Trans- but Not *Cis*-Resveratrol Impairs Angiotensin-II–Mediated Vascular Inflammation through Inhibition of NF-κB Activation and Peroxisome Proliferator-Activated Receptor-γ Upregulation

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Angiotensin II (Ang-II) displays inflammatory activity and is implicated in several cardiovascular disorders. This study evaluates the effect of *cis*- and *trans* (*t*)-resveratrol (RESV) in two in vivo models of vascular inflammation and identifies the cardioprotective mechanisms that underlie them. In vivo, Ang-II–induced arteriolar leukocyte adhesion was inhibited by 71% by *t*-RESV (2.1 mg/kg, i.v.), but was not affected by *cis*-RESV. Because estrogens influence the rennin-angiotensin system, chronic treatment with *t*-RESV (15 mg/kg/day, orally) inhibited ovariectomy-induced arteriolar leukocyte adhesion by 81%, partly through a reduction of cell adhesion molecule (CAM) expression and circulating levels of cytokine-induced neutrophil chemoattractant, MCP-1, and MIP-1α. In an in vitro flow chamber system, *t*-RESV (1–10 μM) undermined the adhesion of human leukocytes under physiological flow to Ang-II–activated human endothelial cells. These effects were accompanied by reductions in monocyte and endothelial CAM expression, chemokine release, phosphorylation of p38 MAPK, and phosphorylation of the p65 subunit of NF-κB. Interestingly, *t*-RESV increased the expression of peroxisome proliferator-activated receptor-γ in human endothelial and mononuclear cells. These results demonstrate for the first time that the in vivo anti-inflammatory activity of RESV is produced by its *t*-RESV, which possibly interferes with signaling pathways that cause the upregulation of CAMs and chemokine release. Upregulation of proliferator-activated receptor-γ also appears to be involved in the cardioprotective effects of *t*-RESV. In this way, chronic administration of *t*-RESV may reduce the systemic inflammatory response associated with the activation of the rennin-angiotensin system, thereby decreasing the risk of further cardiovascular disease. ***The Journal of Immunology*****, 2010, 185: 3718–3727.**

An abundance of epidemiological data confirms a correlation between a low incidence of coronary heart disease and atherosclerosis and the moderate consumption of red wine (1, 2). It has been suggested that this cardioprotective effect is due to the polyphenol fraction (3). Resveratrol (RESV) is a natural polyphenolic compound found in red wine; it is a 3,4′,5-trihydroxystilbene (4) and exists as *cis* and *trans* isomers (5). A wide range of RESV concentrations has been reported in different wines (6). In addition to red wine, RESV is present in grapes, peanuts, and mulberries. Its major biological functions include free-radical scavenging, inhibition of platelet aggregation, anti-inflammatory activity, vasorelaxing activity, increased expression of endothelial NO synthase, modulation of lipid metabolism, and anticancer activity (4, 7, 8). It also displays estrogenic and anti-estrogenic activity, although its affinity for the estrogen receptor is low (9–11). However, despite the extensive body of evidence available, the full nature and extent of the cardiovascular effects of RESV are yet to be determined.

Angiotensin-II (Ang-II), the main effector peptide of the renin-angiotensin system, is implicated in atherogenesis in a way that goes beyond its hemodynamic effects (12). We have previously demonstrated that 4-h exposure to Ang-II in vivo causes arteriolar leukocyte adhesion in the rat mesenteric microcirculation, an effect that is mediated through interaction with its AT_1 receptor subtype (13). In that study, Ang-II selectively promoted the adhesion of mononuclear cells to the arteriolar endothelium despite the fact that the same pattern of cell adhesion molecule (CAM) expression was encountered in both the arteriolar and venular endothelia (13). Interestingly, Ang-II–induced mononuclear leukocyte recruitment was shown to be a consequence of the generation and release of the CXC chemokine IL-8 and various CC chemokines

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Abbreviations used in this paper: Ang-II, angiotensin-II; CAM, cell adhesion molecule; CINC/KC, cytokine-induced neutrophil chemoattractant; *c*-RESV, *cis*-resveratrol; HPBMC, human PBMC; HRT, hormone replacement therapy; HUAEC, human umbilical arterial endothelial cell; MABP, mean arterial blood pressure; OVZ, ovariectomized; p.o., orally; PPARγ, proliferator-activated receptor-γ; RESV, resveratrol; ROS, reactive oxygen species; *t*-RESV, *trans*-RESV.

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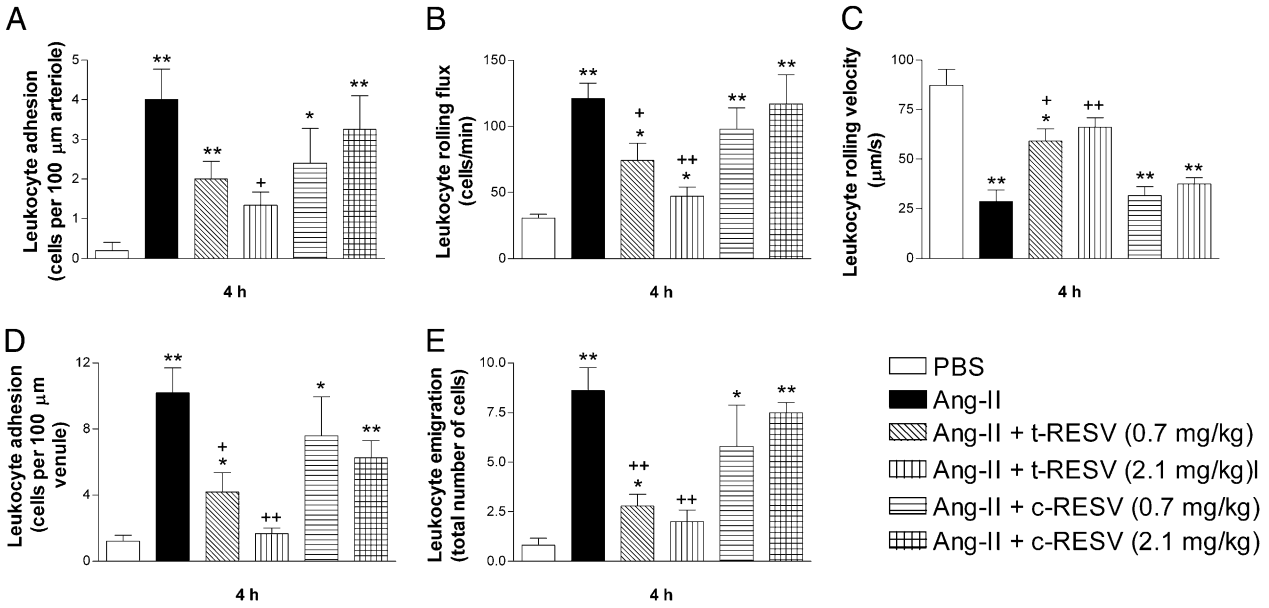

FIGURE 1. Effect of *t*- and *c*-RESV on subacute (4-h) Ang-II–induced leukocyte responses in rat mesenteric arterioles (*A*) and postcapillary venules (*B–E*). Rats were treated i.p. with PBS (buffer, $n = 5$) or 1 nM Ang-II ($n = 5$). Some of these animals were pretreated with *t*-RESV or *c*-RESV (0.7 or 2.1 mg/kg, i.v.) 15 min before administration of Ang-II. Results are expressed as mean ± SEM. $*p < 0.05$ or $**p < 0.01$ relative to the PBS group; $+p < 0.05$ or $++p < 0.01$ relative to the Ang-II group.

(14, 15). More recently, we have demonstrated that this selective mononuclear cell adhesion to Ang-II–stimulated arterioles is largely mediated by TNF-α in cooperation with constitutive IL-4 (16).

