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RESEARCH PAPER

Raloxifene protects endothelial cell function against oxidative stress

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Background and purpose: Maintaining a delicate balance between the generation of nitric oxide (NO) and removal of reactive oxygen species (ROS) within the vascular wall is crucial to the physiological regulation of vascular tone. Increased production of ROS reduces the effect and/or bioavailability of NO, leading to an impaired endothelial function. This study tested the hypothesis that raloxifene, a selective oestrogen receptor modulator, can prevent endothelial dysfunction under oxidative stress.
Experimental approach: Changes in isometric tension were measured in rat aortic rings. The content of cyclic GMP in aortic tissue was determined by radioimmunoassay. Phosphorylation of endothelial NOS (eNOS) and Akt was assayed by Western blot analysis.
Key results: In rings with endothelium, ACh-induced relaxations were attenuated by a ROS-generating reaction (hypoxanthine plus xanthine oxidase, HXXO). The impaired relaxations were ameliorated by acute treatment with raloxifene. HXXO suppressed the ACh-stimulated increase in cyclic GMP levels; this effect was antagonized by raloxifene. The improved endothelial function by raloxifene was abolished by ICI 182,780, and by wortmannin or LY294002. Raloxifene also protected endothelial cell function against H_2O_2. Raloxifene increased the phosphorylation of eNOS at Ser-1177 and Akt at Ser-473; this effect was blocked by ICI 182,780. Finally, raloxifene was not directly involved in scavenging ROS, and neither inhibited the activity of xanthine oxidase nor stimulated that of superoxide dismutase.
Conclusion and implications: Raloxifene is effective against oxidative stress-induced endothelial dysfunction *in vitro* through an ICI 182,780-sensitive mechanism that involves the increased phosphorylation and activity of Akt and eNOS in rat aortae.
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Keywords: raloxifene; nitric oxide; reactive oxygen species; endothelial dysfunction

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; eNOS, endothelial NOS; HXXO, hypoxanthine plus xanthine oxidase; NO, nitric oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; XO, xanthine oxidase

Introduction

Endothelial dysfunction is caused by a disturbance in the balance between beneficial nitric oxide (NO) and harmful reactive oxygen species (ROS) in the vascular wall (Félétou and Vanhoutte, 2006; Yung *et al*., 2006). It signals a start of the atherosclerotic process (Davignon and Ganz, 2004) and is closely associated with the pathological development or events in hypertension, hypercholesterolaemia, diabetes mellitus and heart failure.

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Oestrogen deficiency-associated endothelial dysfunction accounts for the increased cardiovascular incidence in postmenopausal women (Bolad and Delafontaine, 2005). Oestrogen therapy benefits endothelial cell function by enhancing the production and release of NO and reducing oxidative stress (Mendelsohn and Karas, 1999). However, unfavourable side effects have limited the use of natural or synthetic oestrogens in hormone replacement therapy, and this has led to the development of selective oestrogen receptor modulators (SERMs) (Kauffman *et al*., 1997). The results of several recent studies support the potential cardiovascular benefits of raloxifene use (Barrett-Connor *et al*., 2002; Leung *et al*., 2007a). Raloxifene shares a lipid-lowering activity of oestrogen (Goldfrank *et al*., 1999), inhibits cholesterol accumulation in ovariectomized

cholesterol-fed rabbits (Bjarnason *et al*., 1997), improves endothelial function in ovariectomized aging or hypertensive rats (Wong *et al*., 2005; Chan *et al*., 2007), ameliorates hypertension-induced endothelial dysfunction by reducing ROS production (Wassmann *et al*., 2002) and enhances endothelial NO-dependent vasodilatation *in vitro* (Leung *et al*., 2007b). In addition, raloxifene causes direct vasodilatation (Tsang *et al*., 2004; Chan *et al*., 2005; Leung *et al*., 2005, 2007c). Raloxifene benefits women with increased cardiovascular risk (Barrett-Connor *et al*., 2002), although the outcomes of the RUTH trial showed that raloxifene did not affect the overall risk of coronary heart disease in elderly women (Barrett-Connor *et al*., 2006). Nevertheless, the role of SERMs in the prevention and treatment of menopausal syndrome remains to be fully understood. As with oestrogen, raloxifene's effects occur when the drug binds to oestrogen receptors, which alters gene transcription in susceptible cells. In addition, SERMs also induce acute non-genomic beneficial effects, which are less clear. These effects may represent novel targets for the development of a new generation of SERMs.

In view of the reported benefits of raloxifene therapy in many clinical and animal studies as well as the pathological role of oxidative stress in endothelial dysfunction, which can predict the development of hypertension during oestrogen deficiency, we hypothesized that acute treatment with raloxifene could ameliorate or even prevent endothelial dysfunction caused by ROS by preserving the Akt-dependent endothelial NOS (eNOS) activity and thus maintaining the bioavailability of NO.

Methods

Blood vessel 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). Male Sprague-Dawley rats (250–300 g) were killed by cervical dislocation. The thoracic segment of the aorta was dissected out and the surrounding connective tissue was cleaned off. Each aorta was cut into ring segments, ~3 mm in length. Rings were then transferred into 10 mL organ baths containing Krebs solution bubbled with 95% O_2 plus 5% CO_2 at 37 °C. Krebs solution contained (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. Each ring was suspended between two L-shaped stainless steel hooks. One hook was connected to the bottom of the bath while the other connected to FT03 force-displacement transducer. A basal tension of 20 mN was applied to all rings. Twenty minutes after being placed in organ baths, the rings were first contracted with 0.3 μM phenylephrine to test their contractility and then relaxed by 1 μM ACh to assess the integrity of the endothelial layer. Thereafter, they were rinsed in pre-warmed Krebs solution and finally allowed to equilibrate for 60 min. Baseline tension was readjusted if necessary and maintained. In rings with endothelium, ACh at 1 μM normally produced over 85% relaxation and rings were discarded if the relaxation was less than this value. In some rings, the endothelial layer was removed mechanically by gently rubbing the luminal surface with a stainless steel wire. Removal of the endothelium was confirmed by lack of relaxation to 1 μM ACh.

