Endothelial Dysfunction and Enhanced Contractility in Microvessels From Ovariectomized Rats
Roles of Oxidative Stress and Perivascular Adipose Tissue

Dan Wang, Cheng Wang, Xie Wu, Wei Zheng, Kathryn Sandberg, Hong Ji, William J. Welch, Christopher S. Wilcox

Abstract—Ovarian hormone loss increases reactive oxidative species, endothelial dysfunction, and cardiovascular disease. Because perivascular adipose tissue (PVAT) regulates endothelial function, we hypothesized that reactive oxidative species in PVAT mediate adverse microvascular effects of ovarian hormone deficiency. Rats were ovariectomized or sham operated and given vehicle or tempol for 6 weeks. Mesenteric resistance arterioles from ovariectomized compared with sham-operated rats had dysfunctional responses to acetylcholine (ACh) including decreased ACh-induced endothelium-dependent relaxation (50±6% versus 72±2%) and endothelium-dependent relaxation factor (17±4% versus 37±2%) and increased endothelium-dependent contracting factor (27±5% versus 9±3%). OVX rat mesenteric arterioles had increased contractions to the thromboxane/prostanoid receptor agonist U-46619 (58±3% versus 40±5%) and increased reactive oxidative species (tempo-9-AC fluorescence) with U-46619 (0.65±0.17 versus 0.14±0.06 Δ unit) or ACh (0.49±0.09 versus 0.09±0.05 Δ unit) and increased p22 phox protein expression (0.89±0.05 versus 0.18±0.04 Δ unit), whereas nitric oxide activity (DAF-FM [4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate] fluorescence) with ACh was reduced (0.39±0.1 versus 0.70±0.10 Δ unit). No differences were found in endothelium-dependent hyperpolarizing factor or contractile responses to phenylephrine. PVAT enhanced ACh-induced relaxation, endothelium-dependent relaxation factor, and nitric oxide only in sham-operated rats. Tempol prevented ovariectomy-induced endothelial dysfunction and restored the enhancing effects of PVAT on ACh-induced relaxation, endothelium-dependent relaxation factor, and nitric oxide in ovariectomized rat vessels, but both tempol and PVAT were required to normalize the enhanced U-46619 contractions after ovariectomy. In conclusion, ovariectomy redirects endothelial responses from relaxation to contraction by reducing vascular nitric oxide, augmenting thromboxane/prostanoid receptor signaling, and attenuating the vasodilatory effects of PVAT, all of which were dependent on reactive oxidative species. (Hypertension. 2014;63:00-00.)

Key Words: menopause ■ nitric oxide ■ ovariectomy ■ reactive oxygen species ■ tempol ■ thromboxane prostanoid receptors

Ovarian hormone loss in women is associated with increased vascular reactive oxygen species (ROS), reduced endothelial nitric oxide (NO) synthase expression and NO, increased endothelial dysfunction,1,2 and risk of cardiovascular disease (CVD).3 Similar findings have been reported in ovariecetomized animal models.4,5 Endothelial dysfunction entails both impaired relaxation and an endothelium-dependent contracting factor (EDCF) generated by ROS. The EDCF is a prostaglandin or thromboxane product of cyclooxygenase and thromboxane A2, synthase that vasoconstricts vascular smooth muscle cells (VSMCs) by activating thromboxane/prostanoid receptors (TP-Rs).5 Ovariectomy leads to a cyclooxygenase-dependent EDCF response in rat mesenteric arteries6 and in pig coronary arteries.7 Furthermore, endothelial dysfunction in cutaneous blood vessels of postmenopausal women was mediated by cyclooxygenase-2.8 However, the effects of ovariectomy on ROS and TP-R signaling in microvessels remain poorly understood.

Obesity is an independent CVD risk factor6 whose incidence is increased in women with ovarian hormone loss9 or animal models of ovarian hormone deficiency.10 Perivascular adipose tissue (PVAT) improves vascular function12 through generation of NO,13 hydrogen peroxide,12 hydrogen sulphide,14 and poorly characterized adipokine pathways.15 ROS inhibit these protective effects of PVAT in hypertensive models11 and contribute to endothelial dysfunction in animal models15 and in patients with obesity and the metabolic syndrome.16 We tested the hypothesis that ovariectomy impairs PVAT-induced microvascular NO-dependent relaxation and enhances endothelium-dependent contractions through ROS and enhanced TP-R signaling. We reduced ROS using tempol which is a redox cycling nitroxide18 and activated TP-Rs with...
the stable agonist U-46619. These experiments are clinically significant because restoration of endothelial function and NO and abrogation of EDCF and enhanced TP-R signaling could attenuate the enhanced CVD risk after ovarian hormone deficiency, but it is unclear whether the microvessels and the surrounding PVAT should be the preferred therapeutic target.

