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RESEARCH PAPER

Therapeutic concentrations of raloxifene augment nitric oxide-dependent coronary artery dilatation *in vitro*

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Background and purpose: Raloxifene improves cardiovascular function. This study examines the hypothesis that therapeutic concentrations of raloxifene augment endothelium-dependent relaxation via up-regulation of eNOS expression and activity in porcine coronary arteries.
Experimental approach: Isometric tension was measured in rings from isolated arteries. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in arterial endothelial cells were detected by Ca^{2+} fluorescence imaging. Phosphorylation of eNOS at Ser-1177 was assayed by Western blot analysis.
Key results: In arterial rings pre-contracted with 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$ (U46619), treatment with raloxifene (1-3 nM) augmented bradykinin- or substance P-induced relaxation and this effect was antagonized by ICI 182,780, an estrogen receptor antagonist. The enhanced relaxation was abolished in rings treated with inhibitors of nitric oxide/cyclic GMP-dependent dilation, N^G-nitro-L-arginine methyl ester (L-NAME) plus 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ). In contrast, effects of raloxifene were unaffected after inhibition of endothelium-derived hyperpolarizing factors by charybdotoxin plus apamin. Raloxifene (3 nM) did not influence endothelium-independent relaxation to sodium nitroprusside. 17ß-Estradiol (3-10 nM) also enhanced bradykinin-induced relaxation, which was inhibited by ICI 182,780. Treatment with raloxifene (3 nM) did not affect bradykinin-stimulated rise in endothelial cell $[Ca^{2+}]_i$. Raloxifene, 17ß-estradiol, and bradykinin increased eNOS phosphorylation at Ser-1177 and ICI 182,780 prevented effects of raloxifene or 17ß-estradiol but not that of bradykinin. Raloxifene had neither additive nor antagonistic effects on 17ß-estradiol-induced eNOS phosphorylation.
Conclusions and implications: Raloxifene in therapeutically relevant concentrations augmented endothelial function in porcine coronary arteries *in vitro* through ICI 182,780-sensitive mechanisms that were associated with increased phosphorylation of eNOS but independent of changes in endothelial cell $[Ca^{2+}]_i$.
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Keywords: coronary artery; nitric oxide synthase; raloxifene; vasoconstriction/dilation

Abbreviations: CTX, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factors; L-NAME, N^G-nitro-L-arginine methyl ester; ODQ, 1*H*-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one; SERMs, selective estrogen receptor modulators; U46619, 9,11-dideoxy-11α, 9α-epoxy-methanoprostaglandin $F_{2\alpha}$

Introduction

Due to the tissue-specific and mixed estrogen-agonist/antagonist properties, selective estrogen receptor modulators (SERMs), such as raloxifene, exhibit estrogenic activities in bone, cardiovascular system and the central nervous system, while avoiding or opposing harmful effects seen with hormone replacement therapy in breast and uterus. Animal studies indicate that raloxifene exerts estrogen-like cardioprotective actions (Ogita *et al*., 2002, 2004) and improves coronary blood flow partly by releasing endothelial nitric oxide (Zoma *et al*., 2000; Ogita *et al*., 2002). Coronary arteries have greater diameters in ovariectomized sheep receiving

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raloxifene compared with those receiving estrogen or no treatment (Gaynor *et al*., 2000). Raloxifene therapy may reduce the risk of cardiovascular events in women with increased cardiovascular risk (Barrett-Connor *et al*., 2002). However, outcomes of the recent RUTH trial show that raloxifene did not affect the risk of coronary heart disease (Barrett-Connor *et al*., 2006). Nevertheless, the role of SERMs in the prevention and treatment of cardiovascular disease remains to be fully understood.

Long-term oral administration of 60 mg raloxifene to women per day produces a mean maximum plasma concentration of 1.36 $\mu g l^{-1}$ or 2.67 nM raloxifene (Snyder *et al*., 2000; Morello *et al*., 2003). There are no reports that this concentration of raloxifene could produce relaxation in isolated blood vessels. Clinical studies demonstrate that raloxifene therapy results in a positive modulation of endothelium-dependent dilatation in postmenopausal women (Colacurci *et al*., 2003; Sbarouni *et al*., 2003). Raloxifene produces a vasodilator effect when added to isolated blood vessels (Figtree *et al*., 1999; Tsang *et al*., 2004; Chan *et al*., 2005; Leung *et al*., 2005, 2007). Such direct activation of vascular smooth muscle relaxation usually requires high concentrations (0.1–10 μM), which are clearly in excess of therapeutic plasma levels. One explanation for the effectiveness of the low plasma concentrations (nM) of raloxifene in activating nitric oxide (NO)-dependent dilatation *in vivo* is that the SERM interacts with circulating factors. *In vivo*, many different hormone dilators that are secreted from various cells are circulating in the bloodstream and each of these substances are vasoactive, and may modulate the effect of other dilators. Another explanation is that raloxifene is directly able to potentiate vasodilator effectiveness. Interaction between agonists to influence positively endothelial function has been demonstrated (Bell *et al*., 1995; Wang *et al*., 1995, 1996; Chan *et al*., 2002).

