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

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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 (9,11-dideoxy-11\(\alpha\),9\(\alpha\)-epoxyethanoprostaglandin F\(2\alpha\)). 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 [7\(\alpha\]-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17\(\beta\)-diol], inhibition of the 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 because 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 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.

Despite 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 second-generation SERM with antiestrogenic effects on the breast and uterus, represents a promising alternative to HRT because it exerts estrogenic effects on key cardiovascular risk factors (Saitta et al., 2001). Recent clinical studies also 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 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.

ABBREVIATIONS: HRT, hormone replacement therapy; SERM, selective estrogen receptor modulator; NO, nitric oxide; U46619, 9,11-dideoxy-11\(\alpha\),9\(\alpha\)-epoxyethanoprostaglandin F\(2\alpha\); ICI 182,780, 7\(\alpha\)-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17\(\beta\)-diol; AM, acetoxymethyl ester; L-NAME, N\(^{\omega}\)-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one; ANOVA, analysis of variance.
(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 Ca2+ channel antagonism in isolated rat pulmonary arteries and veins and 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 U.S. National Institute of Health (NIH Publication No. 85-23, revised 1996). Female and male Sprague-Dawley rats weighing 250 to 300 g were euthanized. The lungs were carefully dissected out and placed in ice-cold Krebs' solution. Second-order intralobar 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. The individual ring was mounted between two tungsten wires (40 μm in diameter) for the 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, Aarhus, Denmark). The chamber solution was continuously bubbled with 95% O2/5% CO2 at 37°C (pH 7.4). All the rings were placed under an optimal resting tension (−1 mN), which was the minimum level of stretch giving the largest force development in 60 mM K+ solution, as determined by the length-tension relationship (Nyhan et al., 2002). In most experiments, the endothelial layer was removed mechanically 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. After mounting (30 min), rings were first constricted by 100 nM U46619 and subsequently challenged with acetylcholine to confirm the integrity or removal of the endothelium. Then they were washed in Krebs' solution to restore tension to baseline level and exposed for 30 min at 37°C using an OC-4000 optical chopper (Photon Technology International, Monmouth Junction, NJ) that altered wavelengths from 340 to 380 nm with an AM1001 optical shutter (Photon Technology International). A photomultiplier tube collected the emitted light at 510 nm. 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 Ca2+-free, 60 mM K+ perfusion solution. They subsequently were perfused with 60 mM K+ containing CaCl2 (0.1–3 mM) to construct the first concentration-response curve. Rings were rinsed first in Ca2+-free solution and then in Ca2+-free, 60 mM K+ solution to allow returning of the Ca2+ level to baseline and finally incubated for 30 min with 30 μM or 10 μM raloxifene before repeating the second CaCl2 concentration-response curve.

Drugs. Acetylcholine [2-(acetyloxy)-N,N,N-trimethylethanaminium, U46619, t-NNAME, ODQ, and nifedipine ([4,4'-di(2,6-dimethyl-4(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) were purchased from Sigma-Aldrich (St. Louis, MO). ICI 182,780 was purchased from Tocris Cookson Inc. (Ellisville, MO). Raloxifene [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophen-3-yl][4-[2-piperidinethoxy]phenyl]ketone hydrochloride] was a gift from Eli Lilly & Co. (Indianapolis, IN). U46619, raloxifene, and nifedipine were dissolved in dimethyl sulfoxide; others were dissolved in distilled water. Further dilution was made from stock solutions. Krebs' solution contained 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 25 mM NaHCO3, 1.2 mM KH2PO4, and 11 mM 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 the mean ± S.E.M. of rings from n rats. Increases in constractive force were expressed as percentages of the maximal response obtained in the first concentration-dependent constriction to CaCl2. Concentration-response curves were constructed based on responses to cumulative drug concentrations and analyzed by nonlinear curve fitting using GraphPad software version 3.0 (GraphPad Software, Inc., San Diego, CA). The negative logarithm of the dilator (or constrictor) concentration that caused half (pD2 or pEC50) of the maximal response (Emax) was obtained. For statistical analysis, two-tailed Student’s t test or one-way analysis of variance (ANOVA) followed by Newman-Keuls test was used when more than two groups were compared. Individual concentration-
response curves also were compared using a two-way ANOVA 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: \( 3.71 \pm 0.60 \text{ mN} \) with endothelium and \( 3.73 \pm 0.36 \text{ mN} \) without endothelium in female, \( P > 0.05 \); \( 3.13 \pm 0.51 \text{ mN} \) with endothelium and \( 2.98 \pm 0.34 \text{ mN} \) without endothelium in male, \( P > 0.05 \)). On U46619 preconstriction, raloxifene caused relaxations of arteries with and without endothelium (\( \text{pD}_2: 4.78 \pm 0.13 \) versus \( 5.13 \pm 0.13, P > 0.05 \) in female, Fig. 1A; \( 5.34 \pm 0.15 \) versus \( 5.53 \pm 0.08, P > 0.05 \) 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. 1, C and F). ICI 182,780 may act as a partial estrogen receptor agonist (Bracamonte et al., 2002), but this agent (0.1–50 \( \mu \text{M} \)) did not affect U46619-induced constriction (data not shown). Vehicle (dimethyl sulfoxide) did not influence U46619-induced tone in arteries with and without endothelium (Fig. 1, A and D).