Estrogens influence the rennin-angiotensin system by regulating the synthesis of angiotensinogen in hepatocytes (17). Plasma rennin levels and angiotensin-converting enzyme activity are significantly higher in estrogen-deficient versus estrogen-replete rats, and in postmenopausal women not receiving hormone replacement therapy (HRT) versus those receiving it (18, 19). In addition to regulating the components involved in synthesizing Ang-II, estrogens also alter the expression of Ang-II AT_1 receptors in many target tissues (20, 21). In this context, we have encountered an increase in leukocyte adhesion to the arterial endothelium, CAM expression, and circulating chemokine levels in ovariectomized (OVZ) rats without parallel changes in arterial blood pressure (22), indicating that estrogen deficiency results in a low grade of systemic inflammation. Furthermore, chronic administration of low doses of estradiol or inhibition of the rennin-angiotensin system immediately after ovariectomy dramatically reduced this inflammatory state (22). HRT is no longer prescribed, and so new and safer drugs that ameliorate the systemic inflammation that is characteristic of the menopause are essential. Moreover, they would reduce the risk of suffering further cardiovascular complications.

In light of the above mentioned, the aim of the current study was to evaluate the effect of *cis*-RESV (*c*-RESV) and *trans*-RESV (*t*-RESV) on Ang-II–induced leukocyte-endothelial cell interactions in vivo. Because *c*-RESV did not display any significant response in this system, we next investigated the protective effect of chronic treatment with *t*-RESV in an animal model of estrogen deficiency. To extend these findings to humans, we characterized the effect of *t*-RESV on the leukocyte interaction with Ang-II–stimulated arterial and venous endothelia under dynamic flow conditions and its influence on the release of different chemokines and CAM expression. Finally, we set out to determine the mechanisms that contribute to the cardioprotective effects of *t*-RESV.

Materials and Methods

Animal studies

Animal preparation. Male and female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Barcelona, Spain) and fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Ethics Committee of the University of Valencia, and the research complied with the *Guide for the Care and Use of Laboratory Animals*.

Intravital microscopy. The details of the experimental preparation have been described previously (13). In short, 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 optically clear-viewing pedestal 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% CO_2 in nitrogen. An orthostatic microscope (Nikon Optiphot-2, SMZ1) equipped with a ×20 objective lens (Nikon SLDW) and a ×10 eyepiece permitted tissue vi-

Table I. *Hemodynamic parameters*

	MABP (mm Hg)	Arteriolar Shear Rate (s^{-1})	Venular Shear Rate (s^{-1})
PBS	117.8 ± 1.0	1259.2 ± 145.6	533.2 ± 63.2
Ang-II (1 nM)	116.2 ± 3.8	1144.5 ± 174.8	525.1 ± 38.3
Ang-II + *t*-RESV (0.7 mg/kg)	109.6 ± 5.7	1090.1 ± 102.5	540.9 ± 30.2
Ang-II + *t*-RESV (2.1 mg/kg)	112.7 ± 3.8	1115.6 ± 245.2	531.6 ± 65.8
Ang-II + *c*-RESV (0.7 mg/kg)	120.8 ± 7.9	1117.1 ± 160.9	554.0 ± 24.4
Ang-II + *c*-RESV (2.1 mg/kg)	106.5 ± 6.4	1079.9 ± 94.0	522.6 ± 37.7

Parameters (mean ± SEM in animals used for intravital microscopy studies, $n = 5$ rats per group) were measured 4 h after i.p. injection of PBS or Ang-II (1 nM) in animals untreated or pretreated with *t*-RESV or *c*-RESV (0.7 or 2.1 mg/kg, i.v.).

Table II. *Uterine weight and hemodynamic parameters*

	Uterine Weight (g)	MABP (mm Hg)	Arteriolar Shear Rate (s^{-1})	Venular Shear Rate (s^{-1})
Nonoperated females	0.47 ± 0.03	109.0 ± 8.2	1168.6 ± 185.3	491.9 ± 68.7
Sham-operated females	0.44 ± 0.02	112.0 ± 3.1	1135.7 ± 165.7	578.6 ± 48.0
OVZ females	0.13 ± 0.01*	107.8 ± 3.8	1144.8 ± 109.9	566.7 ± 55.9
OVZ females + *t*-RESV (5 mg/kg/day)	0.10 ± 0.01*	104.2 ± 4.9	1228.9 ± 147.1	511.2 ± 54.5
OVZ females + *t*-RESV (15 mg/kg/day)	0.11 ± 0.01*	105.2 ± 4.9	1205.2 ± 97.6	543.0 ± 46.2

Parameters (mean ± SEM in animals used for intravital microscopy studies, n = 5–6 rats per group) were measured in the following experimental groups: nonoperated female rats, sham-operated female rats, female rats after 30 d postovariectomy (OVZ female), OVZ rats treated with *t*-RESV (5 mg/kg/day, OVZ female + *t*-RESV), and OVZ rats treated with *t*-RESV (15 mg/kg/day, OVZ + *t*-RESV). *$p < 0.01$ relative to the sham-operated female group.

sualization. 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 (the final original magnification of the video screen was ×1300). The diameter of the arterioles (15–30 μm diameter) and single unbranched mesenteric venules (25–40 μm diameter) was measured online using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline RBC velocity was also measured online with an optical Doppler velocimeter (Microcirculation Research Institute). Vascular blood flow and wall shear rate were calculated, as previously described (23). The number of rolling, adherent, and emigrated leukocytes was determined off-line during playback analysis of videotaped images.

Experimental protocol. Male rats were sedated and injected i.p. with 5 ml PBS or Ang-II (1 nM). After 4 h, the mesentery was exposed in preparation for measurement of venular leukocyte rolling flux, velocity, adhesion, emigration, arteriolar leukocyte adhesion, mean arterial blood pressure (MABP), venular and arteriolar RBC velocity, shear rate, and diameter. These measurements were performed over a 5-min period. To investigate the effect of *t*- and *c*-RESV in the leukocyte-endothelial cell interactions induced by Ang-II, rats were pretreated 15 min prior to Ang-II injection with *t*- or *c*-RESV (0.7 mg/kg or 2.1 mg/kg, i.v.). The dose of 0.7 mg/kg i.v. *t*-RESV was shown to be effective in another in vivo model of acute inflammation (24).

Because *c*-RESV was not effective in inhibiting the leukocyte-endothelial cell interactions induced by Ang-II, and given that estrogenic activity has been attributed to RESV, we performed an additional set of experiments in which a number of female rats were anesthetized with ~3% isoflurane and underwent bilateral ovariectomization. Some of these rats were chronically treated with *t*-RESV for 1 mo; it was administered via their drinking water, dissolved in 0.5% carboxymethyl cellulose, as previously described (25). A group of sham-operated female rats was also assessed. All measurements were performed 30 d after ovariectomy. The success of ovariectomy was determined by measuring uterine weight.

The following experimental groups were designated: 1) untreated female rats, 2) sham-operated female rats, 3) female rats 30 d postovariectomy (OVZ), 4) OVZ rats treated with *t*-RESV (5 mg/kg/day; OVZ + *t*-RESV), and 5) OVZ rats treated with *t*-RESV (15 mg/kg/day; OVZ + *t*-RESV).

Immunohistochemistry. After 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, E-selectin, and VCAM-1 using a modified avidin and biotin immunoperoxidase technique, as previously described (15). Tissue sections (4 μm thick) were incubated with the following Abs for 24 h at 200 μg/ml: anti-rat P-selectin mAb RP-2, anti-rat E-selectin mAb (RME-1), or anti-rat VCAM-1 mAb 5F10, or their isotype-matched control Ab MOPC 21 (murine IgG1) or UPC 10 (murine IgG2a). Positive staining was defined as an arteriole- or venule-displaying brown reaction product.

Human studies

All research with human samples in the current study complied with the principles outlined in the *Declaration of Helsinki* and was approved by the institutional ethics committee of the University Clinic Hospital of Valencia (Valencia, Spain). Written informed consent was obtained from all volunteers.