Methods

Animal Preparation and Protocols
Female Sprague-Dawley rats (200–220 g; Taconics Lab, Germantown, NY) maintained on tap water and standard chow (Nar 0.3 g · 100 g–1) under conditions approved by the Institutional Animal Care and Use Committee of Georgetown University underwent ovariectomy or sham surgery (SHAM) and received oral temporal (2 mmol·L–1) or vehicle in the drinking water for 6 weeks. After euthanasia by exsanguination, the second branch mesenteric resistance arterioles were isolated with the surrounding PVAT intact (PVAT+) or removed (PVAT−) and mounted in a 4-chamber myograph or snap frozen and stored at −80°C for analysis of p22phox protein.

Vascular ACh-Induced Endothelial Responses, NO, ROS Activities, and Contractions

The media and lumen cross-sectional areas of mesenteric arterioles were measured. For the first series, mesenteric arterioles were pre-constricted with 10–4 mol·L–1 norepinephrine to study: ACh-induced relaxation (endothelium-dependent relaxation [EDR]), endothelium-dependent relaxation factor (EDRF; change in EDR after 10–4 mol·L–1 L-Nitroarginine methyl ester), endothelium-dependent hyperpolarizing factor (EDHF; change in EDRF after 10–4 mol·L–1 apamin and 10–5 mol·L–1 charadotoxin to block small and medium conductance calcium-activated potassium channels), and endothelium-independent responses (relaxation to sodium nitroprusside). To study ACh-induced NO, arterioles were loaded with 5×10–4 mol·L–1 DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate). The change of fluorescence (ΔF/Fo) with 10–5 mol·L–1 ACh quantified vascular NO.

Expression of p22phox

The protein expression of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase regulatory subunit p22phox in lysates of mesenteric arterioles was measured by Western blot and normalized to β-actin.

Statistics

Data are expressed as mean±SEM calculated from 6 rats per group. The concentration–response relationships were analyzed by 2-way repeated measures ANOVA to assess the effects of ovariectomy, PVAT, and the interaction (effects of PVAT on responses to ovariectomy) followed, where appropriate, by Bonferroni tests. Separate analyses were undertaken in the groups given tempol. Significance was defined as P<0.05.

Results

Body Weight, Vascular Structure, and p22phox Protein Expression

Ovariectomy increased body weight by 16%, whereas tempol reduced it by a similar degree (Table 1). Ovariectomy almost doubled the mesenteric arteriole media area without changing the lumen area, resulting in a doubling of the ratio of media to lumen. The p22phox protein expression was increased by 400% in ovariectomized rat vessels (Figure 1). These effects of ovariectomy were prevented by tempol.

Effects of Ovariectomy and PVAT on Endothelial Relaxation and Generation of NO

The maximum ACh-induced relaxation and EDRF responses in preconstricted mesenteric arterioles from ovariectomized rats were reduced by 30±3% and 53±3% (P<0.05), respectively (Figure 2A and 2B), but the EDHF and endothelium-independent responses were maintained (Table 2). Vascular NO activity with ACh was reduced in mesenteric arterioles from ovariectomized rats by ≥2-fold (P<0.001; Table 2; Figure 3A).

The presence of PVAT around mesenteric arterioles from SHAM rats increased their ACh-induced relaxation and EDRF response by 20±4% and 29±3%, respectively (P<0.05; Figure 2A and 2B), and increased their vascular NO activity with ACh by 41±4% (P<0.001; Figure 3A) without changing their EDHF or endothelium-independent responses (Table 2). However, these effects were lost in ovariectomized rat vessels.

EDCF contractions were increased by 200% in vessels from ovariectomized rats (Figure 3C), accompanied by a 500% (P<0.001) increase in vascular ROS activity (Table 2; Figure 3C). PVAT reduced the EDCF responses in ovariectomized rats (P<0.01) without reducing the associated vascular ROS generation.