Measurement of isometric force

After the functional presence of endothelium had been confirmed in each ring, a plateau contraction was induced by 1 μM phenylephrine. After the first concentration-dependent relaxations to ACh had been obtained, rings were rinsed several times in pre-warmed Krebs solution until baseline tension returned and the second concentration-response curve to ACh was obtained. To impair endothelium (NO)-dependent relaxation, the rings were exposed for 30 min to free radicals generated by a chemical reaction between hypoxanthine (100 μM) and xanthine oxidase (0.01 μ mL^{-1}) (HXXO) before repeating the second responses to ACh.

To investigate whether raloxifene treatment could improve endothelial function in rings challenged by HXXO, after the first concentration-response curve to ACh, rings were washed and subsequently exposed for 30 min to 1 μM raloxifene, followed by 30-min incubation with HXXO before the second ACh concentration-response curve was obtained. Individual pharmacological inhibitors were applied to the bathing solution 30 min before the addition of raloxifene. These inhibitors included 1 μM ICI 182,780 (a classical oestrogen receptor antagonist), 1 μM LY294002 and 100 nM wortmannin (putative inhibitors of PI3 kinase), 10 μM actinomycin D (RNA synthesis inhibitor) and 10 μM cycloheximide (protein synthesis inhibitor). The effect of raloxifene on ACh-induced relaxations was also tested on H_2O_2-treated aortae. Some inhibitors (3 μM indomethacin, 1 μM ICI 182,780, 1 μM LY294002 and 100 nM wortmannin) were tested on ACh-induced relaxation in control aortae. For comparison, the effect of 17β-oestradiol was also tested on the impaired relaxations to ACh in HXXO-treated aortae. Finally, the effect of HXXO on A23187 (Ca^{2+} ionophore)-induced relaxation was examined in the absence and presence of raloxifene.

To establish the contribution of the endothelium in mediating the raloxifene effect, endothelium-denuded aortic rings were tested. After the functional removal of endothelium had been confirmed, two consecutive cumulative concentration-response curves to phenylephrine were studied in control and in the presence of HXXO (30 min incubation time). In the last set of experiments using aortae without endothelium, a single cumulative concentration-response curve was obtained for sodium nitroprusside (SNP) (NO donor), forskolin (cyclic AMP-elevator), pinacidil (K^+ channel activator) and nifedipine (L-type Ca^{2+} channel blocker) in the absence and presence of HXXO (30 min incubation time).

Measurement of cyclic GMP

After a 60 min equilibration under an initial passive tension of 20 mN at 37 °C, the rings were pre-incubated with vehicle (dimethyl sulphoxide) or 1 μM raloxifene for 30 min before 30 min exposure to HXXO. Thereafter, rings were exposed to 1 μM phenylephrine for 10 min and then to 1 μM ACh (with endothelium) or 100 nM SNP (without endothelium) for

5 min. This procedure matched with that used for isometric force measurement. At the end of the reaction, rings were rapidly frozen in liquid nitrogen and stored at −70 °C until homogenization in 0.5 mL of ice-cold 0.1 M HCl, using a glass homogenizer. The homogenate was centrifuged at 20 000 *g* for 10 min at 4 °C. The protein content of the supernatant was determined using a protein assay kit with BSA as the standard according to the manufacturer's instructions. The remaining supernatant was lyophilized at 4 °C. The tissue content of cyclic GMP was assayed by radioimmunoassay with a [^{125}I]-cyclic GMP RIA kit. The concentration of cyclic GMP is presented as pmol mg^{-1} protein.

Western blot analysis

At the end of each experiment, as described previously for the measurement of cyclic GMP, aortic rings were snap frozen in liquid nitrogen and subsequently homogenized in ice-cold RIPA lysis buffer. The procedures used to measure the phosphorylation of eNOS and Akt were the same as those described in detail in our recent publication (Leung *et al*., 2007b). Primary antibodies were raised in rabbit against eNOS (1:500), eNOS phospho-eNOS (Ser-1177) (1:1000), Akt (1:1000) and phospho-Akt (Ser-473) (1:1000), whereas the secondary anti-rabbit antibody conjugated to horseradish peroxidase (DakoCytomation, Glostrup, Denmark) was used at a dilution of 1:3000. eNOS and Akt corresponding to 140- and 60-kDa bands, respectively, were visualized with reference to molecular weight markers.

The effect of raloxifene on DPPH-scavenging activity

Vitamin E was used as a reference antioxidant. In brief, 0.5 mL of methanol containing three concentrations (0.1–3 μM) of raloxifene were mixed in a test cuvette with 2.5 mL of methanol containing 75 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a stable free radical and has a deep purple colour in ethalonlic solution and a typical absorbance at 517 nm, but becomes pale yellow when trapped by an antioxidant. The reaction mixture was kept in the dark at room temperature for 90 min and the absorbance at 517 nm was then measured. The free radical-scavenging activity was approximated using the following equation:

$$\text{DPPH- scavenging activity}\,(\%) = [A_a - (A_b - A_c)]/A_a \times 100$$

where A_a is the absorbance of the incubation DPPH solution without drug, A_b is the absorbance of the incubation mixture containing DPPH and drug and A_c is the absorbance of the blank solution without DPPH.

In addition, the effects of raloxifene on activities of xanthine oxidase (XO) and superoxide dismutase, as well as its ability to scavenge superoxide anions and hydroxyl radicals, were investigated using the methods described elsewhere (McCord and Fridovich, 1969; Fried and Fried, 1974; Kong *et al*., 2000; Quick *et al*., 2000; Jansson *et al*., 2003).

