Raloxifene Relaxes Rat Cerebral Arteries In Vitro and Inhibits L-Type Voltage-Sensitive Ca\textsuperscript{2+} Channels

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Background and Purpose—Because of their mixed estrogen-agonist and estrogen-antagonist properties, selective estrogen receptor modulators (SERMs) are considered promising substitutes for hormone replacement therapy. Raloxifene and other SERMs confer estrogen-like cardiovascular protective effects but lack the carcinogenic activity of exogenous estrogen. However, little is known about the cerebrovascular action of raloxifene. Therefore, we studied the effects of raloxifene on the mechanisms regulating rat cerebral artery tone.

Methods and Results—Ring segments of the isolated rat posterior communicating cerebral arteries were mounted in a microvessel myograph for measurement of isometric tension. Whole-cell L-type voltage-sensitive Ca\textsuperscript{2+} currents were recorded using the perforated patch-clamp technique. Raloxifene (0.1 to 10 μmol/L) reduced the contractile responses to U46619, phenylephrine, and endothelin-1 in normal Krebs solution or to CaCl\textsubscript{2} in Ca\textsuperscript{2+}-free, high K\textsuperscript{+}-containing solution. Raloxifene-induced relaxation was identical in endothelium-intact and endothelium-denuded rings. ICI 182780 had no effect on raloxifene-induced relaxation. Raloxifene reduced L-type Ca\textsuperscript{2+} currents with a pD\textsubscript{2} of 5.98±0.06, close to that (6.44±0.09) for raloxifene-induced relaxation of 60 mmol/L K\textsuperscript{+}-contracted rings.

Conclusions—This study demonstrates that raloxifene acutely relaxes rat cerebral arteries largely via an endothelium-independent mechanism, involving inhibition of Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. (Stroke. 2004;35:1709-1714.)

Key Words: vasodilation ■ cerebrovascular circulation ■ rats

A gender-related difference exists in the incidence of cardiovascular diseases with increased risks of cerebrovascular disease such as stroke in postmenopausal women. Improved cerebral vasodilation and recruitment of collateral circulation during cerebral artery occlusion participate partially in the protective effects of estrogen against ischemic stroke. In pooled analysis, observational studies show a 50% reduction in risk of coronary heart disease among users of hormone replacement therapy (HRT) for the primary and secondary prevention of cardiovascular disease.

However, the first randomized trial of HRT for secondary prevention of heart disease found no therapeutic benefit because HRT was associated with increased risks of heart attacks within the first year, as well as deep venous thrombosis and pulmonary embolism (relative risk: 2.89). The first randomized trial of HRT for the primary prevention of heart disease also found no overall benefit. Thus, conventional HRT is no longer recommended for the primary or secondary prevention of cardiovascular disease or stroke.

In the search for more selective agents, molecules retaining the beneficial estrogenic actions on breast, bone, and the cardiovascular system while having antiestrogenic actions on cardiovascular diseases with increased risks of cerebrovascular disease such as stroke in postmenopausal women. Improved cerebral vasodilation and recruitment of collateral circulation during cerebral artery occlusion participate partially in the protective effects of estrogen against ischemic stroke. In pooled analysis, observational studies show a 50% reduction in risk of coronary heart disease among users of hormone replacement therapy (HRT) for the primary and secondary prevention of cardiovascular disease.

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In the search for more selective agents, molecules retaining the beneficial estrogenic actions on breast, bone, and the cardiovascular system while having antiestrogenic actions on breast and endometrium have been developed. Such compounds are known as selective estrogen receptor modulators (SERMs). SERMs exert selective agonistic or antagonistic effects on various estrogen target tissues. Although some members of SERMs have been known for decades, their tissue-specificity has only recently been recognized. SERMs represent a major therapeutic advance for clinical practice in humans.

