

# Raloxifene Elicits Combined Rapid Vasorelaxation and Long-Term Anti-Inflammatory Actions in Rat Aorta

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## ABSTRACT

Previous studies reported the ability of raloxifene to acutely relax arterial and venous vessels, but the underlying mechanisms are controversial. Anti-inflammatory effects of the drug have been reported in nonvascular tissues. Therefore, the aim of this study was to investigate the nature of short- and long-term effects of raloxifene on selected aspects of vascular function in rat aorta. Isometric tension changes in response to raloxifene were recorded in aortic rings from ovariectomized female rats that underwent estrogen replacement, whereas long-term experiments were performed in isolated aortic smooth muscle cells (SMCs). Raloxifene (0.1 pM–0.1 μM) induced acute vasorelaxation through endothelium- and nitric oxide (NO)-dependent, prostanoind-independent mechanisms. The relaxant response to raloxifene was significantly weaker than that to 17β-estradiol and was sensitive to neither the nonselective estrogen receptor antagonist ICI 182,780 [7,17-

9]([4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol] nor a selective estrogen receptor (ER) α antagonist. This rapid vasorelaxant effect was retained in aortic rings from rats treated with 0.1 mg/kg, but not 1 mg/kg, lipopolysaccharide, 4 h before sacrifice. In cultured aortic SMCs, raloxifene treatment (1 nM–1 μM) for 24 h reduced inducible NO synthase activation in response to cytokines. This effect was prevented by the selective ERα antagonist and was associated with up-regulation of ERα protein levels, which dropped markedly upon cytokine stimulation. These findings illustrate the relevance of classic ER-dependent pathways to the vascular anti-inflammatory effects rather than to the nongenomic vasorelaxation induced by raloxifene and may assist in the design of novel ER isoform-selective estrogen-receptor modulators targeted to the vascular system.

Raloxifene is a selective estrogen-receptor modulator (SERM) approved for use in osteoporosis and has been suggested to be cardioprotective in women at high risk for coronary heart disease (Barrett-Connor et al., 2002), although these results were from post hoc analyses. In fact, the results of the recently completed RUTH (Raloxifene Use for The Heart) study indicate that treatment with raloxifene does not significantly affect the risk of coronary events in postmenopausal women at risk for coronary disease (Barrett-Connor et al., 2006). Thus, a more detailed understanding of raloxifene pharmacological action in vascular tissues is required to better define and unravel potential benefits of treatment with raloxifene and other SERMs.

A certain number of studies have examined the direct effects of raloxifene on the vessel wall. It has been consistently shown that the drug acutely relaxes different arterial (Figtree et al., 1999; Tsang et al., 2004; Chan et al., 2005; Leung et al., 2005) and venous (Bracamonte et al., 2002; Chan et al., 2005) vessels from different animal species. A variety of underlying mechanisms, however, can be involved, including enhanced endothelial NO production (Figtree et al., 1999; Bracamonte et al., 2002) and/or inhibition of L-type voltage-sensitive Ca<sup>2+</sup> channels (Tsang et al., 2004; Chan et al., 2005). Although long-term in vitro (Wassmann et al., 2002) and in vivo (Rahimian et al., 2002) studies have confirmed the increased bioavailability of NO afforded by raloxifene, several variables including gender, endothelial status, vessel type, and animal species appear to affect treatment outcomes. For instance, ovarian status is a general variability source to be taken into account when exploring the vas-

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**ABBREVIATIONS:** SERM, selective estrogen-receptor modulator; ER, estrogen receptor; SMC, smooth muscle cell; LPS, lipopolysaccharide; ICI 182,780, 7,17-[9]([4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; MPP, 1,3-bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidylethoxy) phenyl]-1H-pyrazole; NA, noradrenaline; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; E<sub>2</sub>, 17β-estradiol; NO, nitric oxide; iNOS, inducible NO synthase; FCS, fetal calf serum; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; ANOVA, analysis of variance; Ach, acetylcholine; Ral, raloxifene.

cular responses to estrogenic compounds (Bracamonte et al., 2002; Bolego et al., 2005). The contribution of ER isoforms to raloxifene-induced acute vascular relaxation as shown in the above studies is also controversial. Both ER $\alpha$  and ER $\beta$  are expressed in rat aortic smooth muscle cells (SMCs) (Zancan et al., 1999; Maggi et al., 2003), and recent work from our group demonstrates a beneficial role for ER $\alpha$  in the acute vascular effects of estrogenic compounds (Bolego et al., 2005). So far, there is no information as to the involvement of specific ER isoforms in the vascular effects of raloxifene. Beyond the modulation of vascular tone, there have been recent indications that raloxifene is capable of decreasing acute inflammation induced by lipopolysaccharide (LPS) in microglial cells (Suuronen et al., 2005) and by carrageenan in normal and ovariectomized rats (Esposito et al., 2005), but the relevance of these findings to the cardiovascular system is at present unknown.

Therefore, we investigated the vascular effects of raloxifene, yet untested in rat aorta, using a dual approach. First, acute vasomotor effects were tested on aortic rings from estrogen-replaced ovariectomized rats to avoid fluctuations in circulating hormones as seen in gonadally intact females. Second, experiments were performed in isolated aortic SMCs to test potential anti-inflammatory effects following long-term treatment. The role of ER in raloxifene vascular action was assessed using both the established ER antagonist ICI 182,780 and the newly described selective ER $\alpha$  antagonist MPP (Sun et al., 2002). ICI 182,780 is a high-affinity ER antagonist ( $IC_{50} = 0.29$  nM; Wakeling et al., 1991). MPP is a selective, high-affinity silent antagonist at ER $\alpha$  receptors and displays >200-fold selectivity for ER $\alpha$  over ER $\beta$ .  $K_i$  values are 2.7 and 1800 nM at ER $\alpha$  and ER $\beta$  receptors, respectively (Sun et al., 2002).

