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ L-NA to inhibit EDRF/NO. This combination reduced relaxation by $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ Ach to $2\pm6\%$ which was similar to the removal of the endothelium. The EDCF response was calculated as the percentage of a standard contraction with norepinephrine ($10^{-5} \text{ mol} \cdot \text{l}^{-1}$) and KCl (40 mmol • l$^{-1}$) (NAK), which was established at the beginning of the study. The EDCF responses were undertaken without preconstriction in vessels incubated with L-NA, apamin and charybdotoxin to block vasodilator pathways during addition of Ach ($10^{-8}$ to $10^{-4} \text{ mol} \cdot \text{l}^{-1}$) to the bath. Preliminary studies in each series established that endothelial denudation abolished Ach-induced relaxation and contraction. Other preliminary studies detected no effect of blockade of COX with $5\times10^{-5} \text{ mol} \cdot \text{l}^{-1}$ indomethacin on Ach-induced relaxation.

Mediation of EDHF and EDCF: Further studies investigated the mediation of EDHF responses. To assess whether differences in response to H$_2$O$_2$ could account for changes in EDRF, preconstricted vessels from sham- and Ang II-infused rats were relaxed with graded concentrations of H$_2$O$_2$ ($10^{-7}$ to $10^{-4} \text{ mol} \cdot \text{l}^{-1}$). EDHF responses of other vessels from sham rats were repeated in the presence of pegylated (PEG) catalase (125 units • ml$^{-1}$). To assess the role of EETs, preconstricted vessels from sham and Ang II-infused rats were relaxed with ($Z$)-15-(pentyloxy)pentadec-5-enoic acid (JQ-II-30-20, a stable EET analog) ($10^{-8}$ to $10^{-5} \text{ mol} \cdot \text{l}^{-1}$). Other vessels were studied after bath addition of 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE; $10^{-5} \text{ mol} \cdot \text{l}^{-1}$) to block EETs and the EDHF response repeated 1.

To assess the role of COX products in EDCF response, vessels from Ang II-infused rats were incubated with SC-560 ($10^{-7} \text{ mol} \cdot \text{l}^{-1}$) or N-[[4-[(5-methyl-3-phenylisoxazol 4-yl)phenyl]sulfonfonyl]propanamid (parecoxib, $10^{-6} \text{ mol} \cdot \text{l}^{-1}$) to block COX-1 or -2 2 or with [1S-[1$\alpha$,2$\alpha$(Z),3$\alpha$,4$\alpha$]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ-29,548, $10^{-5} \text{ mol} \cdot \text{l}^{-1}$; Bristol Myers Squib, Inc, New Jersey) to block TP-Rs or with ozagrel hydrochloride hydrate (OKY-046, $10^{-5} \text{ mol} \cdot \text{l}^{-1}$) to block thromboxane A$_2$ synthase 2 for 30 minutes prior to testing. These concentrations produced maximal effects on isolated vessels 2.

Fluorescence detection of acetylcholine–induced nitric oxide and ROS in mesenteric resistance arterioles: To assess NO activity, vessels were loaded with 4-amino-5-methoxyamino-2’,7’-difluorofluorescein diacetate (DAFFM-DA; Invitrogen, Carlsbad, CA) for 30 min, and rinsed three times 3,4. DAFFM-DA is taken into cells, trapped by de-esterification and covalently modified by NO to a fluorescent triazolo-fluorescin analog 3. Thirty minutes after loading with excitation at 495 nm, and emissions isolated at 515 nm, the vessels were precontracted with $10^{-5} \text{ mol} \cdot \text{l}^{-1}$ norepinephrine and relaxed with $10^{-4} \text{ mol} \cdot \text{l}^{-1}$ Ach. The change
in fluorescence (ΔF/F₀) was detected by an inverted microscope (Olympus 1x71, Tokyo, Japan) equipped with a 20x objective and an emission photon detection fluorescence system (Ratio Master, Photon Technology International; Lawrenceville, NY) and measured with a photon-counting assembly (D104B; Photon Technology International). Responses were confirmed (Cayman Chemicals, Ann Arber, MI) by showing that 90% of the response to Ach was prevented by 10⁻⁵ mol · L⁻¹ L-NA without affecting the response to the NO donor compound 5×10⁻⁵ mol · L⁻¹ 2-(N,N-diethylamino) diazenolate-2-oxide sodium salt (NONOate; data not shown).

