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Raloxifene Relaxes Rat Pulmonary Arteries and Veins: Roles of Gender, Endothelium and Antagonism of Ca^{2+} Influx

YAU-CHI CHAN, FUNG-PING LEUNG, XIAOQIANG YAO, CHI-WAI LAU, PAUL M. VANHOUTTE and YU HUANG

Department of Physiology (YCC, FPL, XY, CWL, YH), Chinese University of Hong Kong, Hong Kong, China; Department of Pharmacology (PMV), University of Hong Kong, Hong Kong, China

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Corresponding author:

Yu HUANG, Ph.D

Department of Physiology

Faculty of Medicine

Chinese University of Hong Kong

Shatin, NT, Hong Kong

Tel: 852-26096787; Fax: 852-26035022

e-mail: yu-huang@cuhk.edu.hk

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ABBREVIATIONS: L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one; SERM, selective estrogen receptor modulator.

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ABSTRACT

Effects of raloxifene have been documented in the systemic circulation. However, its impact on the pulmonary circulation is unclear. The present study investigated the role of gender, endothelial modulation and Ca^{2+} channel in relaxations evoked by raloxifene in rat pulmonary arteries and veins. Vascular responses were studied on isolated pulmonary blood vessels mounted in a myograph and constricted by U46619. Constrictions to $CaCl_2$ were studied in Ca^{2+}-free, 60 mM K^+ solution. Changes in the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in vascular smooth muscle were measured using a calcium fluorescence imaging method. Raloxifene was more effective in relaxing U46619-constricted pulmonary arteries from male than female rats. Raloxifene-induced relaxation was unaffected by ICI 182,780, inhibition of nitric oxide (NO) pathway, or removal of the endothelium. In arteries without endothelium, raloxifene attenuated $CaCl_2$-induced constriction and $CaCl_2$-stimulated increase in $[Ca^{2+}]_i$ with similar potencies. Raloxifene caused endothelium-independent relaxations in pulmonary veins albeit to a lesser degree than in pulmonary arteries. The venous responses showed a gender difference as raloxifene was more potent in male veins. In summary, raloxifene relaxed rat pulmonary arteries and this effect did not involve the endothelium/NO or ICI 182,780-sensitive estrogen receptors. Raloxifene, like nifedipine, reduced both constriction and $[Ca^{2+}]_i$ increase in response to $CaCl_2$ in high K^+ solution. Raloxifene also relaxed high K^+-constricted pulmonary veins. Our data indicate that raloxifene acutely relaxes rat pulmonary blood vessels primarily via inhibition of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Finally, raloxifene induced more relaxation in blood vessels isolated from male than female rats.

Introduction

In spite of the favorable effects of hormone replacement therapy (HRT) on established cardiovascular risk factors suggested in early observational studies, the findings from a recent HRT clinical trial have questioned its long-term safety (Rossouw et al., 2002). Designer estrogen or selective estrogen receptor modulators (SERMs) have been developed to avoid the clinical disadvantages of HRT. Raloxifene, the 2nd generation SERM with anti-estrogenic effects on breast and uterus, represents a promising alternative to HRT as it exerts estrogenic effects on key cardiovascular risk factors (Saitta et al., 2001). Besides, recent clinical studies support a cardioprotective effect of raloxifene in women at high coronary risk (Barrett-Connor et al., 2002).

Female rats exposed to chronic hypoxia exhibited less pulmonary arterial hypertension (Rabinovitch et al., 1981) and right ventricular hypertrophy (McMurtry et al., 1973) compared with age-matched male rats. Similar to the systemic circulation, one mechanism for estrogen modulation of pulmonary artery relaxation may be mediated by augmented nitric oxide (NO) function (Gonzales et al., 2001).

Many studies have explored the mechanisms for SERM modulation of vascular tone in both systemic arteries and veins (Figtree et al., 1999; Bracamonte et al., 2002; Tsang et al., 2004a). Vasorelaxation to raloxifene in females is influenced by ovarian hormonal status (Bracamonte et al., 2002). Two main mechanisms reported for the vascular action of SERMs in systemic vascular tissues are up-regulation of endothelial NO production (Figtree et al., 1999; Bracamonte et al., 2002) and inhibition of L-type voltage-sensitive Ca^{2+} channels (Tsang et al., 2004b). However, no studies have examined SERM regulation of vasomotor activity in the pulmonary vascular circulation and its potential as a new drug in the treatment of pulmonary

arterial hypertension. Therefore, we investigated the vascular effects of raloxifene, the roles of endothelial modulation, estrogen receptors, and Ca^{2+} channel antagonism in isolated rat pulmonary arteries and veins, as well as gender differences in the action of raloxifene.