If there is an interaction within the endothelium between different intracellular signalling pathways activated by raloxifene and other dilators, it is reasonable to hypothesize that raloxifene at therapeutically relevant concentrations may be able to amplify endothelial function. The purpose of the present study was therefore to test (i) the potentiating effect of raloxifene (1–3 nM) on agonist-induced dilatation; (ii) involvement of endothelial NO; (iii) modulation by raloxifene of vascular smooth muscle reactivity in isolated porcine coronary arteries; and (iv) the effect of raloxifene on endothelial cell $[Ca^{2+}]_i$.

Methods

Artery ring preparation

All experiments were approved by the Animal Research Ethics Committee, Chinese University of Hong Kong. This investigation conformed to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

Pig (~6 month old) hearts were obtained fresh from a local slaughterhouse in Hong Kong. The hearts were placed on a dissecting plate filled with ice-cold Krebs solution containing (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. After removal of connective tissues, each left circumflex coronary artery was cut into ring segments, ~ 3 mm in length. Each ring was suspended in an organ bath filled with Krebs solution that was oxygenated with 95% O_2–5% CO_2 and maintained at 37°C (pH 7.3–7.5) and stretched to an optimal tension of 15 mN. Thereafter, all rings were allowed to equilibrate for 90 min before the start of experiments. In some rings where the endothelial layer was mechanically disrupted with the tips of a pair of forceps, functional removal was confirmed if these rings did not relax in response to the addition of 50 nM bradykinin. Each experiment was performed on rings prepared from different pig hearts.

Isometric force measurement

After an initial equilibration period of 30 min in organ baths, each ring was contracted twice by 60 mM KCl. After washout of KCl, a stable contraction was induced by 30 nM U46619 and the addition of 50 nM bradykinin to confirm the presence of a functional endothelium (>85% relaxation). Rings were thereafter rinsed in pre-warmed Krebs solution several times until baseline tension was restored. Rings with and without endothelium in the absence and presence of inhibitors were studied in parallel. Two consecutive concentration-response curves to bradykinin (1–50 nM) were studied in the absence (control) and presence (30-min incubation) of either raloxifene (1–3 nM) or 17β-estradiol (3–10 nM). To examine the role of NO or endothelium-derived hyperpolarizing factors (EDHF), rings were exposed for 30 min to respective inhibitors before the addition of raloxifene. These inhibitors included N^G-nitro-L-arginine methyl ester (100 μM, L-NAME, NOS inhibitor) plus 1*H*-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (3 μM, ODQ, soluble guanylate cyclase inhibitor) (a combination used to inhibit NO/cyclic GMP-mediated relaxation) or charybdotoxin (CTX; 50 nM, intermediate-conductance Ca^{2+}-activated K^+ channel blocker) plus apamin (50 nM, low-conductance Ca^{2+}-activated K^+ channel blocker) (a combination used to inhibit K^+-mediated EDHF-mediated relaxation). The inhibitory effects of ICI 182780 (1 μM, estrogen receptor antagonist) were tested on vascular responsiveness to raloxifene or 17β-estradiol. Finally, the effect of raloxifene (3 nM) was studied on relaxations to sodium nitroprusside in rings without endothelium.

Western blot analysis

At the end of the force measurement experiments, arterial rings were snap-frozen in liquid nitrogen and subsequently homogenized with ice-cold radioimmunoprecipitation (RIPA) lysis buffer, containing 1 μM leupeptin, 5 μM aprotonin, 100 μM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM EDTA, 1 mM sodium fluoride, and 2 mg ml^{-1} β-glycerolphosphate. The lysates were sonicated on ice for 30 min and then centrifuged at 20 000 *g* for 20 min. The supernatant was collected and analysed for protein concentration using the Lowry method (Bio-Rad, Hercules, CA, USA). Sample buffer containing 5% β-mercaptoethanol was added to the sample, which was then denatured by boiling for 5 min. For each sample, 50 μg of

protein was separated with 7.5% SDS-polyacryamide gel, together with the prestained and biotinylated size marker. The resolved proteins were electrophorectically transferred to a nitrocellulose immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, CA, USA) using a mini-trans-blot cell (Bio-Rad) at 100 V for 60 min at 4°C. The membranes were blocked with 1% bovine serum albumin dissolved in phosphate buffer saline 0.5% Tween-20 (PBST) for 1 h at room temperature. Primary antibodies against total eNOS (1:500) and eNOS phosphorylated at ser-1177 (1:1000) were from Santa Cruz, CA, USA and Upstate (Charlottesville, VA, USA), respectively, while the secondary anti-rabbit antibody conjugated to horseradish peroxidase (DakoCytomation, Produktionsvej, Glostrup, Denmark) was used at a dilution of 1:3000. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) and exposed on X-ray films (Fuji). Densitometry was performed using a documentation program (Flurochem, Alpha Innotech Corp, San Leandro, CA, USA). eNOS corresponding to a 145-kDa band was visualized with reference to molecular weight markers.

In vitro endothelial cell $[Ca^{2+}]_i$ imaging

Porcine coronary artery rings were fluorescently labelled by incubation for 1 h at 22°C in a loading solution containing 10 μM Fura-2 AM, 0.025%. pluronic F-127, and 1 mM probenecid added to prevent Fura-2 leaking into the incubation medium. After this incubation period, the extracellular Fura-2 AM was removed. Native endothelial cells were then perfused for 20 min by Krebs solution at 2 ml min^{-1} (37°C) to allow intracellular cleavage of Fura-2 AM into active Fura-2 by esterases.

