Therapeutically Relevant Concentrations of Raloxifene Dilate Pressurized Rat Resistance Arteries via Calcium-Dependent Endothelial Nitric Oxide Synthase Activation

Yau Chi Chan, Fung Ping Leung, Wing Tak Wong, Xiao Yu Tian, Lai Ming Yung, Chi Wai Lau, Suk Ying Tsang, Xiaojiang Yao, Zhen Yu Chen, Yu Huang

Objective—Selective estrogen receptor modulators (SERMs) inhibit constriction of mammalian conduit arteries. However, it is unknown whether SERMs at therapeutically achievable concentrations could reduce vascular tone in resistance arteries. The present study aimed to examine roles of Ca²⁺ influx in endothelium and endothelial nitric oxide synthase (eNOS) activation in dilatations induced by raloxifene, a second-generation SERM in myogenically active arteries.

Methods and Results—Small mesenteric arteries from Sprague-Dawley rats were isolated and mounted in a pressure myograph for measurement of changes in vessel diameter. [Ca²⁺]i images on native endothelial cells of intact arteries were determined by the fluorescence imaging technique, and phosphorylation of eNOS was assayed by Western blotting. Raloxifene (0.3 to 10 nmol/L) produced dilatations on established steady myogenic constriction. Female rat arteries dilated significantly more in response to raloxifene than male arteries. Raloxifene-induced dilatations of female arteries were blunted by \( \text{N}^\text{G}\)-nitro-L-arginine methyl ester but unaffected by 1400W, charybdotoxin plus apamin, wortmannin, or LY294002. Raloxifene (3 nmol/L) triggered rises in endothelial cell [Ca²⁺]i and increased eNOS phosphorylation at Ser1177. Both effects were greater in arteries from female rats than in arteries from male rats. Increases in endothelial cell [Ca²⁺]i and in eNOS phosphorylation were prevented by removal of extracellular Ca²⁺ ions. Finally, ICI 182,780 did not affect the raloxifene-stimulated rise in endothelial cell [Ca²⁺]i, eNOS phosphorylation, and vasodilatations. Chronic raloxifene treatment reduced myogenic constriction in arteries from female but not male rats.

Conclusion—Raloxifene at therapeutically relevant concentrations inhibits myogenic constriction by an NO-dependent mechanism that causally involves the elevated [Ca²⁺]i in endothelial cells and subsequent eNOS activation. Raloxifene dilates resistance arteries more effectively in female rats, indicating its significant gender-related action on endothelial cells in microcirculation. (Arterioscler Thromb Vasc Biol. 2010;30:992-999.)

Key Words: mesenteric artery ■ myogenic tone ■ endothelial nitric oxide synthase ■ raloxifene ■ selective estrogen receptor modulator

The proven adverse effects of natural and synthetic estrogens have limited their use in hormone replacement therapy, and this has contributed to the development of selective estrogen receptor modulators (SERMs). Recent clinical and animal studies indicate a cardiovascular benefit of raloxifene (a second-generation SERM) use in protecting endothelial function.1–3 In addition, raloxifene produces direct vasodilatations at supratherapeutic doses.4–7 Raloxifene benefits women with high cardiovascular risk,1 although the outcome of the Raloxifene Use for the Heart trial shows that raloxifene has no overall effect in reducing the risk of coronary events in women with established coronary heart disease.8 A most recent analysis taking into account of age and other relevant factors shows that raloxifene can significantly lower the incidence of coronary events in women younger than 60 years of age.9 Nevertheless, the precise role of SERMs in the prevention and treatment of menopausal syndrome remains to be fully investigated, and further study is needed to better define and uncover a potential benefit of therapy with raloxifene.
and other SERMs in a clinically relevant specific subgroup of postmenopausal women. As with estrogen, raloxifene-induced action occurs when it is interacting with the estrogen receptor, which alters gene transcription in affected cells. Besides, SERMs also exert acute nongenomic vascular effects, which are less clear, particularly in small blood vessels that control peripheral resistance to flow. These effects may help to unravel potential novel targets in the circulation for the development of new SERMs.