Methods and Materials

Blood vessel preparation

This study was approved by the Animal Research Ethics Committee of Chinese University of Hong Kong. This investigation conformed to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No.85-23, revised 1996). Both female and male Sprague-Dawley rats weighing 250-300 g were euthanized. The lungs were carefully dissected out and placed in ice-cold Krebs solution. Second order intralobal pulmonary arteries (internal diameter: ~ 200 μm) and main pulmonary veins (internal diameter: ~ 490 μm) were dissected free from lungs and the surrounding connective tissue was cleaned off under a dissecting microscope. Each blood vessel was cut into two ~1-mm ring segments. Individual ring was mounted between two tungsten wires (40 μm in diameter) for measurement of isometric tension in a 5-ml organ chamber filled with Krebs solution. Each wire was fixed to the mounting jaws of the myograph (Danish Myo Technology A/S, Denmark). The chamber solution was continuously bubbled with 95% O_2 - 5% CO_2 at 37 ^oC (pH 7.4.) All rings were placed under an optimal resting tension (approximately 1 mN), which was the minimum level of stretch giving the largest force development in 60 mM K^+ solution as determined on the length-tension relationship (Nyhan et al., 2002). In most experiments, the endothelial layer was mechanically removed by gently rubbing the luminal surface with a tungsten wire and the functional removal was verified by the lack of relaxation to 3 μM acetylcholine.

Protocols

Thirty minutes after mounting, rings were first constricted by 100 nM U46619 and subsequently challenged with acetylcholine to confirm integrity or removal of the endothelium. They were then washed in Krebs solution to restore tension to baseline level and allowed to stabilize for 60 min. The role of endothelium/NO and estrogen receptors was first examined in pulmonary relaxant responses to raloxifene. For this series of experiments, rings were exposed for 30 min to each inhibitor (100 μM N^G-nitro-L-arginine methyl ester, 3 μM 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one, or 10 μM ICI 182,780) before addition of U46619. Once steady blood vessel tone was obtained, raloxifene was applied cumulatively to the bathing solution. Only one concentration-response curve to raloxifene was obtained per ring in the presence of each inhibitor. The ability of raloxifene to modulate Ca^{2+} influx via L-type voltage-sensitive Ca^{2+} channels was studied on constrictions to $CaCl_2$ in rings without endothelium. For this set of experiments, two consecutive concentration-dependent constrictions to $CaCl_2$ were obtained in control and in the presence of raloxifene (0.1-10 μM, 30-min incubation) before repeating the second concentration-response curve. For constructing $CaCl_2$ concentration-response curve, arterial rings were rinsed 3 times in a Ca^{2+}-free solution containing 30 μM Na_2-EGTA, then incubated in Ca^{2+}-free, 60 mM K^+ solution prior to cumulative addition of $CaCl_2$ (0.1-5 mM). The effect of nifedipine (1 μM) was tested as control. The relaxing effect of raloxifene was also studied on 60 mM K^+-constricted rings in normal Ca^{2+}-containing Krebs solution. We finally examined the effect of raloxifene on isolated rat pulmonary veins and the role of the endothelium/NO.

Measurement of vascular smooth muscle $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in Fura-2-loaded pulmonary artery smooth muscle using the ratio imaging. Rings were incubated for 1 hr at 22 ^oC in a Fura-2 loading solution that contained: 10

μM Fura-2 AM and 0.025% pluronic F-127 (to prevent Fura-2 secretion). Thereafter, extracellular Fura-2 AM was removed by repetitive washing in Krebs solution. Rings were then perfused for 20 min with Krebs solution at 2 ml/min (37 ^{o}C) to permit cleavage of intracellular Fura-2 AM into active Fura-2 by esterases. Because of the Fura-2 photosensitivity, precautions were taken to avoid extensive photobleaching. The excitation light was blocked by a shutter when no fluorescence measurement was performed.

The $[Ca^{2+}]_i$ imaging setup was modified from that described by Huang *et al*. (2000). In brief, after Fura-2 loading, cut-open artery rings without endothelium were mounted onto a block of silicone elastomer with stainless steel wires and pins (Sylgard, WPI), which was then fixed into a base plate of a custom-made flow chamber. The base plate was covered with a gasket and cover glass (24 x 32 mm; thickness: no. 1; Menzel-Glaser, Germany) and affixed by screws. There was a 1-mm gap between the vessel and the cover glass to allow flow passage. This arrangement also allows free vessel movement in response to addition of vasoactive drugs. After mounting, the flow chamber was placed on an inverted microscope and perfused at 2 ml/min with Krebs solution at 37 ^{o}C using a six-channel perfusion pump (Watson Marlow Corp., USA).

Fura 2-loaded arterial tissues were viewed through a Nikon CF Fluor 20x objective on an inverted Nikon Eclipse TE300 microscope. Fura-2 was excited using a collimated beam of light from a 75 W xenon arc lamp, which passed through a microscope photometer D-104 (Photon Technology International, NJ, USA) that altered wavelengths from 340 to 380 nm using an optical chopper OC-4000 (Photon Technology International). The emitted light at 510 nm was collected by a photomultiplier tube. Data acquisition and analysis were performed using FELIX 1.21 software (Photon Technology International).