## Methods and Materials

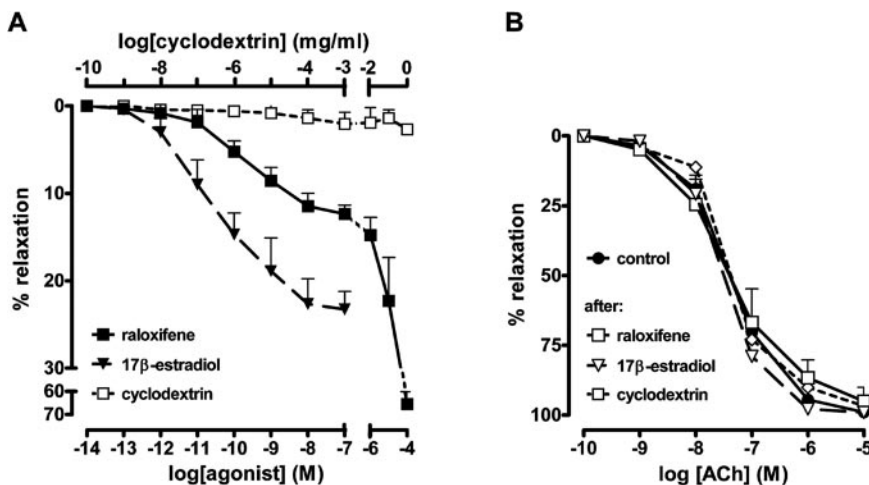
**Drugs and Chemicals.** Noradrenaline (NA) bitartrate, acetylcholine chloride,  $N^G$ -nitro-L-arginine methyl ester (L-NAME), LPS, and 17 $\beta$ -estradiol (E $_2$ ) were purchased from Sigma Chemical (Milan, Italy). The estrogen receptor antagonists ICI 182,780 and MPP were purchased from Tocris Cookson Inc. (Bristol, UK). Raloxifene was kindly provided by Eli Lilly and Co. (Indianapolis, IN). The anti-inducible NO synthase (iNOS) polyclonal antibody was purchased from BD Biosciences Transduction Laboratories (Lexington, KY), the anti-ER $\alpha$  antibody was from Santa Cruz (Santa Cruz, CA), and the

peroxidase-coupled secondary antibody was obtained from Vector (Burlingame, CA). For organ bath experiments, raloxifene was dissolved in 2-hydroxypropyl- $\beta$ -cyclodextrin (Sigma) because dimethyl sulfoxide as a vehicle affected the tone of precontracted aortic rings as assessed in preliminary experiments (data not shown). The cyclodextrin percentage in the tissue bath did not exceed 0.1%. E $_2$  and ICI 182,780 were dissolved in ethanol, whose final concentration in the tissue bath did not exceed 0.01%.

**Animals.** Female Sprague-Dawley rats weighing 200 to 250 g (Charles River Italia, Calco, Italy) were kept in temperature-controlled facilities on a 12-h light/dark cycle and fed normal chow. Bilateral ovariectomy was performed under ketamine (40 mg/kg i.p.) and xylazine (20 mg/kg i.p.) anesthesia. Four weeks later, animals were implanted two silastic capsules containing 25  $\mu$ l of vehicle (peanut oil) or E $_2$  (5.87  $\mu$ g; 0.86 mM) for 5 days (Bolego et al., 1999; Cignarella et al., 2000). Plasma E $_2$  concentrations after this treatment approached the normal rat proestrus level (Lapchak, 1991). This procedure aimed at bypassing estrus cycle-associated variations in vasomotor responses. All procedures conformed to the *Guidelines for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 85-23, revised 1996).

**Isometric Tension Recording Experiments.** Vessel preparations were obtained as described previously (Bolego et al., 2005). In brief, 5- to 6-mm aortic rings were suspended in 5-ml organ baths containing Krebs' solution at 37°C. The Krebs' solution had the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH $_2$ PO $_4$ , 1.1 mM MgSO $_4$ , 2.5 mM CaCl $_2$ , 25 mM NaHCO $_3$ , and 5.5 mM glucose, pH 7.4. The rings were connected to isometric tension transducers (Fort 10; World Precision Instruments, Sarasota, FL) coupled with a digital recording system (PowerLab 8SP; AD Instruments, Basile, Comerio, Italy), equilibrated for 30 min, and contracted with 10 $^{-5}$  M NA to evoke a maximal response. Preparations were then washed with fresh Krebs' solution, and the equilibration period was allowed to continue for a further 30 min. Experiments were carried out on tissues precontracted with NA to 60% of maximal contraction ( $EC_{60} = 10^{-7}$  M). The endothelium was considered functional when relaxation of precontracted vessels to 10 $^{-5}$  M acetylcholine was at least 80%. Care was taken to distinguish drug-induced reduction in tone versus loss of tone over time. To test drug vehicle effects, cumulative additions of equivalent solvent dilutions were performed. Responses were expressed as percentage of relaxation from NA-induced contraction.

**In Vivo LPS Treatment.** Following ovariectomy and E $_2$  capsule implantation, animals were injected i.p. with LPS (0.1 or 1 mg/kg) or saline. At 4 h after injection, when fever reached a plateau and induction of vascular inflammatory enzymes was maximal (Cao et al., 1995; Sunday et al., 2006), the aorta was excised, and aortic rings



**Fig. 1.** A, concentration-response curves of aortic rings from E $_2$ -replaced ovariectomized female rats precontracted with 0.1  $\mu$ M noradrenaline and incubated with raloxifene (full squares) and E $_2$  (triangles). The vehicle curve (cyclodextrin, empty squares) is shown as well. Statistical analysis for the two agonists' curves relates to the concentration range of up to 0.1  $\mu$ M.  $n = 5$  to  $7$ ,  $p < 0.001$  (two-way ANOVA). B, before generating curves as shown in A, a concentration-response curve to Ach was obtained (control, circle). In all three groups, Ach relaxation was attained with similar efficiency after the highest concentration of raloxifene, E $_2$ , and vehicle to demonstrate a functional endothelium.  $n = 3$ .

were mounted in organ baths to obtain concentration-response curves to raloxifene as described above.