Ovariectomy enhances myogenic responses in female rats; these responses can be normalized by chronic estrogen treatment.10 A gender difference has also been demonstrated for myogenic constriction in rat middle cerebral arteries.11 These results indicate the influence of circulating estrogen levels on the myogenic activity of arterioles. Most studies have so far used large conduit arteries for characterizing pharmacological properties of estrogen and SERMs under isometric conditions.4–6 The sensitivity to antihypertensive agents may vary considerably when vascular smooth muscle relaxation is determined by changes in isometric force and in vessel diameter under isobaric conditions.12 Raloxifene and other SERMs relax large arteries in micromolar concentrations in most reported studies,4–7 although raloxifene at low nanomolar concentrations can augment endothelium-dependent dilatations of conduit arteries.13 To our knowledge, only 1 study has examined the effect of SERM in human subcutaneous small arteries, by showing that raloxifene at 50 nmol/L reduces myogenic tone in postmenopausal women.14 However, this concentration is far above the mean maximal plasma level of raloxifene in women who receive long-term oral administration of 60 mg of raloxifene daily, which amounts to 1.36 μg/L, or 2.67 nmol/L.15,16 To be of therapeutic relevance, the aim of this study was therefore to determine (1) whether raloxifene in low nanomolar concentrations (0.3 to 10 nmol/L) could dilate the myogenically active rat mesenteric arteries, (2) whether endothelium-dependent mechanisms were involved, and (3) whether there was a gender difference in vascular responses to raloxifene.

Materials and Methods

Small Artery Preparation

This study was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong and conformed to Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). In experiments using ovariectomized (OVX) rats, adult female Sprague-Dawley rats (200 to 230 g) were anesthetized with sodium pentobarbital (40 mg kg−1 body weight, intraperitoneal injection) and ovariectomy was made via a midabdominal route. OVX rats were euthanized 2 months after ovariectomy. Sprague-Dawley rats (250 to 280 g) were euthanized by CO2 inhalation. The ileum and associated mesentery were quickly dissected out and immersed in iced ice (4°C) Krebs solution oxygenated by a gas mixture of 95% O2 to 5% CO2. The third-order mesenteric artery (2 to 3 mm long), with an outer diameter of ~350 μm, was dissected free of surrounding adipose tissue and cut into ring segments. Each segment was mounted in a Pressure Myograph System (Danish Myo Technology). In brief, each ring was cannulated between 2 glass cannulas with tip diameters <100 μm in a chamber filled with 10 mL of Krebs solution bubbled by 95% O2 plus 5% CO2 and maintained at 37°C (pH 7.4). After 1 end of the segment was secured on a cannula with 2 fine nylon sutures, the luminal content was flushed through with Krebs solution using a perfusion device at a pressure difference <20 mm Hg. The other end was then cannulated. Glass cannulas were pulled on a micropipette puller model P-87 (Shutter Instrument Company, Novato, Calif). Both cannulating pipettes were connected to independent Krebs solution-containing buffer bottles seated on a pressure regulator that controlled the flow rate and intraluminal pressure. A video camera attached to a light-inverted microscope (Zeiss Axiovert 40 Microscope, model 110P) was used to visualize changes in the diameter of cannulated arteries. The outer diameter and luminal pressure were recorded simultaneously using an Myo-View software (version 1.1 P, 2000, Photonics Engineering).

Myogenic Tone Induction

Under no-flow conditions, the artery segment was first subjected to stepwise increments of 20 mm Hg in intraluminal pressure from 20 to 100 mm Hg (3 to 5 minutes at each pressure) at 23°C. After this procedure, the pressure was reduced and kept at 80 mm Hg. Subsequently, the bathing solution was warmed up gradually from 23°C to 37°C, achieved by a built-in heating device. Under these conditions, myogenic tone began to develop as the artery started constricting, and the vessel’s diameter stabilized between 30 and 60 minutes, which was regarded as fully developed myogenic tone. The chamber solution was maintained at 37°C for the rest of the experiment.