After mounting, the arterial tissues were allowed to recover for 30 min at 37 °C and then exposed for 30 min to a Ca^{2+}-free, 60 mM K^+ prefusion solution. Subsequently, they were perfused with 60 mM K^+ containing $CaCl_2$ (0.1-3 mM) to construct the first concentration-response curve. Rings were rinsed first in Ca^{2+}-free solution and then in Ca^{2+}-free, 60 mM K^+ solution to allow returning of the Ca^{2+} level to baseline and finally incubated for 30 min with 300 nM or 10 μM raloxifene before repeating the second $CaCl_2$ concentration-response curve.

Drugs

Acetylcholine [2-(acetyloxy)-*N*,*N*,*N*-trimethylethanaminium], U46619 [9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$], L-NAME [N^G-nitro-L-arginine methyl ester], ODQ [1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one], and nifedipine [1,4-dihydro-2,6-dimethyl-4(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] were purchased from Sigma. ICI 182,780 [7α-[9-[(4,4,5,5,5,-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17ß-diol] was purchased from Tocris. Raloxifene [[6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl][4-[2-piperidinoethoxy]phenyl]ketone hydrochloride] was a gift from Lilly Corporate Center (Indianapolis, USA). U46619, raloxifene, and nifedipine were dissolved in DMSO and others in distilled water. Further dilution was made from stock solutions. Krebs solution contained (in mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. High K^+ solution was prepared by replacing Na^+ with an equimolar amount of K^+ to retain constant ionic strength.

Data analysis

Results are means ± S.E.M. of rings from *n* rats. Increases in constrictive force were expressed as percentages of the maximal response obtained in the first concentration-dependent constriction to $CaCl_2$. Concentration-response curves were constructed based on responses to

cumulative drug concentrations and analyzed by non-linear curve fitting using Graphpad software (Version 3.0). The negative logarithm of the dilator (or constrictor) concentration that caused half (pD_2 or pEC_{50}) of the maximal response (E_{max}) was obtained. For statistical analysis, two-tailed Student's *t* test or one-way analysis of variance followed by Newman-Keuls test was used when more than two groups were compared. Individual concentration-response curves were also compared using a two-way analysis of variance followed by a post hoc test. $P < 0.05$ was considered to be significant.

Results

Effects of raloxifene on pulmonary arteries

U46619 constricted rat pulmonary arteries to a comparable degree in both genders (tension: 3.71 ± 0.60 mN with endothelium and 3.73 ± 0.36 mN without endothelium in female, $P > 0.05$; 3.13 ± 0.51 mN with endothelium and 2.98 ± 0.34 mN without endothelium in male, $P > 0.05$). Upon U46619 pre-constriction raloxifene caused relaxations of arteries with and without endothelium (pD_2: 4.78 ± 0.13 versus 5.13 ± 0.13, $P > 0.05$ in female, Fig. 1A and 5.34 ± 0.15 versus 5.53 ± 0.08, $P > 0.05$ in male, Fig. 1D). Neither L-NAME (NO synthase inhibitor) nor ODQ (guanylate cyclase inhibitor) affected relaxation to raloxifene in female (Fig. 1B) and male (Fig. 1E) arteries. Likewise, ICI 182,780 was without effect on arteries from both genders (Fig. 1C&F). ICI 182,780 may act as a partial estrogen receptor agonist (Bracamonte et al., 2002), but this agent (0.1 – 50 μM) did not affect U46619-induced constriction (data not shown). Vehicle (DMSO) did not influence U46619-induced tone in arteries with and without endothelium (Fig. 1A&D).

There was a gender difference in relaxations to raloxifene in U46619-constricted arteries (pD_2: 4.78 ± 0.13 in female and 5.34 ± 0.15 in male, $P < 0.05$, Fig. 2A), but this difference was

absent in 60 mM K^+-constricted arteries (pD_2: 5.61 ± 0.07 in female and 5.70 ± 0.09 in male, $P > 0.05$, Fig. 2B). The relaxing potency of raloxifene was greater in arteries constricted by 60 mM K^+ than U46619.

Effect of raloxifene on $CaCl_2$-induced constriction in pulmonary arteries

In Ca^{2+}-free, 60 mM K^+ solution, $CaCl_2$ induced constrictions of arteries without endothelium (pEC_{50}: 3.81 ± 0.15 in female and 3.77 ± 0.11 in male, $P > 0.05$). Raloxifene reduced $CaCl_2$-induced constrictions in a non-competitive manner with progressive suppression of the maximal constriction in both female (Fig. 3A) and male (Fig. 3B) arteries. In control experiments, nifedipine (1 μM) abolished constrictions to $CaCl_2$.