**Cell Culture.** SMCs obtained from aortic intimal-medial layers of male Sprague-Dawley rats expressed ER (Zancan et al., 1999; Maggi et al., 2003). These SMCs were used not later than the fifth passage and seeded in six-well plates ( $3 \times 10^5$  cells/well) for experiments. On reaching approximately 70% confluence, the medium was replaced with phenol red-free M199 with 10% FCS for 2 days. SMCs were then synchronized in medium containing 0.4% FCS for 24 h and incubated for a further 24 h with a cytokine mixture comprising 10 ng/ml interleukin-1 $\beta$ , 10 ng/ml interferon- $\gamma$ , and 25 ng/ml tumor necrosis factor- $\alpha$  plus 1  $\mu$ g/ml LPS. Such a mixture, which is likely to occur in vivo in settings of vascular inflammation, consistently induced iNOS protein formation in SMCs (Zancan et al., 1999). Raloxifene (dissolved in dimethyl sulfoxide) was added at the same time as cytokines where indicated, whereas MPP was added 30 min before.

**Cell Viability Assay.** Once  $5 \times 10^3$  or  $8 \times 10^3$  cells/well were plated in a 96-well plate, they were first incubated for 24 h in M199 with 10% FCS and then switched to phenol red-free M199 with 10% FCS for 2 days and phenol red-free M199 with 0.4% FCS for 24 h. Cell viability was assessed after a 24-h incubation with the LPS/cytokine mixture in the presence or absence of 1  $\mu$ M raloxifene. The number of surviving cells was assessed as described elsewhere (Takahashi et al., 2003) by recording the absorbance at 490 nm generated by the bioreduction of the MTS tetrazolium compound into a colored soluble formazan product (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI).

**Western Blot Analysis.** Cells were washed twice with phosphate-buffered saline and extracted directly into the lysis buffer as

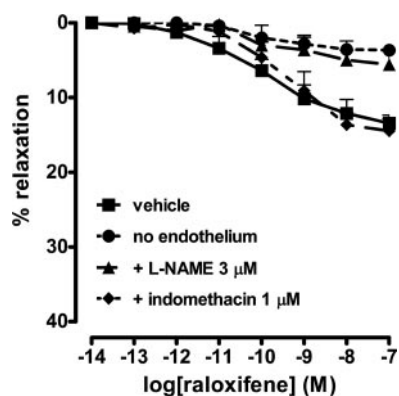
described previously (Idel et al., 2002). At least 30  $\mu$ g of cell protein was loaded onto 10% SDS-acrylamide gels. At the end of the run, proteins were transferred onto Hybond-ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots were incubated with the primary antibody (1:1000) overnight followed by the peroxidase-conjugated secondary antibody for 1 h and, after extensive washing, were developed by chemiluminescence. Sample loading control was performed using  $\beta$ -actin immunodetection.

**Nitrite Assay.** Culture medium was collected and centrifuged at 12,000 rpm for 5 min. Next, 250  $\mu$ l/well medium was treated with 20  $\mu$ l of 6.5 M HCl and 20  $\mu$ l of 37.5 mM sulfanilic acid in a 96-well plate. After incubation for 10 min, 20  $\mu$ l of 12.5 mM *N*-(1-naphthyl)-ethylendiamine was added. Optical density was read at 550 nm after 15 min. Nitrite values were expressed as micromoles of nitrite per milligram of cell protein.

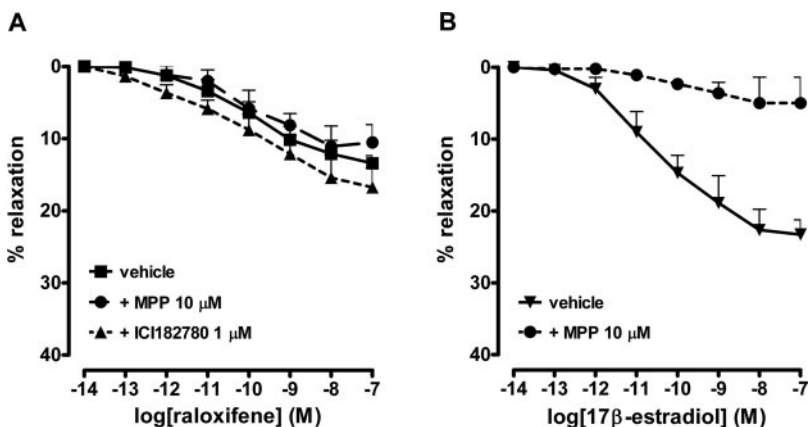
**Statistical Analysis.** All data were expressed as means  $\pm$  S.E.M. and represent unpaired data. Concentration-response curves were obtained using the software Prism (GraphPad Software Inc., San Diego, CA) and compared by means of two-way analysis of variance (ANOVA) followed by a post hoc test.  $pD_2$  values and maximal responses were compared by one-way ANOVA followed by Bonferroni's post hoc test, using the software Minitab (Minitab Inc., State College, PA). Cell culture data were obtained from at least four independent experiments, each value representing mean  $\pm$  S.E.M. of duplicate or triplicate determinations. Statistical evaluation was performed using unpaired Student's *t* test or one-way ANOVA with Fisher analysis when more than two groups were compared. Values of  $p < 0.05$  were considered significant.