Contractions with phenylephrine were unchanged in mesenteric arterioles from ovariectomized rats, but contractions with U-46619 were increased by 45% (P<0.05; Table 2; Figure 4B), accompanied by a 350% increase in vascular ROS (P<0.001; Figure 3E). PVAT moderated the contractions with U-46619 in both SHAM and ovariectomized rat vessels and moderated the accompanying ROS generation in vessels from ovariectomized rats. Nevertheless, both the vascular contractions and the ROS generation with U-46619 in ovariectomized rat vessels with PVAT remained >3-fold higher than in vessels from SHAM rats, despite the moderating effects of PVAT (Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>SHAM</th>
<th>OVX</th>
<th>SHAM+Tempol</th>
<th>OVX+Tempol</th>
<th>By ANOVA, Effect of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRA area (M), μm²</td>
<td>48±5</td>
<td>91±7†</td>
<td>51±5</td>
<td>48±6</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>MRA lumen area (L), μm²</td>
<td>187±12</td>
<td>170±6</td>
<td>178±14</td>
<td>193±5</td>
<td>NS</td>
</tr>
<tr>
<td>MRA M/L ratio (cross-section area), μm²/μm²</td>
<td>1.54±0.29</td>
<td>3.43±0.38*</td>
<td>1.53±0.24</td>
<td>1.63±0.19</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>MRA p22phox expression (relative to β-actin)</td>
<td>0.18±0.04</td>
<td>0.89±0.05†</td>
<td>0.28±0.11</td>
<td>0.26±0.14</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Mean±SEM value (n=6 per group). Compared with SHAM: *P<0.05; †P<0.01. MRA indicates mesenteric arteriole; NS, nonsignificant; and OVX, ovariectomized.
Effects of Tempol on Endothelial Function, Contractility, and Generation of NO and ROS

Tempol did not affect the vascular responses or NO generation of vessels from SHAM rats. However, it improved these responses in ovariectomized rat vessels and prevented the enhanced EDCF responses (Table 2; Figure 2F) and ROS generation (Table 2; Figure 3D). However, although tempol prevented the enhanced vascular ROS generation with U-46619 in ovariec-tomized rat vessels without PV AT, the enhanced contractions to U-46619 persisted. Tempol administered to ovariectomized rats without PVAT restored endothelium-dependent relaxations and EDRF and attenuated EDCF responses (Table 2; Figure 2D–2F). However, the enhanced contractions to U-46619 were not significantly moderated by tempol (Figure 4D), despite prevention of the enhanced generation of ROS. This indicated that there was a ROS-independent vascular effect of ovariectomy to enhance U-46619 contractions.

PVAT reduced the contractions to U-46619 in ovariectomized rat vessels by 40% (Table 2; Figure 4B), but after tempol, PVAT reduced the contractions by 70% (Table 2; Figure 4D). The U-46619 contractions in vessels from ovariec-tomized rats given tempol was 17±4% with PVAT which was similar to the value of 10±2% in vessels with PVAT from SHAM rats (P=NS). Thus tempol+PVAT normalized the enhanced contraction to U-46619 in vessels from ovariectomized rats. Again, the effects of PVAT to moderate U-46619 contraction after tempol occurred independent of ROS which was effectively suppressed by tempol in vessels without PVAT. Thus, neither PVAT nor tempol alone was sufficient to prevent enhanced contractions to U-46619, but the contractions were normalized by their combination. This suggests that ovariectomy enhanced contractions to U-46619 by independent effects of enhanced ROS and diminished blunting by PVAT. Thus, a primary effect of tempol on ovariectomized rat vessels was to restore normal signaling from PVAT which blunted the response to U-46619 in the adjacent blood vessels.

Discussion

This study confirms previous findings that ovariectomy or ovarian hormone deficiency lead to endothelial dysfunction and enhanced vascular oxidative stress. The main new findings are that defects in EDR in ovariectomized rat vessels were specific for EDRF because they did not affect...

Figure 1. Effect of ovariectomy and tempol on p22phox protein expression. Mean±SEM values normalized to β-actin in mesenteric arterioles from sham-operated (SHAM; open bar) or ovariectomized (OVX; hatched bar) rats after 6 weeks of vehicle or tempol treatment (n=6 per group). Top, Representative Western blot used to quantify p22phox expression. MRA indicates mesenteric arteriole.

Figure 2. Effect of perivascular adipose tissue and ovariectomy on acetylcholine (Ach)-induced relaxation, endothelium-dependent relaxation factor (EDRF), and endothelium-dependent contracting factor (EDCF) responses after 6 weeks of vehicle or tempol administration. Mean±SEM (n=6 per group) values for Ach-induced relaxation (A and D), EDRF (B and E), and EDCF (C and F) in mesenteric arterioles with perivascular adipose tissue (PVAT; solid symbol) or without PVAT (open symbol) from sham-operated (SHAM; circles) or ovariectomized (OVX; squares) rats after 6 weeks of vehicle (A–C) or tempol (D–F) treatment. Comparing PVAT+ vs – in same treatment groups (*P<0.05; **P<0.01). Comparing SHAM vs OVX in same treatment groups (†P<0.01; †††P<0.005). NS indicates non significant.
EDHF and were accompanied by reduced NO generation. Ovariectomized rat vessels had an enhanced expression of p22phox, which is a critical regulatory component of NADPH oxidase. Indeed, these vessels developed enhanced ACh- and U-46619-induced ROS generation and contractions.

