Raloxifene Relaxes Rat Cerebral Arteries In Vitro and Inhibits L-Type Voltage-Sensitive Ca\(^{2+}\) 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\(^{2+}\) currents were recorded using the perforated patch-clamp technique. Raloxifene (0.1 to 10 \(\mu\)mol/L) reduced the contractile responses to U46619, phenylephrine, and endothelin-1 in normal Krebs solution or to CaCl\(_2\) in Ca\(^{2+}\)-free, high K\(^+\)-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\(^{2+}\) currents with a pD\(_2\) of 5.98±0.06, close to that (6.44±0.09) for raloxifene-induced relaxation of 60 mmol/L K\(^+\)-contracted rings.

Conclusions—This study demonstrates that raloxifene acutely relaxes rat cerebral arteries largely via an endothelium-independent mechanism, involving inhibition of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) 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.\(^{2,3}\) 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.\(^{4}\)

However, the first randomized trial of HRT for secondary prevention of heart disease found no therapeutic benefit\(^{5}\) 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).\(^{5}\) The first randomized trial of HRT for the primary prevention of heart disease also found no overall benefit.\(^{6}\) Thus, conventional HRT is no longer recommended for the primary or secondary prevention of cardiovascular disease or stroke.\(^{6,7}\)

In the search for more selective agents, molecules retaining the beneficial estrogenic actions on brain, 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).\(^{7}\) 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 thus represent a major therapeutic advance for clinical practice in humans.\(^{7}\)

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.\(^{8-10}\) Raloxifene treatment improves flow-mediated endothelium-dependent vasodilation, increases plasma nitric oxide (NO) concentrations, and decreases plasma endothelin-1 levels in healthy postmenopausal women.\(^{11}\) A significant reduction in carotid artery pulsatility index is observed in healthy subjects receiving raloxifene,\(^{12}\) an effect similar to that reported with estrogen therapy.\(^{13}\)}
istration of raloxifene improves ovarian circulation in post-menopausal women.\textsuperscript{14}

Nongenomic signaling through ERs accounts for much of the estrogen-mediated vascular actions in vitro. Raloxifene relaxes rabbit coronary arteries\textsuperscript{15} and porcine femoral veins,\textsuperscript{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.\textsuperscript{15,17} Raloxifene and estrogen increase NO-mediated coronary and uterine blood flow in vivo in sheep.\textsuperscript{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 independent mechanisms. The effect of raloxifene on endothelium-independent responses to raloxifene were studied in endothelium-intact and endothelium-denuded rings precontracted by U46619 (100 nmol/L).

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 nmol/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,\textsuperscript{20,21} including raloxifene-dependent activation of the ERK1/2, PI3K/Akt pathway in endothelial cells\textsuperscript{17} and raloxifene-induced endothelium-dependent relaxation in rabbit coronary arteries.\textsuperscript{15}

Raloxifene-Induced Inhibition of Ca\textsuperscript{2+} Channel Current
Single smooth muscle cells were enzymatically isolated from rat posterior communicating cerebral arteries by collagenase. Ca\textsuperscript{2+} channel currents in isolated cerebrovascular myocytes were recorded in the whole-cell perforated patch configuration with amphotaerin B\textsuperscript{22} using patch-clamp amplifier EPC 7. Data acquisition and command voltages were controlled with a software program using a CED1401 interface.\textsuperscript{23} 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). BaCl\textsubscript{2} was used as Ca\textsuperscript{2+} channel charge carrier and K\textsuperscript{+} channels were inhibited by intracellular CsCl. The extracellular bathing solution contained (mmol/L): NaCl 125, BaCl\textsubscript{2} 10.8, 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, MgCl\textsubscript{2} 1, Mg-ATP 3, EGTA 10, and Cs-Hepes 10 (pH 7.4). Experiments were performed at 22°C.

Drugs and Solutions
Phenytoin, acetylcholine, endothelin-1, 9,11-dideoxy-11α, 9α-epoxy-methanoprostaglandin F\textsubscript{1α} (U46619) (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 110, KCl 4.7, NaHCO\textsubscript{3} 25, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1, KH\textsubscript{2}PO\textsubscript{4} 1.2, and D-glucose 11. High K\textsuperscript{+} 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\textsuperscript{+}. 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 constrictor (or dilator) concentration that caused 50% (pEC\textsubscript{50} or pD\textsubscript{2}) of the maximum response (E\textsubscript{max}) were 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 pEC<sub>50</sub> of 5.65±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 (pD<sub>2</sub>: 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 (pD<sub>2</sub>: 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 Ca<sup>2+</sup>-Induced Contraction

To study possible inhibition of Ca<sup>2+</sup> influx, the effect of raloxifene was tested on contractions in membrane-depolarized endothelium-denuded rings. In Ca<sup>2+</sup>-free, 60 mmol/L K<sup>+</sup>-containing solution, cumulative addition of CaCl<sub>2</sub> induced contractions with a pD<sub>2</sub> of 3.45±0.11 (n=5). Raloxifene inhibited CaCl<sub>2</sub>-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<sup>+</sup> was reduced by raloxifene (pD<sub>2</sub>: 6.44±0.09, n=6; Figure 4B). The pEC<sub>50</sub> and E<sub>max</sub> values for CaCl<sub>2</sub>-induced contraction are summarized in Figure 4C.