The $[Ca^{2+}]_i$ imaging method has been described by Leung *et al*. (2006). In brief, after Fura-2 loading, rings were longitudinally cut open and pinned (luminal side up) onto a block of silicone elastomer, which was fixed onto a base plate of a custom-made flow chamber. The base plate was then covered with a gasket and cover glass (24 × 32 mm) and affixed by screws. There was a 1-mm gap between vessel lumen and cover glass for flow of the buffer. After tissue mounting, the chamber was placed on an inverted microscope and perfused with Krebs solution at 2 ml min^{-1} (37°C). The specimen was illuminated on the stage of a Ix70 Olympus microscope, fitted with a ×20 Olympus water immersion objective by using a Polychrome IV light source. Fura-2 loaded vascular tissues were sequentially excited at 340 and 380 nm, and images of the respective 510-nm emissions were collected at 1-s intervals using MetaFluor v4.6 software (Universal Imaging Corp., Marlow, Buckinghamshire, UK). The emitted light was transmitted to collecting device and then to a cooled charge coupled-device camera. Illumination through the Polychrome IV light source and acquisition by the charge coupled device camera were controlled by MetaFluor software. Video frames containing images of cell fluorescence were digitized at a resolution of 512 horizontal × 480 vertical pixels. Imaging analysis was also performed with a MetaFluor imaging system. Following background subtraction, the fluorescence ratio (F_{340}/F_{380}) was obtained by dividing, pixel by pixel, the images at 340 and 380 nm. Changes in this ratio reflected changes in $[Ca^{2+}]_i$, eliminating potential artefacts caused by variations in cell thickness, intracellular dye distribution or photobleaching. The vascular tissue was exposed to raloxifene (3 nM) for 30 min before bradykinin (100 nM) application. L-NAME (100 μM) was continuously available to minimize possible inhibitory effects of endogenous NO on Ca^{2+} influx in the endothelium (Yao and Huang, 2003). Bradykinin (100 nM) did not trigger a significant increase in endothelial cell $[Ca^{2+}]_i$ in a Ca^{2+}-free perfusion medium.

Data analysis

Data are mean ± s.e.mean and *n* refers to the number of pigs studied. Several rings prepared from the same artery were examined in parallel, and two consecutive cumulative concentration-response curves were established in each ring. To demonstrate the contribution of endothelial factors to the raloxifene-induced effects, the second concentration-response curve (after inhibition of NO or EDHF) in the absence and presence of raloxifene was subtracted from the first concentration-response curve (control) in each ring. The relaxant response to bradykinin was calculated as percentage reduction of the initial tone in each ring contracted by U46619. Curves were analysed by non-linear curve fitting using Graphpad software (Version 3.0) and statistical difference between curves was analysed by two-way ANOVA followed by Bonferroni post-tests. The negative logarithm of the dilator concentration that produced half (pEC_{50}) of the maximal relaxation was approximated. *P*-values less than 0.05 were regarded as statistically significant.

Chemicals

Bradykinin, U46619, L-NAME, ODQ, CTX, apamin, indomethacin, sodium nitroprusside, 17β-estradiol and substance P were purchased from Sigma. ICI 182780 was purchased from Tocris, and raloxifene hydrochloride was gift from Eli Lilly & Co. U46619, ICI 182780, ODQ and raloxifene were dissolved in dimethyl sulphoxide and stock solutions were diluted to desired concentrations before the experiments. Other chemicals were prepared in double distilled water and further dilutions made in Krebs solution. Treatment with vehicle (dimethyl sulphoxide at 0.1%, v/v) did not affect relaxation to bradykinin.

Results

Effect of raloxifene on endothelium-dependent relaxation

In arterial rings contracted with U46619, the two consecutive bradykinin concentration-relaxation curves were superimposed with pEC_{50} of 7.94 ± 0.19 and 7.98 ± 0.11 for the first and second curves, respectively ($P > 0.05$, Figure 1a). Exposure (30 min) to nanomolar concentrations of raloxifene augmented bradykinin-induced relaxation (Figures 1b and c and Table 1). Raloxifene (1–3 nM) did not affect baseline tone or U46619-induced contraction. In contrast, higher concentrations of raloxifene (> 1 μM) caused

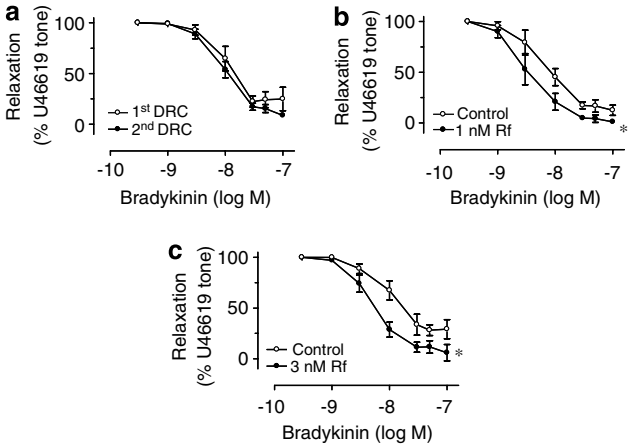

Figure 1 Two consecutive concentration-response curves (DRC) for bradykinin-induced relaxation in U46619-precontracted isolated porcine coronary arteries with endothelium in control (**a**), in the presence of 1 nM (**b**) or 3 nM (**c**) raloxifene (Rf). Results are mean±s.e.mean of 6–8 experiments. *$P<0.05$.

