Wine Polyphenols Improve Endothelial Function in Large Vessels of Female Spontaneously Hypertensive Rats

Rocío López-Sepúlveda, Rosario Jiménez, Miguel Romero, María José Zarzuelo, Manuel Sánchez, Manuel Gómez-Guzmán, Félix Vargas, Francisco O’Valle, Antonio Zarzuelo, Francisco Pérez-Vizcaíno, Juan Duarte

Abstract—Red wine polyphenols (RWPs) have been reported to prevent hypertension and endothelial dysfunction. Several individual RWPs exert estrogenic effects. We analyzed the possible in vivo protective effects on blood pressure and endothelial function of RWPs in female spontaneously hypertensive rats (SHR) and its relationship with ovarian function. RWPs (40 mg/kg by gavage) were orally administered for 5 weeks. Ovariectomized rats showed both increased isoprostaglandin F2α excretion and aortic superoxide production and reduced relaxant response to acetylcholine and contraction to the endothelial nitric oxide synthase (eNOS) inhibitor L-NAME measured in the aorta but similar blood pressure, as compared with sham-operated rats. Moreover, in ovariectomized rats aortic eNOS expression was unchanged, whereas caveolin-1, angiotensin II receptor (AT)-1, and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits p22phox and p47phox expression was increased compared with sham-operated rats. In both ovariectomized and sham-operated SHR, RWPs reduced systolic blood pressure, urinary isoprostaglandin F2α excretion, and aortic O2− production, improving the endothelium-dependent relaxant response to acetylcholine in SHR. These changes were associated with unchanged aortic eNOS expression, whereas caveolin-1 was increased and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits p22phox and p47phox expression was reduced. RWPs had no effect on the AT-1 overexpression found in ovariectomized animals. All these results suggest that a chronic treatment with RWPs reduces hypertension and vascular dysfunction through reduction in vascular oxidative stress in female SHR in a manner independent of the ovarian function. (Hypertension. 2008;51:1088-1095.)

Key Words: red wine polyphenols ■ spontaneously hypertensive rat ■ endothelial dysfunction ■ NADPH oxidase ■ ovariectomy

The incidence of cardiovascular diseases among premenopausal women is lower than age-matched, men but it rises markedly after menopause. Blood pressure is also higher in men than in women at similar ages,1 and this difference is also reduced or even inverted after menopause. The loss of estrogens has been suggested as a major risk factor for postmenopausal hypertension. Estrogen receptor (ER) subtypes ERα and ERβ are expressed in endothelial and smooth muscle cells.2–5 Estrogens increase endothelial-derived NO, modulate the local tissue renin-angiotensin system, and show antioxidant effects.3–10 Long-term estrogen treatment improves endothelial dysfunction, through upregulation of endothelial nitric oxide synthase (eNOS),11,12 posttranslational modulation of eNOS activity,13 or nongenomic effects, including activation of NO synthesis.14,15 However, despite the positive effects on vascular function found in cell culture,12,14 ex vivo15 and in vivo animal16–18 and short-term human7,19–21 studies, estrogen replacement therapy has failed to protect from cardiovascular diseases in large scale randomized controlled trials.22,23

Several classes of polyphenolic compounds which are consumed within our regular diet structurally and functionally resemble the mammalian estrogens and, thus, have been generally termed “phytoestrogens.” These dietary compounds appear to provide protection from cardiovascular diseases.24 Previous works in our laboratory25 and others26,27 demonstrated that soy phytoestrogens can protect estrogen-depleted spontaneously hypertensive rats (SHR). Red wine polyphenols (RWPs) and a grape skin extract also reduced blood pressure in males in several models of experimental hypertension,28–32 which was related to a combination of vasodilator and antioxidant actions. In vitro, several components of RWPs bind and increase the transcriptional activity of ERα
and ERβ. However, there are not studies which analyze the possible in vivo protective effects on blood pressure and endothelial function of RWPs in females and its relationship with ovarian function.