Effect of Raloxifene on Myogenic Constriction

After a stable myogenic tone was established, raloxifene was added cumulatively (0.3 to 30 nmol/L) to the bathing solution at approximately 30-minute intervals without washout of the previously applied drug. By the end of each experiment, the superfusate was replaced with a Ca2+-free Krebs solution containing 2 mmol/L EGTA to obtain a maximal passive dilatation for the purpose of data analysis under isobaric conditions. Finally, the artery bathed in normal Krebs solution was constricted by 1 μmol/L phenylephrine and diluted by 1 μmol/L acetylcholine to confirm the presence of a functional endothelium. The artery was discarded if its dilatation in response to acetylcholine was less than 80%.

Effects of Pharmacological Inhibitors

To study the role of endothelium-derived dilating factors, endothelium-intact arteries with fully developed myogenic tone were incubated for 30 minutes with each of the following inhibitors: Nω-nitro-l-arginine methyl ester (l-NAME, nitric oxide synthase inhibitor, 30 μmol/L) or charybdotoxin (CTX) plus apamin (each in 50 nmol/L, used to inhibit the effect of endothelium-derived hyperpolarizing factor), ICI 182,780 (classic estrogen receptor antagonist, 10 μmol/L), 1400W (inducible nitric oxide synthase inhibitor, 100 nmol/L), wortmannin (phosphatidylinositol 3-kinase [PI3K] inhibitor, 100 nmol/L), and LY 294002 (PI3K inhibitor, 1 μmol/L) before the addition of raloxifene. The time-matched vehicle control experiments were performed to ensure that the induced dilatation was a genuine response.

Western Blot Analysis

Rat small mesenteric arteries including 4-order branches were isolated free of the adhering adipose and connective tissue. After exposure to raloxifene in the presence and absence of extracellular Ca2+ ions, arteries were frozen in liquid nitrogen and subsequently homogenized in ice-cold lysis buffer containing 1 μmol/L leupeptin, 5 μmol/L aprotinin, 100 μmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L sodium fluoride, and 2 mg/mL β-glycerophosphate. The lysates were sonicated for 30 minutes on ice and then centrifuged at 20 000g for 20 minutes. The supernatant was collected and analyzed for determining the
protein concentration using the Lowry method (Bio-Rad, Hercules, Calif). Sample buffer containing 5% β-mercaptoethanol was added and the sample was then denatured by boiling for 10 minutes. For each sample, 80 µg of protein was separated with 7.5% SDS-polyacrylamide gel, together with the pre-stained and biotinylated size markers. The resolved proteins were electro- phoretically transferred to a nitrocellulose Immobilon-P polyvi- nylidene difluoride membrane (Millipore, Billerica, Mass) using a Mini Trans-Blot cell (Bio-Rad, Hercules, Calif) at 100 V for 60 minutes at 4°C. The membranes were blocked with 1% bovine serum albumin dissolved in phosphate buffer saline 0.5%. Then Tween-20 for 1 hour at room temperature. Primary antibodies against total endothelial nitric oxide synthase (eNOS; 1:500, Santa Cruz Biotechnology, Santa Cruz, Calif) and eNOS phos- phorylation at Ser1177 (1:1000, Upstate Biotechnology, Lake Placid, N.Y) were used. The membranes were developed with an enhanced chemilumines- cence detection system (ECL reagents; Amersham Pharmacia Biotech, Uppsala, Sweden) and exposed on X-ray films (Fuji). Densitomet- ry was performed using a documentation program (Flurochem, Alpha Innotech Corp., San Leandro, Calif). eNOS corresponding to a 145-kDa band was visualized with reference to molecular weight markers.

