Stroke
JOURNAL OF THE AMERICAN HEART ASSOCIATION
American Stroke Association
A Division of American Heart Association

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Stroke 2004;35;1709-1714; originally published online May 20, 2004;
DOI: 10.1161/01.STR.0000131479.08005.ca
Stroke is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0039-2499. Online ISSN: 1524-4628

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 μmol/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.[1] 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] Daily admin-

Received February 6, 2004; final revision received March 17, 2004; accepted March 31, 2004.

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***Stroke* is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000131479.08005.ca**

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 arteries[15] 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 Ca^{2+} 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 Kong Hong) 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% O_2 plus 5% CO_2 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 initially 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 nmol/L), or endothelin-1 (1 to 50 nmol/L) in the absence and presence of raloxifene (0.01 to 10 μmol/L, 30-minute preincubation). The ability of raloxifene to modulate Ca^{2+} influx via L-type Ca^{2+} channels was evaluated by studying concentration-dependent responses to $CaCl_2$ (0.1 to 5 mmol/L) in the presence of raloxifene (0.1 to 1 μmol/L). For this set of experiments, rings were rinsed 3 times in a Ca^{2+}-free solution containing 30 μmol/L Na_2-EGTA, then incubated in Ca^{2+}-free, 60 mmol/L K^+ solution (with or without raloxifene, 30-minute incubation) before cumulative addition of $CaCl_2$. 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 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,[20,21] including raloxifene-dependent activation of the ERα/PI3K/Akt pathway in endothelial cells[17] and raloxifene-induced endothelium-dependent relaxation in rabbit coronary arteries.[15]

Raloxifene-Induced Inhibition of Ca^{2+} Channel Current

Single smooth muscle cells were enzymatically isolated from rat posterior communicating cerebral arteries by collagenase. Ca^{2+} channel currents in isolated cerebrovascular myocytes were recorded in the whole-cell perforated patch configuration with amphotericin B[22] 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). $BaCl_2$ was used as Ca^{2+} channel charge carrier and K^+ channels were inhibited by intracellular CsCl. The extracellular bathing solution contained (mmol/L): NaCl 125, $BaCl_2$ 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, $MgCl_2$ 1, Mg-ATP 3, EGTA 10, and Cs-Hepes 10 (pH 7.4). Experiments were performed at 22°C.

Drugs and Solutions

Phenylephrine, acetylcholine, endothelin-1, 9,11-dideoxy-11α, 9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619) (Sigma), ICI 182780 (Tocris), raloxifene hydrochloride (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, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgCl_2$ 1, KH_2PO_4 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 constrictor (or dilator) concentration that caused 50% (pEC_{50} or pD_2) of the maximum response (E_{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.

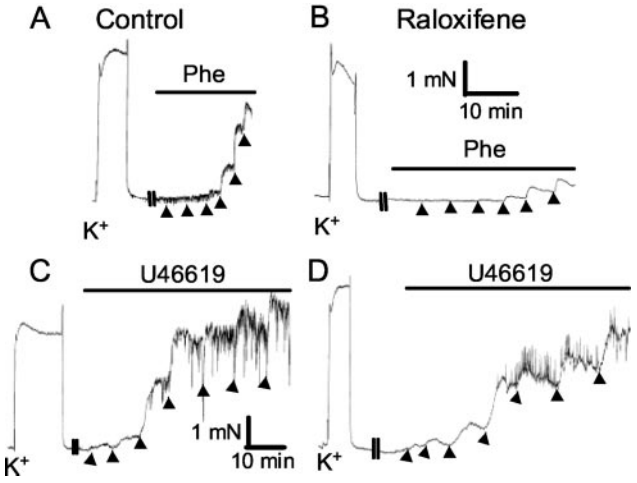

Figure 1. The representative records showing inhibition by raloxifene (1 μmol/L) of contractions to phenylephrine (B) and U46619 (D) and the control contraction to phenylephrine (A) or U46619 (C).

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_{50} 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_2: 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_2: 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^{2+}-Induced Contraction

To study possible inhibition of Ca^{2+} influx, the effect of raloxifene was tested on contractions in membrane-depolarized endothelium-denuded rings. In Ca^{2+}-free, 60 mmol/L K^+-containing solution, cumulative addition of $CaCl_2$ induced contractions with a pD_2 of 3.45±0.11 (n=5). Raloxifene inhibited $CaCl_2$-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 (pD_2: 6.44±0.09, n=6; Figure 4B). The pEC_{50} and E_{max} values for $CaCl_2$-induced contraction are summarized in Figure 4C.