There was a gender difference in relaxations to raloxifene in U46619-constricted arteries (\( \text{pD}_2: 4.78 \pm 0.13 \) in female and \( 5.34 \pm 0.15 \) in male, \( P < 0.05 \); Fig. 2A), but this difference was absent in 60 mM \( K^+ \)-constricted arteries (\( \text{pD}_2: 5.61 \pm 0.07 \) in female and \( 5.70 \pm 0.09 \) 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 CaCl2-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 \pm 0.15 in female and 3.77 \pm 0.11 in male, P > 0.05). Raloxifene reduced CaCl\(_2\)-induced constrictions in a noncompetitive manner with progressive suppression of the maximal constriction in female (Fig. 3A) and male (Fig. 3B) arteries. In control experiments, 1 \(\mu\)M nifedipine abolished constrictions to CaCl\(_2\).

Effects of Raloxifene on Pulmonary Veins. U46619 constricted pulmonary veins (tension: 0.96 \pm 0.12 mN in female and 1.06 \pm 0.23 mN in male, P > 0.05). In U46619-constricted veins from both genders, raloxifene caused small constrictions were demonstrated in the venous responses to raloxifene, with a greater relaxing effect on male than female (Fig. 4, A and B). t-NAME did not modify this relaxation (Fig. 4, B and D). K\(^+\) (60 mM) constricted pulmonary veins (tension: 0.77 \pm 0.12 mN in female and 0.87 \pm 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. 5, A and B).

Effect of Raloxifene on CaCl2-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\(^{2+}\)]\(i\) increase in Ca\(^{2+}\)-free, 60 mM K\(^+\) solution, and the first and second concentration-dependent responses were similar. Changes in [Ca\(^{2+}\)]\(i\) was measured as the fluorescence ratio (F340/F380) before and after treatment with raloxifene (0.3 and 10 \(\mu\)M) in female and male pulmonary arteries are summarized in Fig. 6. The cumulative addition of CaCl\(_2\) caused progressive increases 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, 1 \(\mu\)M nifedipine abolished CaCl\(_2\)-induced increase 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 a 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 on membrane depolarization or constrictor stimulation. 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\(^{2+}\)]\(i\). Like the L-type Ca\(^{2+}\) channel blocker nifedipine, raloxifene at 10 \(\mu\)M almost abolished CaCl\(_2\)-induced increase in vessel tone and [Ca\(^{2+}\)]\(i\). High K\(^+\)-constricted arteries also 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 the 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 hyper-
tension via an endothelial NO synthase-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 nongenomic 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. 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 β-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, nongenomic, and independent of a functional endothelium or ICI 182,780-sensitive estrogen receptors. Such calcium antagonistic action may make raloxifene a potentially useful agent from both genders in the present study. 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 β-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.

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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 seems 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.

References


Brombacher JW, Baribeau J, and Perreault T (2000) Decreased synthesis and release of nitric oxide in male animals. However, it remains to be investigated 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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