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Menopause and Ovariectomy Cause a Low Grade of Systemic Inflammation That May Be Prevented by Chronic Treatment with Low Doses of Estrogen or Losartan

May Abu-Taha,2* Cristina Rius,2* Carlos Hermenegildo,† Inmaculada Noguera,‡ Jose-Miguel Cerda-Nicolas,§# Andrew C. Issekutz,** Peter J. Jose,* Julio Cortijo,§# and Maria-Jesus Sanz3*#

The incidence of cardiovascular diseases in premenopausal women is lower than in men or postmenopausal women. This study reports the discovery of a low grade of systemic inflammation, including monocyte adhesion to arterial endothelium, elicited by menopause or estrogen depletion. Chronic treatment with low dose of 17-β-estradiol or inhibition of the renin-angiotensin system reduced this inflammation. Using an in vitro flow chamber system with human arterial and venous endothelial cells, we found that leukocytes from healthy postmenopausal women were more adhesive to the arterial endothelium than those from premenopausal women regardless of the stimulus used on endothelial cells. Increased circulating levels of IL-8, MCP-1, RANTES, and MIP-1α and monocyte CD11b expression were also encountered in postmenopausal vs premenopausal subjects. This translational data led us to investigate the mechanisms in Sprague-Dawley rats. Using intravital microscopy, we imaged mesenteric arterioles and found significant increases in arteriolar leukocyte adhesion, cell adhesion molecule expression, and plasma levels of cytokine-induced neutrophil chemoattractant (CINC/KC), MCP-1, and MIP-1α in 1-mo ovariectomized rats. Chronic treatment of ovariectomized rats with low dose of 17-β-estradiol, losartan, both, or benazepril inhibited ovariectomy-induced arteriolar mononuclear leukocyte adhesion by 77%, 58%, 92%, and 65% respectively, partly by inhibition of cell adhesion molecule up-regulation and the increase in circulating chemokines. These results demonstrate that menopause and ovariectomy generate a low grade of systemic inflammation. Therefore, administration of low doses of estrogens or inhibition of the renin-angiotensin system, at early stages of estrogen deficiency, might prevent the systemic inflammation associated with menopause and decrease the risk of suffering further cardiovascular diseases. The Journal of Immunology, 2009, 183: 0000 – 0000.

Gender differences in the development of cardiovascular disease are well documented in both human and animal studies. Cardiovascular diseases are more prevalent in men than in premenopausal women, but the incidence increases sharply in postmenopausal women (1, 2). An abundance of epidemiological data supports a role for estrogens in this atheroprotective effect, which led to recommendations for their widespread use in postmenopausal hormone replacement therapy (HRT) both to treat postmenopausal symptoms and to reduce the risk of cardiovascular disease (3, 4). Estrogens reduce the serum levels of several markers for inflammation in postmenopausal women (5). Additionally, observational studies in ovariectomized monkeys show that HRT inhibits coronary atherosclerosis progression by 70% if begun early after ovariectomy (6). However, some clinical trials have failed to demonstrate cardiovascular beneficial effects of HRT and have even suggested increased cardiovascular risk during the initial treatment period (7). These discrepancies between observational and randomized clinical trials suggest the need for research into new forms of postmenopausal HRT using a low dose of estrogen monotherapy, which may relieve cardiovascular alterations without causing the unwanted side effects.

Estrogens influence the renin-angiotensin system, the main effector peptide (i.e., angiotensin-II (Ang-II)) of which is implicated in atherogenesis beyond its hemodynamic effects (8–10). The synthesis of angiotensinogen in hepatocytes is regulated by estrogens (11). Plasma renin levels and angiotensin-converting enzyme (ACE) activity are significantly higher in estrogen-deficient compared with estrogen-replete rats and in postmenopausal women not receiving HRT compared with women on HRT (9, 12). In addition to regulating the components involved in synthesizing Ang-II,
estrogens also alter the expression of Ang-II AT1 receptors in many target tissues (10, 13). Using intravital microscopy to directly observe kinetic events in the rat mesenteric microcirculation, we found that acute (90 min) blockade of estrogen receptors in male rats induces leukocyte-endothelial cell interactions in the postcapillary venules, and these inflammatory responses are significantly attenuated by an AT1 receptor antagonist (14). Later, we demonstrated that exposure to Ang-II in vivo induces arteriolar monocellular cell adhesion (15).