Effects of raloxifene on pulmonary veins

U46619 constricted pulmonary veins (tension: 0.96 ± 0.12 mN in female and 1.06 ± 0.23 mN in male, $P > 0.05$). In U46619-constricted veins from both genders, raloxifene caused small relaxations and this effect was similar in arteries with and without endothelium (Fig. 4A&C). L-NAME did not modify this relaxation (Fig. 4B&D). 60 mM K^+ constricted pulmonary veins (tension: 0.77 ± 0.12 mN in female and 0.87 ± 0.13 mN in male, $P > 0.05$) and this constriction was reduced by raloxifene (Fig. 5B). Gender-related differences were demonstrated in the venous responses to raloxifene with a greater relaxing effect on male than female veins constricted by either U46619 or 60 mM K^+ (Fig. 5A&B)

Effect of raloxifene on $CaCl_2$-stimulated increases in $[Ca^{2+}]_i$ in pulmonary arteries

The effect of raloxifene on $[Ca^{2+}]_i$ was examined in rings without endothelium. $CaCl_2$ induced $[Ca^{2+}]_i$ rise in Ca^{2+}-free, 60 mM K^+ solution and the first and second concentration-dependent responses were similar. Changes in $[Ca^{2+}]_i$ measured as the fluorescence ratio

(F340/F380) before and after treatment with raloxifene (0.3 and 10 μM) in female and male pulmonary arteries are summarized in Fig. 6. The cumulative addition of $CaCl_2$ caused progressive rises in $[Ca^{2+}]_i$ and raloxifene reduced $[Ca^{2+}]_i$ increases. There was no gender difference in the effect of raloxifene (Fig. 6). In control experiments, nifedipine (1 μM) abolished $CaCl_2$-induced rise in $[Ca^{2+}]_i$.

Discussion

The new findings from this study using isolated rat pulmonary arteries and veins are: (1) raloxifene-induced pulmonary vascular relaxation was independent of the presence of endothelium; (2) raloxifene-induced acute effect is unrelated to ICI 182,780-sensitive estrogen receptors; (3) raloxifene reduced $CaCl_2$-induced constriction and Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels; and (4) there was a gender difference in vascular responses to raloxifene. Some of these observations parallel those made in previous studies on the systemic vessels (Figtree et al., 1999; Tsang et al., 2004b). This study of the acute effects of raloxifene suggests that raloxifene may reduce pulmonary pressure by inhibiting the activity of voltage-sensitive Ca^{2+} channels in vascular smooth muscle cells.

Raloxifene inhibited high K^+-induced constrictions, indicating that raloxifene may act as a Ca^{2+} channel inhibitor to cause pulmonary vascular relaxation. Similar effects were reported in systemic arteries (Figtree et al., 1999; Tsang et al., 2004b). Raloxifene also reduced $CaCl_2$-induced constriction in high K^+ solution with progressive reduction of the maximal response; this further suggested that raloxifene interferes with Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in these blood vessels. Ca^{2+} influx is the linker in excitation-contraction coupling in vascular smooth muscle upon membrane depolarization or constrictor stimulation. Indeed, the calcium antagonistic action of raloxifene was proven by the demonstration that raloxifene

inhibits Ca^{2+} influx via Ca^{2+} channels as revealed by $[Ca^{2+}]_i$ imaging measurement in Fura 2-loaded pulmonary vascular tissues without endothelium. The potency was similar for raloxifene between relaxing $CaCl_2$-induced tension and inhibiting $CaCl_2$-stimulated $[Ca^{2+}]_i$ rise. Like the L-type Ca^{2+} channel blocker nifedipine, raloxifene at 10 μM almost abolished $CaCl_2$-induced increase in both vessel tone and $[Ca^{2+}]_i$. Besides, high K^+-constricted arteries exhibited higher relaxing sensitivity to raloxifene than U46619-constricted arteries. These data indicate that inhibition of Ca^{2+} entry via L-type Ca^{2+} channels is an important mechanism by which raloxifene causes pulmonary vascular relaxation.

Endothelial dysfunction characterized by progressive loss of the relaxation to NO-dependent dilators contributes to the development of hypoxic pulmonary hypertension (Adnot et al., 1991; Berkenbosch et al., 2000). Acute treatment with estrogen or phytoestrogens restored endothelial function in pulmonary arteries isolated from chronically hypoxic rats (Karamsetty et al., 2001). In the systemic circulation, raloxifene rapidly relaxed mammalian arteries and veins partly by increasing NO (Figtree et al., 1999; Bracamonte et al., 2002). However, the present study shows that raloxifene induced relaxation to the same extent in pulmonary arteries with and without endothelium. Inhibition of NO pathway did not affect the relaxation. Despite the enhanced NO function described in the systemic arteries from raloxifene-treated rats (Wassmann et al., 2002), the present data make a positive role of endothelium/NO in the acute pulmonary relaxation to raloxifene unlikely. This conclusion agrees with the observation that estrogen attenuated pulmonary hypertension via an eNOS-independent mechanism (Resta et al., 2001).