Cell culture. HUVECs and human umbilical arterial endothelial cells (HUAECs) were isolated by collagenase treatment (26) and maintained in human endothelial cell specific medium (endothelial basal medium-2, EBM-2) supplemented with endothelial growth media (EGM-2) and 10% FCS. Cells were grown to confluence in 24-well culture plates up to passage 2. Prior to every experiment, cells were incubated for 16 h in medium containing 1% FCS and were then returned to the 10% FCS medium before incubation.

Leukocyte-HUVEC and -HUAEC interactions under flow conditions. HUAECs and HUVECs were grown to confluence and stimulated with 1 μM Ang-II for 4 h up to passage 2. Cells were incubated with *t*-RESV (1 or 10 μM; DMSO, final concentration of 0.1%) 1 h prior to stimulation

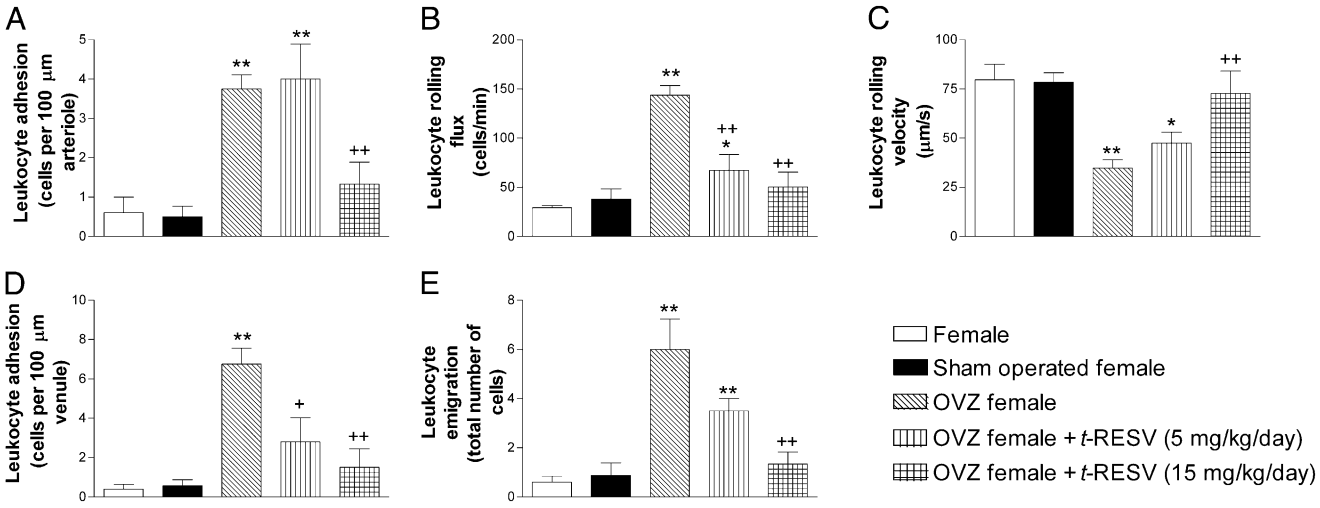

FIGURE 2. Effect of *t*-RESV on ovariectomy-induced leukocyte responses in the rat mesenteric microvessels (*A–E*). Parameters were measured in the following experimental groups: nonoperated female rats, sham-operated female rats, female rats after 30-d postovariectomy (OVZ female), OVZ rats treated with *t*-RESV (5 mg/kg/day, OVZ female + *t*-RESV), and OVZ rats treated with *t*-RESV (15 mg/kg/day, OVZ + *t*-RESV). Results are mean ± SEM for n = 5–6 animals per group. *$p < 0.05$ or **$p < 0.01$ relative to the sham-operated female group; +$p < 0.05$ or ++$p < 0.01$ relative to the OVZ female group.

with Ang-II (1 μM). Human PBMCs (HPBMCs) and neutrophils were obtained from buffy coats of healthy donors by Ficoll-Hypaque density gradient centrifugation, as previously described (16). The Glycotech flow chamber was assembled and placed onto an inverted microscope stage, and diluted whole blood from healthy volunteers (1/10 in HBSS) or freshly isolated HPBMCs or neutrophils (1×10^6/ml) were then perfused across the endothelial monolayers. In all experiments, leukocyte interactions were determined after 5 min at 1 dyn/cm^2. Cells interacting on the surface of the endothelium were visualized and recorded (×20 objective, ×10 eyepiece) using phase-contrast microscopy.

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 stimulated with 1 μM Ang-II for 4 h. Some samples were treated with *t*-RESV (0.1–10 μM) 1 h prior to stimulation with Ang-II. Then they were incubated on ice in the dark for 20 min with saturating amounts (10 μl) of the conjugated mAb anti-human CD11b/CD18 FITC. RBCs were lysed and leukocytes were fixed using an automated EPICS Q-PREP system (Coulter Electronics, Hialeah, Florida). Samples were run in an EPICS XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL). The expression of the surface Ag (FITC fluorescence) was measured in neutrophils, and monocytes were identified by their specific features of size (forward scatter) and granularity (side scatter) in the flow cytometer.

Chemokine levels in rat whole blood and determination of chemokine release from HUAECs, HUVECs, and HPBMCs. Once intravital microscopy measurements had been performed, 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), MCP-1 (MCP-1/CCL2), and MIP-1α (CCL3) ELISAs, as previously described (15).

In another set of experiments, HUAECs, HUVECs, and freshly isolated HPBMCs (5×10^6/ml) were incubated for 4 h with saline or 1 μM Ang-II. Because Ang-II causes the release of the human chemokines IL-8, MCP-1, and RANTES from endothelial cells and IL-8 and MIP-1α from HPBMCs (15, 27), *t*-RESV (0.1–10 μM) was added to some of the wells 1 h prior to Ang-II (1 μM) stimulation. At the end of the experiment, cell-free supernatants were stored at −20°C for chemokine ELISA.

Immunofluorescence. ICAM-1, VCAM-1, and E-selectin were visualized in HUAECs and HUVECs by immunofluorescence. To summarize, confluent endothelial cells were grown on glass coverslips and stimulated with 1 μM Ang-II for 4 h. Some coverslips were incubated with *t*-RESV (1 or 10 μM) 1 h prior to stimulation with Ang-II (1 μM). The cells were then washed with PBS, fixed with 4% paraformaldehyde, and blocked in a 1% PBS/BSA solution. They were subsequently incubated overnight at 4°C with the primary Abs against ICAM-1 (dilution 1/250), VCAM-1 (dilution 1/250), or E-selectin (dilution 1/250) in a 0.1% PBS/BSA solution. Following this, they were incubated again for 1 h at room temperature with an Alexa Fluor 488-conjugated secondary Ab diluted 1/1000 in 0.1% PBS/BSA. Cell nuclei were counterstained with DAPI. Images were captured with an Olympus CAMEDIA-C5060 wide zoom digital camera mounted on an Axiolab stereomicroscope. FITC fluorescence quantification relative to DAPI was carried out using MetaMorph software (MDS Analytical Technologies, Toronto, Ontario, Canada).

Western blot. The effect of *t*-RESV on Ang-II–induced p38-MAPK (p38MAPK) or p65 phosphorylation was determined. HUAECs and HUVECs were incubated for 1 h with saline or 1 μM Ang-II. Cells were incubated with *t*-RESV (1 or 10 μM) 1 h prior to Ang-II (1 μM) stimulation. A similar procedure was used to determine the effect of *t*-RESV on peroxisome proliferator-activated receptor (PPAR) γ upregulation. In this case, endothelial cells and freshly isolated HPBMCs (5×10^6/ml) were stimulated for 4 h with Ang-II (1 μM). After treatment, cells were washed, scraped, collected, and centrifuged at 45,000 × *g* at 4°C for 30 min to yield the whole extract. Protein content was determined by the Bradford method. Samples were denatured, subjected to SDS-PAGE using 10% running gel, and transferred to nitrocellulose membrane. Membranes were blocked with 5% dried milk in TBS containing 0.05% Tween 20, and were then incubated overnight with the corresponding Abs following the manufacturer's recommendations. The blots were washed with a wash buffer (PBS, 0.2% Tween 20) three times for 15 min each time. They were then incubated for 1 h with a secondary HRP-linked anti-rabbit IgG Ab (Cell Signaling Technology, Beverly, MA) and developed using the ECL procedure, as specified by the manufacturer.