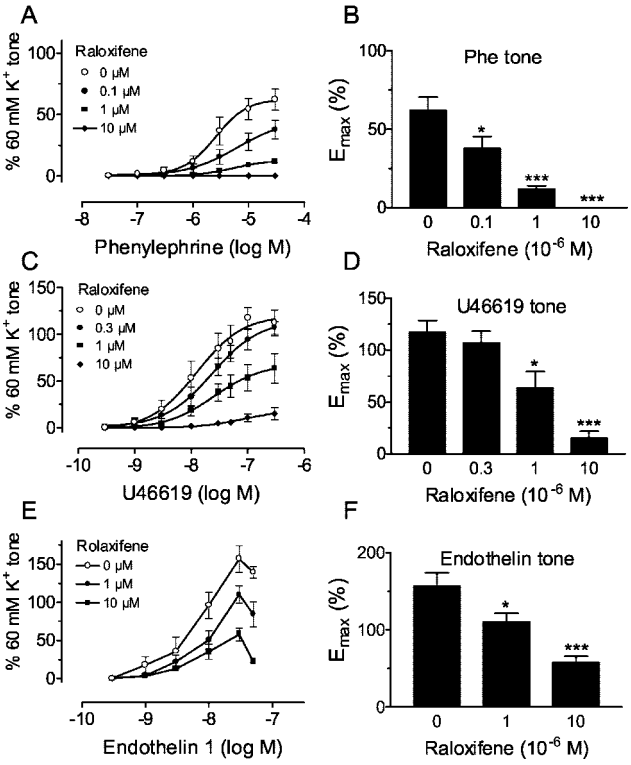

Figure 2. A, Phenylephrine-induced contractions in the absence (○, n=7) and presence of raloxifene (0.1 to 10 μmol/L, n=6 to 7). B, E_{max} values for phenylephrine. C, U46619-induced contraction in the absence (○, n=7) and presence of raloxifene (0.3 to 10 μmol/L, n=6 to 7). D, E_{max} values for U46619. E, Endothelin-1–induced contraction in the absence (○, n=7) and presence of raloxifene (1 to 10 μmol/L, n=6). F, E_{max} values for endothelin-1. Results are expressed as a percentage of 60-mmol/L K^+-induced tone. Data are mean±SEM. Significant differences are indicated from control (without raloxifene treatment): *$P<0.05$, ***$P<0.001$.

For comparison, the effects of L-type Ca^{2+} 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 $CaCl_2$-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^{2+} Current

To confirm that inhibition of the high K^+ response was mediated partly through inhibition of Ca^{2+} influx via Ca^{2+} channels, whole-cell L-type Ca^{2+} currents were recorded in isolated arterial myocytes and characterized using 10.8 mmol/L Ba^{2+} 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 $CdCl_2$ (n=4). To

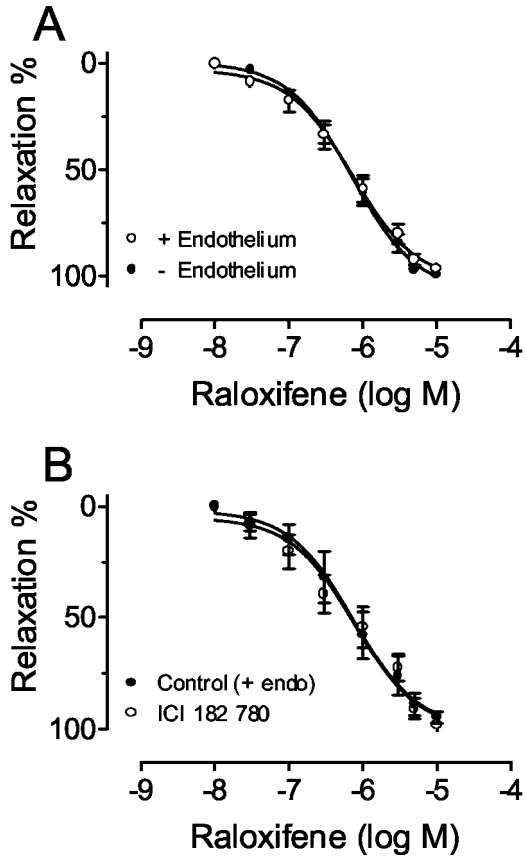

Figure 3. A, Raloxifene-induced relaxation in rings contracted by U46619 (○, with endothelium and ●, without endothelium; n=10 to 11). B, Lack of effect of 10 μmol/L ICI 182780 in endothelium-intact rings (n=5). Data are mean±SEM.