The main new findings related to PVAT signaling are summarized in Figure 5. The presence of PVAT surrounding the mesenteric arterioles from SHAM rats enhanced their EDRF responses and NO generation and diminished their contractions to U-46619 substantially without modifying the modest vascular ROS generation or contractions to phenylephrine. In contrast, PVAT surrounding ovariectomized rat vessels failed to enhance EDRF responses or to restore NO generation. Furthermore, PVAT was less effective in moderating the contractions to U-46619 after ovariectomy and failed to prevent the associated ROS generation.

As in prior studies, tempol did not affect relaxation or contraction responses or NO generation of SHAM vessels, implying that ROS have little effect on normal vascular function. The main new findings related to tempol in ovariectomized rat vessels are that it prevented all of the endothelial dysfunction and diminished NO generation and prevented the augmented EDCF contractions and ACh-induced ROS generation. Moreover, tempol given to ovariectomized rats restored the effects of PVAT to enhance endothelial function. In contrast, tempol failed to prevent the enhanced contractions to U-46619, despite preventing the excessive ROS generation. In fact, a combination of PVAT and tempol was required to reduce U-46619 contractions in ovariectomized rat vessels to SHAM levels.

In contrast, incubation of canine coronary arteries with bradykinin released an adipokine that reduced vascular ROS, yet inhibited vasodilation. Another apparently paradoxical effect of tempol was to reduce bradykinin-induced relaxations. There are important differences in the functions of PVAT between vessels and experimental circumstances. After ovariectomy, tempol enhanced both the intrinsic relaxation to ACh (in vessels without PVAT) and the relaxations mediated by PVAT while reducing ROS generation consistent with many other reports that tempol moderated ROS and contractility and enhanced relaxation during oxidative stress.

Of interest was the finding that ovariectomy increased weight gain similar to the menopause. However, tempol reduced the weight gain in both ovariectomized and SHAM rats. Tempol given to fat-fed mice also prevented weight gain, which was attributed to alteration in the microbiome and signaling via intestinal farnesoid X receptors.

Thus, oxidative stress in microvessels from ovariectomized rats redirected endothelial function from NO-dependent vasorelaxation to ROS-dependent vasoconstriction. About one half of these changes related to effects of ROS mediated by PVAT. The endothelial dysfunction is analogous to the effects of prolonged angiotensin II infusion in rabbits and rats, which also
enhanced \textit{p22}^{phox}\textsuperscript{+} expression. EDCF requires vascular ROS generation after endothelial activation by shear stress,\textsuperscript{28} \textit{ACH},\textsuperscript{5,19} angiotensin II,\textsuperscript{19} or endothelin-1.\textsuperscript{29} Vascular ROS generate vasoconstrictor prostaglandins and thromboxane \textit{A}_{2} from endothelial cyclooxygenase-1 or -2 and thromboxane \textit{A}_{2} synthase that activate TP-Rs on adjacent VSMCs to mediate EDCF responses.\textsuperscript{19} A second effect of vascular ROS is to enhance TP-R responsiveness of VSMCs\textsuperscript{19} by reducing the recycling of TP-Rs.\textsuperscript{30} The enhanced TP-R responsiveness of mesenteric arterioles from ovariectomized rats confirms a prior study.\textsuperscript{6}
However, an enhanced microvascular TP-R responsiveness is an incomplete explanation for the enhanced EDCF responses of mesenteric arterioles from ovariectomized rats because tempol prevented the enhanced EDCF responses without fully correcting the enhanced responses to U-46619. Apparently, an ROS in PVAT changes the release or responsiveness of an adipokine acting on TP-Rs or their signaling in adjacent VSMCs. The moderation of EDCF responses by PVAT in ovariectomized rat vessels cannot be ascribed to upregulation of counterveiling endothelial vasodilation because this was blocked by L-N\textsubscript{G}-nitroarginine methylester, apamin, and charybdotoxin.

Ovariectomized rats had a similar increase in vascular ROS generation with TP-R activation as with Ach, yet neither was prevented by PVAT alone and required coadministration of tempol. Thus, although PVAT enhanced ACh-induced NO generation in ovariectomized rats, it did not restore EDRF responses or abrogate EDCF or U-46619 contractions without first preventing ROS generation with tempol. This extends findings at the whole animal\textsuperscript{31} or cellular level\textsuperscript{32} that TP-R activation is a cause of oxidative stress rather than just its consequence.