In another group of experiments, the effect of *t*-RESV on Ang-II–induced p38MAPK and p65 phosphorylation was determined in HUAECs and HUVECs by flow cytometry and following the protocols described above. The endothelial cells were then fixed and permeabilized with BD Cytofix/Cytoperm solution and were sequentially stained with a 1/100 dilution of PE mouse anti-p65 (pS529) mAb and a 1/100 dilution of Alexa Fluor mouse anti-p38MAPK (pT80/pY182) mAb. Cells were analyzed using a BD FACSCanto Flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis

Group data were submitted to a one-way ANOVA with a Newman-Keuls post hoc correction for multiple comparisons. For human studies, a Student *t* test was employed. All values are reported as mean ± SEM. Statistical significance was determined as $p < 0.05$.

Materials

Ang-II, β-actin, pentobarbital, MOPC21, *t*-RESV, and UPC 10 were purchased from Sigma-Aldrich (Madrid, Spain). Human and rat chemokines, and Abs for all rat chemokine ELISAs were acquired from PeproTech (London, U.K.). The Ab pairs for all human chemokine ELISAs were purchased from R&D Systems (Madrid, Spain). Sodium heparin (5000 U/ml or 50 mg/ml) was from Pharmaceutical Laboratories Rovi SA (Madrid, Spain). Neutravidin-HRP was supplied by Perbio Science (Cheshire, U.K.) and the K-Blue substrate by Neogen (Lexington, KY). The Abs

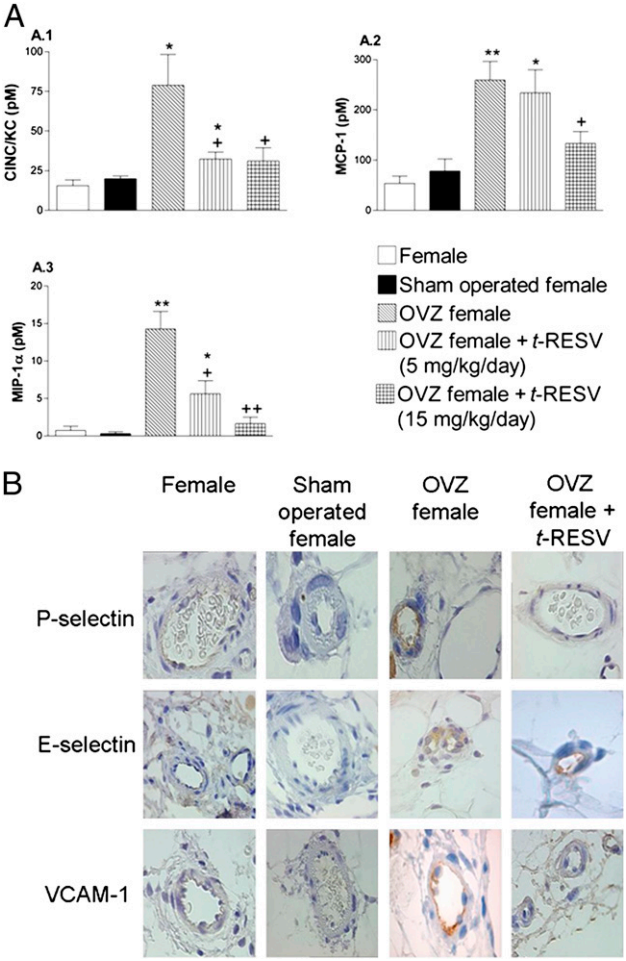

FIGURE 3. Effect of *t*-RESV on the ovariectomy-induced increase in circulating chemokine levels (*A*) and P-selectin, E-selectin, and VCAM-1 expression in the rat mesenteric arterioles (*B*). Plasma levels of CINC/KC (*A.1*), MCP-1 (*A.2*), and MIP-1α (*A.3*) were measured by ELISA. Results (pM in the plasma) were measured in the same rats as those described in the legend of Fig. 2. Results are expressed as mean ± SEM for $n = 5$–6 animals per group. $*p < 0.05$ or $**p < 0.01$ relative to the sham-operated female group; $+p < 0.05$ or $++p < 0.01$ relative to the OVZ female group. Some mesenteries were fixed for staining with anti–P-selectin, anti–E-selectin, and VCAM-1 Abs (*B*). Brown reaction product indicates positive immunoperoxidase localization on the vascular endothelium. All panels are lightly counterstained with hematoxylin. Results are representative of $n = 5$–6 experiments with each treatment.

RP-2, RME-1, and 5F10 were obtained, as previously stated (13). The mouse monoclonal anti-human CD11b FITC (clone ICRF 44) and primary Abs against human ICAM-1, VCAM-1, and E-selectin were from Serotec (Madrid, Spain). *c*-RESV was prepared at the Departamento de Química Orgánica (Universidade de Santiago de Compostela, Spain), following the method previously described (7). DAPI and Alexa Fluor 488-conjugated secondary Ab were from Molecular Probes-Invitrogen (Carlsbad, CA). Primary Abs p-38 MAPK, phospho-p38MAPK (Thr^{180}/Tyr^{182}), NF-κB p65 (c-20), phosph-p65 (Ser^{536}), and the secondary HRP-linked anti-rabbit IgG Ab were supplied by Cell Signaling Technology. The Ab against PPARγ (H-100) was from Santa Cruz Biotechnology (Santa Cruz, CA). ECL developer was purchased from Amersham Biosciences (Barcelona, Spain). BD Cytofix/Cytoperm solution, PE mouse anti-p65 (pS529) mAb (clone K10-895.12.50), and Alexa Fluor mouse anti-p38MAPK (pT80/pY182) mAb (clone 36/p38; pT180/pY182) were from BD Biosciences.

Results

t-*RESV inhibits the leukocyte-endothelial cell interactions induced by Ang-II within the rat mesenteric microcirculation, whereas* c-*RESV does not*

Intravital microscopy was used to evaluate the effect of *t*- and *c*-RESV on Ang-II–induced leukocyte trafficking in the mesentery. Exposure to 1 nM Ang-II for 4 h caused a significant enhancement of arteriolar leukocyte adhesion (Fig. 1*A*). This enhanced adhesion was significantly inhibited by *t*-RESV at a dose of 2.1 mg/kg (71% inhibition), which was three times higher than that previously used in another in vivo model of acute inflammation (24). In contrast, *c*-RESV produced no significant effect on this parameter (Fig. 1*A*).

In the postcapillary venules of the same animals, 4-h exposure to Ang-II induced a significant increase in venular leukocyte rolling flux, adhesion, and emigration, and a concomitant decrease in venular leukocyte rolling velocity (Fig. 1). Interestingly, pretreatment with *t*-RESV, but not with *c*-RESV, significantly reduced Ang-II–induced leukocyte-endothelial cell interactions at the two doses tested (Fig. 1). Neither MABP nor arteriolar or venular shear rates were affected by these treatments (Table I).