Measurements of \([Ca^{2+}]_i\) in Endothelial Cells of Small Mesenteric Artery

The method of \([Ca^{2+}]_i\) detection was described previously. In brief, third-order mesenteric arteries were cut into rings and then opened longitudinally. The vascular strip was loaded with 5 µmol/L Fluo-4 AM (Invitrogen Corp, Carlsbad, Calif) for 45 minutes at room temperature. Subsequently, artery segments were placed on a glass slide and observed using a laser confocal microscope. Fluorometric measurements were performed using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc, Melville, NY) mounted on an inverted IX81 Olympus microscope, equipped with a ×20 water- immersion objective (numerical aperture 0.5). Fluorescence in- tensity excited at 495 nm and emitted at 515 nm was measured for 5 minutes. Alterations in \([Ca^{2+}]_i\), were expressed as \(F/F_0\) ratios, where \(F_0\) and \(F\) were the fluorescence intensity at a specific time point and at the initiation of image recording, respectively. Fluorescence images of the endothelium were obtained before and 3 minutes after the application of raloxifene, which was achieved by dropping 5 µL of HEPES buffer containing 3 nmol/L raloxi- fene onto the slide glass using a micropipette. All measurements were performed in HEPES buffer of the following composition (in mmol/L): 143 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.6 CaCl₂, 1.2 KH₂PO₄, 11 glucose, and 5 HEPES. In some experiments, rings were treated with 10 µmol/L ICI 182,780 before raloxifene was added.

Measurement of Myogenic Tone Generation after Chronic Raloxifene Treatment

Adult female and male Sprague-Dawley rats (weighing 200 to 230 g) were orally administered raloxifene (2.5 mg/kg per day) or vehicle for 4 weeks. After the 4-week treatment, blood pressure was measured by a tail-cuff method, and the myogenic tone was measured by a tail-cuff method..myogenic constriction was calculated as (passive diameter – active diameter)/passive diameter, and passive diameter was regarded as the diameter under Ca²⁺-free conditions.

Acute Effect of Raloxifene on Blood Pressure in Female Rats

Blood pressure on administration of raloxifene to female rats was measured. In brief, rats were anesthetized with sodium pentobar- bital (40 mg kg⁻¹ via intraperitoneal injection) while breathing spontaneously. The right carotid artery was cannulated for the measurement of mean arterial pressure using a pressure trans- ducer. The left jugular vein was cannulated for intravenous infusion of raloxifene or acetylcholine. After baseline mean arterial pressure was obtained during a 1-hour control period, a bolus (200 µL) of raloxifene was injected into the jugular vein at increasing dosages (from 0.01 to 10 µmol). Bolus was given at intervals of between 5 and 10 minutes.

Drugs and Solutions

Acetylcholine, phenylephrine, L-NAME, apamin, and CTX were purchased from Sigma (St. Louis, Mo.). ICI 182,780, 1400W, L-NAME, and CTX were from Tocris (Avonmouth, Bristol, United Kingdom). Raloxifene was a gift from Eli Lilly Corporate Center (Indianapolis, Ind).

Raloxifene, ICI 182,780, L-NAME, and CTX were dissolved in dimethyl sulfoxide and others in double-distilled water. Further dilution was made from stock solutions. The Krebs solution contained (mmol/L): 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 d-glucose. A Ca²⁺⁻free solution was identical to the Krebs solution with the exclusion of Ca²⁺ and the addition of 2 mmol/L EGTA.

Expression of Results and Data Analysis

Percentage dilatation was calculated using the following equation: \(100\% \times \frac{[D_d - D_0]/[D_{Ca-free} - D_0]]}{D_{Ca-free}}\), where D is the diameter of the stabilized vessel after drug exposure (d), baseline (b), or Ca²⁺⁻free conditions.

Results are means±SEM of n arteries from different rats. The 2-tailed Student t test or analysis of variance (ANOVA) was used for statistical evaluation when more than 2 groups were com- pared. Concentration-response curves were analyzed by 2-way ANOVA followed by Bonferroni post tests. P<0.05 was taken as statistically significant.

Results

The average outer diameter under isobaric conditions (80 mm Hg) was 361.30±4.31 µm for female rat mesen- teric artery rings (n=103) and 353.40±10.33 µm (n=29) for male rings (P<0.05).