Drugs and materials

Phenylephrine, ACh, hypoxanthine, XO, A23187, SNP, forskolin, pinacidil, nifedipine, DPPH, actinomycin D, cycloheximide, N^G-nitro-L-arginine methyl ester, catalase, ebselen, dimethylthiourea (DMTU), MnTBAP, and vitamin E (St Louis, MO, USA); ICI 182,780 ((7a,17b)-7-[9-[(4,4,5,5,5 pentafluoropentyl)sulphinyl]nonyl]estra-1,3,5(10)-triene-3, 17-diol; Tocris, Ellisville, Missouri, USA); raloxifene (Eli Lilly Company, Indianapolis, Indiana, USA); L-arginine (RBI, Natick, MA, USA); LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; Sigma-Aldrich, St Louis, MO, USA). Phenylephrine, ACh, L-arginine and SNP were prepared in distilled water, DPPH in methanol and the others in dimethyl sulphoxide. Dimethyl sulphoxide at 0.2% (v/v) did not affect phenylephrine-induced tone. The protein assay kit was from Sigma; [^{125}I]-cyclic GMP RIA kit (DuPont, Wilmington, DE, USA). The primary antibodies raised in rabbit against eNOS and eNOS phospho-eNOS (Ser-1177) and Akt and phospho-Akt (Ser-473) were obtained from Santa Cruz (Santa Cruz, CA, USA) and Upstate (Billerica, MA, USA), respectively.

Data analysis

All data are expressed as mean ± s.e.mean of *n* rings. Replicates of each experiment were performed on aortic rings prepared from different animals. The vasorelaxing effect was expressed as percentage reduction of evoked contraction. Nonlinear regression curve fitting was performed on individual cumulative concentration-response curves (GraphPad Prism software, Version 4.0) to estimate E_{max} as the maximal response and pD_2 values as negative logarithm of drug concentration that induced 50% of the maximal response (E_{max}). Statistical significance was determined by Student's two-tailed *t*-test or one-way ANOVA followed by Bonferroni post-tests when more than two treatments were compared. Concentration-response curves were analysed by two-way ANOVA followed by Bonferroni post-tests. A *P*-value of less than 0.05 was regarded as significant.

Results

Impairment of endothelium-dependent relaxation

In aortic rings with endothelium, ACh caused similar relaxations in the two consecutive concentration-response curves (pD_2 of 7.72 ± 0.11 and E_{max} of 92.1 ± 2.8% for the first curve and 7.67 ± 0.08 and E_{max} of 92.3 ± 2.6% for the second curve ($n = 5$, Table 1). ACh-induced relaxations were abolished by N^G-nitro-L-arginine methyl ester (100 μM) or by removal of the endothelium (data not shown) but not by 3 μM indomethacin (Table 1). HXXO impaired the relaxations to ACh (Figure 1a). ACh-induced relaxations were inhibited slightly by XO, whereas unaffected by hypoxanthine (Figure 1b, Table 1). Incubation with 1 mM L-arginine (NO precursor) protected endothelial function in HXXO-treated aortae (Table 1). The maximal relaxation to ACh was recovered after 1 h repetitive washout of HXXO although the relaxing sensitivity was still reduced (Table 1).

Hypoxanthine plus xanthine oxidase did not modify contractile responses to phenylephrine in rings without endothelium (pD_2: 6.99 ± 0.25 in control and 7.06 ± 0.08 in

Table 1 pD_2 and E_{max} values for ACh- and A23187-induced relaxations

Treatment	*pD_2*	*E_{max} (%)*	n
ACh relaxation			
Control	7.43 ± 0.05	97.3 ± 1.3	8
Rf	7.36 ± 0.07	102.8 ± 2.3	6
HXXO	6.25 ± 0.19#	48.4 ± 5.8#	13
HXXO washout	6.70 ± 0.08#	99.2 ± 2.9	6
XO	6.96 ± 0.09#	101.9 ± 3.9	6
HX	7.61 ± 0.14	95.6 ± 3.5	6
Rf + HXXO	6.52 ± 0.08§	83.9 ± 3.8§	10
L-arginine + HXXO	7.42 ± 0.21§	75.9 ± 7.4§	6
Rf + HXXO	6.78 ± 0.07	77.4 ± 1.1	4
+ ICI 182,780	6.57 ± 0.08	52.1 ± 1.9&	4
Rf + HXXO	6.45 ± 0.10	77.4 ± 5.5	10
+ Wortmannin	6.48 ± 0.24	48.2 ± 4.5&	7
+ LY294002	6.32 ± 0.13	53.3 ± 4.8&	6
Control (first curve)	7.72 ± 0.11	92.1 ± 2.8	5
Control (second curve)	7.67 ± 0.08	92.3 ± 2.6	5
Control	7.59 ± 0.07	89.5 ± 2.3	5
+ ICI 182,780	7.48 ± 0.09	92.7 ± 1.2	5
Control	7.82 ± 0.13	90.8 ± 2.7	5
+ Wortmannin	7.51 ± 0.10	89.1 ± 4.6	5
Control	7.83 ± 0.15	89.7 ± 2.7	5
+ LY294002	7.82 ± 0.22	94.6 ± 2.1	5
Control	7.60 ± 0.14	90.3 ± 2.5	5
+ Indomethacin	7.46 ± 0.20	93.5 ± 2.1	5
Rf + HXXO	6.52 ± 0.08	83.1 ± 3.9	12
+ Actinomycin D	6.37 ± 0.14	72.3 ± 7.3	4
+ Cycloheximide	6.78 ± 0.12	66.9 ± 6.5	4
ACh relaxation	7.55 ± 0.16	98.3 ± 1.6	5
HXXO	6.59 ± 0.10#	50.1 ± 4.3#	4
+ 17β-oestradiol	6.61 ± 0.18#	50.4 ± 7.4#	4
A23187 relaxation			
Control	7.85 ± 0.11	90.1 ± 6.1	7
HXXO	7.47 ± 0.11#	83.5 ± 4.5	10
Rf + HXXO	7.94 ± 0.12§	91.4 ± 3.3	5

Abbreviations: HXXO, hypoxanthine plus xanthine oxidase; Rf, raloxifene; XO, xanthine oxidase.
Data are mean ± s.e.mean of *n* separate experiments. Statistical difference ($P<0.05$) is indicated from the control (#), from HXXO (§) and from Rf-HXXO (&).