Ovariectomy induces microvascular inflammation, which is prevented by chronic treatment with t-*RESV*

Estrogens affect the rennin-angiotensin system at different points of the cascade (17–19, 21, 28, 29), and we have recently reported that inhibition of the rennin-angiotensin system improves the microvascular inflammation associated with estrogen deficiency (22). In addition, several studies have attributed estrogenic properties to RESV (9–11). In light of this evidence, we next investigated the effect of chronic administration of *t*-RESV (orally [p.o.]) in an animal model of estrogen deficiency. Ovariectomy caused a significant reduction in the uterine weight of rats with respect to that of

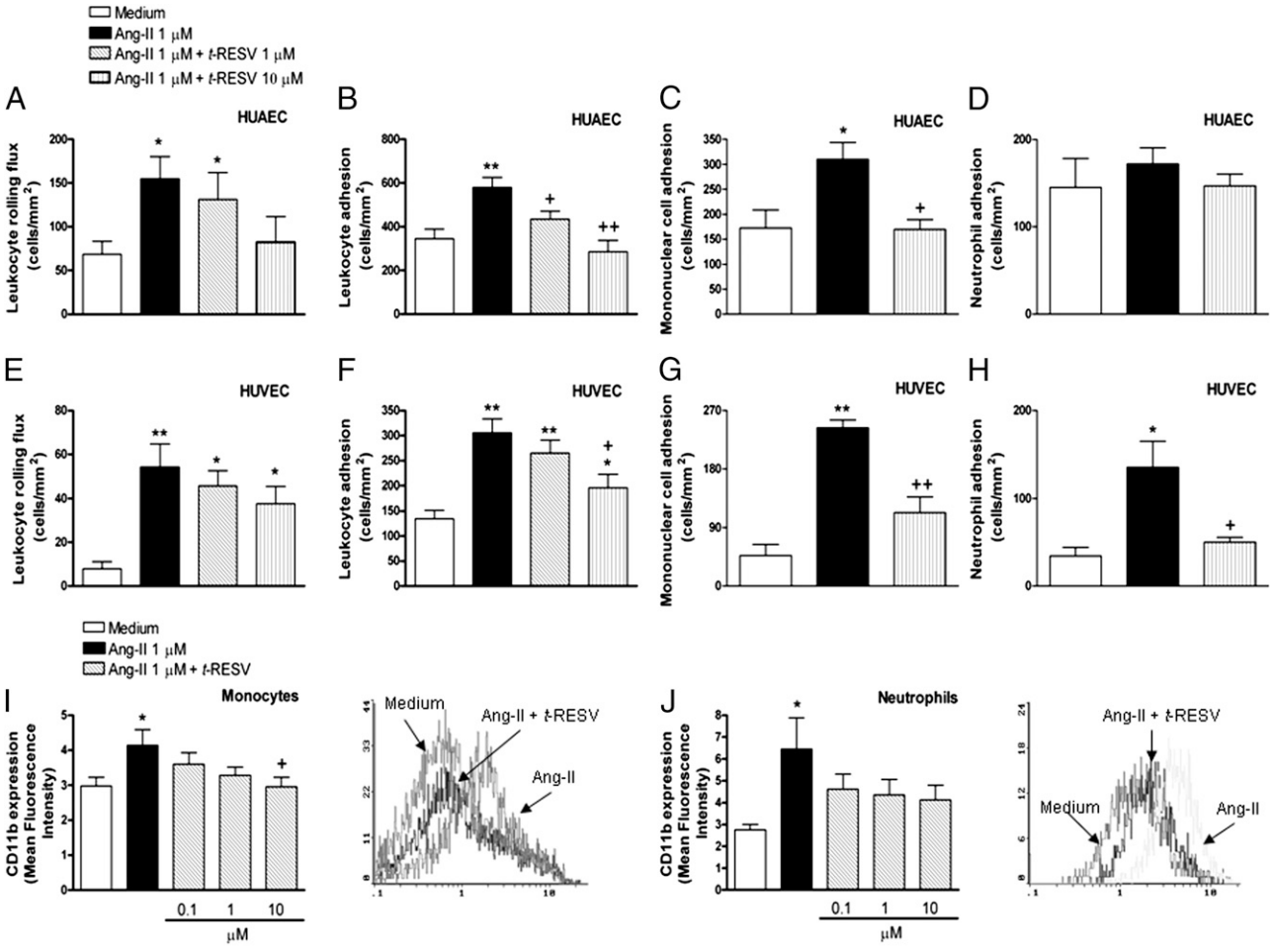

FIGURE 4. Effect of *t*-RESV on leukocyte-endothelial cell interactions induced by Ang-II–stimulated endothelial cells and CD11b expression in human leukocytes. HUAECs and HUVECs were incubated for 4 h with medium or Ang-II (1 μM). Some cells were pretreated with 1 or 10 μM *t*-RESV for 1 h prior to Ang-II stimulation. Whole blood from healthy volunteers, freshly isolated HPBMCs (mononuclear cells), or neutrophils (1×10^6/ml) was perfused over the endothelial monolayers for 5 min at 1 dyn/cm^2, and leukocyte rolling (*A*, *E*) and adhesion (*B–D*, *F–I*) were quantified. Human whole blood was also stimulated for 4 h with Ang-II (1 μM). Some samples were pretreated with 1-10 μM *t*-RESV before Ang-II stimulation. Then leukocytes were stained with a conjugated mAb for CD11b and analyzed by flow cytometry (*J*, *K*). Results are the mean ± SEM for $n = 4$–9 experiments. $*p < 0.05$ or $**p < 0.01$ relative to values in the control group; $+p < 0.05$ or $++p < 0.01$ relative to the 1 μM Ang-II group.

nonoperated and sham-operated animals (Table II). Chronic 1-mo treatment with *t*-RESV had no affect on this loss of uterine mass (Table II).

In line with previously reported data (22), OVZ rats exhibited a higher level of leukocyte adhesion to the arteriolar endothelium than sham-operated animals (Fig. 2*A*). *t*-RESV at the highest dose assayed inhibited this response by 81%, whereas the lowest dose produced no significant activity (Fig. 2*A*).

Ovariectomy provoked a significant increase in leukocyte recruitment in the postcapillary venules (Fig. 2*B–D*). When OVZ animals were treated with *t*-RESV, ovariectomy-induced venular leukocyte-endothelial cell interactions were inhibited in a dose-dependent manner (Fig. 2). None of these treatments affected MABP, arteriolar, or venular shear rate (Table II).

Given that chemokines are involved in the regulation of leukocyte trafficking (30), we decided to determine the circulating levels of different CXC and CC chemokines. Ovariectomy caused significant increases in the levels of CINC/KC, MCP-1, and MIP-1α (Fig. 3*A*). Chronic treatment with *t*-RESV at 15 mg/kg/day inhibited the increase of CINC/KC, MCP-1, and MIP-1α caused by ovariectomy by 81, 70, and 91%, respectively (Fig. 3*A*). At the lowest dose assayed, *t*-RESV did not affect circulating plasma levels of MCP-1 (Fig. 3*A.2*).

To examine the effect of *t*-RESV on ovariectomy-induced endothelial CAM up-regulation, we performed immunohistochemical studies of the mesenteric microvasculature. P-selectin and VCAM-1 were clearly up-regulated in OVZ rats, whereas E-selectin was expressed only weakly (Fig. 3*B*). Interestingly, chronic treatment of these animals with *t*-RESV (15 mg/kg/day, p.o.) inhibited P-selectin and VCAM-1 upregulation in the arterial endothelium, but did not affect E-selectin expression (Fig. 3*B*). Similar results were observed in postcapillary venules.

t-*RESV inhibits leukocyte adhesiveness to Ang-II–stimulated HUAECs and HUVECs and Ang-II–induced CD11b upregulation*

To extend these findings to humans, whole blood from healthy volunteers was perfused across unstimulated or Ang-II–stimulated HUAECs and HUVECs for 5 min at a shear force of 1 dyn/cm^2. Fig. 4 shows leukocyte rolling (Fig. 4*A*, 4*E*) and adhesion (Fig.