We have investigated whether RWPs exerted an in vivo protection against the decline in vascular function in an experimental model of endothelial dysfunction induced by both blood pressure increase and ovariectomy in female rats. It was hypothesized that RWPs would alter vascular reactivity involving changes in eNOS expression and its regulatory proteins (caveolin) and/or changes in oxidative status in isolated thoracic aortas.

**Methods**

**Animals and Experimental Groups**

Experiments followed our Institutional Guidelines for the ethical care of animals. Female SHR aged 24 weeks (Harlan Laboratories, Barcelona, Spain) were maintained (5 per cage, 24±1°C, 12-hour dark/light cycle) on soy-free chow (AIN 76, American Institute of Nutrition). SHR is an inbred rat strain derived from Wistar rats which is genetically predisposed to develop hypertension spontaneously resembling human essential hypertension. Rats were ovariec-
tomized (OVX) or sham-operated under anesthesia (2.5 mL/kg ethanol; 4.86 mg nembutal; 198 mL propylene glycol; 10.63 g MgSO4; distilled water). Three weeks after surgery animals were divided into the following groups (n=6 to 9 in each group): Sham-placebo, OVX-placebo, Sham-RWPs (40 mg/kg, per day, by gavage), and OVX-RWPs and followed for 5 weeks. Placebo was 1 mL of tap water daily by gavage. RWPs treatment was stopped 2 days before the end of experiments, to study their long-term effects without the involvement of acute administration effects. All rats of each group were then housed in metabolic cages with free access to food and their respective drinking fluids to measure urine output during 24-hours.

**Blood Pressure Measurements**

Systolic blood pressure (SBP) was measured weekly 18 to 20 hours after administration of the drugs in conscious, prewarmed, restrained rats by tail-cuff plethysmography.22

**Cardiac and Renal Weight Indices**

At the end of the experimental period, animals were anesthetized with 2.5 mL/kg equitensin (IP) and blood was collected from the abdominal aorta. Animals were euthanized and kidneys and hearts excised, cleaned, and weighed. The atria and the right ventricle were then removed and the remaining left ventricle weighed. The cardiac, left ventricular, and renal weight indices were calculated by dividing the heart, left ventricle, and kidney weight by the body weight.

**Urinary Determinations**

For total 8-iso-prostaglandin (PG) (iso-PGF2α) determination, 50 μL of urine was used for assay. The total iso-PGF2α concentration was measured by competitive enzyme immunoassay kit (Cayman Chem-

**Vascular Reactivity Studies**

Descending thoracic aortic rings (3 mm) were dissected and mounted in organ chambers filled with Krebs solution (composition in mmol/L: NaCl 118, KCl 4.75, NaHCO3 25, MgSO4 1.2, CaCl2 2, KH2PO4 1.2, and glucose 11) at 37°C and gassed with 95% O2 and 5% CO2. Rings were stretched to 2 g of resting tension by means of 2 L-shaped stainless-steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (Letigraph 2000), respectively, as previously described.32 The concentration-relaxation response curves to acetylcholine (ACH) (10−6 mol/L to 10−4 mol/L) were performed in rings pre-contracted by 10−5 mol/L phenylephrine. The concentration-relaxation response curves to nitroprusside (10−5 mol/L to 10−3 mol/L) were performed in the dark in rings pre-contracted by 10−6 mol/L phenylephrine. In some rings without endothelium, a concentration-response curve to angiotensin II (10−10 mol/L to 10−7 mol/L) was carried out by cumulative addition of the drugs.