## Results

Raloxifene induced acute concentration-dependent relaxation of endothelium-intact NA-precontracted aortic rings from  $E_2$ -replaced ovariectomized rats (Fig. 1A). In the concentration range of up to 0.1  $\mu$ M, which is likely to be relevant in vivo, the maximal response was modest but significant (15% relaxation). At higher concentrations, the magnitude of raloxifene-induced relaxation sharply increased starting at 10  $\mu$ M and approaching 70% at 100  $\mu$ M. Cyclodextrin solvent controls had no effect on vascular tone. As shown in Fig. 1A, raloxifene was significantly less potent and effective than  $E_2$  at relaxing precontracted aortic rings ( $pD_2$ ,  $9.9 \pm 0.4$  versus  $10.4 \pm 0.3$ ;  $E_{max}$ ,  $13.4 \pm 1.1\%$  versus  $23.3 \pm 2.1\%$ ;  $n = 5-7$ ,  $p < 0.001$ ). As shown in Fig. 1B, acetylcholine (ACh) relaxation was attained with similar efficiency in the three groups both prior to generating curves and after the highest agonist concentration to demonstrate that the endothelium was functional throughout the experiment.



**Fig. 2.** Concentration-response curves of aortic rings from 17 $\beta$ -estradiol-replaced ovariectomized female rats precontracted with 0.1  $\mu$ M noradrenaline and incubated with raloxifene alone (squares), in the presence of the NO synthase inhibitor L-NAME (3  $\mu$ M; triangles) or the cyclooxygenase inhibitor indomethacin (1  $\mu$ M; diamonds) and in the absence of endothelium (circles).  $E_{max}$  L-NAME and  $E_{max}$  no endothelium,  $p < 0.001$  versus raloxifene;  $E_{max}$  indomethacin, N.S.;  $n = 5$ , two-way ANOVA.



**Fig. 3.** A, concentration-response curves of aortic rings from 17 $\beta$ -estradiol-replaced ovariectomized rats precontracted with 0.1  $\mu$ M noradrenaline and incubated with raloxifene in the absence (squares) or presence of the antiestrogen ICI 182,780 (1  $\mu$ M, triangles) and the selective ER $\alpha$  antagonist MPP (10  $\mu$ M; circles). The three curves are not statistically different. B, concentration-response curves of aortic rings from 17 $\beta$ -estradiol-replaced ovariectomized rats precontracted with 0.1  $\mu$ M noradrenaline and incubated with 17 $\beta$ -estradiol alone (triangles) or in the presence of the selective ER $\alpha$  antagonist MPP (10  $\mu$ M; circles). A,  $n = 4$ , N.S.; B,  $n = 5$ ,  $p < 0.001$  versus vehicle (two-way ANOVA).



Raloxifene-induced relaxation of precontracted aortic rings was virtually abolished by incubation with 3  $\mu\text{M}$  L-NAME and by endothelium removal (Fig. 2), as previously shown in different vascular beds (Figtree et al., 1999; Bracamonte et al., 2002). To determine whether raloxifene-induced vasodilation was related to any extent to prostanoid release, inhibiting cyclooxygenase with indomethacin (1  $\mu\text{M}$ ) had no effect on relaxations to raloxifene in aortic rings with endothelium (Fig. 2;  $E_{\text{max}}$  L-NAME,  $5.6 \pm 2.0\%$ ,  $n = 4$ ,  $p < 0.001$

versus raloxifene;  $E_{\text{max}}$  no endothelium,  $3.7 \pm 0.6\%$ ,  $n = 3$ ,  $p < 0.001$  versus raloxifene;  $E_{\text{max}}$  indomethacin,  $14.5 \pm 1.7\%$ ,  $n = 5$ , N.S.).

When testing the role of ER in raloxifene-induced acute relaxation of aortic tissues, pretreatment with ICI 182,780 (1  $\mu\text{M}$ ) failed to affect the response (Fig. 3A;  $pD_2$ ,  $9.9 \pm 0.7$  versus  $10.0 \pm 0.3$ ;  $E_{\text{max}}$ ,  $13.4 \pm 1.1\%$  versus  $16.8 \pm 2.7\%$ ,  $n = 7$ , N.S.). The same was true when tissues were preincubated with the selective ER $\alpha$  antagonist MPP (10  $\mu\text{M}$ ; Fig. 3A;  $pD_2$  MPP,  $10.0 \pm 0.5$ ,  $n = 4$ , N.S.). In contrast, this selective antagonist significantly impaired  $E_2$ -evoked vasodilator responses (Fig. 3B;  $pD_2$  MPP,  $9.8 \pm 1.3$  versus  $10.4 \pm 0.3$ ,  $n = 5$ ,  $p < 0.001$ ), suggesting that the pharmacological action profiles of raloxifene and  $E_2$  do not fully overlap.