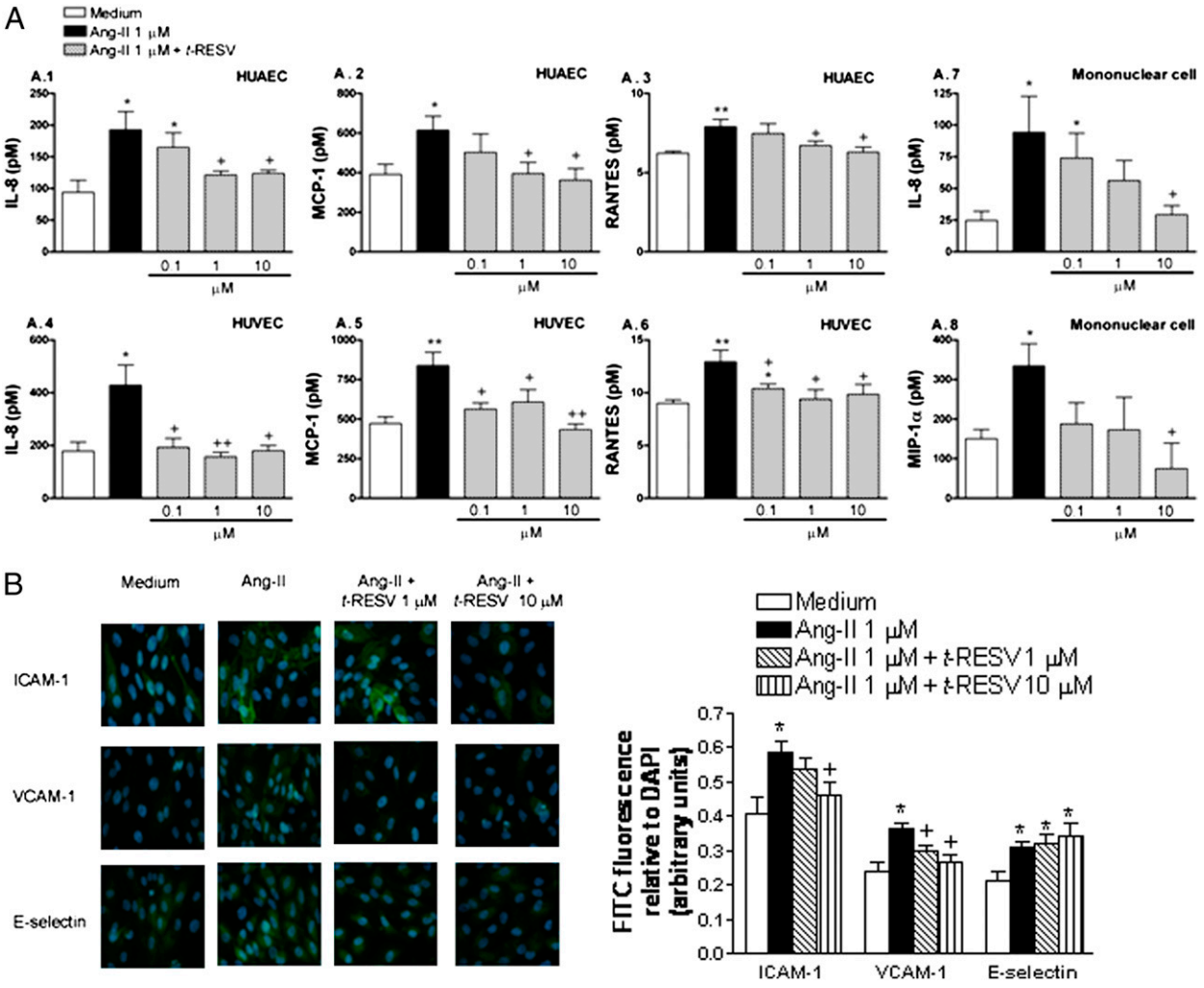

FIGURE 5. Effect of *t*-RESV on Ang-II–induced IL-8, MCP-1, and RANTES release from HUAECs and HUVECs and IL-8 and MIP-1α release from HPBMCs (*A*). Effect on Ang-II–induced ICAM-1, VCAM-1, and E-selectin expression in HUAECs (*B*). Cells were stimulated with Ang-II (1 μM) for 4 h. Some cells were pretreated with 1–10 μM *t*-RESV prior to Ang-II stimulation. The release of chemokines (pM in the cell supernatant) in response to Ang-II was measured by ELISA and is expressed as mean ± SEM of $n = 4$–6 experiments: $*p < 0.05$ or $**p < 0.05$ relative to values in the control group; $+p < 0.05$ or $++p < 0.01$ relative to the 1 μM Ang-II group. Following a similar protocol, ICAM-1, VCAM-1, and E-selectin were visualized in nonpermeabilized HUAECs by immunofluorescence (green). Nuclei were counterstained with DAPI. Results are representative of $n = 3$ experiments with each treatment. Images were captured and FITC fluorescence quantification of DAPI was performed using MetaMorph software. $*p < 0.05$ relative to values in the control group; $+p < 0.05$ relative to the 1 μM Ang-II group.

4*B*, 4*F*) in either the medium or 4-h Ang-II–treated HUAECs and HUVECs. Significant increases were observed in leukocyte-endothelial cell interactions when whole blood was perfused across both endothelia (Fig. 4*A*, 4*B*, 4*E*, 4*F*), but was more pronounced in the Ang-II–stimulated arterial endothelium. When mononuclear cells were perfused across Ang-II–stimulated endothelial cells, a significant adhesion was detected (Fig. 4*C*, 4*H*). In contrast, adhered neutrophils were encountered in Ang-II–stimulated HUVECs, but not in HUAECs.

Whereas preincubation with 10 μM *t*-RESV did not significantly modify the increase of leukocyte rolling flux induced by Ang-II, it did significantly inhibit leukocyte adhesion to Ang-II–stimulated HUAECs and HUVECs by 100 and 67%, respectively (Fig. 4*B*, 4*F*). Similarly, at the highest dose assayed (10 μM), *t*-RESV significantly inhibited mononuclear cell interactions with HUAECs or HUVECs and neutrophil/HUVEC adhesion (Fig. 4*C*, 4*D*, 4*H*, 4*I*). Additionally, *t*-RESV reduced Ang-II–induced CD11b upregulation in human monocytes in a concentration-dependent manner (Fig. 4*J*). In contrast, *t*-RESV had no significant effect on the levels of this CAM when human neutrophils were stimulated by Ang-II (Fig. 4*K*).

t-*RESV inhibits the chemokine release and endothelial CAM expression induced by Ang-II*

We have previously shown that Ang-II causes the release of IL-8, MCP-1, and RANTES from HUVECs and HUAECs, and of IL-8 and MIP-1α from HPBMCs (15, 27). In the current study, significant increases in IL-8, MCP-1, and RANTES were detected when HUAECs and HUVECs were stimulated for 4 h with 1 μM Ang-II (Fig. 5*A*). Pretreatment of the cells with *t*-RESV reduced the release of these chemokines in a concentration-dependent manner (Fig. 5*A*). Similarly, when HPBMCs were stimulated for 4 h with 1 μM Ang-II, a significant release of IL-8 and MIP-1α was observed (Fig. 5*A.7*, 5*A.8*). Preincubation of the cells with *t*-RESV also decreased the release of Ang-II–induced IL-8 and MIP-1α, but differences were significant only at the highest dose assayed (Fig. 5*A.7*, 5*A.8*).

