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Raloxifene lowers ischaemia susceptibility by increasing nitric oxide generation in the heart of ovariectomized rats in vivo

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Abstract

We studied the effects of a 2-week period of oral raloxifene therapy on the cardiac level of nitric oxide (NO) and on the susceptibility to angina in ovariectomized rats. Ovariectomy decreased the activity of Ca^{2+}-dependent nitric oxide synthase (NOS) in the left ventricle, an effect restored by raloxifene (0.2–5 mg kg^{-1} day^{-1}) or 17β-oestradiol (0.3 mg kg^{-1} day^{-1}). Ovariectomy led to a significant ST segment depression after the injection of (1) ornithine-vasopressin (0.5 IU kg^{-1}, i.v.) or (2) epinephrine (10 μg kg^{-1}, i.v.), followed 30 s later by phentolamine (15 mg kg^{-1}, i.v.); both effects were reversed by raloxifene or 17β-oestradiol treatment. Inhibition of nitric oxide synthase (with N^G-nitro-L-arginine methyl ester [L-NAME]; 5 mg kg^{-1}, s.c.) augmented the ST segment depression in the ovariectomized rat and abolished the anti-ischaemic effect of 17β-oestradiol or raloxifene. Thus, an oestrogen deficiency down-regulates the cardiac constitutive nitric oxide synthase, which increases the susceptibility of the heart to ishaemia because both actions can be blocked by exogenous administration of the natural oestrogen 17β-oestradiol or the selective oestrogen-receptor modulator (SERM) raloxifene. In the present in vivo system, raloxifene exerts oestrogen-agonist properties.

Keywords: Nitric oxide synthase regulation; Oestrogen; Selective oestrogen-receptor modulator; Raloxifene; Heart; Cardiac ischaemia

1. Introduction

Raloxifene, a selective oestrogen-receptor modulator (SERM), has oestrogen-agonistic properties towards the bone and the cardiovascular system, but it exerts oestrogen-receptor-antagonist actions in the breast and uterus (Riggs and Hartmann, 2003). In recent years, raloxifene has been approved for human use in the prevention of osteoporosis in postmenopausal women.

Endogenous oestrogen protects the circulation. In the reproductive years, men are more susceptible than women to hypertension and cardiovascular diseases (Barrett-Connor, 1997). However, when the ovulation cycle stops and the endogenous production of oestrogens falls (either naturally or following surgery), a dramatic increase occurs in the development of cardiovascular diseases in women. The frequency of cardiovascular events in postmenopausal women approaches the level for men of the same age (Clarkson et al., 1997; Barrett-Connor, 1997). Moreover, 24-h ambulatory blood pressure monitoring has recently demonstrated that the blood pressure of postmenopausal women is higher than that of men (Reckelhoff, 2001). Numerous epidemiological (e.g., the Framingham Study), observational and cohort studies (e.g., the Nurses Health Cohort Study) have been made on the association between hormone replacement therapy and cardiovascular disease, and conflicting results have been reported (Grodstein et al., 2000; for a review, see Humphrey et al., 2002). However, large secondary or primary prevention randomized clinical trials such as the Heart and Estrogen/Progestin Replacement Study or the Women's Health Initiative revealed no overall effect or a significant worsening action of hormone replacement ther-

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apy on the coronary events in postmenopausal women (Furberg et al., 2002; Manson et al., 2003). In contrast, raloxifene therapy significantly reduced the risk of cardiovascular events in women at increased cardiovascular risk (Barrett-Connor et al., 2002).

Clinical and preclinical studies have demonstrated that oestrogen and SERMs exert their favourable cardiovascular effects through a number of mechanisms. They improve the profile of such cardiovascular risk factors as serum lipids (low-density lipoprotein cholesterol and apolipoprotein A_1 and B) and homocysteine, and lower the accumulation of cholesterol and cholesterol degradation products in the vascular wall (for a review, see Barrett-Connor et al., 1999). Oestrogen and SERMs have beneficial actions on the vascular endothelial function (Wassmann et al., 2002), the vascular smooth muscle cell proliferation (Takahashi et al., 2003) and direct injury of the vascular wall (Kauffman et al., 2000). However, oestrogen and raloxifene increase the risk of venous thromboembolism to a similar extent. Although the absolute risk is small, this side effect might limit their usage in clinical practice (for a review, see Riggs and Hartmann, 2003).