The contribution of estrogen receptors to vascular responses to estrogen or SERMs remains controversial and undefined. ICI 182,780, a selective estrogen receptor antagonist inhibited the non-genomic effects of raloxifene on the endothelium (Figtree et al., 1999), but not

on vascular smooth muscle (Figtree et al., 1999; Tsang et al., 2004b). The present study shows that ICI 182,780 failed to influence raloxifene-induced pulmonary artery relaxation. ICI 182,780 had no effect on relaxation to raloxifene in porcine femoral veins (Bracamonte et al., 2002). Instead, ICI 182,780 may act as a partial estrogen receptor agonist in femoral veins by causing relaxation (Bracamonte et al., 2002). However, ICI 182,780 did not induce significant relaxation in pulmonary arteries from both genders in the present study. Taken together, like its effects on some arteries in the systemic circulation (Bracamonte et al., 2002; Tsang et al., 2004b), the acute relaxation caused by raloxifene in pulmonary arteries *in vitro* does not involve ICI 182,780-sensitive estrogen receptor stimulation. However, it is unclear how raloxifene may act on Ca^{2+} channels in vascular smooth muscle if its effect is not mediated by estrogen receptors. Estrogen was shown to activate Ca^{2+}-activated K^+ channels by a direct interaction with the ß-subunit of the channel protein (Valverde et al., 1999). It is yet to elucidate whether the Ca^{2+} channel could provide such an interactive site for raloxifene. It should be noted that the present data do not preclude the chronic action of raloxifene on vascular estrogen receptors, which could contribute to long-term effects of raloxifene in the pulmonary circulation.

There is a gender difference in hypoxia-induced pulmonary hypertension (Rabinovitch et al., 1981) and right ventricular hypertrophy (McMurtry et al., 1973), but it is unknown whether this difference is influenced by the direct vascular effects of sex hormones. The gender difference was observed in relaxation to raloxifene of pulmonary arteries constricted by the receptor-dependent constrictor U46619, but not by the receptor-independent constrictor K^+. The relaxing potency was higher in male than female arteries. This sexual dimorphism in raloxifene relaxation is more significant in pulmonary veins regardless of the type of constrictors used.

Finally, the present study shows that raloxifene was less effective in pulmonary veins than arteries although the mechanism underlying that discrepancy is unclear.

In conclusion, the present findings provide experimental evidence for a key mechanism by which raloxifene relaxes rat pulmonary vessels. Raloxifene acts primarily on vascular smooth muscle of pulmonary arteries by inhibiting Ca^{2+} entry via L-type Ca^{2+} channels. This action is acute, non-genomic and independent of a functional endothelium or ICI 182,780-sensitive estrogen receptors. Such calcium antagonistic action may make raloxifene a potentially useful agent in the pulmonary arterial hypertension like an oral Ca^{2+} channel blocker if this effect also occurs *in vivo*. Raloxifene is clinically used to treat menopausal women, but the present *in vitro* data show that raloxifene appears to be more effective in causing pulmonary vascular relaxation in male than female animals. However, it remains to be investigated whether raloxifene could exert similar gender-related effects *in vivo* on the pulmonary circulation.

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Footnotes

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Address correspondence to: Dr. Yu Huang, Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, NT, Hong Kong. E-mail: yu-huang@cuhk.edu.hk

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Legends for Figures

Fig. 1. Concentration-dependent relaxations to raloxifene in female (A) and male (D) rat pulmonary arteries with and without endothelium, in arteries with endothelium treated by 100 μM L-NAME or 3 μM ODQ (B and E) or by 10 μM ICI 182,780 (C and F). Arteries were constricted by U46619 following 30-min incubation with each inhibitor. Results are means ± S.E.M. of 7-10 experiments.

Fig. 2. Relaxation to raloxifene in (A) U46619- or (B) 60 mM K^+-constricted arteries with endothelium from both female and male rats. Results are means ± S.E.M. of 7-8 experiments.

Fig. 3. $CaCl_2$-induced contraction in Ca^{2+}-free, 60 mM K^+ solution in the absence and presence of raloxifene (0.1 to 10 μM) in arteries without endothelium from female (A) and male (B) rats. Results are means ± S.E.M. of 6 experiments.

Fig. 4. Effects of raloxifene on U46619-constricted pulmonary veins from (A) female and (C) male rats. Lack of effect of 100 μM L-NAME on relaxation to raloxifene (B, female and D, male). Results are means ± S.E.M. of 7-8 experiments.

Fig. 5. Gender differences in relaxant responses to raloxifene in pulmonary veins constricted by (A) U46619 or (B) 60 mM K^+. Results are means ± S.E.M. of 7-8 experiments. Statistical difference is indicated between two curves (*** $P < 0.001$, two-way ANOVA).