Experimental studies have demonstrated that leukocyte adhesion and infiltration into the arterial wall, regulated by leukocyte and endothelial cell adhesion molecules (CAMs) and chemokines, represent an essential step in atherosclerotic lesion formation (16–20). Estrogens are associated with improved endothelial function, by elevation of NO and prostacyclin levels, enhanced vasodilatation, inhibition of vascular smooth muscle cell proliferation, and reduction of platelet aggregation, and they are therefore thought to attenuate atherosclerosis (4). In the present study, we have used whole blood from both healthy premenopausal and postmenopausal women and determined leukocyte interaction with arterial and venous endothelia under dynamic flow conditions. The levels of different circulating markers of systemic inflammation were also determined. These translational data led us to investigate in rats the effects of estrogen depletion by ovariectomy on leukocyte-endothelial cell interactions, CAM expression, and circulating chemokine levels. We have also investigated the protective effects displayed by chronic treatment of ovariectomized (OVZ) rats with low doses of 17-β-estradiol in monotherapy, the Ang-II AT1 receptor antagonist, losartan, and the ACE inhibitor benazepril. Finally, since estrogens influence the renin-angiotensin system, the leukocyte-endothelial cell interactions elicited by the intraperitoneal administration of Ang-II in nonoperated and OVZ female rats as well as in male animals were also studied.

Materials and Methods

Human studies

Healthy 6 premenopausal and 13 postmenopausal women received a basic physical examination and a routine blood analysis. The inclusion criteria were: nonsmokers, nonhysterectomized women, and not taking any medication, including HRT or contraceptives, steroids, antidiabetic drugs, statins, or antihypertensive drugs. The postmenopausal women were with at least 2 years of amenorrhoea in association with elevated serum follicle-stimulating hormone levels (>30 mIU/ml) and low density lipoprotein cholesterol. The postmenopausal women were taking HRT or contraceptives or antihypertensive drugs. The premenopausal women were smokers, nonhysterectomized women, and not taking any medication. A routine analysis, including glucose, complete lipid profile, and concentrations of estradiol and follicle-stimulating hormone, was performed. The clinical characteristics of the human subjects are shown in Table I. This investigation conforms with the principles outlined in the Declaration of Helsinki, was approved by the institutional ethics committee at the University Clinic Hospital of Valencia, Spain, and written informed consent was obtained from all volunteers.

Leukocyte-HUVEC and leukocyte-HUAEC interactions under flow conditions. HUAEcs and HUVEcs were isolated by collagenase as previously described (21). Cells up to passage 2 were used. A routine analysis, including glucose, complete lipid profile, and concentrations of estradiol and follicle-stimulating hormone, was performed. The clinical characteristics of the human subjects are shown in Table I. This investigation conforms with the principles outlined in the Declaration of Helsinki, was approved by the institutional ethics committee at the University Clinic Hospital of Valencia, Spain, and written informed consent was obtained from all volunteers.

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Determination of surface expression of CD11b/CD18 integrins. The expression of CD11b/CD18 integrins was determined on human neutrophils and monocytes in heparinized whole blood. Duplicate samples (100 μl) were incubated for 20 min on ice in the dark with saturating amounts (10 μl) of the conjugated mAb anti-human-CD11b/CD18-FITC or RBCs were lysed and leukocytes fixed using an automated Epics Q-Prep system (Coulter Electronics). Samples were run in an Epics XI-MCL flow cytometer (Beckman-Coulter). The expression of the surface Ag (FITC-fluorescence) was measured in neutrophils and monocytes identified by their specific features of size (FS) and granularity (SS) in the flow cytometer. Determination of chemokine and Ang-II levels in human whole blood. Plasma from whole blood (10 U heparin/ml, from healthy volunteers) was collected. An aliquot of the blood sample (2–3 ml) was collected into tubes containing a cocktail of protease inhibitors. The blood was centrifuged, and the plasma (1–2 ml) was then extracted with Sep-Pak C18 cartridges. Plasma Ang-II levels were determined with a high-sensitivity enzyme immunoassay kit. Before centrifugation to obtain plasma, further heparin was added to the blood sample (to 100 U/ml). This procedure was used to help dissociate chemokines from blood cells. Plasma samples were stored at −80°C. Human chemokines (growth related oncogene-α, GROα/CXCL1; IL-8, IL-8/CXCL8; MCP-1, MCP-1/CCL2; MCP-3/CCL7; regulated on activation normal T expressed and secreted RANTES/CCL5 and MIP-1α/CCL3) were measured in plasma by ELISA, as previously described (21).

Animal studies

Animal preparation. Male and female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories and fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Ethics Committee at the University of Valencia, and the investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Ovariectomy and treatments. Some rats were subjected to ovariectomy and randomly divided into two groups for immediate s.c. implantation of osmotic minipumps. The first group was implanted with a minipump that allowed the release of 17-β-estradiol (5 μg/kg/day; OVZ plus estradiol). This dose of estradiol is equivalent to 0.35 mg/day in an adult postmenopausal woman and is considered as a low dose in HRT (22). The second group received a minipump filled with vehicle (50% DMSO in saline; OVZ plus vehicle). To establish the effect of an Ang-II type 1 (AT1) receptor blocker, the third group of ovariectomized rats received losartan in the drinking water (10 mg/kg/day; OVZ plus losartan). Another group of animals was treated with both 17-β-estradiol and losartan (OVZ plus estradiol plus losartan). Since ACE activity is increased in estrogen-deficient rats, a group of animals was implanted with a minipump to be treated with an ACE inhibitor, benazepril (5 mg/kg/day; OVZ plus benazepril). A group of sham-operated female rats was also included. All the subsequent measurements were performed 30 days after ovariectomy. The success of ovariectomy, and the response to hormonal treatments, was determined by measuring uterine weight.