To evaluate the formation of basal NO, the contraction induced phenylephrine (10−5 mol/L to 10−6 mol/L) was measured 30 minutes after aortic incubation with the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10−4 mol/L).25,32

**In Situ Detection of Vascular Superoxide Anion (O2−) Production**

Unfixed thoracic aortic rings were cryopreserved (PBS 0.1 mol/L, plus 30% sucrose for 1 to 2 hours), included in OCT, frozen (−80°C), and 10 μm cross sections were obtained in a cryostat (Microm International Model HM500 OM). Sections were incubated for 30 minutes in Hepes buffered solution containing dihydroethidium (DHE, 10−5 mol/L), counterstained with the nuclear stain DAPI, and in the following 24 hours examined on a fluorescence microscope (Leica DM IRB). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j). NIH, http://rsb.info.nih/i j/). O2− production was estimated from the ratio of ethidium/DAPI fluorescence.25 In preliminary experiments, DHE fluorescence was almost abolished by the O2− scavenger tiron, indicating the specificity of this reaction.

**Western Blotting Analysis**

Aortic homogenates were run on a sodium dodecyl sulfate (SDS)-polyacrilamide electrophoresis. Proteins were transferred to polyvi-

**Reverse Transcriptase-Polymerase Chain Reaction Analysis**

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted from aorta by homogenization and converted to cDNA by standard methods. PCR was performed with a Techne Techgene thermocycler (Techne). Initial denaturation was done at 95°C for 3 minutes and followed by 28 to 40 (30 for caveolin-1, p22phox and eNOS, 32 for AT-1 and 40 for p47phox) cycles of amplification. Each cycle consisted of 1 minute of denaturation at 94°C, 45 s of annealing at 60°C for p47phox, 55°C for p22phox, 55°C for caveolin-1, 77°C for AT-1, or 63°C for eNOS, and 1 minute for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 minutes to allow reannealing of amplified products. RT-PCR products were then size-fractionated through a 1.5% agarose gel, and the bands were visualized with ethidium bromide and quantified by densitometric analysis performed on the scanned images using Scion Image-Release Beta 4.02 software (http:// www.scioncorp.com).28 Samples were repurposed for expression of smooth muscle α-actin.
Long-term RWP administration induced a progressive reduction in SBP (9% at the end of the 5 weeks, \(P<0.01\) versus sham-operated SHR). SBP did not differ between OVX and sham-operated SHR.

### Urinary Isoprostane Excretion

Ex Vivo Aortic Reactivity

ACh induced a relaxant response that was abolished by endothelium removal as described elsewhere.\(^3\) Depletion of most of the endogenous estrogen (via ovariectomy) and exogenous estrogens (dietary soy phytoestrogens, via diet AIN 76) impaired relaxation evoked by ACh as compared with sham-operated rats (Figure 3A). The relaxant response induced by ACh was augmented by RWPs significantly (Figure 3A). In both groups of rats, RWPs treatment decreased significantly urinary isoprostane excretion.

#### Ex Vivo Aortic Reactivity

ACh-induced relaxation of aortic rings from both groups of SHR, independently of the estrogens levels (Figure 3A). The relaxant response induced by ACh was augmented by RWPs significantly (Figure 3A).

### Table. Body and Organ Weights and Cardiac and Renal Indices

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW, g</th>
<th>HW, mg</th>
<th>LVW, mg</th>
<th>KW, mg</th>
<th>LVW/HW Ratio</th>
<th>LW/BW Ratio</th>
<th>KW/BW Ratio</th>
<th>UW, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-placebo</td>
<td>205±3</td>
<td>883±13</td>
<td>688±7</td>
<td>614±12</td>
<td>0.78±0.01</td>
<td>3.37±0.06</td>
<td>3.00±0.07</td>
<td>598±43</td>
</tr>
<tr>
<td>Sham-RWPs</td>
<td>204±6</td>
<td>854±21</td>
<td>670±19</td>
<td>594±14</td>
<td>0.78±0.01</td>
<td>3.29±0.05</td>
<td>2.92±0.06</td>
<td>627±57</td>
</tr>
<tr>
<td>OVX-placebo</td>
<td>245±10</td>
<td>929±43</td>
<td>735±29</td>
<td>677±25</td>
<td>0.79±0.01</td>
<td>3.00±0.11†</td>
<td>2.75±0.07*</td>
<td>176±11*</td>
</tr>
<tr>
<td>OVX-RWPs</td>
<td>239±3†</td>
<td>890±24</td>
<td>697±17</td>
<td>625±16</td>
<td>0.78±0.02</td>
<td>2.91±0.09†</td>
<td>2.61±0.05†</td>
<td>208±25*</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; BW, body weight; HW, heart weight; LVW, left ventricle weight; KW, kidney weight; UW, uterus weight. Values are expressed as mean±SEM of 6 to 9 rats.