Raloxifene, a second-generation SERM, is of considerable interest because of its tissue-specific agonist–antagonist effects on the estrogen receptor (ER). Raloxifene therapy in postmenopausal women decreases serum levels of total and low-density lipoprotein cholesterol, fibrinogen, and homocysteine. Raloxifene treatment improves flow-mediated endothelium-dependent vasodilation, increases plasma nitric oxide (NO) concentrations, and decreases plasma endothelin-1 levels in healthy postmenopausal women. A significant reduction in carotid artery pulsatility index is observed in healthy subjects receiving raloxifene, an effect similar to that reported with estrogen therapy. Daily admin-
istration of raloxifene improves ovarian circulation in postmenopausal women.14

Nongenomic signaling through ERs accounts for much of the estrogen-mediated vascular actions in vitro. Raloxifene relaxes rabbit coronary arteries15 and porcine femoral veins,16 probably via both endothelium-dependent and endothelium-independent mechanisms. The effect of raloxifene on endothelial function is inhibited by classical ER antagonists, including ICI 182780.15,17 Raloxifene and estrogen increase NO-mediated coronary and uterine blood flow in vivo in sheep.18 Treatment with raloxifene improves hypertension-induced endothelial dysfunction by increasing bioavailability of NO in hypertensive rats. The underlying mechanisms may involve an increased activity of endothelial NO synthase and an ER-dependent reduction in production of reactive oxygen species.19

Raloxifene and other SERMs have potentials as novel drugs in the prevention and treatment of cerebrovascular disease such as vasospasm and ischemia. However, the effects of raloxifene on cerebrovascular events and cerebral artery tone are unknown. Therefore, we investigated the cerebrovascular effects of raloxifene and the roles of the endothelium, vascular L-type Ca2+ channels, and ERs in isolated rat posterior communicating cerebral arteries.

Methods and Materials

Vessel Preparation

Adult male Sprague-Dawley rats (20- to 24-week-old, weighing between 300 and 350 grams; Laboratory Animal Services Center, Chinese University of Hong Kong) were euthanized by cervical dislocation. The brain was rapidly removed and immersed in a dissection dish filled with Krebs solution. The posterior communicating cerebral arteries from both sides were dissected free from surrounding connective tissues under a dissection microscope. Two 2-mm-long ring segments were prepared from each rat. The rings were mounted in a Multi Myograph System (Danish Myo Technology A/S) and changes in isometric tension were recorded. Briefly, 2 tungsten wires were passed through the segment’s lumen and each wire was fixed to the jaws of the myograph. Rings were bathed in Krebs solution aerated with 95% O2 plus 5% CO2 at 37°C (pH 7.4). Each ring was stretched to a previously determined optimal tension of 0.3 mN. In some experiments, the endothelium was mechanically removed by rubbing the luminal surface several times with a stainless steel wire. Thirty minutes after mounting, each ring was initialized contracted by phenylephrine (10 μmol/L) and subsequently relaxed by acetylcholine (1 μmol/L) to test the vessel’s contractility and the integrity of its endothelium. The rings were then rinsed until baseline tone was restored and allowed to stabilize for 60 minutes. This study was conducted after approval had been obtained from CUHK Animal Ethical Committee.

Effect of Raloxifene on Agonist-Induced Contractions

Each endothelium-intact ring was contracted with 60 mmol/L K+ at 30-minute intervals until 2 consecutive contractions were similar in amplitude (<10%). Thereafter, concentration–response relationships were examined in response to phenylephrine (0.1 to 30 μmol/L), U46619 (1 to 300 mmol/L), or endothelin-1 (1 to 50 mmol/L) in the absence and presence of raloxifene (0.01 to 10 μmol/L, 30-minute preincubation). The ability of raloxifene to modulate Ca2+ influx via L-type Ca2+ channels was evaluated by studying concentration-dependent responses to CaCl2 (0.1 to 5 mmol/L) in the absence of raloxifene (0.1 to 1 μmol/L). For this set of experiments, rings were rinsed 3 times in a Ca2+-free solution containing 30 μmol/L Na2-EGTA, then incubated in Ca2+-free, 60 mmol/L K+ solution (with or without raloxifene, 30-minute incubation) before cumulative addition of CaCl2. The effects of nifedipine (1 μmol/L) were also tested on agonist-induced contractions.