Oestrogen increases nitric oxide (NO) generation in the vascular tissue (Chambliss and Shaul, 2002), NO having an important physiological role in the maintenance of the vascular integrity (Moncada and Higgs, 1995). Less information has accumulated as concerns the actions of SERMs on NO regulation and its relation to the in vivo cardiovascular pathology. In the present study, therefore, we investigated the effects of ovariectomy and raloxifene substitution on the cardiac NO synthase activity in conjunction with the susceptibility of the rat heart to ischaemia in vivo. Finally, we compared the action of the SERM raloxifene with that of the natural oestrogen 17β-oestradiol.

2. Materials and methods

2.1. Study groups

10- to 12-week-old female Wistar rats were used. Each group consisted of at least six animals. Ovariectomy and sham operation were performed under ether anaesthesia. The animals were then allowed to recover during 1 month. In separate groups of ovariectomized animals, oestrogen (17β-oestradiol, 0.3 mg kg^{-1} day^{-1}, orally, once daily) or raloxifene (0.2–5 mg kg^{-1} day^{-1}, orally, once daily) replacement therapy was introduced for a 2-week period. The doses of oestrogen and raloxifene were selected on the basis of the findings of previous studies (Morschl et al., 2000; Pávó et al., 2000). Neither 17β-oestradiol nor raloxifene was administered on the day of any experimentation. All manipulations were performed in accordance with the standards of the European Community guidelines on the care and use of laboratory animals and had been approved by the institutional ethics committee.

2.2. Ca^{2+}-dependent nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was determined via the conversion of L-[^{14}C]arginine monohydrochloride to L-[^{14}C]citrulline, on the basis of a method described previously (Salter et al., 1991) with minor modifications aimed mostly at the determination of the activity of Ca^{2+}-dependent constitutive NOS (cNOS) (Weiner et al., 1994; Morschl et al., 2000). The animals were sacrificed by decapitation, and immediately after autopsy, fresh tissue was prepared from the left ventricle of the heart for NOS measurements. Cardiac tissue samples were homogenized (15 s) in buffer (250 mg ml^{-1}, 4 °C, 10 mM HEPES, 32 mM sucrose, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 2 μg ml^{-1} aprotonin, pH 7.4), followed by centrifugation for 20 min at 10,000 × *g* at 4 °C. Samples were mixed with Dowex resin (AG 50W-8; 200–400, 8% cross-linked, Na^+ form), followed by centrifugation for 10 min at 10,000 × *g* at 4 °C. A sample supernatant (40 μl) was incubated for 10 min at 37 °C in a reaction buffer comprising (final concentrations) of 50 mM KH_2PO_4, 10 μg/ml calmodulin, 2.5 mM $CaCI_2$, 50 mM valine, 1 mM DTT, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 μM FAD, 3 μM flavin mononucleotide (FMN), 3 μM tetrahydrobiopterin and 0.17 μM [^{14}C]L-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex/water. After the addition of 0.85 ml distilled water and the suspension had been allowed to settle for 30 min, the supernatant was removed for scintillation counting. Protein content was determined by spectrophotometric assay (Bio-Rad Protein Assay), and the NOS activity was expressed as pmol min^{-1} mg^{-1} protein.

The total NOS activity was defined as the extent of citrulline formation abolished by incubation in vitro with N^G – nitro-L-arginine (L-NNA, 1 mM). The basal L-NNA-sensitive activity that was abolished by EGTA was taken as the Ca^{2+}-dependent cNOS activity.

2.3. Measurement of cardiac ischaemia

The animals were anaesthetized with urethane (1.25 g kg^{-1}, i.p.). The core body temperature was maintained at 37 °C with a homeothermic control unit (Harvard Instruments, UK). Cannulae were inserted into the tail vein, trachea and right carotid artery for the administration of drugs, spontaneous respiration and blood pressure measurement, respectively. After a stabilization period of 15 min, the mean arterial blood pressure and the standard lead II surface electrocardiogram (ECG) were recorded simultaneously by means of the HAEMOSYS computerized complex haemodynamic analysis system (Experimetria, UK, London). As a measure of cardiac ischaemia, the changes in the ST segment were used. The value of the ST segment was defined as the mean voltage 13 ms after the S wave, according to the method of Mori et al. (1995). The differ-

ence between the amplitudes of the ST segment before and after the administration of ischaemia-provoking agents was taken as the ST segment change.