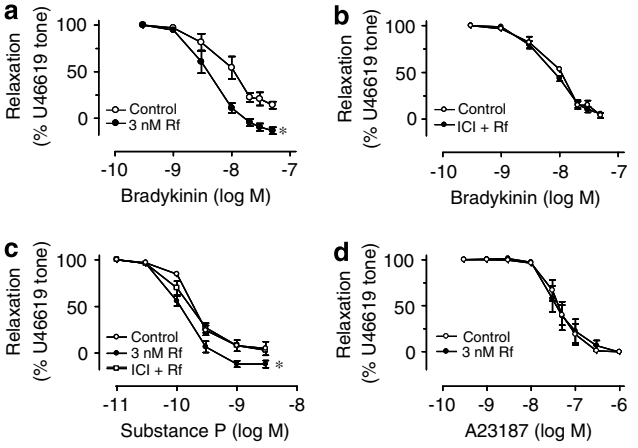

Figure 2 The effect of 3 nM raloxifene (Rf) on bradykinin-induced relaxation in rings with endothelium, in the absence (**a**) and presence of ICI 182780 (ICI, **b**). (**c**) Inhibition by ICI 182780 on the enhancing effect of raloxifene (3 nM) on substance P-induced relaxation. (**d**) Lack of effect of raloxifene on A23187-induced relaxation. Results are mean±s.e.mean of 6–8 experiments. *$P<0.05$ between Rf and other groups.

Table 1 pEC_{50} values for bradykinin-induced relaxation

Treatment	*pEC_{50}*
Control for raloxifene (Rf)	7.96±0.10
Rf (1 nM)	8.50±0.11*
Rf (3 nM)	8.31±0.07*
L-NAME + ODQ + Rf (3 nm)	8.03±0.20
Control for 17β-estradiol (E_2)	8.15±0.06
E_2 (3 nM)	8.60±0.03*
E_2 (10 nM)	8.69±0.06*
ICI 182780 (1 μm) + E_2 (10 nm)	8.12±0.31

Abbreviations: L-NAME, N^G-nitro-L-arginine methyl ester; ODQ, 1*H*-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one.
Data are mean±s.e.mean of five experiments (*$P<0.05$ compared with control).

endothelium-independent relaxation (pEC_{50} 4.61±0.16 with endothelium and 4.49±0.16 without endothelium, $n=6$, $P>0.05$, data not shown). Raloxifene (3 nM)-induced enhancement of relaxation to bradykinin (Figure 2a) was inhibited by 1 μM ICI 182780, a classic estrogen receptor antagonist (Figure 2b). Both substance P (receptor-dependent, Figure 2c) and A23187 (receptor-independent, Figure 2d) caused relaxations, which were absent upon removal of the endothelium. Raloxifene (3 nM, 30-min incubation) augmented substance P-induced dilatation, and this effect was inhibited by 1 μM ICI 182780 (Figure 2c). In contrast, raloxifene at 3 nM did not affect dilation to A23187, a calcium ionophore (Figure 2d).

Bradykinin induced relaxations of porcine coronary arteries (E_{max}: 91.1±2.1%, $n=6$), which were inhibited by 100 μM L-NAME plus 3 μM ODQ (E_{max}: 31.6±10.4%, $n=6$, $P<0.05$ versus control, Figure 3a) or CTX plus apamin (each at 50 nM, E_{max}: 35.8±11.5%, $n=6$, $P<0.05$ versus control, Figure 3b). Bradykinin-induced relaxation was eliminated following a combined treatment with L-NAME plus ODQ, CTX and apamin (data not shown). In contrast, indomethacin (1 μM, inhibitor of prostacyclin formation) had no effect ($n=5$) (data not shown).

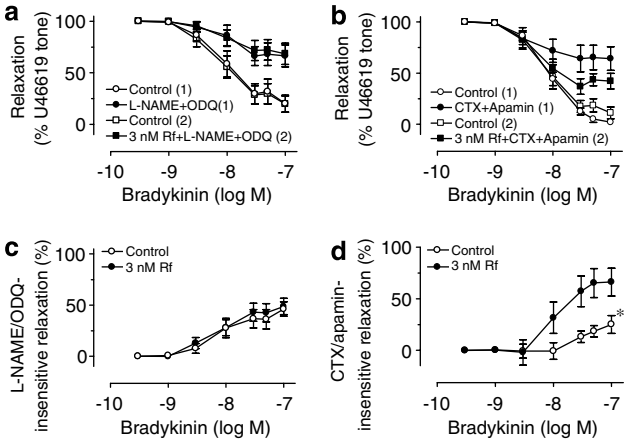

Figure 3 Effect of 3 nM raloxifene (Rf) on bradykinin-induced relaxation in rings with endothelium, in the absence and presence of treatment with N^G-nitro-L-arginine methyl ester(L-NAME) plus 1*H*-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ) (**a**) or with charybdotoxin (CTX) plus apamin (**b**). (**c**) L-NAME/ODQ- or (**d**) CTX/apamin-insensitive component of bradykinin relaxation. Results are mean±s.e.mean of six experiments. *$P<0.05$ between control and Rf groups (two-way ANOVA).