HXXO, $n=7$, $P>0.05$), suggesting that the HXXO-derived free radicals do not modify contractile properties of vascular smooth muscle. To exclude the nonspecific action of HXXO, its effect was tested on relaxations induced by endothelium-independent dilators. In rings without endothelium, HXXO did not affect relaxations to SNP (pD_2: 7.62 ± 0.04 in control and 7.41 ± 0.03 in HXXO, $P>0.05$), forskolin (pD_2: 7.50 ± 0.09 in control and 7.32 ± 0.08 in HXXO, $P>0.05$), pinacidil (pD_2: 6.09 ± 0.06 in control and 5.95 ± 0.06 in HXXO, $P>0.05$) or nifedipine (pD_2: 7.64 ± 0.10 in control and 7.66 ± 0.08 in HXXO, $P>0.05$) ($n=4$–5 in each case).

Effect of raloxifene on HXXO-induced endothelial dysfunction
The representative records in Figure 1a show that exposure to HXXO resulted in markedly reduced relaxations to ACh in rings with endothelium compared with untreated rings. The reduced relaxations were partially prevented by 30 min prior

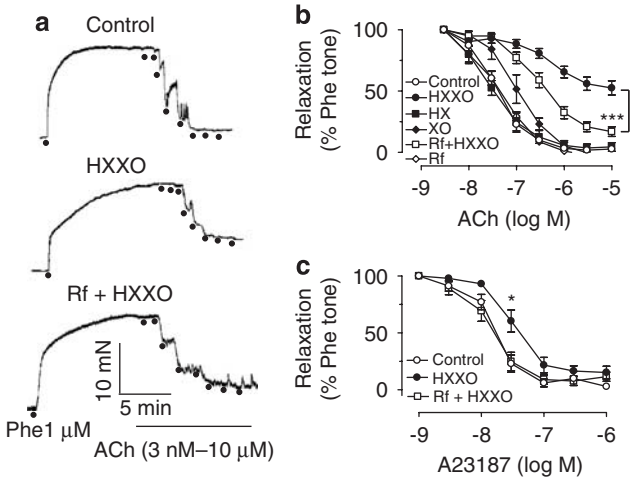

Figure 1 (**a**) Traces showing ACh-mediated relaxations in aortic rings with endothelium in control (upper) and in the presence of hypoxanthine plus xanthine oxidase (HXXO) without (middle) or with treatment (bottom) with 1 μM raloxifene (Rf). Scale bars apply to all records. (**b**) Concentration-response curves for ACh under different pharmacological treatments. (**c**) Effect of 1 μM raloxifene on A23187-induced relaxations in HXXO-treated aortae. Data are mean ± s.e.mean of 6–13 experiments. Significant difference between HXXO and raloxifene-treated groups is indicated by $*P<0.1$ and $***P<0.001$.

treatment with 1 μM raloxifene (Figures 1a and b). Prolonged exposure (120 min) to raloxifene did not give rise to further improved relaxation (data not shown). HXXO also attenuated A23187-induced relaxations and this inhibition was prevented by 1 μM raloxifene (Figure 1c, Table 1). By contrast, 17β-oestradiol (1 μM) did not affect the impaired relaxation by HXXO (Table 1).

Effects of PI3 kinase inhibitors
The protective effect of raloxifene was antagonized by 1 μM ICI 182,780 (Figure 2a, Table 1), and inhibited by 100 nM wortmannin and 1 μM LY294002, putative inhibitors of PI3 kinase (Figure 2b, Table 1). In control experiments, ICI 182,780, wortmannin and LY294002 did not modify ACh-induced relaxations (Table 1). By contrast, incubation (3 h) with 10 μM actinomycin D and 10 μM cycloheximide did not inhibit raloxifene's ability to attenuate the HXXO-impaired relaxations to ACh (Figure 2c, Table 1).

Effects of raloxifene on phosphorylation of eNOS and Akt
Densitometry analysis of the Western blots showed that HXXO did not affect the total level of eNOS protein (Figure 3b), but significantly reduced the phosphorylation level of eNOS at Ser-1177 in response to 1 μM ACh in rings with endothelium and such changes were largely normalized by treatment with raloxifene at 1 μM (Figure 3a). In the same preparations, HXXO treatment also reduced the phosphorylation level of Akt at Ser-473, and this was reversed by raloxifene (Figure 3c) without affecting the total Akt levels (Figure 3d). The protective effect of raloxifene on the phosphorylation of Akt and eNOS was antagonized by ICI