Intravital microscopy. The details of the experimental preparation have been described previously (15). Briefly, male or female Sprague-Dawley rats (200–250 g) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and the trachea, right jugular vein, and carotid artery were cannulated. After performing a midline abdominal incision, a segment of the midjejunum was exteriorized and placed over an objective window. The window was maintained at 37°C, which facilitated tissue transillumination. The exposed mesentery was continuously superfused with warmed bicarbonate-buffered saline (pH 7.4) equilibrated with 5% CO₂ in nitrogen. An orthostatic

Table I. Clinical characteristics of the pre and postmenopausal women

<table>
<thead>
<tr>
<th>Variable</th>
<th>Postmenopausal Women</th>
<th>Premenopausal Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Smokers</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.3 ± 2.4</td>
<td>25.8 ± 0.9</td>
</tr>
<tr>
<td>Years of menopause</td>
<td>10.6 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.2 ± 4.5</td>
<td>54.1 ± 0.5</td>
</tr>
<tr>
<td>Circulating leukocytes (cells/μl)</td>
<td>6485.7 ± 407.1</td>
<td>7117 ± 654</td>
</tr>
<tr>
<td>Glucose (g/L)</td>
<td>95.3 ± 1.7</td>
<td>86.7 ± 1.4</td>
</tr>
<tr>
<td>Total cholesterol (g/L)</td>
<td>240.5 ± 8.1</td>
<td>192.0 ± 8.7</td>
</tr>
<tr>
<td>Triglycerides (g/L)</td>
<td>77.5 ± 7.7</td>
<td>62.7 ± 4.9</td>
</tr>
<tr>
<td>HDL cholesterol (g/L)</td>
<td>75.0 ± 3.6</td>
<td>70.5 ± 5.2</td>
</tr>
<tr>
<td>LDL cholesterol (g/L)</td>
<td>147.8 ± 8.2</td>
<td>128.0 ± 5.4</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>77.6 ± 9.7</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>30.1 ± 4.7</td>
<td>5.8 ± 1.3</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>13.5 ± 1.5</td>
<td>164.3 ± 5.3</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>101.3 ± 3.8</td>
<td>84.4 ± 3.4</td>
</tr>
</tbody>
</table>

a HDL indicates high density lipoprotein; LDL, low density lipoprotein; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MABP, mean arterial blood pressure.
microscope (Nikon Optiphot-2, SMZ1) equipped with a ×20 objective lens (Nikon SLDW) and a ×10 eyepiece permitted tissue visualization. A video camera (Sony SSC-C350P) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E), and these images were captured on a videotape (Sony SVT-S3000P) for playback analysis (final magnification of the video screen was ×1300). Arterioles (15- to 30-μm diameter) and single unbranched mesenteric venules (25- to 40-μm diameter) were selected and the diameters measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline RBC velocity was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute). Venular blood flow and wall shear rate were calculated as previously described (21). The number of rolling, adherent, and emigrated leukocytes was determined off-line during playback analysis of videotaped images.

**Experimental protocol.** Animals were anesthetized and the mesentery was exposed in preparation for measurements of arteriolar leukocyte adhesion and venular events of leukocyte rolling flux, rolling velocity, adhesion and emigration, and hemodynamic parameters of mean arterial pressure, arteriolar and venular centerline RBC velocity, shear rate, and diameter, which were determined during a 5-min period.

Experimental groups were allocated as follows: 1) untreated female rats, 2) sham-operated female rats, 3) female rats 30 days after ovariectomy (OVZ plus vehicle), 4) ovariectomized rats treated with estradiol (OVZ plus estradiol), 5) ovariectomized rats treated with losartan (OVZ plus losartan), 6) ovariectomized rats treated with both 17β-estradiol and losartan (OVZ plus estradiol plus losartan), 7) ovariectomized rats treated with benazepril (OVZ plus benazepril), and 8) untreated male rats.

In another set of experiments, male, female, or OVZ females were sedated and i.p. injected with 5 ml of PBS or 5 ml of 1 nM Ang-II, and leukocyte-endothelial cell interactions and hemodynamic parameters were evaluated 4 h later. Since this dose of Ang-II caused no effect on nonoperated female rats, two additional groups were i.p. injected with Ang-II either 10 or 100 nM.