Fig. 6. Concentration-dependent increases in Ca^{2+}/Fura 2-AM fluorescence in response to $CaCl_2$ in a Ca^{2+}-free, 60 mM K^+-containing solution and inhibitory effects of raloxifene (30 min incubation time) on rings from female (A&B) and male (C&D) rats. All experiments were performed on pulmonary artery rings without endothelium that were first incubated with Fura 2-AM for 45 min and perfused with Ca^{2+}-free, 60 mM K^+ solution for 15 min prior to perfusion of 60 mM K^+ solution supplemented with $CaCl_2$ (0.1-3 mM). Changes in $[Ca^{2+}]_i$ were recorded as the ratio (F_{340}/F_{380}) of Fura-2 fluorescence at excitation wavelengths of 340 and 380 nm. Data are expressed in means ± S.E.M. of rings from four separate animals. Statistical difference is indicated between two curves (* $P < 0.05$; *** $P < 0.001$, two-way ANOVA).

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Fig. 1.

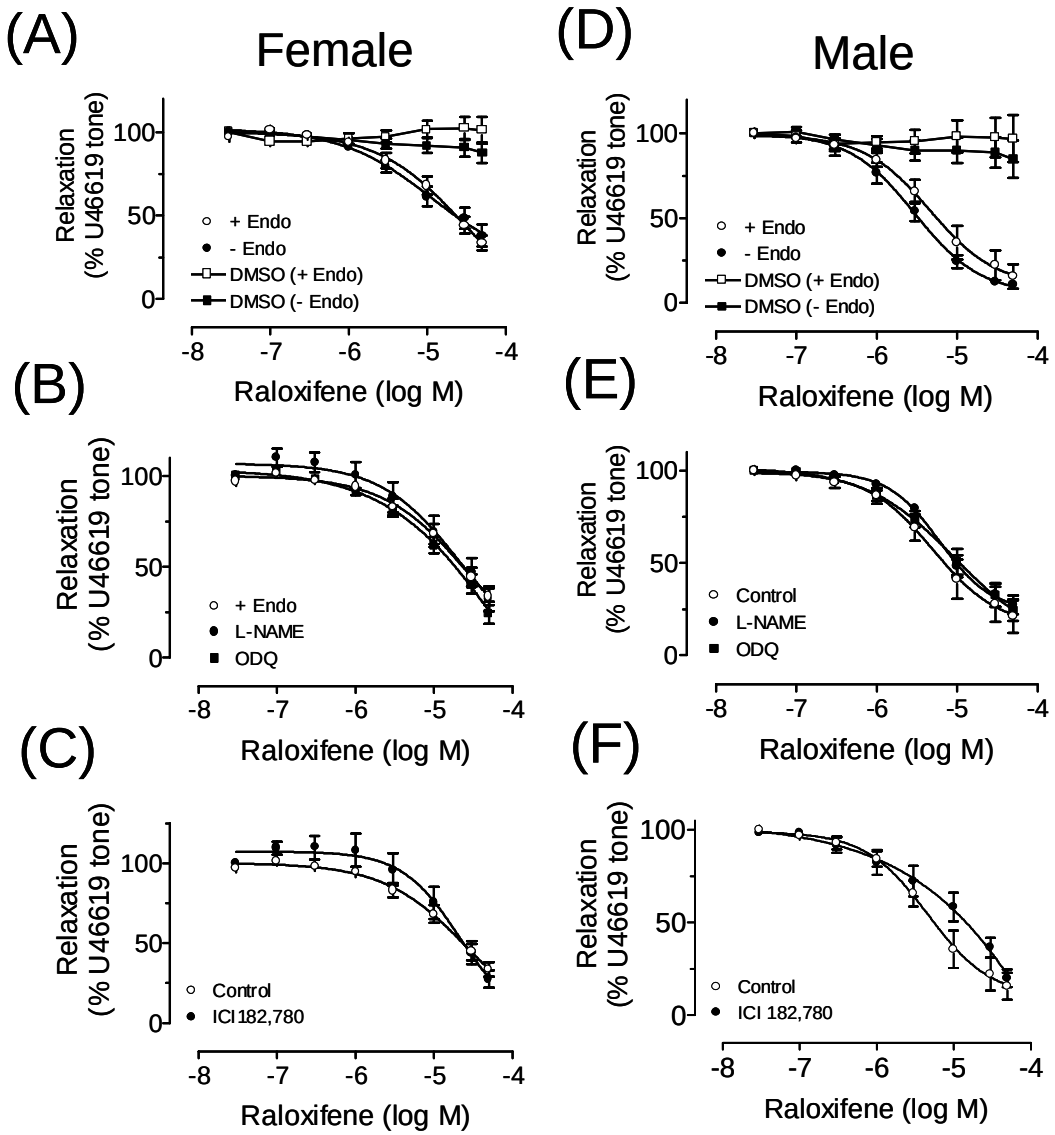

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Fig. 2.