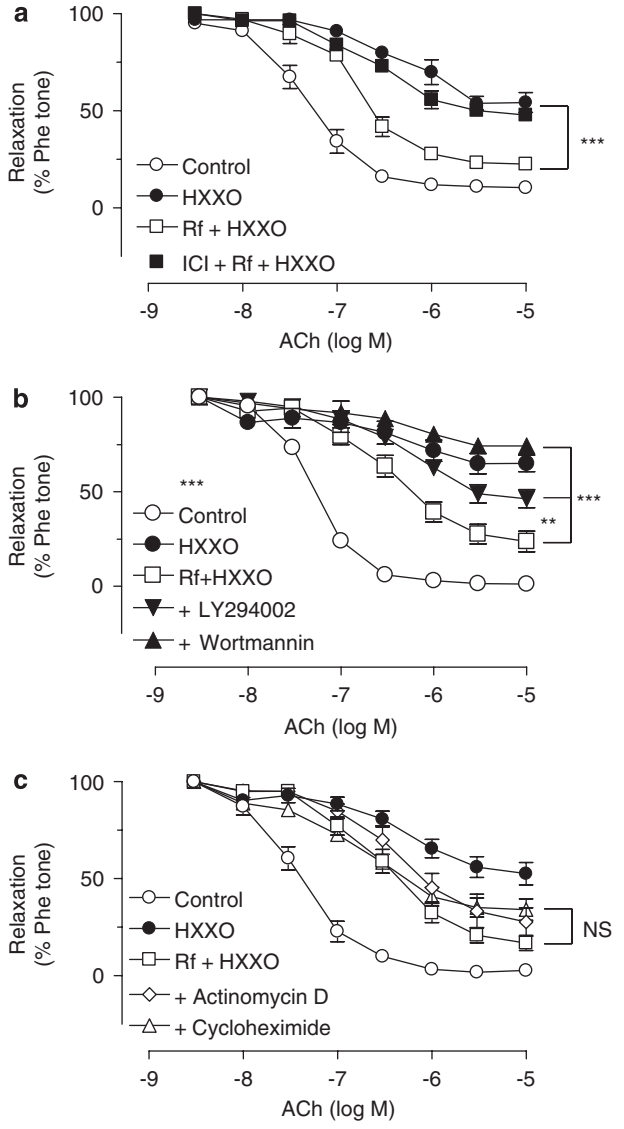

Figure 2 Effects of 1 μM ICI 182,780 (**a**), 100 nM wortmannin and 1 μM LY294002 (**b**), as well as cycloheximide and actinomycin D (each at 10 μM, (**c**)) on the protective effect of raloxifene (Rf) against hypoxanthine plus xanthine oxidase (HXXO)-induced impairment of relaxations. Data are mean ± s.e.mean of 4–10 experiments. Significant difference between raloxifene-treated group and group treated with inhibitor plus raloxifene is indicated by $^{**}P<0.01$ and $^{***}P<0.001$.

182,780 (Figures 3a and c). Raloxifene (1 μM) alone did not affect the protein levels for total eNOS or Akt and phospho-eNOS or phospho-Akt in phenylephrine-treated rings ($n=4$, data not shown). In rings without endothelium, eNOS protein level was very low (data not shown).

Effects on cyclic GMP content

ACh at 1 μM caused a significant rise of the total tissue content of cyclic GMP in rings with endothelium. This increase was markedly reduced by HXXO. Treatment with 1 μM raloxifene inhibited the effect of HXXO (Figure 4a). By contrast, the SNP (100 nM)-stimulated increase in the level of cyclic GMP in rings without endothelium was unaffected by HXXO (Figure 4b).

Effect of raloxifene in H_2O_2-treated aortas

Exposure (30 min) of aortae to 300 μM H_2O_2 impaired ACh-induced relaxations with over 50% of reduction in the maximal response, and treatment with 1 μM raloxifene inhibited the effect of H_2O_2 (Figure 5a, Table 2). H_2O_2-induced impairment in relaxations was abolished by calatase (1000 μ mL^{-1}) and DMTU (5 mM) (Figure 5b, Table 2). Similarly, both calatase and DMTU prevented the effects of HXXO on the ACh-induced relaxations (Figure 5c, Table 2). By contrast, ebselen (0.5 μM) and MnTBAP (10 μM) did not have protective effects (Figure 5d, Table 2).

Free radical-scavenging activity of raloxifene

The concentrations of raloxifene that protected endothelial cell function did not exert a free radical-scavenging effect (Figure 6). Under the same assay conditions, vitamin E produced concentration-dependent increases in the free radical-scavenging activity (Figure 6b). In comparison, raloxifene (1–50 μM) did not alter the activity of XO and superoxide dismutase, or scavenge superoxide anions and hydroxyl radicals (data not shown).

Discussion

Even though hormone replacement therapy in postmenopausal women results in an increased risk of coronary artery disease, both epidemiological in premenopausal women and experimental studies have suggested or clearly demonstrated a major atheroprotective action of oestradiol and SERMs. A better understanding of the deleterious and beneficial effects of oestrogens and SERMs is thus required. Oxidative stress is one of the key contributors to the increased cardiovascular risk, and ROS are known to harm the normal vascular function by limiting the beneficial effects of endothelium-derived NO (Yung *et al*., 2006). The enhanced production and release of ROS and/or the diminished bioavailability of NO within vascular wall leads to endothelial dysfunction that is widely believed to be the early key event in the pathogenesis of various vascular complications (Félétou and Vanhoutte, 2006). Endothelial dysfunction precedes the expression of atherosclerosis in postmenopausal women in an oestrogen-deficient state (Bolad and Delafontaine, 2005). The major findings of the present investigation *in vitro* include (i) raloxifene ameliorates ROS-induced endothelial dysfunction in the rat aortae by increasing the bioavailability of NO; (ii) the increased endothelial NO-dependent relaxations result from the increased phosphorylation and hence activity of Akt and eNOS; (iii) the beneficial effect of raloxifene appears to be non-genomic and is mediated by ICI 182,780-sensitive mechanisms and (iv) raloxifene does not scavenge ROS or affect the activity of the ROS-generating and antioxidant enzymes.

In this study, a ROS-generating reaction (HXXO) was used to release superoxide anions (Greenwald, 1985). ROS