The Dilating Effect of Raloxifene

The original trace in Figure 1A shows changes in diameter of a ring segment (≈350 µm in outer diameter) from a female rat mesenteric vascular bed. Shortly after full development of myogenic tone at an applied intraluminal pressure of 80 mm Hg, the addition of raloxifene (0.3 to 30 nmol/L) produced concentration-dependent dilatations, with a pD₂ value of 8.91±0.18 and a maximal response at ≈10 nmol/L (Figure 1). The raloxifene-induced dilatation was significantly greater in female (Figure 1A) than male (Figure 1B) or OVX rat rings (Figure 1C) (E⁻max(%)= 93.9±4.6 in female, 36.7±2.2 in male, and 30.5±15.96 in OVX rats, P<0.001 compared with female; Figure 2A).

Treatment with 10 µmol/L ICI 182,780 did not affect raloxifene-induced dilatations in female rat rings (pD₂: 8.91±0.18 in control and 8.81±0.29 in ICI 182,780 with similar values of E⁻max, P>0.05; Figure 2B).

Effects of Nitric Oxide Synthase Inhibitor

To assess the possible involvement of endothelium-derived NO, the presence of 30 µmol/L L-NAME attenuated dilatations to raloxifene (E⁻max(%)= 85.9±7.3 in control and 35.7±15.1 in L-NAME, P<0.05; Figure 3A). By con- trast, 1400W, the inducible nitric oxide synthase inhibitor,
did not modify raloxifene-induced dilatations (P > 0.05, Figure 3B).

**Effect of CTX Plus Apamin**

A combined treatment with CTX plus apamin (each at 50 nmol/L) did not reduce raloxifene-induced dilatations (P > 0.05, Figure 3C). Raloxifene at 0.3 nmol/L, which caused a small dilatation (Figure 1A), failed to modify acetylcholine-induced dilatation in female rat rings, and this acetylcholine dilatation was largely mediated by endothelium-derived hyperpolarizing factor, as it was prevented by CTX plus apamin (each in 50 nmol/L, n = 3, data not shown).

**Effects of Wortmannin and LY 294002**

Acute 30-minute treatment with wortmannin (100 nmol/L) or LY294002 (1 μmol/L) did not influence raloxifene-induced dilatations in female rat rings (n = 4 to 5, Figure 3D).

**Effects on Phosphorylation of eNOS**

eNOS was posttranslationally activated by 3 nmol/L raloxifene, as reflected by significantly elevated phosphorylation of eNOS at Ser 1177 (p-eNOS) in female rat small mesenteric arteries with endothelium, as compared with untreated arteries without affecting the p-eNOS level in male rat mesenteric arteries (Figure 4A). By contrast, the total amount of eNOS protein was unaffected by raloxifene (Figure 4A). The increased eNOS phosphorylation was abolished by removal of extracellular Ca²⁺ (Figure 4B), whereas treatment with 10 μmol/L ICI 182,780 did not prevent a raloxifene-stimulated increase in eNOS phosphorylation (Figure 4B). Finally, acute treatment with 3 nmol/L raloxifene did not change phosphorylation of Akt or the total Akt in both female and male rat small mesenteric arteries (data not shown).

**Effects on In Situ [Ca²⁺]i in Endothelial Cells of Intact Arteries**

Ca²⁺ fluorescence images from viable individual endothelial cells in longitudinally cut open endothelium-intact...
had no effect (Figure 5).

In each case, Figure 5C and 5D). Vehicle dimethyl sulfoxide (DMSO) increased 

Second-order mesenteric arteries were clearly visible (Figure 5A). Perfusion of 3 nmol/L raloxifene triggered a rapid increase of endothelial cell Ca\(^{2+}\) (peak rise in 1 minute (data not shown). The effect of raloxifene was greater in endothelial cells from female rats (peak rise in Ca\(^{2+}\) in vehicle-treated rats (3.08) than in cells from male rats (peak rise in Ca\(^{2+}\); 1.15±0.04 at 1 minute and 1.14±0.01 at 5 minutes (female versus male, P<0.001, n=5). This stimulatory effect of raloxifene was unchanged by pretreatment of 10 μmol/L ICI 182,780 in arteries from both genders, but it was lost in the absence of extracellular Ca\(^{2+}\) (n=5 in each case, Figure 5C and 5D). Vehicle dimethyl sulfoxide had no effect (Figure 5).