Role of Endothelium in Raloxifene-Induced Relaxation

To elucidate a role of endothelium in raloxifene-mediated relaxation, concentration-dependent responses to raloxifene were studied in endothelium-intact and endothelium-denuded rings precontracted by U46619 (100 mmol/L).

Effect of ICI 182780 on Raloxifene-Induced Relaxation

To examine the possible involvement of the classical ER, raloxifene-induced relaxations were compared in the absence and presence of ICI 182780, a specific ER antagonist. Rings were incubated with 10 μmol/L ICI 182780 for 30 minutes before precontraction in U46619. ICI 182780 in concentrations between 1 and 10 μmol/L has been shown to effectively block ER activation and intracellular signaling,20 including raloxifene-dependent activation of the ERK/PI3K/Akt pathway in endothelial cells17 and raloxifene-induced endothelium-dependent relaxation in rabbit coronary arteries.15

Raloxifene-Induced Inhibition of Ca2+ Channel Current

Single smooth muscle cells were enzymatically isolated from rat posterior communicating cerebral arteries by collagenase. Ca2+ channel currents in isolated cerebrovascular myocytes were recorded in the whole-cell perforated patch configuration with amphotericin B22 using patch-clamp amplifier EPC 7. Data acquisition and command voltages were controlled with a software program using a CED1401 interface.22 Currents were recorded from holding potentials of −80 mV during linear voltage ramps at 0.67 V/s from −100 mV to +100 mV or 300-ms step pulses to different potentials (pulse frequency: 0.2 Hz). Analysis of whole-cell ionic currents was performed by using Ced Patch and Voltage Clamp Software (version 6.08). BaCl2 was used as Ca2+ channel charge carrier and K+ channels were inhibited by intracellular CsCl. The extracellular bathing solution contained (mmol/L): NaCl 125, BaCl2 10, 5, CsCl 5.4, glucose 10, and Na-Hepes 10 (pH 7.3 to 7.4 at 37°C). The pipette solution contained (mmol/L): CsCl 120, MgCl2 1, Mg-ATP 3, EGTA 10, and Cs-Hepes 10 (pH 7.4). Experiments were performed at 22°C.

Drugs and Solutions

Phenytoin, acetylsalicylic acid, endothelin-1, 9,11-dideoxy-11α, 9α-epoxy-methanoprostaglandin F1α (Sigma), ICI 182780 (Tocris), raloxifene hydrochloride (I-Lilly Corporate Center), U46619, raloxifene, nifedipine, and ICI 182780 were dissolved in DMSO, and other chemicals were suspended in double-distilled water. DMSO at 0.1% (v/v) did not affect the contractile responses. Krebs solution contained (mmol/L): NaCl 119, KCl 4.7, NaHCO3 25, CaCl2 2.5, MgCl2 1, KH2PO4 1.2, and D-glucose 11. High K+ solution (60 mmol/L) was prepared by substituting equimolar amounts of NaCl with KCl.

Data Analysis

The contractile force was presented as percentage of the mean value of 2 consecutive responses to 60 mmol/L K+. Concentration–response curves were constructed based on responses to cumulative concentrations of drugs and analyzed by nonlinear curve fitting using Graphpad software (Version 3.0). The negative logarithm of the maximum response (Emax) was calculated. For statistical analysis, 2-tailed Student t test or 1-way analysis of variance followed by Newman–Keuls test was used when >2 treatments were compared. Statistical significance was accepted when P<0.05. The results are mean±SEM of n rings from different rats.
Results

Relaxant Effect of Raloxifene
Traces in Figure 1 show the inhibition by raloxifene (1 μmol/L) of contractile responses to phenylephrine (Figure 1A and 1B) and U46619 (Figure 1C and 1D) in endothelium-intact rings. Phenylephrine, U46619, and endothelin-1–contracted endothelium-intact arteries (expressed as a percentage of 60 mmol/L K⁺-induced tone) with pEC₅₀ of 6.65±0.15% (n=6), 7.91±0.19% (n=7), and 8.10±0.21% (n=7), respectively. At concentrations >0.1 μmol/L, raloxifene caused inhibition, reducing the magnitude of the maximal contraction (Figure 2B, 2D, and 2F) and slopes of the concentration–contraction curves for the 3 agonists (Figure 2A, 2C, and 2E). Raloxifene at 10 μmol/L eliminated contractions to phenylephrine or U46619 (Figure 2A, 2C). The order of effectiveness for raloxifene inhibition of tone was phenylephrine > U46619 > endothelin-1 (Figure 2).