2.4. Provocation of cardiac ischaemia

An ST segment depression was induced by the administration of a bolus injection of the vasopressin V_1-receptor agonist ornithine-vasopressin (ornipressin; 0.5 IU kg^{-1}) into the tail vein. The dose of ornipressin was selected on the basis of previous studies (Mori et al., 1995).

In a separate study, for induction of the ST segment depression, the animals received an intravenous injection of epinephrine (10 μg kg^{-1}) and 30 s later the α-adrenoceptor antagonist phentolamine (15 mg kg^{-1}, i.v.). The dose of epinephrine was chosen on the basis of our pilot studies, where epinephrine caused a similar increase in mean arterial blood pressure to that observed with ornipressin. The dose of phentolamine was selected so as to induce a minimum 80 mm Hg fall in mean arterial blood pressure, when administered after epinephrine.

2.5. Effects of NOS inhibition on ST segment depression

Groups of animals were injected with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 5 mg kg^{-1}, s.c.) 15 min before the provocation of an ST segment depression by the administration of ornipressin or epinephrine + phentolamine.

2.6. Chemicals

L-[U-^{14}C]Arginine monohydrochloride, ornithine-vasopressin, raloxifene and phentolamine were from Amersham International (UK), Sandoz (Switzerland), Eli Lilly (USA) and Novartis (Switzerland), respectively. All other compounds were from Sigma.

2.7. Statistics

The data are expressed as the means ± S.E.M. of the results for n rats per experimental group. The data were analysed with the Mann–Whitney nonparallel U-test and the Tukey–Kramer Multiple Comparisons test, where appropriate. A difference of $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of ovariectomy, raloxifene and oestrogen on cardiac Ca^{2+}-dependent NOS activity

Ovariectomy was found to lead to a significantly decreased cardiac cNOS enzyme activity in the rat (by 51 ± 7%; $n = 5$; $P < 0.005$). 17β-Oestradiol (0.3 mg kg^{-1}) or raloxifene (5 mg kg^{-1}) supplementation (2 weeks, orally, once daily) in the ovariectomized rat completely restored the cNOS activity to the level observed in the heart of the ovary-intact females. Data are shown in Fig. 1.

3.2. Effects of ovariectomy, raloxifene and oestrogen on cardiac ischaemia

Administration of ornipressin (0.5 IU kg^{-1}) or epinephrine (10 μg kg^{-1}) alone caused a rapid 80–90% increase in mean arterial blood pressure in all groups (Fig. 2, upper panels). The administration of phentolamine (15 mg kg^{-1}) 30 s after epinephrine led to a rapid drop in mean arterial blood pressure by 100–110% (30 s after phentolamine) and caused a slow further decrease over the investigation period (Fig. 2, upper left panel). There was no significant intergroup difference in blood pressure changes throughout the study.

Sixty seconds after ornipressin administration, a significant ST segment depression was observed only in ovariectomized rats. In the ovary-intact females and in the oestrogen- or raloxifene-treated ovariectomized groups, an ST segment depression did not develop (Fig. 2, lower right panel).

Epinephrine alone did not cause an ST segment change in any of the groups investigated ($n = 5–12$, data not shown). The administration of phentolamine 30 s after epinephrine resulted in a significant ST segment depression only in the ovariectomized rat. In ovary-intact females and in oestrogen- or raloxifene-treated ovariectomized rats, no ST segment depression could be detected (Fig. 2, lower left panel).

3.3. Action of NOS inhibition on the susceptibility of the heart to ischaemia

The administration of L-NAME (5 mg kg^{-1}) increased the mean arterial blood pressure by 60–70% during 15 min