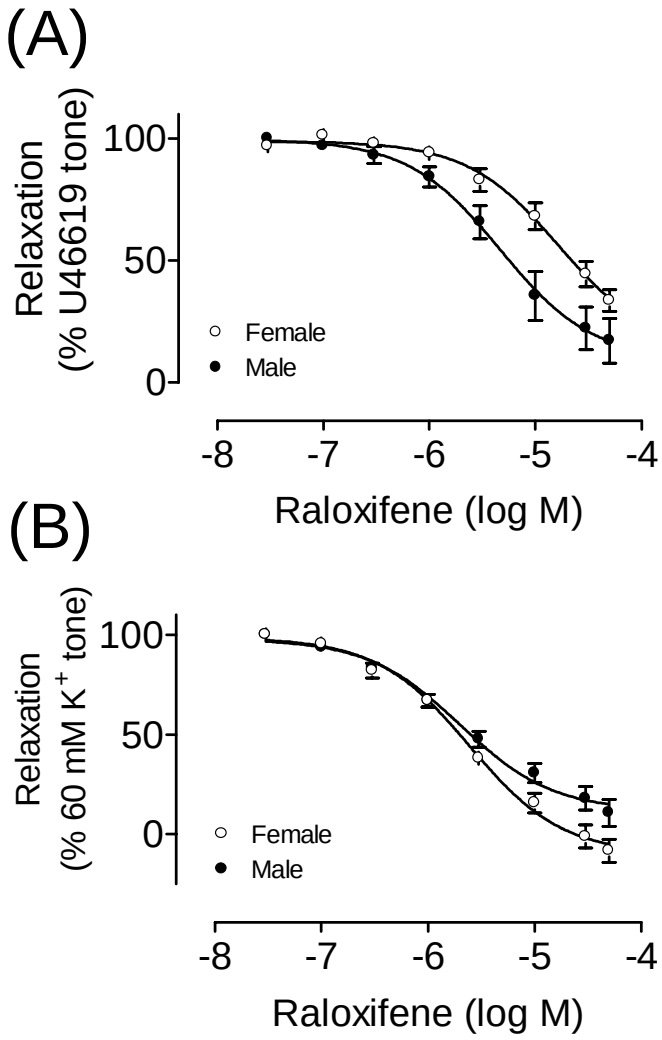

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Fig. 3.

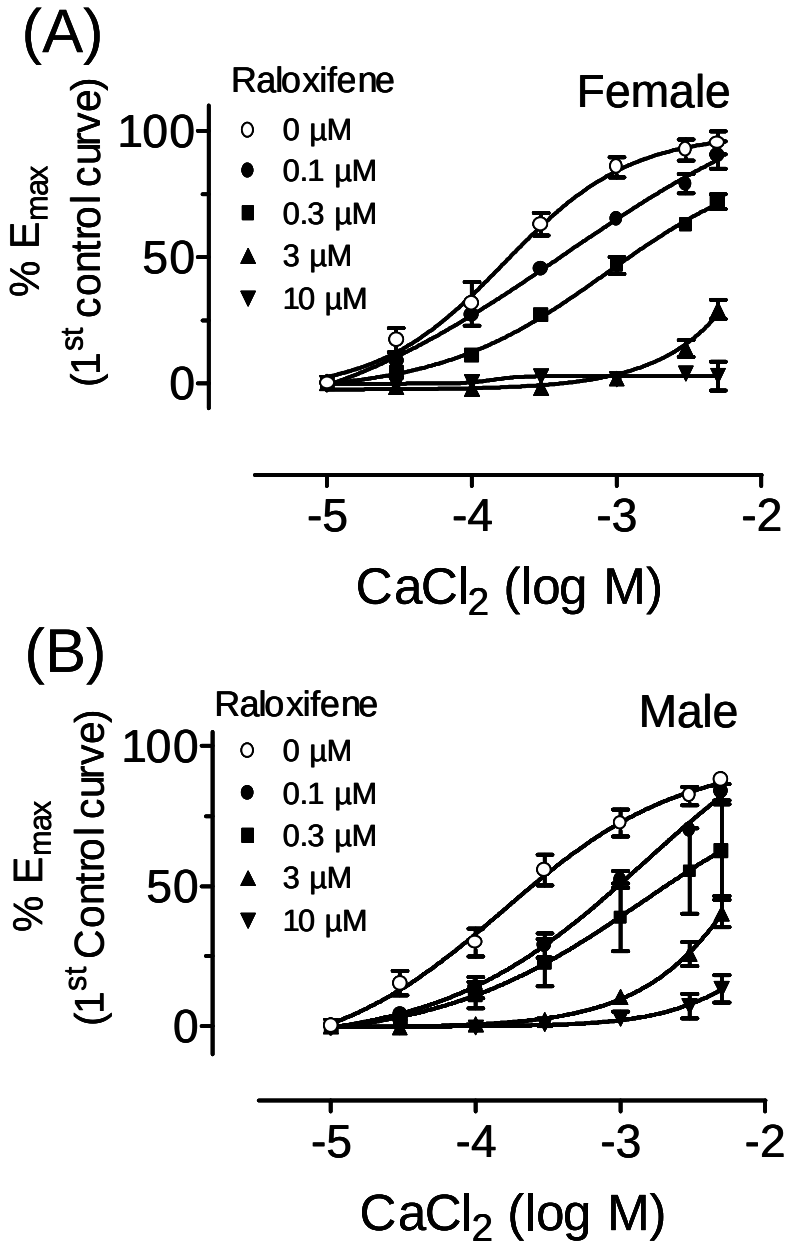

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Fig. 4.