The L-NAME/ODQ-insensitive relaxations to bradykinin were identical in the absence and presence of raloxifene (Figure 3c). In contrast, in rings treated with CTX plus apamin, raloxifene (3 nM) was still able to enhance the relaxation as reflected by a significant increase in CTX/apamin-insensitive relaxation in raloxifene-treated rings ($P<0.05$, Figures 3b and d).

Effect of 17β-estradiol on bradykinin-induced relaxation

Like raloxifene, low concentrations of 17β-estradiol (3–10 nM) also increased bradykinin-induced relaxation (Figure 4b and Table 1) and this effect was antagonized by

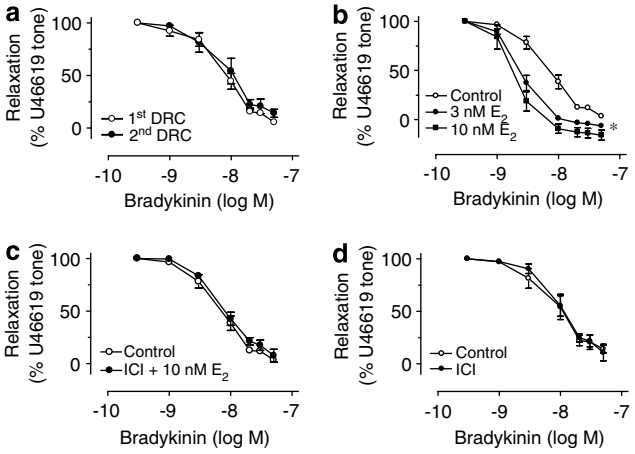

Figure 4 (**a**) Two identical relaxation curves to bradykinin in rings with endothelium. (**b**) Enhancing relaxation to bradykinin in the presence of 3 or 10 nM 17β-estradiol (E_2). (**c**) Abolition by ICI 182780 of 17β-estradiol (10 nM)-induced enhanced relaxation to bradykinin. (**d**) Lack of effect of ICI 182780 alone on bradykinin-induced relaxation. Results are mean$\pm$s.e.mean of 5–7 experiments. *$P<0.05$ between control and estrogen groups (two-way ANOVA).

1 μM ICI 182780 (Figure 4c and Table 1). ICI 182780 alone did not influence bradykinin-induced relaxation (Figure 4d).

Effect of raloxifene on endothelium-independent relaxation

Raloxifene (3 nM) did not modulate endothelium-independent relaxation to sodium nitroprusside in rings without endothelium (pEC_{50}: 6.50$\pm$0.17 in control and 6.65$\pm$0.20 in raloxifene, $n=6$, $P>0.05$, data not shown). ODQ at 3 μM abolished NO donor-mediated relaxation ($n=5$).

Effect of raloxifene on bradykinin-stimulated endothelial cell $[Ca^{2+}]_i$

An increase in endothelial cell $[Ca^{2+}]_i$ is believed to serve an early trigger in agonist-induced endothelium-dependent dilatation. Treatment with 3 nM raloxifene did not affect bradykinin (100 nM)-stimulated rise in endothelial cell $[Ca^{2+}]_i$ ($P>0.05$, Figure 5).

Effect of raloxifene on eNOS protein expression

eNOS was post-translationally activated by 50 nM bradykinin as reflected by significantly increased phosphorylation of eNOS at Ser-1177 in arteries with endothelium, as compared with unstimulated arteries. Raloxifene (3 nM) also increased phosphorylation of eNOS at Ser-1177 with and without bradykinin stimulation. This additional eNOS phosphorylation induced by raloxifene was normalized by 1 μM ICI 182780 to a level stimulated by bradykinin (Figure 6b). Raloxifene did not have either additive or antagonistic effect on 17β-estradiol-induced eNOS phosphorylation (Figure 6b). The increased eNOS phosphorylation induced by 17β-estradiol (3 nM) was also abolished by ICI 182780 (1 μM). In contrast, the total amount of eNOS protein was unaffected by raloxifene, 17β-estradiol and raloxifene plus 17β-estradiol or bradykinin (Figure 6a).