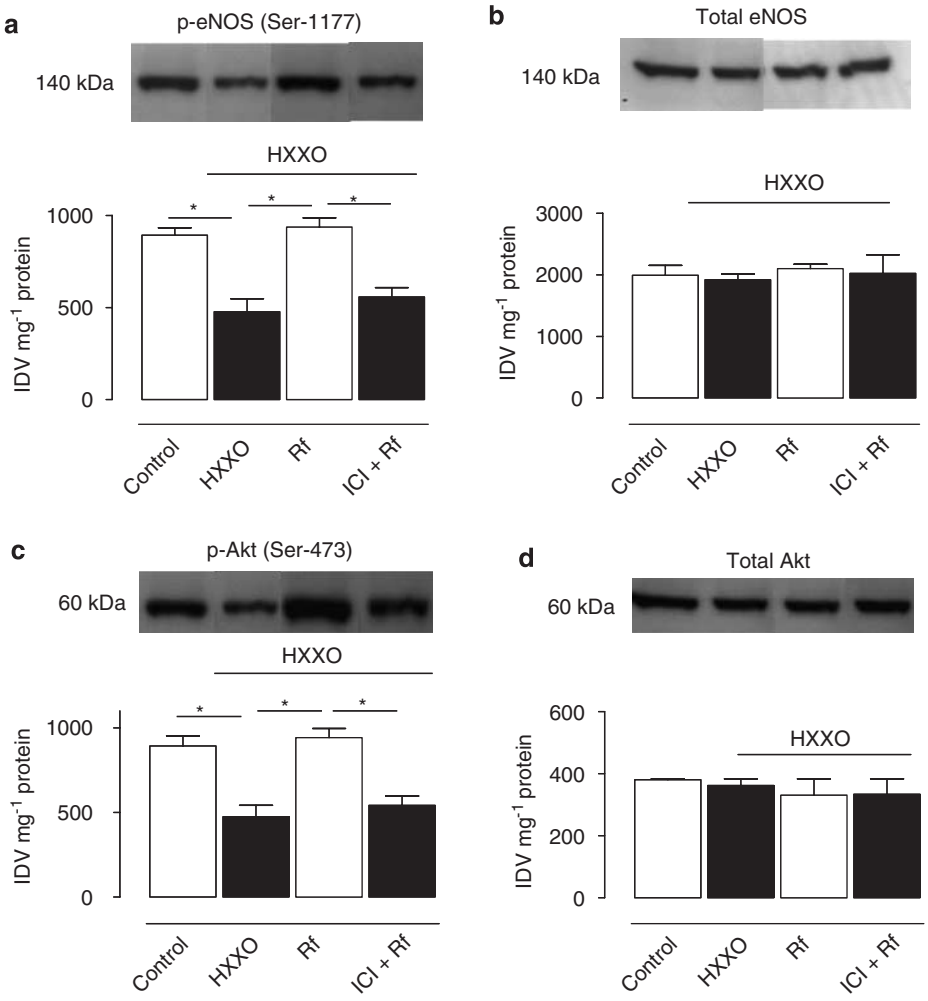

Figure 3 The effects of raloxifene (1 μM, Rf) on hypoxanthine plus xanthine oxidase (HXXO)-induced alterations in the level of phospho-endothelial NOS (eNOS) (Ser-1177) (**a**), the total eNOS protein levels (**b**), and the level of phospho-Akt (Ser-473) (**c**) with and without 1 μM ICI 182,780 (ICI) and the total Akt protein levels (**d**). Data are mean ± s.e.mean of five experiments on arteries from different rats. Statistical difference is indicated by $*P<0.05$ between treatment groups. IDV, integrated density value.

produced from a brief exposure to HXXO profoundly impaired NO-mediated relaxations to ACh (a receptor-mediated dilator) and to A23187 (a receptor-independent dilator), suggesting that free radicals are likely to impair vasorelaxation at a more distal step common to both the ACh- and A23187-induced pathways, and not through interference of ACh binding to its receptors. To establish the specificity of HXXO's action, endothelium-independent dilators were tested. HXXO did not affect relaxations induced by SNP (exogenous NO donor). Furthermore, HXXO did not modify the contractile or relaxating properties of vascular smooth muscle, as it did not affect contractions induced by phenylephrine or relaxations induce by pinacidil, nifedipine and forskolin. When L-arginine was added together with HXXO, endothelium-dependent relaxations were significantly improved. These results further indicate that a loss of production of NO accounts for HXXO-induced impairment of ACh-induced relaxations. Similarly, L-arginine effectively antagonized N^G-nitro-L-arginine methyl ester-induced inhibition of endothelium-dependent relaxations in the same preparations (data not shown). However, it remains to be determined whether ROS could reduce the availability of the eNOS substrate (L-arginine) and/or cofactor levels. Such effects could also contribute to the reduced NO bioavailability.

Clinical studies indicate that raloxifene improves endothelial function in healthy postmenopausal women (Saitta *et al*., 2001; Sarrel *et al*., 2003) and that intra-arterial infusion of an NOS inhibitor negates the effect of raloxifene (Colacurci *et al*., 2003). However, the latter was not observed in patients with coronary artery disease (Griffiths *et al*., 2003), indicating that a healthy endothelium is essential for beneficial vascular effects of raloxifene therapy. Raloxifene activates eNOS in human cultured endothelial cells in a non-genomic manner (Simoncini *et al*., 2002). Although unproven, this effect may partly account for endothelial cell protection by chronically administered raloxifene.

This study demonstrates that the HXXO-attenuated relaxations were ameliorated by raloxifene. This effect is associated with the increased activity of eNOS on tha basis of