\(*P<0.05, \dagger P<0.01\) as compared to the sham group.
by ACh in all experimental groups was abolished by L-NAME (10^{-4} \text{ mol/L}; not shown). The endothelium-independent relaxation induced by sodium nitroprusside was not different among groups (Figure 3B; Table S1).

No differences were found among all experimental groups in the concentration-contractile response induced by phenylephrine in intact aortic rings (Figure 4A; Table S1). However, this response was significantly reduced in aorta from OVX as compared with sham-operated than animals (Figure 4B; Table S1) when the rings were incubated previously with the NO synthase inhibitor L-NAME, indicating a reduced basal NO formation in OVX SHR. RWPs increased this contractile response only in rings from OVX rats, suggesting a higher NO formation in these vessels.

**In Situ Detection of O_{2}^{-} Production in Rat Aorta**

To characterize and localize O_{2}^{-} production within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE. DHE is oxidized by O_{2}^{-} to yield ethidium which stains DNA. Positive red nuclei could be observed in adventitial, medial, and endothelial cells (Figure 5A). Nuclear red ethidium fluorescence, indicative of O_{2}^{-} production, was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from OVX SHR showed marked increased staining in adventitial, medial, and endothelial cells as compared with sham-operated rats which was significantly reduced by RWPs in both sham and OVX groups (Figure 5A and 5B).

**Gene and Protein Expression of eNOS, Caveolin-1, p47phox, p22phox, and AT-1 Receptor in Rat Aorta**

eNOS gene and protein expression was unchanged in ovariectomized rats as compared with sham-operated rats (Figure 6A and 6B). We next examined changes in expression of caveolin-1, an allosteric negative regulator of eNOS. The expression of caveolin-1 was markedly higher in aortae from OVX SHR than sham-operated rats (Figure 6C and 6D). After treatment of animals with RWPs for 5 weeks, eNOS gene and protein expression was unchanged (Figure 6A and 6B), whereas caveolin-1 was increased (Figure 6C and 6D) in both control and OVX rats.

Significant mRNA and protein overexpression of NADPH oxidase subunits, p22phox and p47phox, were observed in aortic tissue from OVX SHR as compared with sham rats. RWP treatment was able to reduce gene and protein expression of both subunits in SHR independently of ovarian function (Figure 7).

Both AT-1 mRNA and AT-1 protein expression were increased in aorta from OVX rats as compared with sham-operated rats (Figure 8). Treatment with RWPs in Sham or OVX rats did not modify AT-1 gene and protein expression. Moreover, the contractions induced by angiotensin II, which were greater in OVX than in sham-operated rats, were also unmodified by chronic RWPs treatment (Figure 8C; Table S1).

**Discussion**

The major new findings of this study are that chronic treatment with RWPs reduced SBP, systemic oxidative stress, and the endothelial dysfunction in female SHR, and that this effect seems to be independent to ovarian function and related to attenuation of vascular O_{2}^{-} production mediated by NADPH oxidase inhibition.

This study confirms and extends previous evidence about antihypertensive effects and the improvement in endothelial function of RWPs in male hypertensive rats.28-32 According to previous studies we also found that the antihypertensive effects of RWPs seem to be related to attenuation of oxidative stress, because the urinary iso-PGF_{2alpha}, a PG-like compound produced in a nonenzymatic reaction of arachidonic acid and O_{2}^{-}, was significantly reduced by RWPs in both sham and OVX rats.