EDRF responses of normal rodent mesenteric arterioles depend on NO but not on prostaglandins.\textsuperscript{39} The reduced EDRF responses and NO generation after ovariectomy, and their restoration by tempol, imply a ROS-dependent bioinactivation of NO or oxidation of the NO synthase cofactor, tetrahydrobiopterin.\textsuperscript{33} The maintenance of EDHF responses is consistent because EDHF does not normally depend on NO. PVAT enhanced EDRF responses and NO only in mesenteric arterioles from SHAM rats, but an enhancing effect of PVAT in ovariectomized rats was restored by tempol administration. This implies that ROS in PVAT surrounding ovariectomized rat vessels impaired the adipokine signaling that normally enhanced NO generation and EDRF responses or that ROS in blood vessels prevented the signaling effects of the adipokine. Thus, ROS in PVAT were implicated in both enhancing the EDCF responses and reducing the EDRF/NO responses in vessels from ovariectomized rats.

This study has some limitations. It was confined to mesenteric arterioles. However, their function parallels that of renal afferent arterioles and systemic vessels.\textsuperscript{5,31} We did not test the effects of ovarian hormone replacement in ovariectomized rats. However, prior studies have reported that concomitant 17β-estradiol replacement at the time of ovariectomy prevents endothelial dysfunction and enhances TP-R signaling.\textsuperscript{6} The adipokines released from PVAT were not identified. However, these studies suggest that it was not hydrogen sulfide that activates vascular ATP-dependent K+ channels and leads to hyperpolarization of VSMCs because we did not detect any effect of PVAT on EDHF responses.

**Perspective**

TP-R signaling not only mediates EDCF responses but also contributes to hypertension, vascular remodeling, renal vasoconstriction, oxidative stress, and platelet aggregation, as observed during angiotensin\textsuperscript{11} or Goldblatt renovascular hypertension.\textsuperscript{34,35} Thus, enhanced TP-R signaling may contribute to the microvascular remodeling, endothelial dysfunction, and oxidative stress observed in ovariectomized rats and also to the loss of protection from CVD observed in women with ovarian hormone deficiency. The finding that microvessels from ovariectomized rats have defects in endothelial relaxation, an EDCF response, and enhanced TP-R signaling that were mediated by adverse ROS-dependent signaling in the vessels and the surrounding PVAT suggests that full restoration of the beneficial signaling from PVAT would be a valuable therapeutic approach for reducing CVD risk in women with ovarian hormone deficiency.

**Acknowledgments**

We thank Sarah Stulnaker for preparing the article.

**Sources of Funding**

This work was supported by grants to D. Wang from the National Kidney Foundation Capital Area and the Marriott Cardiovascular Research Fellowship Award, to K. Sandberg from National Institutes of Health (AG/HL-19291, AG-039779, and AG-16902), to C.S. Wilcox and W.J. Welch from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-049870 and DK-036079) and to the National Heart, Lung, and Blood Institute (HL-68686), to C.S. Wilcox by funds from the George E. Schreiner Chair of Nephrology and the Hypertension, Kidney and Vascular Research Center, and to C. Wang by a Chinese Government Scholarship Program.

**Disclosures**

None.

**References**

Novelty and Significance

What Is New?
- Microvascular endothelial dysfunction and reduced nitric oxide in this model of ovarian hormone deficiency derives from reactive oxidative species and impaired reactive signaling from perivascular adipose tissue. Correction of oxidative stress and presence of perivascular adipose tissue are both required to normalize the greatly enhanced thromboxane/prostanoid receptor signaling after ovariectomy.

What Is Relevant?
- Correction of vascular reactive oxidative species alone is insufficient to correct microvascular dysfunction in full. Vascular thromboxane/prostanoid receptor and perivascular adipose tissue are novel targets to correct menopausal microvascular dysfunction.

Summary
Microvascular endothelial dysfunction and enhanced thromboxane/prostanoid receptor signaling after ovariectomy originate from interactive effects of oxidative stress in blood vessel and adverse adipokine signaling from perivascular adipose tissue.
Endothelial Dysfunction and Enhanced Contractility in Microvessels From Ovariectomized Rats: Roles of Oxidative Stress and Perivascular Adipose Tissue
Dan Wang, Cheng Wang, Xie Wu, Wei Zheng, Kathryn Sandberg, Hong Ji, William J. Welch and Christopher S. Wilcox

Hypertension, published online March 3, 2014;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2014/03/03/HYPERTENSIONAHA.113.02284

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/