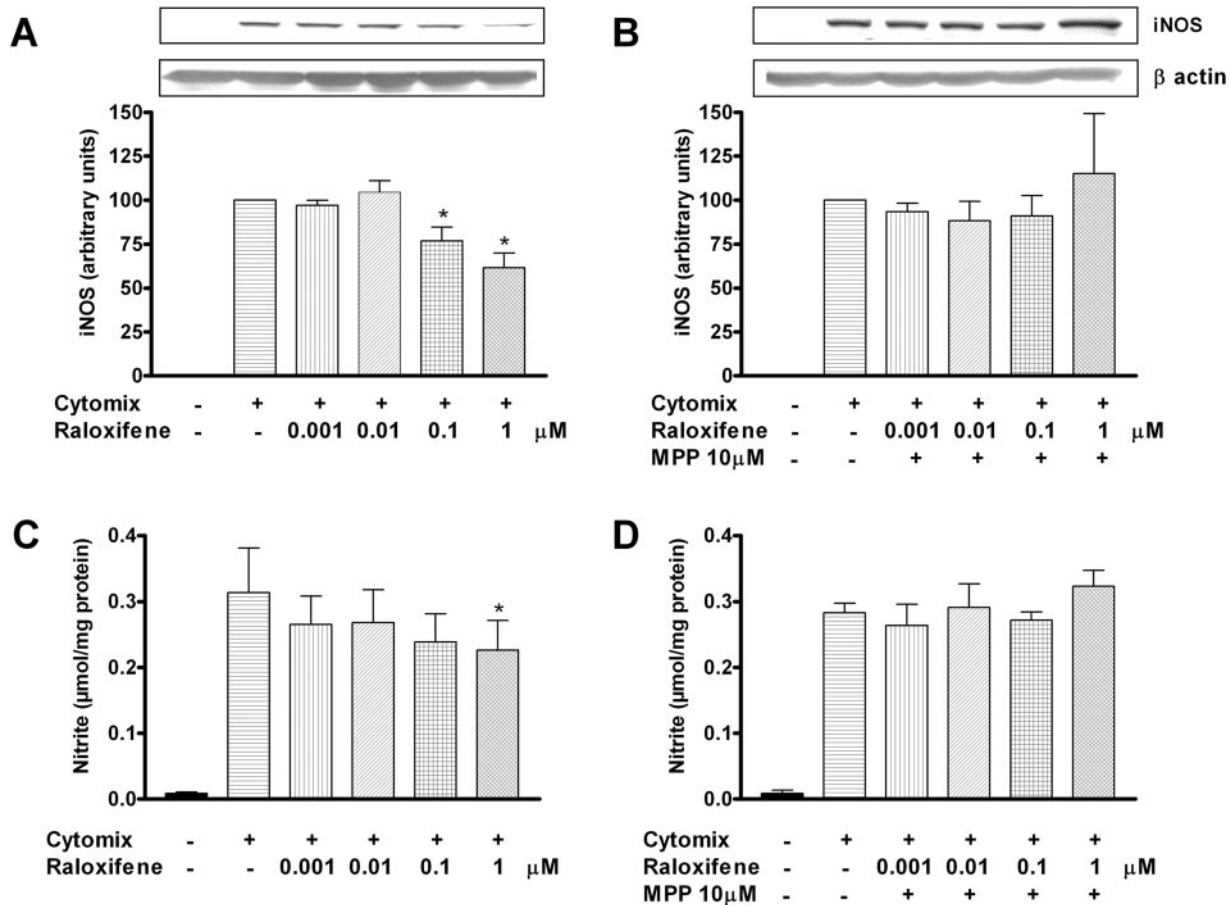
The reported decrease in inflammatory responses after raloxifene treatment in vitro and in vivo (Esposito et al., 2005; Suuronen et al., 2005) led us to test potential anti-inflammatory effects of the drug in the vessel wall. Therefore, the effect of raloxifene on vascular tone was investigated in ex vivo aorta from rats under inflammatory challenge. As shown in Table 1, raloxifene retained its relaxant activity in aortic rings isolated from rats treated with 0.1 mg/kg LPS

TABLE 1

Rapid effects of raloxifene on the relaxation of precontracted aortic rings from estrogen-replaced ovariectomized rats under inflammatory challenge

$E_2$ -replaced ovariectomized rats were treated with 0.1 or 1 mg/kg LPS for 4 h before sacrifice. Tension recording experiments in response to raloxifene were performed as described under *Materials and Methods*. Results indicate the percent relaxation of NA-precontracted tone and are expressed as mean of  $\pm$  S.E.M. ( $n = 3$  for each group).

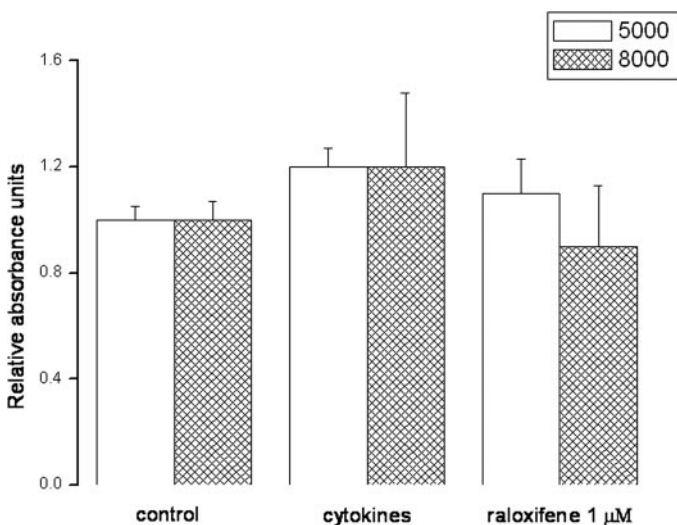
Raloxifene	LPS		
	0	0.1	1
<i>log M</i>	<i>mg/kg</i>		
-11	$1.9 \pm 1.5$	$3.0 \pm 1.9$	$3.3 \pm 0.8$
-9	$8.6 \pm 1.5$	$10.1 \pm 4.2$	$3.9 \pm 2.4$
-7	$12.4 \pm 1.0$	$13.4 \pm 3.5$	$4.6 \pm 2.0$