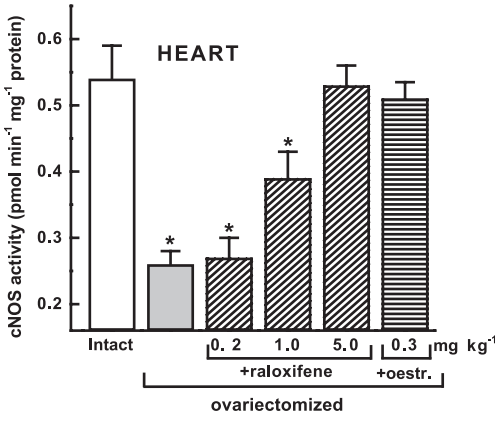

Fig. 1. Ca^{2+}-dependent nitric oxide synthase activities (cNOS; expressed as pmol min^{-1} mg^{-1} protein) in the cardiac tissue of ovary-intact female, ovariectomized and raloxifene- or 17β-oestradiol-treated (oestr.; 2 weeks, orally for each) ovariectomized rats. Data are expressed as means ± S.E.M. of the results on a minimum of eight rats per group. *$P < 0.05$ versus ovary-intact females.

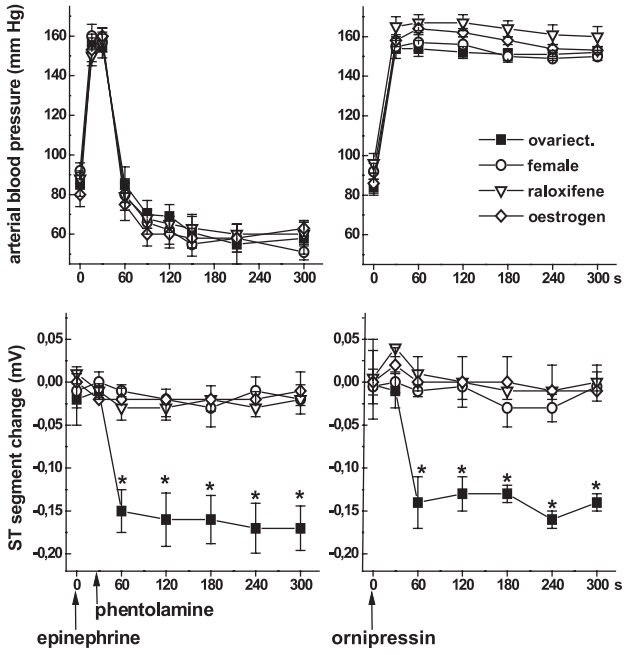

Fig. 2. Time course of mean arterial blood pressure (measured from the right carotid artery; expressed in mm Hg; upper panels) and ST segment (measured in lead II standard surface ECG; expressed in mV; lower panels) changes following a bolus intravenous (tail vein) injection of (1) epinephrine (10 μg kg^{-1}) and 30 s later phentolamine (15 mg kg^{-1}; left panels) or (2) ornithine-vasopressin (ornipressin; 0.5 IU kg^{-1}; right panels) over a 5-min period in ovary-intact female, ovariectomized and raloxifene (5 mg kg^{-1} day^{-1})- or 17β-oestradiol-treated (oestrogen; 0.3 mg kg^{-1} day^{-1}; 2 weeks, orally for each) ovariectomized rats. Data are expressed as means ± S.E.M. of the results on a minimum of six rats per group. * $P<0.05$ versus ovary-intact females.

before the provocation of cardiac ischaemia. There were no differences between the groups in the enhancement of the blood pressure by L-NAME ($n=6–8$, data not shown).

Following L-NAME pretreatment, a significant ST segment depression developed in the ovary-intact females and in the oestrogen- or raloxifene-supplemented ovariectomized rats after either ornipressin or epinephrine + phentolamine administration (Fig. 3). Following both cardiac ischaemic attacks, L-NAME augmented the ST segment depression in the ovariectomized rats (Fig. 3).

4. Discussion

The present study revealed a significantly lower Ca^{2+}-dependent constitutive NOS activity in the cardiac tissue of the ovariectomized rats as compared with the ovary-intact control females. This result confirms our previous findings in relation to the heart (Morschl et al., 2000) and the observations of other investigators concerning the vascular tissue (Weiner et al., 1994; Rahimian et al., 1997, 2002; Morschl et al., 2000; Pávó et al., 2000). The oestrogen-deficient state might account for the down-regulation of the Ca^{2+}-dependent NOS because a 2-week treatment with 17β-oestradiol or raloxifene restored the NOS activity to the level to be found in the heart of the ovary-intact females. In this system, raloxifene seems to exert an oestrogen-agonist action in the regulation of the cardiac NOS.