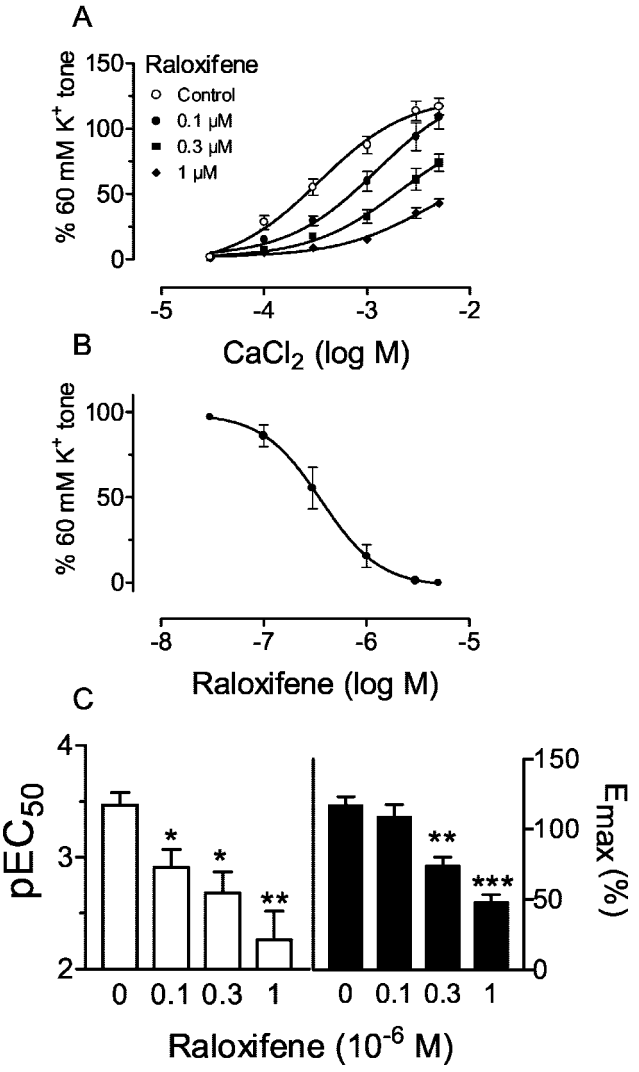

Figure 4. A, $CaCl_2$-induced contraction in Ca^{2+}-free, 60 mmol/L K^+-containing solution in the absence (○, n=7) and presence of raloxifene (0.1 to 1 μmol/L, n=6). B, Concentration-dependent relaxation induced by raloxifene in 60 mmol/L K^+-contracted rings (n=6). C, pEC_{50} and E_{max} values for $CaCl_2$-induced contractions. All experiments were conducted in endothelium-denuded rings. Results are expressed as a percentage of 60 mmol/L K^+-induced tone. Data are mean±SEM. Significant differences are indicated from control (without raloxifene treatment): *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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 ≈−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.[15] 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