Role of Endothelium
Raloxifene induced relaxations in endothelium-intact or endothelium-denuded rings contracted with U46619. There was no difference in the relaxation under either condition (pD₂: 6.14±0.09 with endothelium and 6.14±0.06 without endothelium, n=10 to 11; P>0.05; Figure 3A).

Effect of ER Antagonist
Treatment with ICI 182780 (10 μmol/L) failed to affect raloxifene-induced relaxation in endothelium-intact rings (pD₂: 6.13±0.14, n=5 in control and 6.12±0.14, n=5 in ICI 182780; P>0.05; Figure 3B).

Effect of Raloxifene on Ca²⁺-Induced Contraction
To study possible inhibition of Ca²⁺ influx, the effect of raloxifene was tested on contractions in membrane-depolarized endothelium-denuded rings. In Ca²⁺-free, 60 mmol/L K⁺-containing solution, cumulative addition of CaCl₂ induced contractions with a pD₂ of 3.45±0.11 (n=5). Raloxifene inhibited CaCl₂-induced contraction in a noncompetitive manner with progressive reduction of maximal contraction with increasing concentrations (Figure 4A). In separate experiments, the maintained tone developed by 60 mmol/L K⁺ was reduced by raloxifene (pD₂: 6.44±0.09, n=6; Figure 4B). The pEC₅₀ and Eₘₐₓ values for CaCl₂-induced contraction are summarized in Figure 4C.

For comparison, the effects of L-type Ca²⁺ channel blocker nifedipine were tested on rings contracted by 30 μmol/L phenylephrine, 100 nmol/L U46619, 30 nmol/L endothelin-1, or 60 mmol/L K⁺ (n=6 in each case; Figure 5A through 5D). Nifedipine at 1 μmol/L abolished CaCl₂-induced contraction and markedly suppressed receptor-dependent contractions (Figure 5E compared with the effect of raloxifene shown in Figure 5F).

Effect of Raloxifene on L-Type Ca²⁺ Current
To confirm that inhibition of the high K⁺ response was mediated partly through inhibition of Ca²⁺ influx via Ca²⁺ channels, whole-cell L-type Ca²⁺ currents were recorded in isolated arterial myocytes and characterized using 10.8 mmol/L Ba²⁺ ions as divalent charge carriers with a perforated patch configuration of the patch-clamp method. The inward current was enhanced by 1 μmol/L (±) –Bay K 8644 (n=4) but inhibited by 100 μmol/L CdCl₂ (n=4). To
examine the current–voltage relationship, a linear potential ramp pulse was applied from −100 mV to +100 mV from a holding potential of −80 mV. The current recorded during voltage ramp pulses was U-shape and revealed a peak current at around 2 ± 6 mV. The apparent threshold potential was estimated at −31 ± 6 mV and the reversal potential was estimated at 52 ± 7 mV (n = 6).

Figure 6A shows that raloxifene at 1 μmol/L rapidly inhibited Ca\(^{2+}\) channel currents elicited by linear voltage ramp pulses. The effect of raloxifene was reversed by washing (Figure 6A, 6B). Raloxifene caused reductions of currents at potentials between −30 and 50 mV. Raloxifene did not affect the potential at which the peak inward currents were recorded (Figure 6B). Figure 6C shows the average concentration–response curve for current inhibition by raloxifene after 5 minutes of application. The pD\(_2\) value was 5.98 ± 0.06 (n = 6) for raloxifene-induced reduction in Ca\(^{2+}\) current.