Ca^{2+}-dependent constitutive NOS consists of two distinct isoenzymes: the endothelial (eNOS) and the neuronal NOS (nNOS) (Moncada and Higgs, 1995). The cellular sources of NO synthesised by eNOS are the vascular endothelial and smooth muscle cells in the coronary circulation and the cardiac myocytes themselves (Balligand and Cannon, 1997; Chambliss and Shaul, 2002). The expression of nNOS can be detected in cholinergic and nonadrenergic–noncholinergic nerve terminals, in specialized conduction tissue of the heart and in sympathetic nerve terminals, where it has been postulated to play a role in catecholamine release and reuptake (Schmidt et al., 1993; Schwarz et al., 1995). Recent studies demonstrated that eNOS and nNOS independently affect the cardiac function (Barouch et al., 2002) and that oestrogen selectively up-regulates both constitutive NOS isoforms (Chambliss and Shaul, 2002; Rosenfeld et al., 2003). The expression of oestrogen receptor α and β in the heart is a further indication of the importance of oestrogen-mediated NOS regulation in cardiac physiology (Grohé et al., 1998). Accordingly, it seems that the increase of NO formation in the heart by the Ca^{2+}-dependent NOS following 17β-oestradiol and raloxifene treatment of ovariectomized rats in the present study

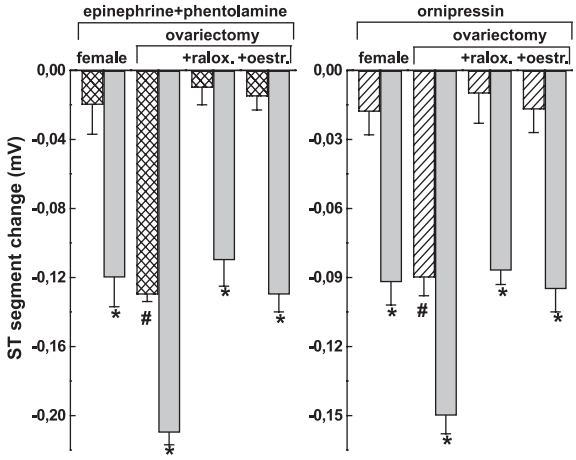

Fig. 3. ST segment changes (measured in lead II standard surface ECG; expressed in mV) following a bolus intravenous (tail vein) injection of (1) epinephrine (10 μg kg^{-1}) and 30 s later phentolamine (15 mg kg^{-1}; left panel; crosshatched columns) or (2) ornithine-vasopressin (ornipressin; 0.5 IU kg^{-1}; right panel; hatched columns) in ovary-intact female, ovariectomized and raloxifene (ralox.; 5 mg kg^{-1} day^{-1})- or 17β-oestradiol-treated (oestr.; 0.3 mg kg^{-1} day^{-1}; 2 weeks, orally for each) ovariectomized rats. The ST segment change was determined 3 min after the challenge. Grey columns show the actions of N^G-nitro-L-arginine methyl ester (L-NAME; 5 mg kg^{-1}, s.c.) pretreatment (15 min before challenge) on the ST segment changes. Data are expressed as means ± S.E.M. of the results on a minimum of six rats per group, where $^{\#}P<0.05$ denotes a significant difference compared to the ovary-intact females and $*P<0.05$ a significant difference between groups with and without L-NAME pretreatment.

involves the up-regulation of both eNOS and nNOS in the cardiac tissue.

The NO generated by eNOS and nNOS plays an important role in the maintenance of the vascular integrity (Moncada and Higgs, 1995; Chambliss and Shaul, 2002; Morishita et al., 2002; Al-Shabrawey et al., 2003). The cardiovascular protection exerted by eNOS is well characterized. It relaxes the vascular smooth muscle and inhibits platelet activation and platelet and leukocyte adhesion to the vascular endothelium (Moncada and Higgs, 1995). The beneficial actions of nNOS on the vasculature, however, came into focus only in very recent work in which genetically manipulated mice were used. In eNOS-deficient mice, the normal microvasculature develops via processes mediated by nNOS originating from the perivascular nerves (Al-Shabrawey et al., 2003). Moreover, NO, synthesized by nNOS, decreases macrovascular injury in various in vivo models (Morishita et al., 2002). Hence, it is strongly suspected that the attenuation of the susceptibility to cardiac ischaemia by endogenous oestrogen in the ovary-intact female rats and by 17β-oestradiol or raloxifene treatment in the ovariectomized rats in the present study involves both oestrogen-mediated eNOS and nNOS up-regulation. Following the administration of the nonselective NO synthase inhibitor L-NAME, we observed a significant aggravation of cardiac ischaemia in the ovariectomized rats, in the ovary-intact females and in the 17β-oestradiol or raloxifene-treated ovariectomized rats, which supports the role of oestrogen-mediated NOS regulation in the cardiovascular defence. This finding also suggests the oestrogen-agonistic properties of raloxifene.