**Histology and immunohistochemistry.** After the completion of the intravital microscopy measurements, the mesentery was isolated, fixed in 4% paraformaldehyde, dehydrated using graded acetone washes at 4°C, and embedded in paraffin wax for localization of P-selectin and VCAM-1 using a modified avidin and biotin immunoperoxidase technique as previously described (21). Tissue sections (4 μm thick) were incubated for 24 h with Ab at 200 μg/ml: anti-rat-P-selectin mAb RP-2 or with anti-rat-VCAM-1 mAb 5F10, or their isotype-matched control Ab MOPC21 (murine IgG1) or UPC10 (murine IgG2a). Positive staining was defined as an arteriole or venule displaying brown reaction product.

To elucidate the type of leukocyte adhered to the endothelium of arterioles and venules, some mesenteric sections were stained with H&E. On the other hand, some aortas were also fixed with 4% paraformaldehyde. After carefully removing the adventitia, the aorta was excised, fixation was continued overnight, and vessels were opened longitudinally and stained with Oil Red O solution (0.2% in 80% methanol). Specimens were pinned onto a flat surface and photographed to determine the possible extent of the macroscopic atherosclerotic lesion. In another set of animals, the heart and the proximal aorta were fixed, paraffin-embedded, and 3-μm-thick sections were taken from the three valve cusps, the aortic arch, and the proximal aorta for localization of macrophage infiltration. Tissue sections were incubated for 24 h with a mouse anti-rat-CD68 mAb.

**FIGURE 1.** Recruitment of leukocytes by stimulated endothelial cells and CD11b expression using whole blood from premenopausal and postmenopausal women. HUAECs and HUVECs were incubated with medium or Ang-II (1 μM) for 4 h. Whole blood from healthy premenopausal and postmenopausal women was perfused over the endothelial monolayers for 5 min at 1 dyn/cm², and leukocyte rolling (A and C) and adhesion (B and D) were quantified. Leukocytes were also stained with a conjugated mAb for CD11b and analyzed by flow cytometry (E). Results are the means ± SEM for n = 6–13 experiments. *p < 0.05 or **p < 0.01 relative to values in the medium group. +p < 0.05 or ++p < 0.01 relative to the values in premenopausal women.
FIGURE 2. A–F, Circulating chemokines in human whole blood from premenopausal and postmenopausal women. GROα, IL-8, MCP-1, MCP-3, RANTES, and MIP-1α plasmatic levels were measured by ELISA. Results (pM in the plasma) are means ± SEM of n = 6–13 experiments. *, p < 0.05 or **, p < 0.01 relative to values in premenopausal women.

**Chemokine, cholesterol, and Ang-II levels in rat whole blood.** At the end of intravital microscopy measurements, rat whole blood was collected into sodium heparin, the concentration of which was adjusted to 100 U/ml 10 min before centrifugation to obtain plasma. Plasma samples were stored at −80°C for cytokine-induced neutrophil chemoattractant (CINC/KC), MIP-2, MCP-1, RANTES, and MIP-1α ELISAs, as previously described (21).

In some plasma samples additional heparin was not added and such samples were used for determination of total cholesterol and Ang-II circulating levels. Total cholesterol was measured by spectrometry (Chol-H-L-Type). Circulating Ang-II levels were determined as described above.

**Statistical analysis**

Data between groups were analyzed using a one-way-ANOVA with a Newman–Keuls post hoc correction for multiple comparisons. For human studies, a Student’s t test was employed. All values are reported as mean ± SEM. Statistical significance was set as p < 0.05.

**Materials**

Ang-II, estradiol, pentobarbital, MOPC21 Oil Red O solution, and UPCI10 were purchased from Sigma-Aldrich. Losartan was donated by Merck Sharp & Dohme. Human and rat chemokines and Abs for all rat chemokine ELISAs were from PeproTech. The Ab pairs for all human chemokine ELISAs were from R&D Systems. Alzet 2004 osmotic minipumps were purchased from Perbio Science, and the K-Blue substrate was from Neogen. Chol-H-L-Type was from Wako Pure Chemical Industries. The Ang-II enzyme immunoassay kit was from SPI-BIO. Sep-Pak C18 cartridges were from Waters Chromatography Division. The Abs RP-2 and 5F10 were obtained as previously stated (15). The mouse monoclonal anti-human-CD11b-FITC (clone ICRF 44) and the anti-rat CD68 (clone ED1) were from Serotec.