When HUAECs were stimulated with Ang-II for 4 h, clear increases were detected in the expression of ICAM-1, VCAM-1, and E-selectin (Fig. 5*B*). Pretreatment of the cells with 10 μM *t*-RESV reduced Ang-II–induced ICAM-1– and VCAM-1–increased expression, whereas E-selectin upregulation remained virtually unaffected (Fig. 5*B*). Similar results were observed in HUVECs (data not shown).

t-*RESV inhibits Ang-induced NF-κB and p38MAPK activation in endothelial cells*

To investigate the mechanisms involved in the inhibitory responses produced by *t*-RESV, HUAECs and HUVECs were stimulated with Ang-II for 1 h in the presence or absence of *t*-RESV. As illustrated in Fig. 6, *t*-RESV inhibited the Ang-II–induced phosphorylation of both p38MAPK and the p65 subunit of NF-κB. However, the expression of nonphosphorylated p38MAPK and p65 was unaffected by Ang-II or Ang-II plus *t*-RESV (Fig. 6).

t-*RESV increases the expression of PPARγ in Ang-stimulated HUAECs, HUVECs, and HPBMCs*

An elevated expression of PPARγ exerts strong antioxidative and anti-inflammatory effects. To investigate the possible effect of *t*-RESV on PPARγ expression, cells were preincubated with *t*-RESV and then stimulated with Ang-II for 4 h. This did not affect PPARγ expression (Fig. 7). Interestingly, when the cells

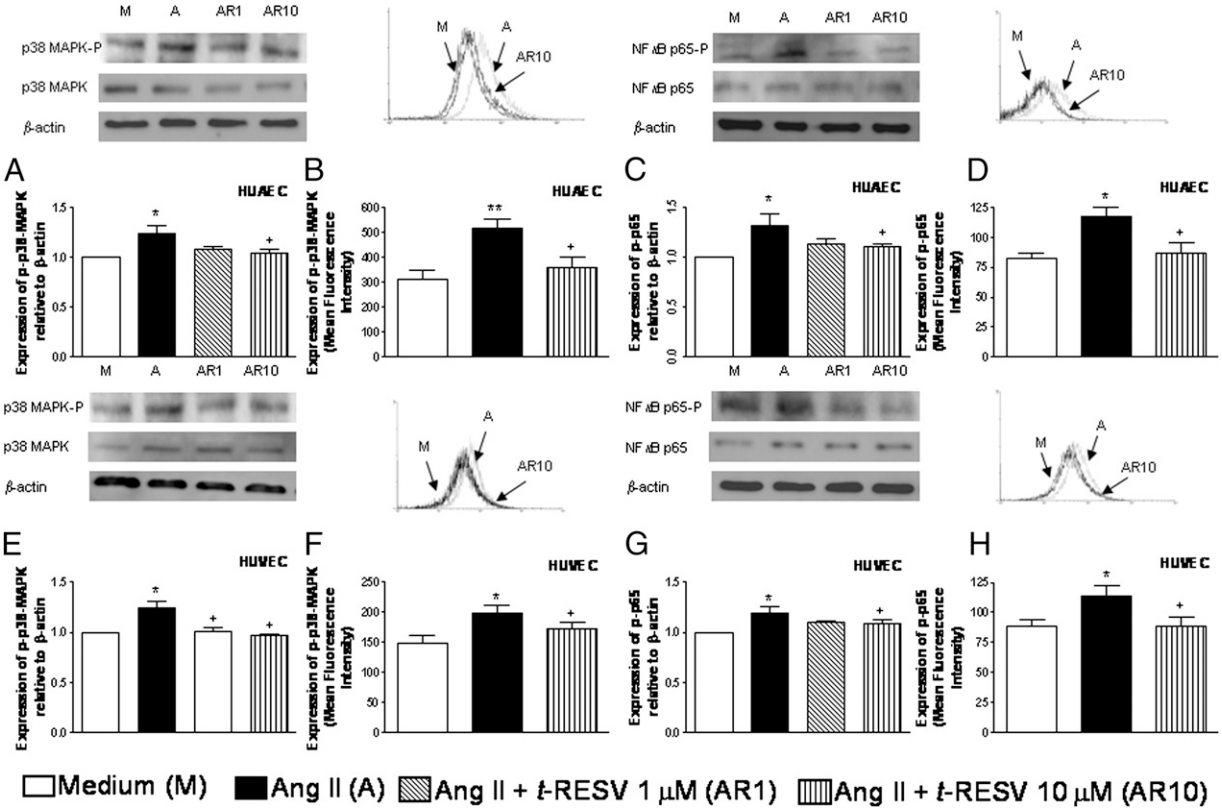

FIGURE 6. Effect of *t*-RESV on Ang-II–induced NF-κB and p38MAPK activation in HUAECs and HUVECs (*A*–*H*). Cells were stimulated for 1 h with Ang-II (1 μM). Some cells were pretreated with 1–10 μM *t*-RESV before Ang-II stimulation. NF-κB and p38 MAPK activation was determined by Western blotting and flow cytometry. Results (mean ± SEM of at least four independent experiments) are expressed as fold increase of the phospho-p38MAPK or phospho-p65:β-actin or as mean fluorescence intensity. Representative gels and histograms are also shown. $*p < 0.05$ or $**p < 0.01$ relative to values in the control group; $+p < 0.05$ relative to the 1 μM Ang-II group.

were pretreated with *t*-RESV, significant increases in PPARγ were detected (Fig. 7), with effects being more pronounced in HPBMCs than in human endothelial cells, because a concentration of 1 μM *t*-RESV was sufficient to increase the expression of this transcription factor in this leukocyte subtype.

Discussion

RESV has been proposed as one of the principal actors in the cardioprotective effects of red wine, and two of its isomers, *trans* and *cis*, are found in nature. To date, the majority of studies concerning the pharmacological effects of RESV have focused on its *trans* isomer, whereas knowledge about the pharmacological activity of its *cis* isomer is far more limited, probably due to the fact that the latter (unlike the former) is not available commercially. This has led us to carry out the current study, in which we have evaluated the in vivo effects of *t*- and *c*-RESV in a model of vascular inflammation. Although there are some reports indicating that RESV inhibits Ang-II–induced responses in vascular smooth muscle cells and myocytes (31, 32), little is known regarding its effect on the in vivo inflammatory response induced by this peptide hormone. Proinflammatory and prothrombotic events precede vasomotion alterations in endothelial dysfunction (33), and we have demonstrated that exposure to Ang-II for 4 h produces a clear enhancement of arteriolar leukocyte adhesion without affecting wall shear rate. This effect is inhibited by *t*-RESV, but not by *c*-RESV, and similar results are observed in postcapillary venules. Although a number of in vitro studies have revealed quantitative differences in the activity of the two forms, with the *cis* isomer appearing to be less active than its *trans* isomer (34–36), to our knowledge this is the first report to show that *t*-RESV inhibits the leukocyte-endothelial cell interactions induced by Ang-II in vivo, whereas *c*-RESV does not. Based on this finding, *t*-RESV was used in all subsequent experiments to elucidate and characterize the cardiovascular effects of RESV.

HRT is no longer prescribed to postmenopausal women (37), a population among which the incidence of cardiovascular complications increases sharply after menopause (38, 39). The literature contains controversial data regarding the effect of *t*-RESV on estrogen receptors. Whereas some studies point to the estrogenic activity of *t*-RESV (9, 11), others demonstrate its antagonistic activity in reproductive and nonreproductive estrogen target tissues (10, 40). In the current study, *t*-RESV lacked any significant effect on the loss of uterine mass induced by ovariectomy, indicating that the protective effects displayed by *t*-RESV in this model were not linked to its potential estrogenic activity.

To investigate its cardioprotective effect in a model of estrogen deficiency, *t*-RESV was chronically administered to OVZ rats. In these experiments, the only significant effect of a 5 mg/kg/day p.o. dose of *t*-RESV was observed in leukocyte parameters in postcapillary venules and circulating levels of CINC/KC and MIP-1α. It was necessary to increase the dose by 3-fold to detect significant functional effects at the arterial level. Indeed, at this dose, the expression of P-selectin and VCAM-1 was undermined, and significant reductions in the plasma levels of MCP-1 were achieved.