The most characteristic feature of endothelial dysfunction is a diminished bioactivity of endothelium-derived...
NO. In the rat aorta, endothelium-dependent vasodilatation relies almost entirely on the endothelial release of NO. The SHR is a well-known and widely used animal model of endothelial dysfunction which aggravates after ovariectomy, resembling that observed in postmenopausal hypertensive women. More specifically, OVX SHR showed a reduced relaxant response to ACh, an endothelium- and NO-dependent vasodilator, and unchanged response to nitroprusside, an NO donor that relaxed arteries in an endothelium-independent manner. In addition, endothelium-intact aortic rings from OVX rats showed a reduced contraction to phenylephrine in presence of L-NAME, an inhibitor of eNOS, as compared with sham-rats. Taken together, these data indicate that OVX rats show endothelial dysfunction characterized by a reduced NO bioactivity. RWP s were able to improve the endothelium-dependent vasodilator response to ACh in both sham- and OVX-SHR without affecting the response to nitroprusside, and restored the contraction evoked by L-NAME in OVX rats. These data strongly suggest that RWPs improve endothelial function in SHR by increasing NO bioactivity.

Several potential mechanisms would be involved in the RWP-induced increase of endothelial-derived NO responses, such as changes in the activity or expression of eNOS, changes in the vascular levels of $\text{O}_2^-$ and thus $\text{O}_2^-$-driven NO inactivation, and changes in the sensitivity to NO-cGMP.
pathway in vascular smooth muscle cells. Because the responses to nitroprusside were not modified by RWPs, the third potential mechanism can be ruled out. Reduced NO synthesis associated to endothelial dysfunction may be caused by impaired expression of eNOS, posttranslational modification of the enzyme (eg, phosphorylation or fatty acid modifications), interactions with heat shock protein 90 (hsp90) and caveolin, or suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin (BH4). In agreement with our previous study, we found and nox 4] and p22phox) and 3 cytoplasmic subunits, p47phox, and its allosteric regulator, caveolin-1.41 In fact, in the face of exerts a negative feedback on regulation of eNOS expression consistent with early studies, which demonstrated that NO RWPs did not change eNOS expression but increased endothelial dysfunction after ovariectomy. Interestingly, eNOS modulator are consistent with the widely observed decrease of caveolin-1 (increase) in SHR which seems related to the vasodilatory functions.42 RWPs induced expressional changes in eNOS (increase) and its negative allosteric regulator, caveolin-1.43 In fact, in the face of increased oxidative stress and endothelial dysfunction, such as SHR, reduced vascular NO levels induced coordinated expres- sional changes in eNOS (increase) and its negative allosteric regulator, caveolin-1 (reduction), may even be viewed as a compensatory mechanism to maintain the production of bioactive NO.42 RWPs induced expressional changes of caveolin-1 (increase) in SHR which seems related to the reduced vascular oxidative stress found in aorta from RWPs treated animals and subsequent increase in NO bioactivity.

NADPH oxidase is a multi-subunit enzymatic complex responsible for the mono-electronic reduction of oxygen to produce O2•− at the expense of NADPH. Similarly to the neutrophil oxidase, vascular NADPH oxidase comprises a membrane-bound flavocytochrome b558 heterodimer (formed by gp91phox [nox 2] or gp91phox homologues [nox 1 and nox 4] and p22phox) and 3 cytoplasmic subunits, p47phox, p67phox, and p40phox. This enzyme complex is considered to be the most important source of O2•− in the vessel wall.43 Estrogen deficiency led to an increased NADPH oxidase activity, associated with increased vascular expression of p22phox and p67phox subunits in the aorta of OVX mice, and supplementation of estrogen prevented this effect. In our experimental conditions, we also found in OVX rats an increased O2•− production, associated with increased p22phox and p47phox expression in the vascular wall. RWPs reduced the expression of these subunits in both sham-operated and estrogen-depleted SHR.