Effects of Chronic Raloxifene Treatment on Blood Pressure and Myogenic Constriction

Chronic administration of raloxifene did not affect blood pressure in female rats (systolic blood pressure in mm Hg: 110.4±3.08 in vehicle-treated rats and 113.6±2.73 in raloxifene-treated rats) or in male rats (systolic blood pressure in mm Hg: 112±4.01 in vehicle-treated rats and 113.4±3.46 in raloxifene-treated rats). Raloxifene treatment significantly suppressed the generation of myogenic tone in mesenteric resistance arteries from female rats (myogenic constriction: 21.30±2.97% in vehicle-treated rats and 7.38±2.42% in raloxifene-treated rats, n=5, P<0.001), but not in those from male rats (myogenic constriction: 24.33±2.42% in vehicle-treated and 22.30±2.43% in raloxifene-treated rats, n=5, P>0.05, Figure 6).

Acute Effect of Raloxifene on Blood Pressure in Female Rats

Raloxifene did not cause an acute reduction of blood pressure in vivo in female rats (n=4, Supplemental Figure I, available online at http://atvb.ahajournals.org).

Discussion

The novel findings of the present investigation in pressurized rat resistance mesenteric arteries with endothelium include the following: (1) raloxifene at therapeutically achievable concentrations reduces myogenic constriction in a concentration-dependent manner; (2) dilatations to raloxifene are rapid and nongenomic in nature, unrelated to IC1 182,780-sensitive estrogen receptor; (3) endothelium-derived NO accounts in large part for raloxifene-induced dilatations, as they are inhibited by L-NAME; (4) eNOS activation is causally controlled by Ca\(^{2+}\) influx stimulated by raloxifene without involving PI3K/Akt-related mechanisms; (5) raloxifene dose not modulate CTX/apamin-sensitive endothelium-derived hyperpolarizing factor–mediated dilatations; and (6) there is a marked gender difference in dilator responses to raloxifene, which is more effective on female arteries. The present study has provided the first line of experimental evidence exhibiting a potent vasodilating effect of raloxifene in female resistance arteries in therapeutically relevant concentrations.

Small resistance arteries regulate total peripheral resistance to blood flow. If the vasomotor activity of these arteries can be favorably modulated by SERMs at therapeutic concentrations, increased blood pressure in postmenopausal women could be partially controlled. If the dilating effect is proven in vivo in resistance vascular beds in humans, raloxifene is not only used currently for the prevention and treatment of postmenopausal osteoporosis, but also useful against hypertension associated with estrogen deficiency. Long-term oral ingestion of 60 mg/day of raloxifene in patients resulted in a mean maximal plasma concentration of 1.36 μg/L or the equivalent to 2.67 nmol/L of raloxifene.\(^7\) The present results clearly show that low concentrations of raloxifene produce potent vasodilator responses in resistance arteries, highlighting an important mechanism by which raloxifene benefits the vascular function. More importantly, this benefit also occurs in vivo. Most published studies examined the effect of SERMs or estrogen in large mammalian arteries for the measurement of isometric tone, and much higher concentrations of raloxifene were usually required to induce dilatations.\(^4\)\(^-\)\(^6\) However, such high levels of raloxifene are unlikely to build up after normal dosing.

Raloxifene-induced dilatations involved an endothelium-derived NO-related mechanism, because dilatations were largely attenuated by L-NAME, a nitric oxide synthase inhibitor. By contrast, 1400W, the inducible nitric oxide synthase inhibitor, was without effect. Raloxifene-induced dilatations were unaffected by treatment with CTX plus apamin, a recipe widely used to block K\(^+\)-dependent endothelium-derived hyperpolarizing factor–mediated response, such as acetylcholine-induced dilatation in resistance arteries, 90% of which was inhibited by CTX plus...
apamin in the present preparations; similar results were also reported by others.\textsuperscript{18,19} In addition, treatment with raloxifene (0.3 nmol/L) did not modify acetylcholine-induced endothelium-derived hyperpolarizing factor–mediated dilatations, further suggesting that raloxifene acts differently from acetylcholine in rat resistance arteries.