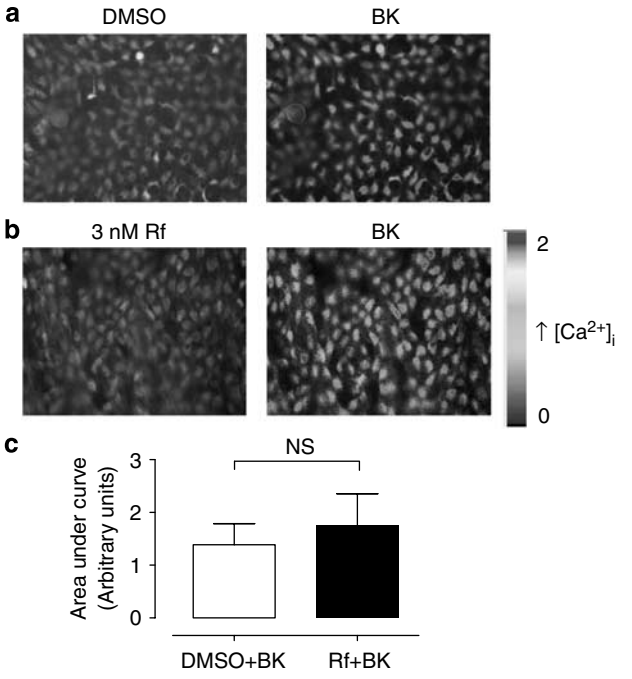

Figure 5 Calcium levels in *en face* preparations of endothelial cells of intact porcine coronary arteries measured by ratiometric fluorescence imaging. Images show increases in $[Ca^{2+}]_i$ *in vitro* induced by bradykinin (BK, 100 nM) in the absence (**a**) and presence (**b**) of 3 nM raloxifene (Rf). Increases in $[Ca^{2+}]_i$ are expressed as averaged area under curve in response to bradykinin with and without raloxifene treatment (**c**). Results are mean$\pm$s.e.mean of experiments from six different pigs.

Discussion

This study shows, for the first time, that raloxifene amplifies endothelium-dependent dilatation to bradykinin in isolated porcine coronary arteries in a non-genomic manner. This effect occurred with concentrations (1–3 nM) that approximate to plasma levels detected in patients taking a daily oral dosage of 60 mg raloxifene (Snyder *et al*., 2000; Morello *et al*., 2003). The main findings include: (i) raloxifene-augmented NO-mediated relaxation was antagonized by ICI 182780; (ii) raloxifene increased phosphorylation of eNOS at Ser-1177 and this effect was prevented by ICI 182780; (iii) raloxifene did not have either additive or antagonistic effect on 17β-estradiol-induced eNOS phosphorylation and (iv) low concentrations of 17β-estradiol also enhanced endothelial function and that effect was also sensitive to inhibition by ICI 182780.

Clinical studies reveal that raloxifene therapy improves endothelial function in healthy post-menopausal women (Saitta *et al*., 2001; Sarrel *et al*., 2003) and that intra-arterial infusion of the NOS inhibitor N^G-nitro-L-arginine inhibits the effect of raloxifene (Colacurci *et al*., 2003). However, this effect was not observed in patients with coronary artery disease (Griffiths *et al*., 2003), indicating that a healthy endothelium is required for vascular benefits of raloxifene treatment. Raloxifene activates eNOS in human endothelial cells in a non-genomic manner (Simoncini *et al*., 2002). Although unproven, this effect may partly account for

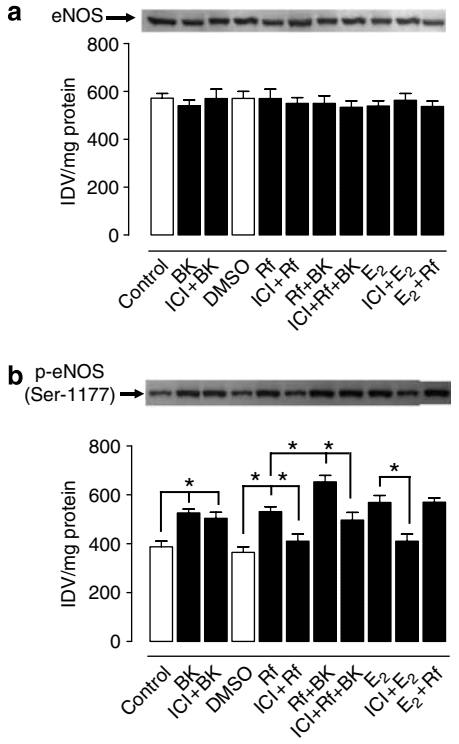

Figure 6 Effects of raloxifene (3 nM), 17β-estradiol (3 nM), raloxifene (3 nM) plus 17β-estradiol (3 nM), and bradykinin (50 nM) on total eNOS protein expression levels (**a**) and eNOS phosphorylation at Ser-1177 (**b**) with and without treatment with 1 μM ICI 182780. Results are mean$\pm$s.e.mean of five experiments on arteries from different pigs. Statistical difference is indicated by *($P<0.001$) between groups.

endothelial cell protection by chronically administered raloxifene.

Bradykinin-induced dilatation of porcine coronary arteries is mediated by NO and EDHF (Zhang *et al*., 2004), as this dilatation was prevented by a combined treatment with L-NAME (NOS inhibitor) and CTX plus apamin (inhibitors of EDHF) as shown in the present study. The potentiating effect was absent after exposure of rings to L-NAME plus ODQ. In contrast, the effect of raloxifene was unchanged after treatment with CTX plus apamin, indicating that effect of raloxifene is likely to be associated with enhanced eNOS activity. Indeed, both raloxifene (3 nM) and bradykinin (50 nM) increased the phosphorylation of eNOS. However, bradykinin (50 nM) but not raloxifene (3 nM) caused relaxations of coronary arteries, implicating that raloxifene may increase the bioavailability of NO in response to bradykinin via enhancing the eNOS activity and subsequent NO synthesis and release. Raloxifene did not have either additive or antagonistic effects on eNOS phosphorylation in the presence of 17β-estradiol, suggesting that raloxifene and 17β-estradiol may act on the same target.