To assess ROS, the vessels were preloaded with 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin- 1-oxyl free radical (TEMPO-9-AC; 10⁻⁵ mol · L⁻¹) 5. The vessels were prepared as for EDCF studies and stimulated with 10⁻⁴ mol · L⁻¹ Ach with excitation at 360 nm and emission at 460 nm. Preliminary studies in vessels pretreated with PEG superoxide dismutase (125 units · ml⁻¹) prevented 92% of the fluorescent response to Ach in vessels from Ang II-infused rats.

**Plasma malondialdehyde:** Plasma ultrafiltrate was injected (127 nanoliter under 1 psi for 10 seconds) into an uncoated fused silica capillary (75 micron ID, length to detector 40 cms, total length 50.2 cms) on a Beckman Coulter MDQ system (Fullerton, CA). The background electrolyte solution contained borate 50 mM, spermine 1 mM, and tetradecyltrimethylammonium bromide 2 mM at a pH of 9.7. UV detection was carried out at 260 nm. The MDA peak (migration time 5.2 minutes) was identified by spiking the samples. The separation was carried out at -12kV and 25°C. Intra- and inter-assay coefficients of variation in plasma were 2.1% and 4.3%, respectively.

**L-arginine, asymmetric - and symmetric - dimethylarginine concentrations in plasma and tissue:** Separate groups of rats (n=6 per group) were used for these studies. Under pentobarbital anesthesia (50 mg · kg⁻¹ ip), a carotid artery was cannulated, 1 ml of blood removed, and the plasma separated with care to avoid hemolysis. Mesenteric vessels, the aorta, the renal cortex, and the liver were removed, washed rapidly in isotonic saline, snap-frozen in liquid nitrogen and stored at -80°C prior to analysis for L-arginine, ADMA and symmetric dimethylarginine (SDMA) concentrations 6,7. The isolated aortas, mesenteric vessels, liver and renal cortex from Sprague-Dawley rats were washed three times with ice-cold PBS, transferred to a Lysing Matrix D tube (MP Biomedicals, Solon, OH) containing 1:10 volume of 0.6 mol/L ice-cold perchloric acid (Sigma, St. Louis, MO) and homogenized in a FastPrep (Thermo Electron Corporation, Waltham, MA). After homogenization, 20µl of each sample of tissue homogente was placed in a tube for protein measurement. The remainder was centrifuged for 10 minutes at 2,000 g at 4°C, the clear supernatant collected and stored at -80 °C. This avoided artefactual increases (by proteinolysis) or decreases (by DDAH) of ADMA. ADMA, SDMA and arginine are stable in the clear supernatant, which was neutralized shortly before HPLC analysis. Concentrations of L-arginine, ADMA and SDMA in plasma and supernatant were measured by HPLC with fluorescence detection as previously described 6 using modified chromatographic separation conditions 7. Supernatants obtained after deproteinization of tissue homogenate with perchloric acid were neutralized with two volumes of 0.5 M Na₂HPO₄ to one volume of the supernatant. Supernatant and plasma were spiked with monomethylarginine as an internal standard and purified by solid-phase extraction on polymeric cation-exchange columns. Thereafter, the samples were dried with nitrogen, and derivatized with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid. Analytes were separated by isocratic reversed-
phase HPLC with fluorescence detection. A human plasma pool was analyzed in all analytical series as a quality control. For all analytes the intra- and inter-assay coefficients of variation were <1.5% and <3.5%, respectively. Supernatant concentrations were normalized to protein content.