For comparison, the effects of L-type Ca<sup>2+</sup> 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<sup>+</sup> (n=6 in each case; Figure 5A through 5D). Nifedipine at 1 μmol/L abolished CaCl<sub>2</sub>-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 Ca<sup>2+</sup> Current

To confirm that inhibition of the high K<sup>+</sup> response was mediated partly through inhibition of Ca<sup>2+</sup> influx via Ca<sup>2+</sup> channels, whole-cell L-type Ca<sup>2+</sup> currents were recorded in isolated arterial myocytes and characterized using 10.8 mmol/L Ba<sup>2+</sup> 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 CdCl<sub>2</sub> (n=4).
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 \(\pm 6\) mV. The apparent threshold potential was estimated at \(-31\pm 6\) mV and the reversal potential was estimated at \(52\pm 7\) mV (n=6).

Figure 6A shows that raloxifene at \(1\, \mu\text{mol/L}\) rapidly inhibited \(\text{Ca}^{2+}\) 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 \(5.98\pm 0.06\) (n=6) for raloxifene-induced reduction in \(\text{Ca}^{2+}\) 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 \(\text{CaCl}_2\)-mediated contraction and inhibited L-type \(\text{Ca}^{2+}\) currents; and (3) raloxifene-induced relaxation was similar in endothelium-intact and endothelium-denuded rings.

Voltage-sensitive \(\text{Ca}^{2+}\) channels are activated by depolarization in vascular smooth muscle cells when the extracellular \(\text{K}^+\) concentration is raised. Raloxifene markedly reduced the contractile responses to high \(\text{K}^+\) as well as \(\text{CaCl}_2\)-induced contractions in \(\text{Ca}^{2+}\)-free, high \(\text{K}^+\) 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 \(\text{Ca}^{2+}\) currents as recorded on single smooth muscle cells isolated from the cerebral arteries. Raloxifene inhibited \(U46619\)-induced and high \(\text{K}^+\)-induced contraction with \(IC_{50}\) of 756 and 360
nmol/L, respectively. Raloxifene reduced L-type Ca\(^{2+}\) current with an IC\(_{50}\) of \(\approx 1\) μmol/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 μmol/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.\(^{15}\) In contrast, this antagonist inhibited endothelium-dependent relaxation to raloxifene in rabbit coronary arteries without an effect on endothelium-denuded rings\(^{15}\) and blocked raloxifene-induced NO production in human endothelial cells.\(^{17}\) Similarly, ICI 182780 inhibited only the endothelium-dependent portion of the vasorelaxant response to tamoxifen, another SERM member.\(^{23}\) It appears that SERMs-induced (nongenomic) effects on the endothelium-

Figure 5. Inhibitory effect of nifedipine (1 μmol/L) on contractions induced by phenylephrine (A), U46619 (B), or endothelin-1 (C) in normal Krebs solution, and by CaCl\(_2\) in 60 mmol/L K\(^+\) solution (D). Reduction in maximal contraction (E\(_{max}\)) in the presence of 1 μmol/L nifedipine (E) or 1 μmol/L raloxifene (F). Data are mean±SEM (n=6).

Figure 6. Inhibition of L-type Ca\(^{2+}\) currents by raloxifene in smooth muscle cells isolated from rat cerebral arteries. A, Time course of raloxifene-induced (1 μmol/L) inhibition of maximal current. B, Average traces from 10 recordings of Ca\(^{2+}\) channel currents of the same cell as shown in (A) (in control; in the presence of 1 μmol/L raloxifene, and after washout of raloxifene). C, Raloxifene-induced inhibition of Ca\(^{2+}\) currents. Data are mean±SEM (n=6).
um are more likely mediated through ERs on the endothelium, whereas their acute effects on vascular smooth muscle cells are probably independent of classical ER.

In conclusion, we identified a key mechanism by which raloxifene induces cerebrovascular relaxation. Raloxifene is able to act directly on the vascular smooth muscle cells of the rat cerebral arteries by inhibiting Ca\(^{2+}\) influx through L-type voltage-sensitive 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 µg/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, in 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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