The potentiating effect of raloxifene was not confined to bradykinin-induced relaxation. Indeed, raloxifene (3 nM) also augmented relaxations to substance *P*, another receptor-mediated endothelium-dependent dilator. It is unlikely that raloxifene modulated NO-mediated cyclic GMP-dependent effects on vascular smooth muscle since dilatation to sodium nitroprusside was unaffected in rings without endothelium. These results are consistent with modulatory effects of raloxifene on endothelial NO production, as shown in human studies (Colacurci *et al*., 2003; Sbarouni *et al*., 2003).

Various endothelium-dependent dilators stimulate NO release by increasing endothelial cell $[Ca^{2+}]_i$. Raloxifene at 3 nM, a concentration that enhanced relaxations to bradykinin, did not significantly affect bradykinin-stimulated rise in $[Ca^{2+}]_i$ in native endothelial cells. Thus, the increased Ca^{2+} influx is unlikely to contribute to the augmented NO function. Likewise, raloxifene did not modulate endothelium-dependent relaxations to A23187 (a calcium ionophore used to cause receptor-independent release of NO), suggesting that raloxifene may interact with receptor-mediated intracellular signaling pathways leading to the activation of eNOS.

The present study demonstrated that raloxifene augmented endothelial NO-mediated coronary artery dilatation via ICI 182780-sensitive mechanism(s) based on the following two observations: (i) ICI 182780 normalized the enhanced dilatation by raloxifene and (ii) ICI 182780 inhibited the upregulation of eNOS phosphorylation in response to raloxifene but not to bradykinin. ICI 182780 inhibits primarily the endothelium-dependent relaxation to raloxifene in rabbit and canine coronary arteries (Figtree *et al*., 1999; Ogita *et al*., 2004), while this anti-estrogenic agent does not affect endothelium-independent relaxation to raloxifene in several vascular beds (Tsang *et al*., 2004; Chan *et al*., 2005; Leung *et al*., 2005).

Estrogen therapy increased endothelium-mediated dilatation in coronary arteries (Gilligan *et al*., 1994) through enhanced production and release of NO (Guetta *et al*., 1997). Others have reported that supramaximal concentrations of 17β-estradiol (3 μM) increased bradykinin-induced dilatation of human coronary arteries (Barton *et al*., 1998). However, it is unclear whether bradykinin-mediated relaxation could be augmented by 17β-estradiol at near physiological concentrations. Indeed, 17β-estradiol (3–10 nM) also potentiated dilatations to bradykinin, which is related to the increased eNOS phosphorylation because both effects were abolished by ICI 182780.

In summary, the present study provides evidence for significant synergistic interaction between raloxifene and other endothelial NO-dependent dilators in isolated porcine coronary arteries. Acute exposure to therapeutically relevant concentrations of raloxifene augmented bradykinin-induced relaxation; this effect was mediated through ICI 182780-sensitive estrogen receptor and occurred via the upregulation of eNOS activity. Enhanced endothelial NO function can contribute to the reported cardioprotective effect of raloxifene even though we cannot attribute this observation with certainty to an *in vivo* condition in which raloxifene therapy improves endothelial function in postmenopausal women (Colacurci *et al*., 2003; Sbarouni *et al*., 2003). Thus crosstalk between raloxifene and other dilator agonists in the endothelium is likely to represent an important target site for the vascular benefits of raloxifene (and perhaps other SERMS) in the coronary circulation.

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Conflict of interest

The authors state no conflict of interest.

References

Barrett-Connor E, Grady D, Sasheyi A, Anderson PW, Cox DA, Hoszowski K, *et al.*, Harper KD MORE Investigators (Multiple Outcomes of Raloxifene Evaluation) randomized trial (2002). Raloxifene and cardiovascular events in osteoporotic postmenopausal women: four-year results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized trial. *JAMA* **287**: 847–857.

Barrett-Connor E, Mosal L, Collins P, Geiger MJ, Grady D, Kornitzer M, *et al.*, Raloxifene Use for the Heart (RUTH) Trial Investigator (2006). Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *New Engl J Med* **355**: 125–137.

Barton M, Cremer J, Mügge A (1998). 17β-Estradiol acutely improves endothelium-dependent relaxation to bradykinin in isolated human coronary arteries. *Eur J Pharmacol* **362**: 73–76.

Bell DR, Rensberger HJ, Koritnik DR, Koshy A (1995). Estrogen pretreatment directly potentiates endothelium-dependent vasorelaxation of porcine coronary arteries. *Am J Physiol* **268**: H377–H383.

Chan HY, Yao X, Tsang SY, Bourreau JP, Chan FL, Huang Y (2002). Isoproterenol amplifies 17β-estradiol-mediated vasorelaxation: role of endothelium/nitric oxide and cyclic AMP. *Cardiovasc Res* **53**: 627–633.

Chan YC, Leung FP, Yao X, Lau CW, Vanhoutte PM, Huang Y (2005). Raloxifene relaxes rat pulmonary arteries and veins: roles of gender, endothelium and antagonism of Ca^{2+} influx. *J Pharmacol Exp Ther* **312**: 1266–1271.

Colacurci N, Manzella D, Fornaro F, Carbonella M, Paolisso G (2003). Endothelial function and menopause: effects of raloxifene administration. *J Clin Endocrinol Metab* **88**: 2135–2140.