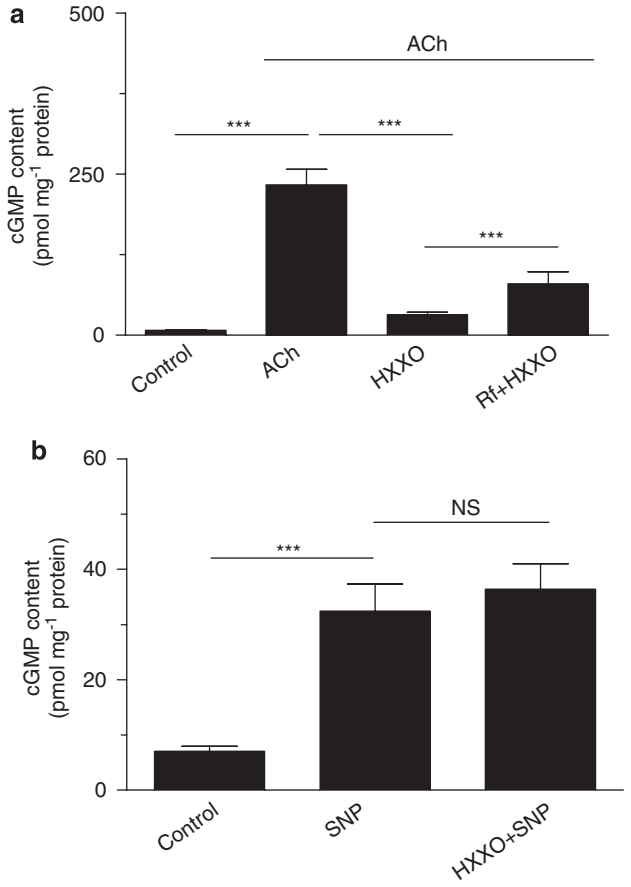

Figure 4 Effect of hypoxanthine plus xanthine oxidase (HXXO) on the tissue level of cyclic GMP in response to 1 μM ACh in rings with endothelium (**a**) to sodium nitroprusside (SNP) (100 nM) in rings without endothelium (**b**). Data are mean ± s.e.mean of five experiments. Significant difference from control group, from ACh group and between HXXO and raloxifene-treated group is indicated by ***$P<0.001$.

the following three observations. First, HXXO reduced the ACh-stimulated rise in the tissue cyclic GMP levels, whereas raloxifene reversed this effect. To determine whether HXXO also inhibits endothelial NO-mediated responses through inhibition of GC, the effects of HXXO on exogenous NO donor-mediated relaxation in rings without endothelium were investigated. Unlike its effect on ACh-mediated relaxations, HXXO did not inhibit relaxations to SNP (NO donor) or affect SNP-stimulated cellular production of cyclic GMP. These results clearly indicate that HXXO mainly targets the endothelial cells in intact aortae. Second, Western blot analysis showed that raloxifene prevents HXXO-induced decrease in eNOS phosphorylation without affecting the total amount of eNOS protein. Hisamoto *et al*. (2001) demonstrated that raloxifene-induced eNOS phosphorylation is mediated by an Akt-dependent cascade, which is an important regulator of cellular processes including cell survival (Hisamoto *et al*., 2001; Shiojima and Walsh, 2002). Indeed, in this study, exposure to HXXO reduced phosphorylation of Akt in rat aortae with endothelium. The decreased Akt phosphorylation was abolished by raloxifene. These effects were blocked by the oestrogen receptor antagonist, ICI 182,780. The role of PI3K-Akt in mediating the beneficial effect of raloxifene on endothelial function was further supported by the observation that PI3 kinase inhibitors, wortmannin and LY294002 negated the effect of raloxifene in arteries that had previously been exposed to ROS. Third, the beneficial effect of raloxifene was not confined to ACh-induced relaxations. Indeed, raloxifene also ameliorated the impaired relaxations to A23187 in the presence of HXXO. We have thus provided novel evidence suggesting that by acting on endothelium but not vascular smooth muscle cells, HXXO causes endothelial dysfunction by inhibition of NO production/release and raloxifene attenuates such an effect by an endothelium-dependent mechanism, that may involve maintaining the levels of phosphorylated eNOS and, hence, the eNOS activity used to generate NO that 'neutralizes' ROS.

Treatment with ICI 182,780 antagonized the effect of raloxifene. Similarly, this anti-oestrogen agent antagonized raloxifene-induced augmentation of endothelium-dependent dilatation of the porcine coronary artery (Leung *et al*., 2007b). This study, however, shows that raloxifene exerts mainly a rapid and non-genomic vascular effect, as neither actinomycin D (RNA synthesis inhibitor) nor cycloheximide (protein synthesis inhibitor) affected the effects of raloxifene on the relaxation responses. Our results are consistent with previous observations that the ICI 182,780-sensitive action of raloxifene does not involve new synthesis of RNA or protein (Simoncini *et al*., 2002). Therefore, it is likely that raloxifene interacts with an undefined putative membrane binding site(s) by a non-transcriptional signalling mechanism to generate its protective action. In actual fact, raloxifene can increase the eNOS activity acutely in cultured endothelial cells (Simoncini *et al*., 2002; Wassmann *et al*., 2002). Similarly, ICI 182,780 but not actinomycin D inhibits the acute phosphorylation of eNOS caused by LY117018 in human cultured endothelial cells (Hisamoto *et al*., 2001), indicating that such an acute regulation of eNOS does not involve transcriptional control by the classical intracellular oestrogen receptors. The ICI 182,780-sensitive effect of raloxifene appears to be unique as the same concentration of 17β-oestradiol did not inhibit the HXXO-induced impairment in ACh relaxation, suggesting some different cellular mechanisms of action for these two agents in intact arteries.

Raloxifene at concentrations that improve endothelial function did not exhibit an apparent free radical-scavenging activity, as revealed by DPPH-scavenging assay. In addition, raloxifene did not affect the activity of XO or superoxide dismutase and was unable to scavenge superoxide anions or hydroxyl radicals at concentrations that enhanced endothelium-dependent relaxations in ROS-treated aortae. Taken together, the present results indicate that the direct removal of ROS is not involved in the protective effects of raloxifene on endothelial cell function.

H_2O_2 appears to be one of the species involved in HXXO-impaired relaxations to ACh (Dowell *et al*., 1993). Indeed, the addition of H_2O_2 resulted in significantly reduced relaxations to ACh. This effect is comparable with that of HXXO. The effect of H_2O_2 was prevented by treatment with catalase (H_2O_2 scavenger) or DMTU (hydroxyl radical