**Results**

*Increased adhesiveness of leukocytes from postmenopausal women to unstimulated or Ang-II–stimulated arterial endothelium*

Since estrogens influence the renin-angiotensin system, whole blood from premenopausal and postmenopausal women was perfused across unstimulated or Ang-II–stimulated HUAECs and HUVECs at a shear force of 1 dyn/cm² for 5 min. Fig. 1 shows leukocyte rolling (Fig. 1, A and C) and adhesion (Fig. 1, B and D) on either medium or 4 h Ang-II–treated HUAECs and HUVECs. Increased and significant differences in leukocyte interactions were observed when whole blood from these two women populations was perfused across arterial endothelium (Fig. 1, A and B), being more marked in the postmenopausal group. Furthermore, leukocyte rolling and adhesion were significantly enhanced in both populations when HUAECs were stimulated with Ang-II (Fig. 1, A and B). When whole blood from postmenopausal women was perfused across HUVEC monolayers, a significant increase in leukocyte rolling was observed when HUVECs were stimulated with

**Table II. Uterus weight and hemodynamic parameters***

<table>
<thead>
<tr>
<th></th>
<th>Uterus Weight (g)</th>
<th>MABP (mmHg)</th>
<th>Arteriolar Shear Rate (s⁻¹)</th>
<th>Venular Shear Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonoperated female</td>
<td>0.45 ± 0.03</td>
<td>109.8 ± 6.5</td>
<td>1255.7 ± 198.5</td>
<td>495.4 ± 63.2</td>
</tr>
<tr>
<td>Sham-operated female</td>
<td>0.49 ± 0.02</td>
<td>108.8 ± 4.1</td>
<td>1392.4 ± 158.3</td>
<td>568.6 ± 48.5</td>
</tr>
<tr>
<td>OVVZ female</td>
<td>0.12 ± 0.01**</td>
<td>112.8 ± 4.8</td>
<td>1305.0 ± 100.9</td>
<td>612.2 ± 42.5</td>
</tr>
<tr>
<td>OVVZ female + estradiol</td>
<td>0.36 ± 0.03+++</td>
<td>112.5 ± 3.0</td>
<td>1436.3 ± 201.4</td>
<td>622.1 ± 77.8</td>
</tr>
<tr>
<td>OVVZ female + losartan</td>
<td>0.15 ± 0.04**</td>
<td>109.4 ± 6.4</td>
<td>1291.5 ± 261.8</td>
<td>509.6 ± 43.9</td>
</tr>
<tr>
<td>OVVZ female + estradiol + losartan</td>
<td>0.34 ± 0.03+++</td>
<td>107.3 ± 1.5</td>
<td>1198.6 ± 168.5</td>
<td>546.6 ± 36.2</td>
</tr>
<tr>
<td>OVVZ female + benazepril</td>
<td>0.12 ± 0.02**</td>
<td>103.6 ± 4.3</td>
<td>1260.7 ± 150.6</td>
<td>579.1 ± 52.9</td>
</tr>
<tr>
<td>Male</td>
<td>114.2 ± 2.9</td>
<td>1514.2 ± 211.8</td>
<td>578.2 ± 75.5</td>
<td></td>
</tr>
</tbody>
</table>

*Results are means ± SEM for n = 5–13 animals/group. **, p < 0.01 relative to the sham-operated female group; +, p < 0.05 or ++, p < 0.01 relative to the OVVZ female group. MABP indicates mean arterial blood pressure.*
Ang-II (Fig. 1C). In contrast, no differences in the leukocyte rolling and adhesion were detected between the premenopausal and the postmenopausal group (Fig. 1C and D). Additionally, CD11b up-regulation was detected in circulating monocytes of postmenopausal women (Fig. 1E).

**Circulating chemokine profile in premenopausal and postmenopausal women**

Chemokines are involved in leukocyte adhesion and infiltration into the tissues. Therefore, we next investigated the circulating levels of different CXC and CC chemokines in premenopausal and postmenopausal women. In the plasma of postmenopausal women significant increases in the levels of IL-8, RANTES, and MIP-1α were detected (Fig. 2B, E, and F). While elevated plasma levels of MCP-1 were also found in postmenopausal women, they did not significantly differ from those encountered in the premenopausal group (Fig. 2C). No significant differences between both groups were found in the circulating levels of GRO-α and MCP-3 (Fig. 2A and D). Finally, Ang-II plasma levels did not differ between the two groups investigated (20.4 ± 0.8 pg/ml in the premenopausal group vs 23.7 ± 3.1 in the postmenopausal group).

Ovariectomy induces microvascular inflammation, which is prevented by chronic treatment with low doses of estradiol or losartan

These translational data then led us to investigate the mechanisms in rats, and for this purpose some animals were ovariectomized and levels of different CXC and CC chemokines in premenopausal and postmenopausal women. In the plasma of postmenopausal women significant increases in the levels of IL-8, RANTES, and MIP-1α were detected (Fig. 2, B, E, and F). While elevated plasma levels of MCP-1 were also found in postmenopausal women, they did not significantly differ from those encountered in the premenopausal group (Fig. 2C). No significant differences between both groups were found in the circulating levels of GRO-α and MCP-3 (Fig. 2, A and D). Finally, Ang-II plasma levels did not differ between the two groups investigated (20.4 ± 0.8 pg/ml in the premenopausal group vs 23.7 ± 3.1 in the postmenopausal group).