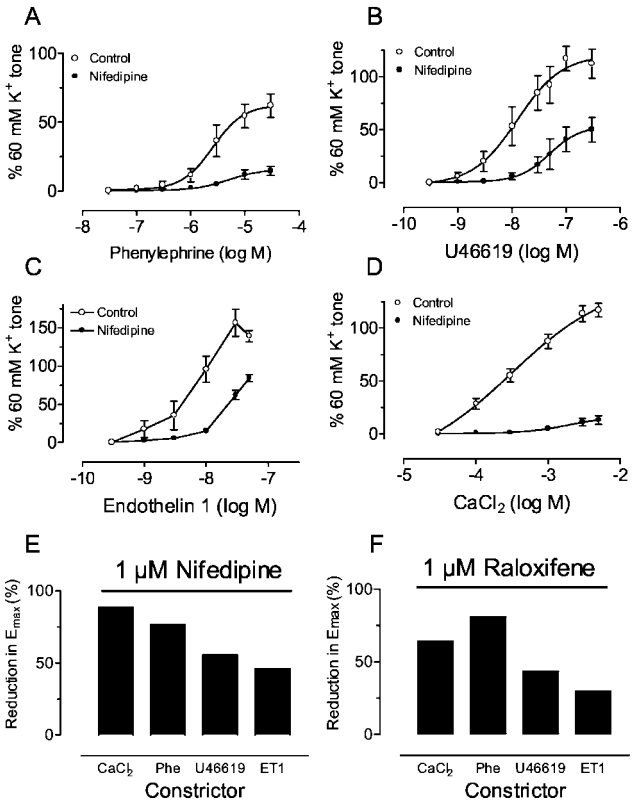

Figure 5. Inhibitory effect of nifedipine (1 μmol/L) on contractions induced by phenylephrine (A), U46619 (B), or endothelin-1 (C) in normal Krebs solution, and by $CaCl_2$ in 60 mmol/L K^+ solution (D). Reduction in maximal contraction (E_{max}) in the presence of 1 μmol/L nifedipine (E) or 1 μmol/L raloxifene (F). Data are mean±SEM (n=6).

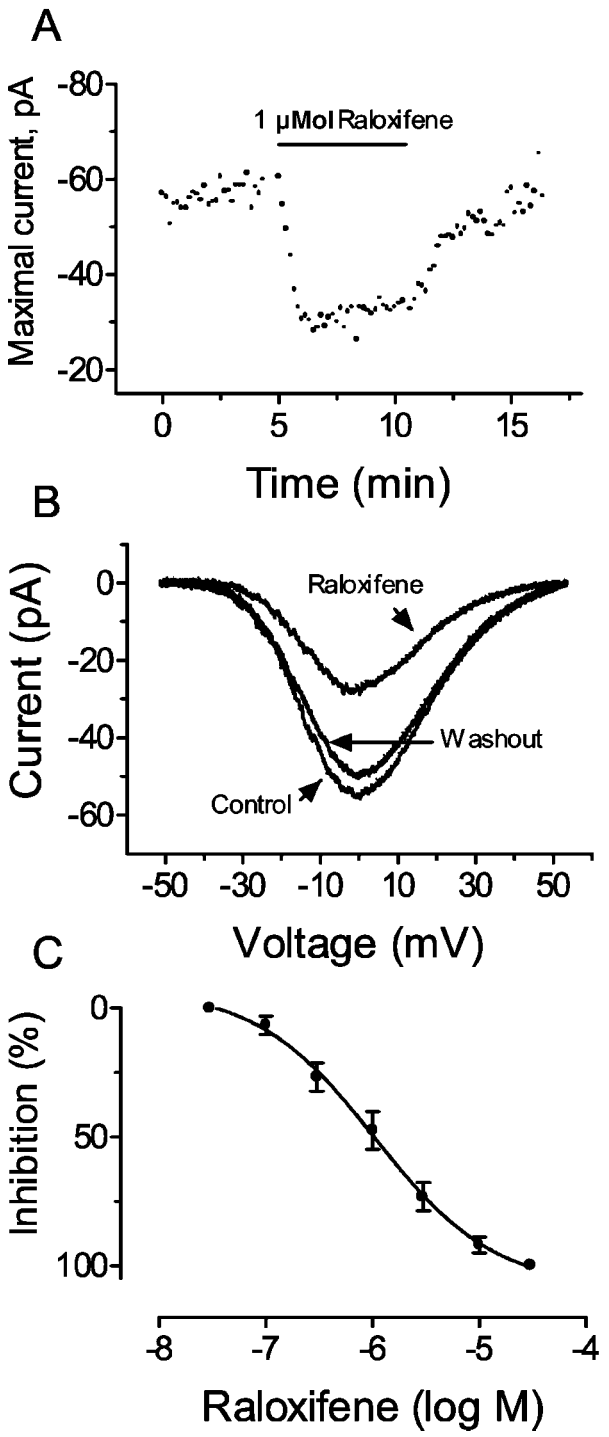

Figure 6. Inhibition of L-type Ca^{2+} currents by raloxifene in smooth muscle cells isolated from rat cerebral arteries. A, Time course of raloxifene-induced (1 μmol/L) inhibition of maximal current. B, Average traces from 10 recordings of Ca^{2+} channel currents of the same cell as shown in (A) (in 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).

nmol/L, respectively. Raloxifene reduced L-type Ca^{2+} current with an IC_{50} of ≈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 ≈81% but had lower potency in rings contracted with U46619 (≈46%) or endothelin 1 (≈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.[16] 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 endotheli-

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,[24,25] 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 ≈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.

Acknowledgments

This study was supported by Hong Kong Research Grants Council (CUHK 4366/03M) and the Deutsche Akademische Austauschdienst.

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