Figtree GA, Lu Y, Webb CM, Collins P (1999). Raloxifene acutely relaxes rabbit coronary arteries in vitro by an estrogen receptor-dependent and nitric oxide-dependent mechanism. *Circulation* **100**: 1095–1101.

Gaynor JS, Monnet E, Selzman C, Parker D, Kaufman L, Bryant HU *et al.* (2000). The effect of raloxifene on coronary arteries in aged ovariectomized ewes. *J Vet Pharmacol Ther* **23**: 175–179.

Gilligan DM, Quyyumi AA, Cannon RO (1994). Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. *Circulation* **89**: 2545–2551.

Griffiths KA, Sader MA, Skilton MR, Harmer JA, Celermajer DS (2003). Effects of raloxifene on endothelium-dependent dilation, lipoproteins, and markers of vascular function in postmenopausal women with coronary artery disease. *J Am Coll Cardiol* **42**: 698–704.

Guetta V, Quyyumi AA, Prasad A, Panza JA, Waclawiw M, Cannon RO (1997). The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* **96**: 2795–2801.

Leung FP, Yao X, Lau CW, Ko WH, Lu L, Huang Y (2005). Raloxifene relaxes rat intrarenal arteries by inhibiting Ca^{2+} influx. *Am J Physiol Renal Physiol* **289**: F137–F144.

Leung HS, Seto SW, Kwan YW, Leung FP, Au ALS, Yung LM *et al.* (2007). Endothelium-independent relaxation to raloxifene in porcine coronary artery. *Eur J Pharmacol* **555**: 178–184.

Leung HS, Yao X, Leung FP, Ko WH, Chen ZY, Gollasch M *et al.* (2006). Cilnidipine, a slow-acting Ca^{2+} channel blocker, induces relaxation in porcine coronary artery: role of endothelial nitric oxide and $[Ca^{2+}]_i$. *Br J Pharmacol* **147**: 55–63.

Morello KC, Wurz GT, DeGregorio MW (2003). Pharmacokinetics of selective estrogen receptor modulators. *Clin Pharmacokinet* **42**: 361–372.

Ogita H, Node K, Asanuma H, Sanada S, Liao Y, Takashima S *et al.* (2002). Amelioration of ischemia- and reperfusion-induced myocardial injury by the selective estrogen receptor modulator, raloxifene, in the canine heart. *J Am Coll Cardiol* **40**: 998–1005.

Ogita H, Node K, Liao Y, Ishikura F, Beppu S, Asanuma H *et al.* (2004). Raloxifene prevents cardiac hypertrophy and dysfunction in pressure-overloaded mice. *Hypertension* **43**: 237–242.

Saitta A, Altavilla D, Cucinotta D, Morabito N, Frisina N, Corrado F *et al.* (2001). Randomized, double-blind, placebo-controlled study on effects of raloxifene and hormone replacement therapy on plasma no concentrations, endothelin-1 levels, and endothelium-dependent vasodilation in postmenopausal women. *Arterioscler Thromb Vasc Biol* **21**: 1512–1519.

Sarrel PM, Nawaz H, Chan W, Fuchs M, Katz DL (2003). Raloxifene and endothelial function in healthy postmenopausal women. *Am J Obstet Gynecol* **188**: 304–309.

Sbarouni E, Flevari P, Kroupis C, Kyriakides ZS, Koniavitou K, Kremastinos DT (2003). The effects of raloxifene and simvastatin on plasma lipids and endothelium. *Cardiovasc Drugs Ther* **17**: 319–323.

Simoncini T, Genazzani AR, Liao JK (2002). Nongenomic mechanisms of endothelial nitric oxide synthase activation by the selective estrogen receptor modulator raloxifene. *Circulation* **105**: 1368–1373.

Snyder KR, Sparano N, Malinowski JM (2000). Raloxifene hydrochloride. *Am J Health Syst Pharm* **57**: 1669–1675.

Tsang SY, Yao X, Essin K, Wong CM, Chan FL, Gollasch M *et al.* (2004). Raloxifene relaxes rat cerebral arteries in vitro and inhibits L-type voltage-sensitive Ca^{2+} channels. *Stroke* **35**: 1709–1714.

Wang X, Chu W, Lau F, van Breemen C (1995). Bradykinin potentiates acetylcholine induced responses in native endothelial cells from rabbit aorta. *Biochem Biophys Res Commun* **213**: 1061–1067.

Wang X, Chu W, van Breemen C (1996). Potentiation of acetylcholine-induced responses in freshly isolated rabbit aortic endothelial cells. *J Vasc Res* **33**: 414–424.

Yao X, Huang Y (2003). From nitric oxide to endothelial cytosolic Ca^{2+}: a negative feedback control. *Trends Pharmacol Sci* **24**: 263–266.

Zhang RZ, Yang Q, Yim AP, Huang Y, He GW (2004). Different role of nitric oxide and endothelium-derived hyperpolarizing factor in endothelium-dependent hyperpolarization and relaxation in porcine coronary arterial and venous system. *J Cardiovasc Pharmacol* **43**: 839–850.

Zoma WD, Baker RS, Clark KE (2000). Coronary and uterine vascular responses to raloxifene in the sheep. *Am J Obstet Gynecol* **182**: 521–528.