Ovariectomy induces microvascular inflammation, which is prevented by chronic treatment with low doses of estradiol or losartan

These translational data then led us to investigate the mechanisms in rats, and for this purpose some animals were ovariectomized and
the success of this procedure was determined by the decrease in uterine mass. Ovariectomy promoted a significant decrease (71–75%) in uterus weight compared with nonoperated and sham-operated female rats (Table II). Chronic treatment of OVZ rats with estradiol resulted in a much smaller reduction (25–30%) of uterus mass. In contrast, losartan or benazepril treatment for 1 mo did not affect the loss of uterus weight provoked by ovariectomy (Table II).

Intravital microscopy was used to examine leukocyte trafficking in the mesenteric microcirculation. We imaged arterioles and unexpectedly found that ovariectomized rats showed increased leukocyte adhesion to arteriolar endothelium when compared with that found in sham-operated animals (Fig. 3). No differences in this leukocyte parameter were encountered between nonoperated female, sham-operated female, or male rats (Fig. 3). Estradiol, losartan, and both estradiol plus losartan and benazepril treatments inhibited ovariectomy-induced arteriolar leukocyte adhesion by 77%, 58%, 92%, and 65%, respectively (Fig. 3). Neither ovariectomy nor any treatment affected hemodynamic parameters such as mean arterial blood pressure or arteriolar shear rate (Table II).

In the postcapillary venules of the same animals, ovariectomy provoked a significant increase in leukocyte recruitment (Fig. 4) without modifying venular shear rate (Table II). Treatment of OVZ rats with estradiol, losartan, or both estradiol plus losartan or benazepril inhibited ovariectomy-induced venular leukocyte-endothelial cell interactions (Fig. 4). None of these treatments affected venular shear rate (Table II).

Histological examination was conducted in the exposed tissue to determine the type of leukocytes recruited by ovariectomy (Fig. 5). Mononuclear leukocytes were the predominant cells adhered to the arteriolar endothelium of OVZ rats (Fig. 5), whereas in the postcapillary venules neutrophils were the predominant cells (Fig. 5). Despite these findings, no macroscopic or microscopic atherosclerotic lesions were detected in the aortas of sham-operated or OVZ females rats (Fig. 5).

To examine the potential contribution of ovariectomy to endothelial CAM up-regulation, we performed immunohistochemical studies on the mesenteric microvasculature. We have focused our study on P-selectin and VCAM-1 since these two endothelial CAMs are clearly up-regulated by Ang-II (15). In control sham-operated females, no arteriolar staining for P-selectin and VCAM-1 was detected (Fig. 6). These endothelial adhesion molecules were clearly up-regulated in OVZ animals (Fig. 6). Interestingly, chronic treatment of OVZ rats with estradiol or losartan inhibited P-selectin and VCAM-1 up-regulation in the arterial endothelium (Fig. 6). Similar results were observed in the postcapillary venules.
As previously done in humans, we next investigated the circulating levels of different CXC and CC chemokines. Ovariectomy induced significant increases in the levels of CINC/KC, MCP-1, and MIP-1/β2, while no significant differences were encountered between the basal levels of these circulating chemokines in non-operated female, sham-operated female, or male rats (Fig. 7). Chronic treatment with estradiol inhibited the increase of CINC/KC, MCP-1, and MIP-1/β2 caused by ovariectomy by 64%, 100%, and 69%, respectively (Fig. 7). Losartan treatment also reduced the plasma levels of these chemokines by 64%, 66%, and 89%, respectively (Fig. 7). The administration of both drugs resulted in further reductions in the levels of these chemokines by 64%, 66%, and 89%, respectively (Fig. 7). The administration of both drugs resulted in further reductions in the levels of these chemokines by 64%, 66%, and 89%, respectively (Fig. 7). Additionally, when an ACE inhibitor was given, a similar reduction to that detected in the losartan-treated group was observed (Fig. 7). Although plasma levels of RANTES were measurable, there were no significant differences in the amounts of this chemokine between the different groups (Fig. 7C). MIP-2 could not be detected in any of the plasma samples.

Additionally, while plasma cholesterol levels were increased in OVZ animals compared with sham-operated rats (170.7 ± 16.7 vs 121.2 ± 10.1 mg/dl, respectively; \( p < 0.05 \)), Ang-II plasma levels remained unchanged (20.2 ± 1.7 vs 22.8 ± 2.1 pg/ml, respectively).