**Discussion**

In this study, we examined the cerebrovascular effects of raloxifene in the isolated rat posterior cerebral communicating arteries, with and without a functional endothelium. Our main findings are: (1) raloxifene reduced cerebrovascular contractions to receptor-dependent and receptor-independent agents; (2) raloxifene reduced CaCl\(_2\)-mediated contraction and inhibited L-type Ca\(^{2+}\) currents; and (3) raloxifene-induced relaxation was similar in endothelium-intact and endothelium-denuded rings.

Voltage-sensitive Ca\(^{2+}\) channels are activated by depolarization in vascular smooth muscle cells when the extracellular K\(^+\) concentration is raised. Raloxifene markedly reduced the contractile responses to high K\(^+\) as well as CaCl\(_2\)-induced contractions in Ca\(^{2+}\)-free, high K\(^+\) solution. Similar effects were also observed in rabbit coronary arteries. These results indirectly suggest that raloxifene exerts a direct muscle relaxation, probably by acting as a calcium antagonist. Indeed, raloxifene inhibited the L-type Ca\(^{2+}\) currents as recorded on single smooth muscle cells isolated from the cerebral arteries. Raloxifene inhibited U46619-induced and high K\(^+\)-induced contraction with IC\(_{50}\) of 756 and 360.
nmol/L, respectively. Raloxifene reduced L-type Ca\(^{2+}\) current with an IC\(_{50}\) of \(\approx 1 \mu\text{mol/L}\). These values are relatively similar, indicating that inhibition of Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels contributes to raloxifene-induced cerebrovascular relaxation. We have thus provided the first line of evidence showing direct antagonism of vascular L-type Ca\(^{2+}\) channels by raloxifene.

Raloxifene reduced contractile responses to 3 receptor-dependent constrictors, phenylephrine, U46619, and endothelin-1, in a concentration-dependent manner but with varying potency. Raloxifene at 1 \(\mu\text{mol/L}\) reduced phenylephrine-induced maximal contraction by \(\approx 81\%\) but had lower potency in rings contracted with U46619 (\(\approx 46\%\)) or endothelin 1 (\(\approx 30\%\)). A similar pattern was observed for nifedipine-induced inhibition of contractions to these 3 agonists (Figure 5E, 5F). These results indicate that raloxifene-induced relaxation may not only involve an agonist receptor-associated mechanism. Instead, raloxifene, like nifedipine, acts as a calcium antagonist.

Genomic effects of estrogens are mediated through activation of nuclear receptors. The selective ER antagonist ICI 182780 did not affect raloxifene-induced relaxation. This blocker also fails to antagonize raloxifene-induced relaxation in porcine femoral veins. In contrast, this antagonist inhibited endothelium-dependent relaxation to raloxifene in rabbit coronary arteries without an effect on endothelium-denuded rings and blocked raloxifene-induced NO production in human endothelial cells. Similarly, ICI 182780 inhibited only the endothelium-dependent portion of the vasorelaxant response to tamoxifen, another SERM member. It appears that SERMs-induced (nongenomic) effects on the endotheli-
Raloxifene induces cerebrovascular relaxation. Raloxifene is able to act directly on the vascular smooth muscle cells of the rat cerebral arteries by inhibiting $\mathrm{Ca}^{2+}$ influx through L-type voltage-sensitive $\mathrm{Ca}^{2+}$ channels. The actions demonstrated in this study are short-term and nongenomic effects of raloxifene. However, long-term and in vivo genomic effects of raloxifene may differ. Long-term oral administration of 60 mg/d of raloxifene hydrochloride in women is expected to result in a mean maximum plasma concentration of 1.36 $\mu$g/L, equivalent to 2.67 nmol/L of raloxifene. In the present study, the threshold concentration shown to be effective in relaxing male cerebral arteries is $\approx 30$ nmol/L for raloxifene. However, our in vitro assay does not include the effects of other circulating hormones and dilating factors, which may be enhanced by raloxifene in vivo and may differ in females. Because raloxifene is clinically used to treat females (premenopausal and postmenopausal), we cannot attribute this observation with certainty to this population. Nevertheless, the cerebrovascular effects of raloxifene we describe greatly enhance the perspectives of raloxifene and other SERMs as novel drugs in cerebrovascular disease.

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