**Results**

Figure S1 depicts how endothelium-dependent responses were assessed. Panels A and B are from vessels from sham rats, and panel C from Ang II infused rats (which had a robust EDCF). The EDRF responses were the differences between acetylcholine-induced relaxations alone and during blockade of NOS with $10^{-4}$ mol · l$^{-1}$ L-NA (Panel A). The EDHF responses were the differences between the L-NA pretreated vessel alone and during apamin and charybdotoxin (Ap + Ctx; panel B). The latter almost abolished the relaxation, similar to high KCl solution (40 mmol · l$^{-1}$ KCl). Contraction responses were obtained in vessels under spontaneous tone pretreated with L-NA, Ap and Ctx to prevent confounding effects of relaxation responses. Endothelium removal (Endo-) prevented the relaxation and contraction responses.
Reference List


Supplemental Table

Table S1. Effects of angiotensin II infusion alone or during tempol administration on L-arginine, ADMA or SDMA in aorta, kidney and liver homogenates

<table>
<thead>
<tr>
<th>Condition</th>
<th>Aorta (nmol · mg protein(^{-1}))</th>
<th>Kidney (pmol · mg protein(^{-1}))</th>
<th>Liver (pmol · mg protein(^{-1}))</th>
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</thead>
<tbody>
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<td>A. L-arginine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sham</td>
<td>21 ± 5</td>
<td>90 ± 5</td>
<td>918 ± 86</td>
</tr>
<tr>
<td>Ang II</td>
<td>19 ± 2</td>
<td>112 ± 10</td>
<td>886 ± 140</td>
</tr>
<tr>
<td>Tempol</td>
<td>18 ± 2</td>
<td>63 ± 15</td>
<td>830 ± 96</td>
</tr>
<tr>
<td>Ang II+Tempol</td>
<td>17 ± 1</td>
<td>71 ± 3</td>
<td>947 ± 101</td>
</tr>
<tr>
<td>B. ADMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>69 ± 10</td>
<td>1,330 ± 220</td>
<td>200 ± 32</td>
</tr>
<tr>
<td>Ang II</td>
<td>126 ± 17*</td>
<td>2,360 ± 240†</td>
<td>416 ± 74*</td>
</tr>
<tr>
<td>Tempol</td>
<td>84 ± 8</td>
<td>1,410 ± 375</td>
<td>217 ± 11</td>
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<tr>
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<td>80 ± 8</td>
<td>1,880 ± 290</td>
<td>300 ± 43</td>
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<td>C. SDMA</td>
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<td></td>
</tr>
<tr>
<td>Sham</td>
<td>37 ± 6</td>
<td>301 ± 47</td>
<td>159 ± 21</td>
</tr>
<tr>
<td>Ang II</td>
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<td>461 ± 29</td>
<td>118 ± 17</td>
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<tr>
<td>Tempol</td>
<td>20 ± 5</td>
<td>382 ± 68</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>Ang II+Tempol</td>
<td>14 ± 3</td>
<td>546 ± 65</td>
<td>43 ± 7</td>
</tr>
</tbody>
</table>

Mean ± SEM values (n = 6 per group). Effects of Ang II: *, P<0.05; †, P<0.01.
Figure S1: Mean ± SEM values for endothelium derived relaxing factor (Panel A), endothelium derived hyperpolarizing factor (Panel B), both in vessels from normal rats, and endothelium derived contracting factor (Panel C) in vessels from angiotensin infused rats. L-NA, L-Nitro arginine; Endo-, endothelium removal; Ap, apomin; Ctx, charybdotoxin.
**Figure S2:** Relaxation responses to H$_2$O$_2$ (Figure A) or the stable epoxyeicosatrienoic acid derivative, (Z)-15-(pentyloxy)pentadec-5-enoic acid (JQ-II-30-20) (Figure B) comparing results in sham-infused rats (open circles) or rats infused with Ang II (solid circles).