The present results clearly show that stimulated rise in endothelial cell \([\text{Ca}^{2+}]\) may causally account for raloxifene-induced NO-dependent dilatations in female rat resistance arteries on the basis of the following observations. First, raloxifene-stimulated phosphorylation of eNOS depends on the presence of extracellular Ca\textsuperscript{2+} ions. Second, raloxifene at 3 nmol/L, a concentration that increases eNOS phosphorylation and causes \textit{l}-NAME-sensitive dilatations, triggers a rapid and sustained elevation of \([\text{Ca}^{2+}]\), in endothelial cells of intact female rat mesenteric arteries, and this effect is prevented with \textit{l}-NAME-free bathing solution. This novel action of raloxifene has not been described before in fresh endothelial cells in situ in intact resistance arteries.

Neither LY294002 (a selective PI3K inhibitor) nor wortmannin (an irreversible PI3K inhibitor) inhibited raloxifene-induced dilatations, thus minimizing the involvement of PI3K pathway, although raloxifene in micro-molar concentrations has been shown previously to stimulate NO production in cultured human endothelial cells via estrogen receptor–associated PI3K activation.\textsuperscript{20} However, most of the studies in endothelium-intact arteries in vitro show that high concentrations of raloxifene produce endothelium-independent vasodilating effects, as this

\begin{figure}
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\caption{The stimulatory effect of raloxifene (3 nmol/L) on endothelial cell \([\text{Ca}^{2+}]\), in situ in cut-open female (A) and male (B) rat mesenteric artery. Also shown is the time course for the raloxifene-stimulated rise in endothelial cell \([\text{Ca}^{2+}]\), in female (C) and male (D) artery in normal Krebs solution without and with pretreatment by 10 \textmu mol/L ICI 182,780, in dimethyl sulfoxide (DMSO) vehicle, and in \textit{l}-NAME-free solution. Results are means±SEM of 5 experiments from different rats.}
\end{figure}
SERMs at such concentrations can inhibit the activity of voltage-sensitive \( \text{Ca}^{2+} \) channels. Treatment with 3 nmol/L raloxifene resulted in significant enhancement in bradykinin-induced endothelium-dependent dilatations via eNOS activation in porcine coronary arteries, whereas endothelium-independent relaxations can be observed by raloxifene only at concentrations greater than 1 \( \mu \)mol/L.

Raloxifene improves coronary perfusion and cardiac contractility by the release of PI3K/Akt-dependent NO in the ischemic heart; however, the source of NO is unknown in this study. Raloxifene at 1 \( \mu \)mol/L can increase Akt phosphorylation and NO formation in cultured human endothelial cells. On the other hand, the raloxifene analogue LY171018 does not stimulate phosphorylation of Akt in bovine endothelial cells. The present results do not indicate significant involvement of Akt pathway, as raloxifene did not increase phosphorylation of these markers. Taking these data together, it appears that the endothelial NO-dependent or -independent mechanism underlying the relaxant effect of raloxifene may vary considerably depending on type (conduit or resistance) and anatomic origin of arteries or even species. Besides, the amount of NO generated in response to raloxifene may be different in cultured endothelial cells and in freshly isolated intact arteries. In the latter situation, the released NO may be insufficient to trigger a noticeable NO-dependent relaxant effect, as reported previously in many intact arteries. In addition, the method used for measurement of vascular reactivity (under isometric or isobaric conditions) may also have an impact on the way an artery responds to a dilator.

Nongenomic effects of raloxifene may be mediated through activation of the estrogen receptor. However, the classical estrogen receptor antagonist ICI 182,780 did not antagonize raloxifene-induced dilatations, eNOS phosphorylation, and rise in \([\text{Ca}^{2+}]\), in endothelial cells of intact resistance arteries. Likewise, several previous studies do not indicate a positive role for ICI 182,780-sensitive estrogen receptor in acutely induced dilatations of large arteries. Previous studies reported that raloxifene-induced NO production in human endothelial cells is associated with an estrogen receptor-dependent acute stimulation of eNOS activity.