**Decreased responsiveness to Ang-II in nonoperated female rats**

Since Ang-II seems to be involved in ovariectomy-induced leukocyte-endothelial cell interactions, we next investigated the leukocyte recruitment elicited by 1 nM Ang-II i.p. injection in non-operated female, OVZ female, and male rats. As illustrated in Fig. 8, 4 h after the i.p. administration of 1 nM Ang-II, no changes in arteriolar leukocyte adhesion in female animals (nonoperated and ovariectomized) were observed, whereas this parameter was significantly increased in male rats (Fig. 8). Of note, the administration of 1 nM Ang-II to male animals provoked a similar degree of leukocyte infiltration to that encountered in OVZ female rats injected with PBS (Fig. 8). Interestingly, it was necessary to increase 100-fold the dose of Ang-II to cause similar arteriolar leukocyte adhesion to that found in OVZ female or in 1 nM Ang-II-injected male rats (Fig. 8). A similar behavior was found in the postcapillary venules exposed for 4 h to this peptide hormone (Fig. 9).

**Discussion**

Endothelial function has been assessed primarily in terms of endothelium-dependent vasomotion, largely based on the assumption that impaired endothelium-dependent vasomotion reflects alterations of other functions of the endothelium as well. Indeed, endothelial dysfunction leads to a proinflammatory and prothrombotic phenotype of the endothelium (24) and thus provokes the attachment and the subsequent migration of leukocytes, events that are linked with the onset of the atherosclerotic lesion formation.
Estrogens reduce endothelial dysfunction. Therefore, to examine human leukocyte responses in menopause, the interactions of leukocytes from both premenopausal and postmenopausal women to unstimulated and Ang-II-stimulated arterial and venous endothelial cells was investigated using a dynamic in vitro model. Interestingly, we first found marked and significant differences in leukocyte rolling and adhesion when the blood was perfused across the arterial endothelium, while in HUVECs there were no significant differences between both women groups. In fact, this enhanced adhesiveness was encountered under both basal and stimulated conditions, and these effects were accompanied by significant increases in CD11b expression on monocytes from postmenopausal women.

Another striking observation of the present study is the effect of menopause on the circulating levels of different CXC and CC chemokines. Increased levels of IL-8, RANTES, and MIP-1α were encountered in the plasma of postmenopausal women. Although MCP-1 was also increased in this population, there were no significant differences with the levels found in the premenopausal group. Taken into account that the number of subjects in each group was not identical and was relatively small, it is likely that these differences reach significance in larger studies. Nevertheless, other studies have found increased plasmatic amounts of MCP-1 in postmenopausal women (25, 26).

Since there are not animal models showing the proinflammatory state developed after ovariectomy in the absence of other comorbidities, in this study we have found that ovariectomy in rats causes microvascular inflammation. This is of relevance for testing different therapeutic agents whose use in humans remains controversial at present. In this context, the low grade of systemic inflammatory response was characterized by leukocyte-endothelial cell interactions in the mesenteric arterioles, which resembled the responses found in the in vitro system using human samples. Additionally, increased expression of CAMs (P-selectin and VCAM-1) on the microvascular endothelium as well as of chemokines (CINC/KC, MCP-1, and MIP-1α) in the circulation were also detected. Despite these findings no even early atherosclerotic lesions were detected in the aortas of OVZ animals. Furthermore, arterial blood pressure and wall shear rate remained unchanged in all of the experimental groups.

Most of these results are in agreement with those found in postmenopausal women, since rats do not synthesize IL-8 or GROα but instead synthesize CINC/KC, which is widely assumed to be the rat functional homolog of human IL-8 (27). Indeed, KC seems to be more relevant than MCP-1 in inducing arterial mononuclear leukocyte recruitment (28). With regard to RANTES, in our study there are clear species differences in the RANTES pattern between postmenopausal women and ovariectomized rats. In this context, the circulating RANTES levels in humans are higher than those in rodents (nanogram range vs picogram range) (29, 30). Additionally, since it is now well known that platelets store RANTES in their α-granules and release it during acute stages of inflammation (31–33), it is also possible that the number of circulating platelets or the amount of RANTES stored in them differs between humans and rats. Therefore, the recruitment of mononuclear cells by the arterial endothelium may be the result of a cooperation between the CXCR2-stimulating mediators (CINC/KC/IL-8) and CC chemokines (MCP-1, MIP-1α, and RANTES in humans) acting at CXCR2,CCR1, and CCR5.

Taken together, these findings suggest that either menopause or ovariectomy causes a low grade of systemic inflammation that is characterized by increased adhesiveness of leukocytes, being in human samples mainly adhered to the arterial endothelium. However, the findings also indicate that postmenopausal women or ovariectomized animals might be more prone to the development of atherosclerotic lesion formation than are premenopausal subjects or nonovariectomized rats since inappropriate monocyte adhesion can affect arteriolar function in an adverse manner. Therefore, the coexistence of other risk factors for atherosclerosis with menopause may explain the increased incidence of cardiovascular disorders in this state in women.