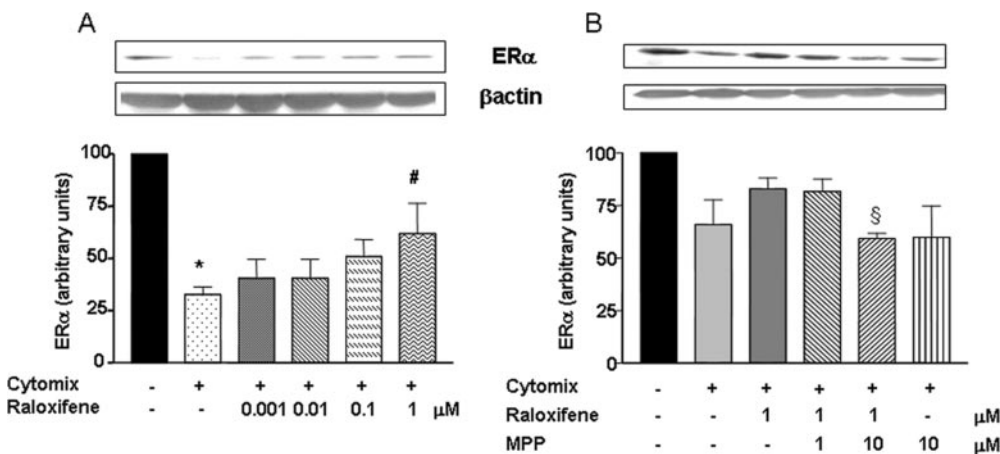
**Fig. 4.** Effects of raloxifene and the estrogen receptor- $\alpha$  antagonist MPP on cytokine-induced iNOS protein synthesis (A and B) and activity (C and D) in rat aortic smooth muscle cells. Cells were grown in phenol red-free M199 medium for 2 days, synchronized in 0.4% FCS for 24 h, and stimulated with a cytokine cocktail (10 ng/ml interleukin-1 $\beta$ , 10 ng/ml interferon- $\gamma$ , 25 ng/ml tumor necrosis factor- $\alpha$  plus 1  $\mu\text{g/ml}$  LPS) for 24 h. Increasing concentrations of raloxifene (1 nM–1  $\mu\text{M}$ ) were added where indicated at the same time as cytokines in the absence (A and C) or presence (B and D) of 1  $\mu\text{M}$  MPP added 30 min before. The amount of iNOS in cell lysates was measured by scanning densitometry, whereby the intensity of the cytomix band was set arbitrarily to 100. Representative Western blots are shown. The culture media were harvested and samples thereof assayed for nitrite accumulation using the Griess reaction. Data are expressed as means  $\pm$  S.E.M. of four to eight independent experiments. \*,  $p < 0.05$  compared with cytokines alone, ANOVA.

but not from those treated with 1 mg/kg LPS 4 h before sacrifice.

The possibility that raloxifene could also modulate vascular function through long-term effects was investigated in isolated SMCs from rat aorta. After stimulation with cytokine plus LPS mix for 24 h, iNOS became detectable in aortic SMCs (Fig. 4A). This cytokine-elicited rise in iNOS protein, however, was significantly attenuated by incubation with 0.1 and 1  $\mu$ M raloxifene (Fig. 4A). As shown in Fig. 4B, this effect was mediated by ER $\alpha$  because MPP pretreatment blocked raloxifene action on iNOS protein over 24 h. Neither raloxifene nor MPP promoted iNOS synthesis in the absence of cytokines (data not shown). Raloxifene treatment for 24 h significantly decreased cytokine-stimulated iNOS activity, as measured by nitrite accumulation in the culture medium (Fig. 4C), at the highest concentration tested of 1  $\mu$ M ( $p < 0.05$ ). This effect was again abolished by preincubation with MPP (Fig. 4D). Under the same experimental conditions, raloxifene treatment failed to affect the functional expression



**Fig. 5.** Raloxifene does not affect the viability of rat aortic smooth muscle cells under inflammatory challenge. Cells were seeded at 5000 or 8000/well in a 96-well plate and cultured in M199 medium plus 10% FCS for 24 h. Thereafter, cells were treated as described in the legend to Fig. 4. After the final 24-h incubation, cell viability was evaluated by the MTS assay after 1.5 to 3 h as described under *Materials and Methods*. Cell growth is expressed as the relative absorbance obtained in the control condition. Data are expressed as means  $\pm$  S.E.M. of three independent experiments, each with  $n = 8$  to 24 in all groups.



**Fig. 6.** Concentration-dependent effect of raloxifene (A) and modulation by the selective antagonist MPP (B) on the accumulation of estrogen receptor- $\alpha$  protein in rat aortic smooth muscle cells stimulated with a cytokine/LPS mixture for 24 h. Cell incubation and Western blotting procedures were as described in the legend to Fig. 4. Data are expressed as means  $\pm$  S.E.M. of six (A) or four (B) independent experiments. \*,  $p < 0.01$  compared with basal, Student's  $t$  test; #,  $p < 0.05$  compared with cytokines alone, ANOVA; §,  $p < 0.05$  compared with raloxifene alone, one-way ANOVA.

of cyclooxygenase-2, another molecular target indicative for vascular inflammation (data not shown). The observed reduction in iNOS expression and activity was not merely the result of cell death because no significant growth inhibitory effect by raloxifene treatment at 1  $\mu$ M was observed after 24 h in SMCs plated at two different initial density values ( $5 \times 10^3$  and  $8 \times 10^3$  cells/well) (Fig. 5).

Based on the above findings, the negative regulation of iNOS functional expression induced by raloxifene treatment appeared to be mediated by ER $\alpha$ . Hence, we investigated if the drug had any influence on ER $\alpha$  protein formation over the course of the anti-inflammatory action. In agreement with previous studies (Maggi et al., 2003), the cytokine/LPS mixture reduced the amount of immunodetectable ER $\alpha$  by approximately 70% in vascular SMCs (Fig. 6A). Yet, treatment with increasing concentrations of raloxifene partially restored ER $\alpha$  protein levels under these experimental conditions (Fig. 6A). In a different set of experiments, preincubation with MPP at 10  $\mu$ M but not 1  $\mu$ M was found to prevent the rise in ER $\alpha$  mediated by raloxifene while being ineffective when added alone (Fig. 6B). This suggests that raloxifene is capable of interacting with and regulating the amount of ER $\alpha$ .