The present study reveals that raloxifene-induced vasodilation was markedly blunted in OVX rats and in male rats, implying that the vasodilator response to raloxifene may depend on the circulating estrogen level. However, the present results show that raloxifene at a therapeutically achievable concentration elicits endothelial NO-dependent vasodilation in intact mesenteric resistance artery, which is extracellular calcium-dependent but ICI-182780-insensitive, indicating that the vasodilator response to raloxifene in myogenically active arteries is independent of the estrogen receptor. The contrasting role of the estrogen receptor in the effect of raloxifene on endothelial cells might be related to different species (human versus rat), vascular beds (veins versus arteries), and preparations (cultured endothelial cells and resistance intact arteries under isobaric conditions).

A gender difference was demonstrated unambiguously in raloxifene-induced dilatations, eNOS phosphorylation, and an increase in endothelial cell \([\text{Ca}^{2+}]\); the 3 responses were much greater in female rat resistance arteries. Because a \([\text{Ca}^{2+}]\) rise is required for the increased eNOS phosphorylation in response to 3 nmol/L raloxifene in female rat mesenteric arteries, it is likely that poor dilating responses in male rat arteries may be associated with only a small elevation in endothelial cell \([\text{Ca}^{2+}]\), which is inadequate to trigger a significant eNOS activity in male arteries. Although the precise cause of such a discrepancy in \([\text{Ca}^{2+}]\), response is at present unclear, other possibilities cannot be dismissed, including the possibility that the expression of an unknown membrane-bound raloxifene-binding site may differ between female and male rat arteries, as the estrogen receptor expression was reported to be gender-related in nonvascular tissues during aging. The present results show that estrogen receptor-\( \alpha \) but not estrogen receptor-\( \beta \) is expressed in rat mesenteric resistance arteries and that the estrogen receptor-\( \alpha \) level detected in mesenteric arteries is in the following descending order: OVX rats > male > female rats (Supplemental Figure II). Thus, it is less likely that a greater dilator effect of raloxifene correlates with the expression level of estrogen receptor-\( \alpha \).

In conclusion, the present study has provided novel findings that therapeutically achievable concentrations (0.3 to 10 nmol/L) of raloxifene potently dilate rat resistance arteries with spontaneous myogenic tone. This dilatation is mediated primarily through extracellular \([\text{Ca}^{2+}]\)-dependent eNOS activation and NO production, but it is independent of pathways related to ICI 182,780-sensitive estrogen receptor, PI3K/Akt, or inducible nitric oxide synthase. The present results demonstrate a marked gender difference in the action of raloxifene with being much more effective in female rat arteries both in vitro and in vivo. Although the randomized controlled trial (Raloxifene Use for the Heart) shows minimal effect of the use of raloxifene in lowering the risk of coronary events in postmenopausal women at risk for coronary disease, recent studies do support vascular benefit of raloxifene, as this SERM effectively retards the progression of intima-media thickness in postmenopausal women, improves...
ovariectomy-induced endothelial dysfunction, and decreases the risk of coronary events in younger postmenopausal women. Undoubtedly, more careful analysis and further pharmacological characterisation are needed to identify clinically relevant particular subgroups of postmenopausal women with hypertension that may benefit from the use of raloxifene and other safe SERMs with optimization of the dose and timing of therapy.

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Disclosures
None.

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**Supplemental Figure SI.** Raloxifene did not induce an acute reduction of blood pressure *in vivo* (n=4). By contrast, the infusion of acetylcholine (ACh) transiently reduced blood pressure.
Supplemental Figure SII. Protein expressions of ER-α and ER-β in mesenteric arteries from female rats, ovariectomized (OVX) rats and male rats; and in porcine coronary arteries (PCA). Results are means ± SEM of 4 experiments.