AT-1 receptor activation induces vasoconstriction and cellular growth and leads to free radical release in the vessel wall.44 It has been reported that estrogen causes downregulation of vascular AT-1 receptors and that estrogen deficiency is accompanied by AT-1 receptor overexpression.45 In ovariectomized SHR, we also found an increased AT-1 receptor expression at the level of mRNA and protein and a clear functional correlate, ie, parallel changes in angiotensin II–induced vasoconstriction. The increased AT-1 receptor expression in OVX may also contribute to the increase in O2•− production and the impaired endothelial function. However, RWPs did neither change AT-1 overexpression nor the vasoconstriction induced by angiotensin II in OVX rats. Therefore, the improvement of endothelial function and the reduction in O2•− production induced by RWPs is independent of AT-1 receptor expression regulation.

In conclusion, our results clearly demonstrated that RWP treatment reduces the elevated blood pressure, the endothelial dysfunction, and the vascular oxidative stress in this model of genetic hypertension. These effects seem to be independent of ovarian function and related to the increased NO bioactivity, resulting from reduced NADPH-oxidase mediated O2•− production.

Perspectives
Our present results in female rats confirm and extend previous data showing antihypertensive and vasoprotective effects of RWPs in male animals. Moreover, the relevance of NADPH oxidase system as a potential target for controlling hypertension and endothelial dysfunction is also enhanced. However, the exact mechanism involved in the NADPH oxidase subunits downregulation induced by RWPs continues unclarified. The present findings may help to explain the potential benefits of RWPs as a therapeutic agent for preventing the menopausal vascular complications, especially in hypertensive women.
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Disclosures

None.

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Running title: Polyphenols and female SHR.
Table S1. Parameters of the concentration-response curves to vasoactive factors

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Sham-placebo (n=9)</th>
<th>Sham-RWP (n = 9)</th>
<th>OVX-placebo (n = 6)</th>
<th>OVX-RWP (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>E\textsubscript{max}</td>
<td>pD2</td>
<td>E\textsubscript{max}</td>
</tr>
<tr>
<td>Acetylcholine (10\textsuperscript{-9} - 10\textsuperscript{-4} mol/L)</td>
<td>7.58 ± 0.21</td>
<td>35.0 ± 2.6</td>
<td>7.29 ± 0.15</td>
<td>46.1 ± 4.6 †</td>
</tr>
<tr>
<td>Sodium nitroprusside (10\textsuperscript{-9} - 10\textsuperscript{-5} mol/L)</td>
<td>7.43 ± 0.11</td>
<td>93.6 ± 3.6</td>
<td>7.72 ± 0.11</td>
<td>95.9 ± 2.4</td>
</tr>
<tr>
<td>Phenylephrine (without L-NAME) (10\textsuperscript{-9} - 10\textsuperscript{-6} mol/L)</td>
<td>7.13 ± 0.05</td>
<td>1.4 ± 0.1</td>
<td>7.15 ± 0.13</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Phenylephrine (with L-NAME) (10\textsuperscript{-9} - 10\textsuperscript{-6} mol/L)</td>
<td>7.59 ± 0.11</td>
<td>2.3 ± 0.1</td>
<td>7.51 ± 0.10</td>
<td>2.35 ± 0.3</td>
</tr>
<tr>
<td>Angiotensin II (10\textsuperscript{-10} - 10\textsuperscript{-6} mol/L)</td>
<td>8.96 ± 0.33</td>
<td>0.12 ± 0.04</td>
<td>9.04 ± 0.19</td>
<td>0.17 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± s.e. mean. E\textsubscript{max} (maximal effect) for phenylephrine and angiotensin II are expressed as g of contraction/mg tissue and for acetylcholine and sodium nitroprusside as a percentage of relaxation of the pre-contraction with phenylephrine. pD\textsubscript{2} is the drug concentration exhibiting 50% of the E\textsubscript{max} expressed as negative log molar. *P<0.05 as compared to the sham-placebo group. †P<0.05 RWPs as compared to the respective placebo group.