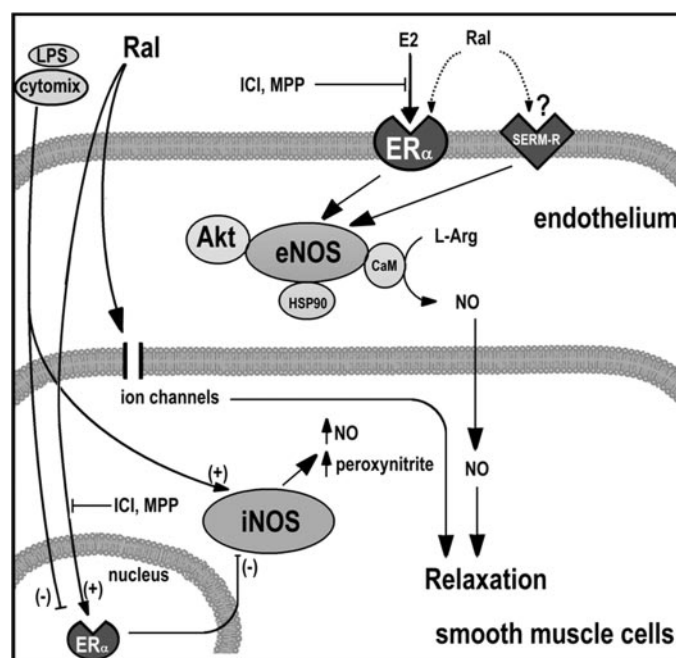
## Discussion

The present study describes novel aspects of the vascular pharmacology of raloxifene. We herein report a nongenomic vasorelaxant effect in aortic rings from female rats with steady estrogen concentrations, thereby ruling out the confounding factors of estral cycle and hormone deprivation; the maintenance of this rapid effect in aortic preparations *ex vivo* from animals under inflammatory challenge; and an anti-inflammatory action in isolated aortic SMCs. The last two effects, to the best of our knowledge, have not been described before. The present findings confirm that raloxifene has the potential to reproduce selected genomic and, to a lesser extent, nongenomic actions of E $_2$  in the vascular wall. Distinct mechanisms account for the nongenomic as opposed to long-term effects of raloxifene. In particular, the involvement of classic ERs in raloxifene action was not apparent for the nongenomic vasorelaxant effect but could be shown for the long-term anti-inflammatory action, indicating that multiple mechanisms of action drive raloxifene action in the arterial wall as depicted in Fig. 7. The therapeutic relevance of these findings needs to be determined in further studies, but ex-

periments in the present work were performed at raloxifene concentrations not too distant from possible peak plasma concentrations in women after long-term oral administration (Morello et al., 2003).

In agreement with previous studies in rabbit coronary arteries (Figtree et al., 1999) and pig femoral veins (Bramante et al., 2002), raloxifene-induced acute relaxation of rat aortic rings was endothelium- and NO-dependent. This finding, however, was not confirmed in rat cerebral (Tsang et al., 2004) or intrarenal arteries (Leung et al., 2005) and pulmonary vessels (Chan et al., 2005), wherein the relaxant effect has been attributed primarily to inhibition of voltage-sensitive  $Ca^{2+}$  channels. Therefore, the nature of this rapid effect of raloxifene is controversial. In fact, gender, endothelial status, precontracting agent, and regional distribution of molecular targets (ER, ion channels) may sway the onset of different pathways in response to raloxifene treatment. Evidence suggests that raloxifene is capable of evoking vascular relaxation via both endothelium-dependent and -independent pathways, which may not be mutually exclusive though. Accordingly, our data suggest two components of action of raloxifene (Fig. 1), which were also able to markedly relax endothelium-denuded aortic rings at pharmacological concentrations above 10  $\mu$ M (data not shown). Although the latter effect is consistent with inhibition of  $Ca^{2+}$  channels, acute relaxant responses to estrogen-related agents at submicromolar concentrations may be secondary to activation of  $ER\alpha$  located on the plasma membrane and downstream up-regulation of endothelial NO synthase (Chen et al., 1999; Kim et al., 1999). Our results only partially fit with this paradigm because neither ICI 182,780 nor the selective  $ER\alpha$  antagonist MPP affected the acute relaxant response to raloxifene. To reconcile these conflicting results, it may be assumed that raloxifene bound to a novel membrane SERM receptor (Mercier et al., 2003) or that ER antagonists inhibited only specific components of raloxifene action. In fact, endothelium-dependent relaxation to tamoxifen is only partially inhibited by ICI 182,780 (Figtree et al., 2000). In other studies, ICI 182,780 failed to block all responses mediated by estrogenic compounds (Dudley et al., 2000). It is also conceivable that ICI 182,780 and MPP are effective antagonists of full-length  $ER\alpha$  but not of such truncated variants thereof expressed in rats as the splice variant ER46 (Longo et al., 2004; Staub et al., 2005), which might still be activated by raloxifene leading to increased endothelial NOS function (Haynes et al., 2000). The mechanisms of raloxifene-mediated acute vasorelaxation, however, did differ from those of  $E_2$  under our experimental conditions (Fig. 7) because the latter was significantly more effective and more potent than the former (Fig. 1) and was sensitive to antagonism by both ICI 182,780 (Bolego et al., 2005) and MPP (Fig. 4). This pattern may be further affected by gender and ovarian status (Bramante et al., 2002; Bolego et al., 2005), but  $E_2$  replacement to ovariectomized rats as used in the present study rules out the confounding variables of estrus cycle and hormone deprivation.