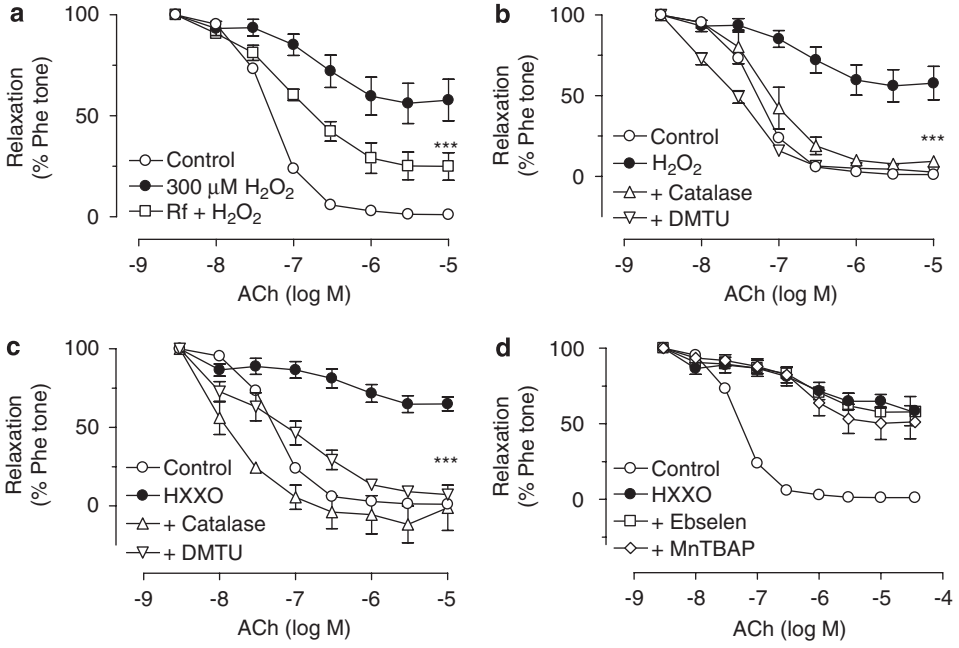

Figure 5 (**a**) Protective effect of raloxifene (1 μM) on H_2O_2-induced impairment in relaxations to ACh. Preventive effects of catalase and dimethylthiourea (DMTU) on the impaired relaxations to ACh caused by H_2O_2 (**b**) and by HXXO (**c**). (**d**) Lack of effects of ebselen and MnTBAP on HXXO-impaired relaxations. Data are mean ± s.e.mean of 3–10 experiments. Significant difference between HXXO and treatment groups is indicated by ***$P<0.001$.

Table 2 pD_2 and E_{max} values for ACh-induced relaxations in the presence of ROS inhibitors

ACh relaxation	*pD_2*	*E_{max} (%)*	n
Control	7.38 ± 0.03	98.2 ± 0.7	10
H_2O_2	6.74 ± 0.34	38.1 ± 10.4$^{\#}$	9
Rf + H_2O_2	7.07 ± 0.11	75.0 ± 6.4§	4
Catalase + H_2O_2	7.23 ± 0.11	90.6 ± 2.5§	3
DMTU + H_2O_2	7.74 ± 0.05	93.4 ± 0.4§	3
Control	7.38 ± 0.03	98.8 ± 0.7	10
HXXO	6.33 ± 0.28$^{\#}$	35.1 ± 4.4$^{\#}$	9
Catalase + HXXO	8.11 ± 0.20§	101.1 ± 14.4§	3
DMTU + HXXO	7.02 ± 0.12	92.8 ± 2.5§	7
Ebselen + HXXO	6.23 ± 0.10	42.3 ± 2.6	4
MnTBAP + HXXO	6.28 ± 0.25	49.2 ± 10.6	5

Abbreviations: DMTU, dimethylthiourea; HXXO, hypoxanthine plus xanthine oxidase; ROS, reactive oxygen species; Rf, raloxifene; XO, xanthine oxidase. Data are mean ± s.e.mean of *n* separate experiments. Statistical difference ($P<0.05$) is indicated from the control (#) and from HXXO or H_2O_2 (§).

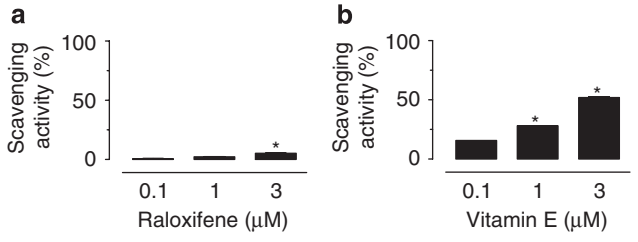

Figure 6 Lack of free radical-scavenging activity of raloxifene (**a**) and free radical-scavenging effect of vitamin E (**b**). Results are mean ± s.e.mean of six separate measurements. Significant difference in comparison with a concentration of 0.1 μM is indicated by *$P<0.05$.

scavenger). Similarly, catalase and DMTU also inhibited HXXO-induced endothelial dysfunction. By contrast, the impaired relaxations by HXXO were unaffected by ebselen (glutathione peroxidase mimetic) and MnTBAP (SOD mimetic), suggesting that H_2O_2 and its metabolite, hydroxyl radical, are most likely responsible for the impaired endothelial function caused by HXXO. The limitation of the use of HXXO should be noted; HXXO is known to yield urate, which is the scavenger of peroxynitrite (Houston *et al.*, 1998), another highly reactive ROS that damages endothelial function (Yung *et al.*, 2006). If this were the case, a part of HXXO's effect may have been masked by the removal of peroxynitrite due to XO-derived urate accumulation around the aortic tissue.

In conclusion, this study on intact rat aortae demonstrates a protective effect of raloxifene against oxidative stress-associated endothelial dysfunction, a key vascular condition that progresses towards the occurrence of hypertension and atherosclerosis in humans. The vascular benefit of raloxifene stems from a rapid and non-genomic ICI 182,780-sensitive that involves the maintenance of the levels of phosphorylated eNOS needed to generate NO. These findings can be added to the expanding list of beneficial cellular mechanisms of raloxifene and possibly other SERMs. As ROS is closely coupled to the initiation and development of cardiovascular disease, raloxifene as well as other new SERMs could be potentially useful in the prevention or even treatment of oxidative stress-related vascular dysfunction during oestrogen deficiency. Our findings might be helpful in the search for the 'ideal' SERM, which would have beneficial oestrogenic anti-atherosclerotic effects and an improved safety profile.

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Conflict of interest

The authors state no conflict of interest.

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