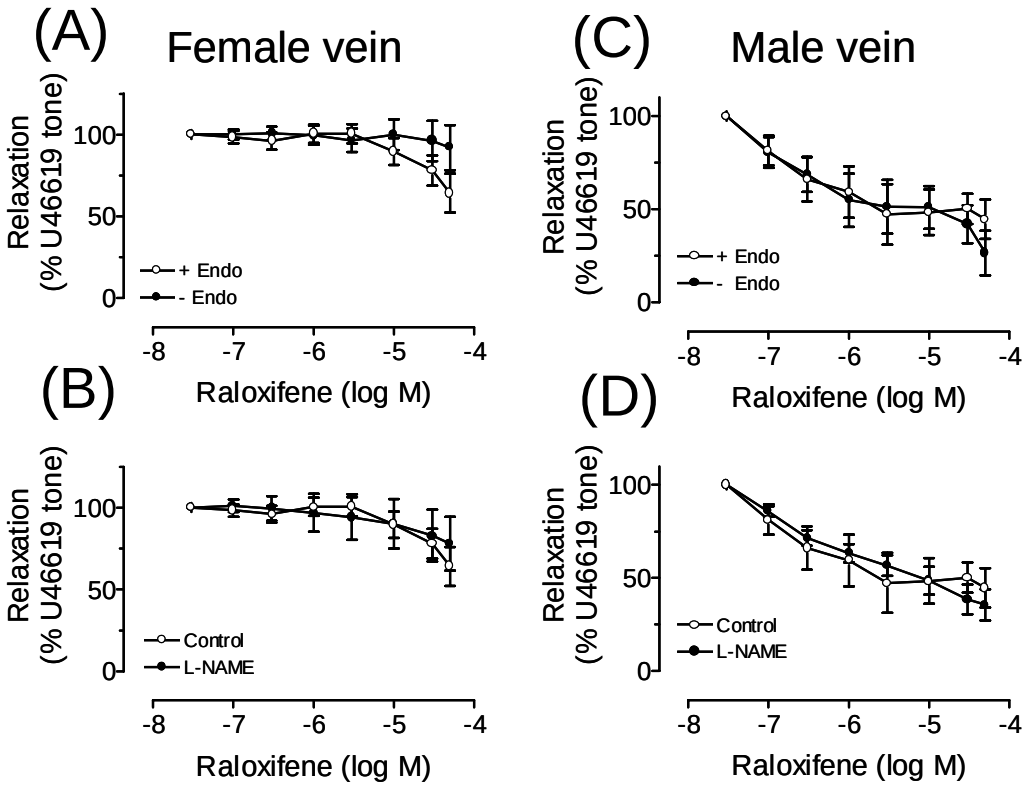

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Fig. 5.

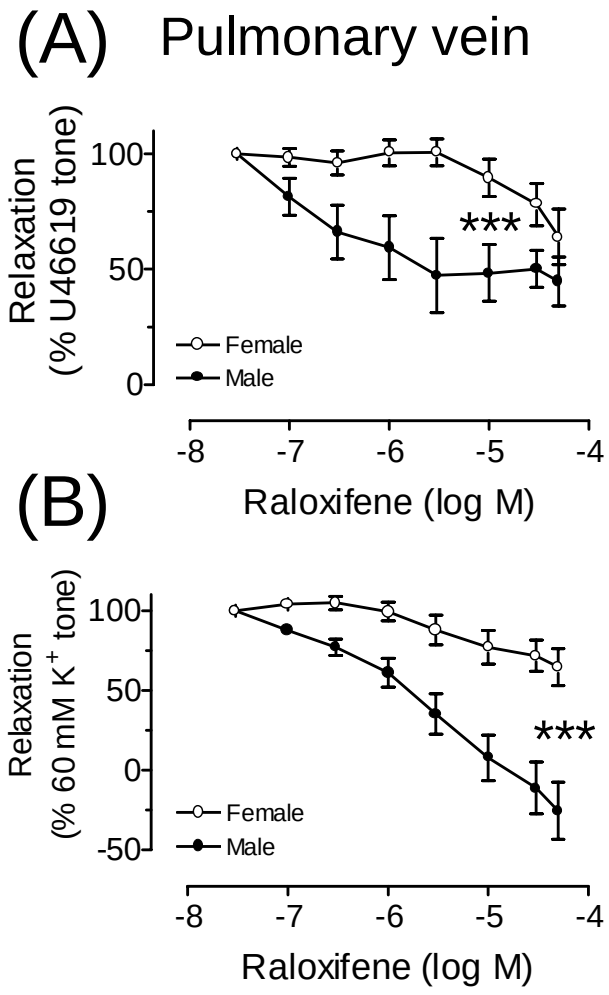

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Fig. 6.