The duration of exposure to raloxifene as well as other estrogenic compounds is a critical determinant of activation of different mechanisms in reproductive tissues (Zheng et al., 2004). Therefore, short-term nongenomic effects of raloxifene as discussed above may be associated with long-term genomic effects in the vessel wall. On testing this hypothesis,



**Fig. 7.** Schematic diagram of the mechanism of regulation of vascular tone and iNOS expression by raloxifene. At nanomolar concentrations, both  $E_2$  and raloxifene (Ral) induce rapid L-arginine (L-Arg)-derived NO-dependent vasorelaxation. Although it is established that  $E_2$  acts via membrane  $ER\alpha$  by activating Akt and endothelial NOS (eNOS) phosphorylation, a pathway that is blocked by selective or nonselective ER antagonists [ICI 182,780 (ICI), MPP], raloxifene-driven eNOS activation is insensitive to ER antagonists and may occur through a different  $ER\alpha$  domain or variant with respect to  $E_2$  or through a putative SERM receptor. This acute vasorelaxant effect of raloxifene is retained after challenge with 0.1 mg/kg LPS *in vivo* (not shown). At micromolar concentrations (Ral), raloxifene may relax vascular smooth muscle cells by modulating ion channels. Upon activation of inflammatory signaling pathways,  $ER\alpha$  levels are decreased, and iNOS production is enhanced in smooth muscle cells, leading to accumulation of NO as well as of harmful derivatives thereof such as peroxynitrite. By acting through intracellular  $ER\alpha$ , Ral restores, at least in part,  $ER\alpha$  protein levels and down-regulates iNOS functional expression. Although the nature of the association between  $ER\alpha$  activation and iNOS function remains to be established, this pathway does not involve direct transcriptional modulation because the iNOS gene lacks estrogen response elements (data not shown). CaM, calmodulin.

we detected novel anti-inflammatory properties of raloxifene in isolated vascular SMCs in culture, consistent with those shown in microglial cells (Suuronen et al., 2005) and on carrageenan-induced paw edema and pleurisy after *in vivo* treatment (Esposito et al., 2005). In addition, the present study reports that the rapid vasorelaxation mediated by raloxifene was retained in aortic tissues from rats under moderate inflammatory challenge as induced by low-dose LPS. By contrast, no effect of raloxifene on vascular tone was detected in tissues from animals challenged with high-dose LPS, possibly because of a massive systemic vascular NO release (Vo et al., 2005) that outweighed the acute release triggered by the drug in the organ bath. To allow more predictive conclusions on the relevance of these mechanisms, further studies with long-term raloxifene treatment should be performed using *in vivo* models of vascular inflammation.

At the cellular level, the decrease in iNOS functional expression induced by raloxifene after 24-h treatment (Fig. 4A) resembles that induced by  $E_2$  in vascular SMCs (Zancan et al., 1999). At variance with acute vasorelaxation, iNOS inhibition by raloxifene was blocked by MPP, suggesting the



onset of ER $\alpha$ -dependent mechanisms. Raloxifene binds to and activates ER $\alpha$  and ER $\beta$  (Brzozowski et al., 1997; Barkhem et al., 1998; Pike et al., 1999), both of which are expressed in rat aortic SMCs (Zanac et al., 1999; Maggi et al., 2003). ER contribution to the anti-inflammatory effect of SERM in microglial cells has not been assessed, although the authors argue that the SERM-induced response is due to interference with the signaling cascade activated by LPS treatment (Suuronen et al., 2005). To the best of our knowledge, we here report for the first time that iNOS inhibition by raloxifene was ER $\alpha$ -dependent and was associated with up-regulation of ER $\alpha$  protein levels that had been decreased by cytokine challenge (Fig. 6). This is an autologous regulation pathway because it is inhibited by MPP and likely involves binding of liganded ER to the ER gene (Barchiesi et al., 2004), thus enhancing transcription at least when inflammatory signaling pathways are operating (Fig. 7). Although the relationship between ER $\alpha$  protein expression and iNOS-mediated NO formation remains to be established, increased concentrations of E<sub>2</sub> also up-regulate ER $\alpha$  in rat vascular SMCs (Maggi et al., 2003). Thus, a potential amplification of ER $\alpha$  action occurs with prolonged ligand activation in vascular SMCs. These findings are also in agreement with the anti-inflammatory role of ER $\alpha$  as described in other tissues (Evans et al., 2002; Vegeto et al., 2003) and instead hint at a pro-inflammatory role for genomic ER $\beta$  activation in the vascular wall as that induced, for instance, by the relatively ER $\beta$ -selective phytoestrogen genistein (Cignarella et al., 2006). This fits with the current paradigm of the yin/yang relationship between the two cloned ER isoforms (Weihua et al., 2003). Consequently, any shift in the relative ER expression pattern in vascular and other tissues may be potentially associated with changes in the anti-inflammatory action of estrogenic compounds.

In conclusion, raloxifene rapidly relaxed aortic tissues of E<sub>2</sub>-replaced ovariectomized female rats also following inflammatory challenge and prevented cytokine-driven iNOS activation in isolated aortic SMCs after 24-h treatment. These effects were raised at concentrations that may be relevant in vivo and involved, at least in part, ER $\alpha$  activation. This combined action of raloxifene on vascular function could contribute to explain its antiatherosclerotic properties in animal models (Bjarnason et al., 2001) and may lay the ground for the development of novel ER isoform-selective SERMs with targeted action to the vascular system (Bolego et al., 2006).

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