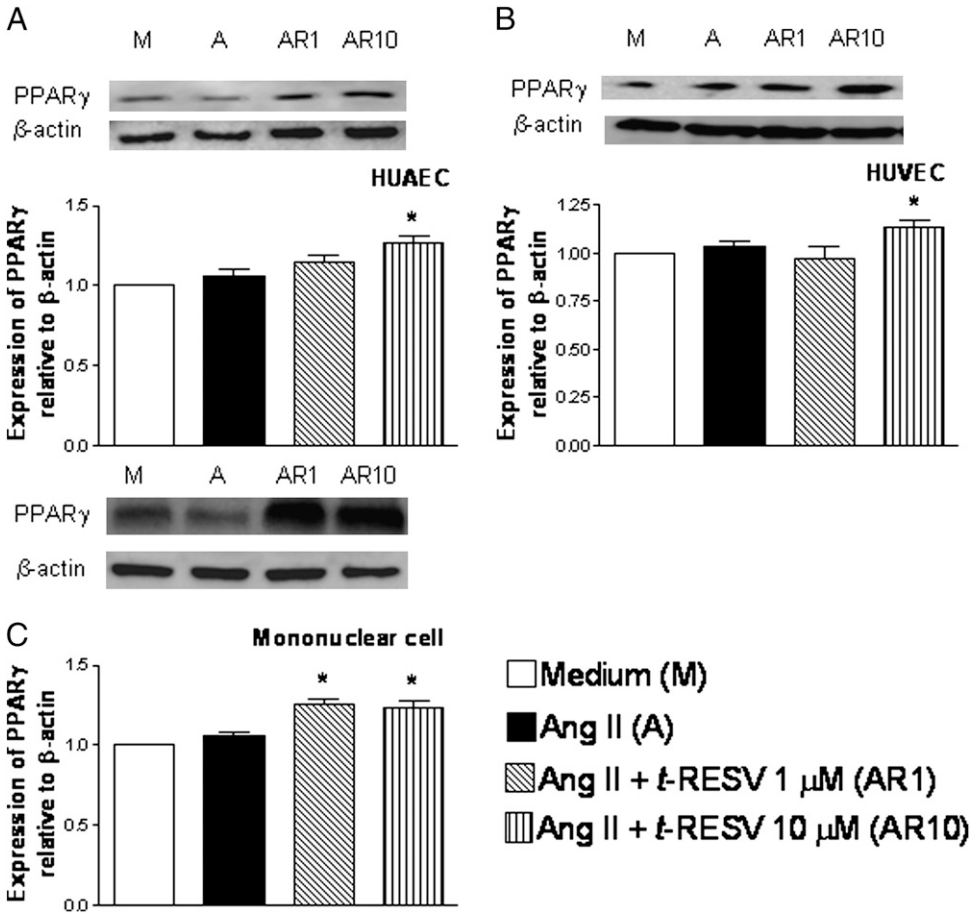

FIGURE 7. Effect of *t*-RESV on PPARγ expression in HUAECs (*A*), HUVECs (*B*), and HPBMCs (*C*). Cells were stimulated for 4 h with Ang-II (1 μM). Some cells were pretreated with 1–10 μM *t*-RESV before Ang-II stimulation. PPARγ expression was determined by Western blotting. Results (mean ± SEM of at least four independent experiments) are expressed as fold increase of the PPARγ:β-actin. Representative gels are shown above. $*p < 0.05$ relative to values in the control group.

Whereas in vitro studies have shown the capacity of *t*-RESV to downregulate the ICAM-1 and VCAM-1 expression induced by other stimuli (41, 42), the effect of *t*-RESV on P- and E-selectin expression has barely been investigated. We can now report that *t*-RESV inhibits the Ang-II–induced increase of P-selectin expression.

In light of these in vivo findings, and because estrogens influence the rennin-angiotensin system, we next examined the effect of *t*-RESV on human cells in vitro. As a result, we found that *t*-RESV inhibited leukocyte adhesion to HUAECs and HUVECs, and was most effective in inhibiting mononuclear leukocyte-HUAEC interaction in this dynamic system. Additionally, the expression of CD11b on Ang-II–stimulated human monocytes was significantly reduced by pretreatment of the cells with *t*-RESV, whereas the expression of this integrin on human neutrophils was not significantly affected, a finding that contradicts previously published evidence (43). Most relevantly, such effects occurred at low micromolar concentrations within a concentration range that would be expected after nutritional intake from a Mediterranean diet (42).

The strong antioxidant properties of RESV are perhaps its best-known feature. RESV can act on NAD (NADPH)-dependent oxidases, hypoxanthine/xanthine oxidase, 15-lipoxygenase, myeloperoxidase, and NO synthases (35, 44). Its actions on these enzymes contribute to a reduction in the formation of intracellular reactive oxygen species (ROS). In contrast, Ang-II–induced hypertension is mediated in part by superoxide anion production, whereas free radical generation is also involved in the acute inflammatory response elicited by Ang-II, and *t*-RESV decreases Ang-II–induced intracellular ROS levels, albeit in vascular smooth muscle cells (32, 45, 46). ROS generation induces NF-κB activation, and this NF has been shown to activate the transcription of several target genes implicated in the initiation and progression of pathogenesis in atherosclerosis, inflammation, and cancer (44). In our study, pretreatment of human cells with *t*-RESV reduced Ang-II–induced ICAM-1 and VCAM-1 expression and chemokine release (in a concentration-dependent manner). Some of these effects were also observed when endothelial cells were stimulated with TNF-α or LPS (41, 42). Given that *t*-RESV is capable of inhibiting the activation of NF-κB by Ang-II, it is possible that RESV acts at a step in which most of these agents converge in the signal transduction pathway, leading to ROS generation and NF-κB activation. In this context, upregulation of p300 expression and its histone acetyltransferase activity promotes acetylation of NF-κB p65, which results in NF-κB–specific transcriptional upregulation. Activation of sirtuin-1 by RESV induces sirtuin-1–p300 association and acetyltransferase inactivation, which may lead to inhibition of NF-κB–specific transcriptional activation, as suggested by previous reports (47–49).

MAPKs play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stressors. Moreover, they are known to be important for the activation of NF-κB. We have found that *t*-RESV inhibits Ang-II–induced p38MAPK phosphorylation in both mononuclear leukocytes and endothelial cells. These results are in accordance with previous reports that RESV downregulates the MAPK cascade by inhibiting the tyrosine phosphorylation of ERK1/2/JNK/p38 (44). Furthermore, Rac, a member of the Rho family of GTPases, regulates the actin cytoskeleton and focal adhesion turnover during cell migration. Therefore, it is tempting to speculate that the inhibition of Ang-II–induced leukocyte recruitment by *t*-RESV observed in the current study was due to Rac. That said, it has been demonstrated that RESV at 5 μM promotes Rac activity and lamellipodia formation, whereas at 50 μM it inhibits both of these parameters (50). This suggests that the inhibition of Rac signaling is not involved in the inhibitory response produced by *t*-RESV in the current study, because the concentrations employed were not within the range of inhibition.

Finally, many effects of *t*-RESV are similar to those of PPARγ agonists, which belong to the PPAR family of ligand-dependent transcription factors. PPARγ is highly expressed in macrophages and other atheroma constituent cells and exerts strong antioxidative and anti-inflammatory effects by negatively regulating the transcription of related genes in these cells (51). Genes inhibited by both *t*-RESV and PPARγ ligands include proinflammatory cytokines, such as TNF-α, and CAMs, such as VCAM-1 or ICAM-1. Some reports have demonstrated that *t*-RESV activates PPARγ in cell types other than those studied in this work (52, 53). In the current study, *t*-RESV increased PPARγ expression in human mononuclear and endothelial cells. Inhibition of NF-κB activation is one of the underlying mechanisms of the negative regulation of the PPARγ agonist, and it seems that this mechanism is also involved in the protective effects exerted by *t*-RESV.

In conclusion, this is the first report to demonstrate in vivo that the anti-inflammatory activity of RESV depends on its *trans* isomer. *t*-RESV was effective in subacute and chronic models of rennin-angiotensin system-dependent vascular inflammation. Consistent with these findings, we have also observed that *t*-RESV inhibits the Ang-II–induced increase in the adhesiveness of leukocytes to the endothelium, CAM expression, and chemokine release in human cells. These effects appear to be mediated through the inhibition of p38MAPK and NF-κB activation and the upregulation of PPARγ. Considered as a whole, these results indicate that the effects exerted by *t*-RESV occur through attenuation of AT_1 receptor signaling. In this way, the chronic administration of *t*-RESV would appear to reduce the systemic inflammation associated with activation of the rennin-angiotensin system, and therefore, it may diminish the risk of further cardiovascular diseases.

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Disclosures

The authors have no financial conflicts of interest.

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