Ovariectomy-induced responses were markedly reduced by chronic treatment with low doses of estradiol, losartan, or benazepril. Several clinical trials with menopausal HRT have demonstrated the reduction of soluble CAMs such as E-selectin, ICAM-1, and VCAM-1, and of circulating MCP-1 levels (5, 6, 26). However, there have been fewer observations on the effects of estrogens on cellular recruitment. Our results show that estradiol inhibits the leukocyte/endothelial interactions associated with estrogen deficiency and decreased the expression of P-selectin and VCAM-1 in the mesenteric microvasculature. Moreover, these inhibitory effects were achieved with low-dose estrogen treatment, suggesting that this potential therapy at early stages of estrogen deficiency may prevent leukocyte adhesion and reduce the risk of suffering further cardiovascular diseases.

Estrogens affect the renin-angiotensin system at different points of the cascade (9–12, 34, 35), and we now report that inhibition of the renin-angiotensin system improves the microvascular inflammation associated with estrogen deficiency. Either losartan or benazepril were capable of inhibiting ovariectomy-induced leukocyte-endothelial cell interactions and associated changes in chemokines and adhesion molecules. This inhibition was achieved at doses lacking a hypotensive effect and were of similar degree to that seen with estradiol. Interestingly, the effect of combined estrogens and losartan was greater than that of losartan or estradiol in most of the parameters measured. In agreement with our data, ovariectomy in spontaneously hypertensive female rats leads to increased Ang-II-induced responses via increased vascular AT1 receptor expression, and estrogen administration prevents these pathological changes (10). Additionally, we have found no differences in the circulating levels of Ang-II between the estrogen-deficient and the estrogen-replete subjects. These findings are consistent with previous studies in which tissue Ang-II levels were increased in postmenopausal women without changes in Ang-II circulating levels (36, 37).

Angiotensin-dependent inflammatory mechanisms have also been implicated in hypercholesterolemia-associated inflammation (38, 39) and small, but increased plasma levels of cholesterol have been found in both postmenopausal women and ovariectomized rats. First, it is noteworthy that the levels of plasma cholesterol are in the normal range for healthy postmenopausal women. In fact, our group of postmenopausal women has even a better lipid profile than that shown in other studies for healthy postmenopausal women (40–43). Second, it is well known that the administration of an ACE inhibitor or an AT1 receptor antagonist do not affect the lipid profile. Furthermore, some studies have reported that a reduction in total and LDL cholesterol is unrelated to endothelial function according to the results of a multivariate analysis (44), and it has been suggested that nonlipid-related mechanisms contribute to the cardioprotective effects of HRT. Therefore, it is likely that blockade of the renin-angiotensin system could be beneficial in improving endothelial dysfunction associated with menopause regardless of the moderated hypercholesterolemia associated with women in this state.

Finally, sexual dimorphism has been observed in the development of cardiovascular disease (45–47). In agreement with these observations, we have found that while nonoperated female rats do...
not shown enhanced leukocyte-endothelial cell interactions in response to 1 nM Ang-II, males do. Indeed, a 100-fold increase in the dose of Ang-II was necessary to find a similar degree of leukocyte recruitment in nonoperated female rats to that encountered in male animals. Furthermore, ovariectomized animals show similar leukocyte recruitment in the mesenteric arterioles to that found in male animals exposed to 1 nM Ang-II for 4 h. It is noteworthy that these results are also consistent with the results obtained in the in vitro model of leukocyte-endothelial cell interactions used in the present study, since leukocytes from premenopausal women were less adhesive to Ang-II-stimulated HUAECs than were leukocytes from postmenopausal women.

In conclusion, using whole blood from healthy premenopausal and postmenopausal women we have found increased adhesive-ness of leukocytes to the arterial endothelium and circulating lev-

els of different chemokines such as IL-8, MCP-1, RANTES, and MIP-1α in the postmenopausal group. The human data led us to set up a rat model of estrogen depletion by ovariectomy to study the associated microvascular inflammation. Increased leukocyte adhes-

ion to the arterial endothelium, CAM expression, and circulating chemokine levels were also detected in OVZ rats without affecting arterial blood pressure. These results provide further evidence to the possible link between estrogen deficiency and the subsequent low grade of systemic inflammation. Chronic administration of low doses of estradiol or inhibition of the renin-angiotensin system just after ovariectomy dramatically reduced this inflammatory state. Therefore, the administration of low doses of estrogens in monotherapy or, for a wider section of the population, inhibition of the renin-angiotensin system may reduce the systemic inflammation that occurs in menopause and decrease the risk of suffering further cardiovascular diseases.

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Disclosures

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