Oestrogen modulates NO synthase by both genomic and nongenomic mechanisms (Simoncini et al., 2002; Chambliss and Shaul, 2002). The acute administration of oestrogen has beneficial effects on exercise-induced myocardial ischaemia in women with coronary artery disease (Rosano et al., 1993). Moreover, in animal models, oestrogen and raloxifene acutely increase the coronary blood flow (Zoma et al., 2000) and reduce ischaemia- and reperfusion-provoked myocardial injury in a partially NO-dependent mechanism (Ogita et al., 2002), effects reflecting the nongenomic pathways of cardiac protection by oestrogen and SERM. On the other hand, the chronic treatment of ovariectomized animals with oestrogen or raloxifene stimulates eNOS expression and increases NO generation in the cardiac and vascular tissue (Rahimian et al., 1997, 2002; Pávó et al., 2000; Morschl et al., 2000; Wang and Abdel-Rahman, 2002). This leads to a decrease in ovariectomy-provoked enhanced vasoconstriction and impaired baroreflex sensitivity (Rahimian et al., 1997, 2002; Pávó et al., 2000; Morschl et al., 2000; Wang and Abdel-Rahman, 2002). These latter findings explain the cardiovascular defence through NO-mediated oestrogen and SERM actions at a transcriptional level. Our present results may indicate that oestrogen and raloxifene lower the susceptibility of the heart to ischaemia by the genomic modulation of NOS expression. It should be mentioned that the doses of oestrogen and raloxifene in the present study and in many of the previous works mentioned above are high, limiting the drawing of conclusions of value from a clinical aspect.

We demonstrated that the nonselective inhibition of NOS isoenzymes by L-NAME alone caused similar increases in the mean arterial blood pressure but did not generate cardiac ischaemia in the ovary-intact, ovariectomized and 17β-oestradiol or raloxifene-treated rats. However, L-NAME aggravated the vasopressin or epinephrine + phentolamine-induced ST segment depression in the ovariectomized rats and abolished the anti-ischaemic action of oestrogen or raloxifene therapy. Our observations are in agreement with the recent findings that L-NAME administration alone did not affect the size of myocardial infarct provoked by ischaemia–reperfusion but partially attenuated the infarct size-limiting effect of raloxifene (Ogita et al., 2002). These results might suggest that besides NO regulation, other NO-independent mechanisms must also be involved in oestrogen-mediated cardiac protection. The proposed pathways include protection against oxidative injury (Sack et al., 1994; Wassmann et al., 2002), the inhibition of vascular smooth muscle cell proliferation (Takahashi et al., 2003), the opening Ca^{2+}-activated K^{+} channels (Ogita et al., 2002) and modulation of the renin–angiotensin system (Brosnihan et al., 1997).

In summary, it is suggested that an oestrogen deficiency down-regulates the cardiac constitutive NOS, which, at least in part, increases the susceptibility of the heart to ischaemia because both actions can be restored by the exogenous administration of the natural oestrogen 17β-oestradiol or the selective oestrogen-receptor modulator raloxifene. In the present in vivo system, raloxifene exerts oestrogen-agonist properties.

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References

Al-Shabrawey, M., El-Remessy, A., Gu, X., Brooks, S.S., Hamed, M.S., Huang, P., Caldwell, R.B., 2003. Normal vascular development in mice deficient in endothelial NO synthase: possible role of neuronal NO synthase. Mol. Vis. 9, 549–558.

Balligand, J.L., Cannon, P.J., 1997. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler. Thromb. Vasc. Biol. 17, 1846–1858.

Barouch, L.A., Harrison, R.W., Skaf, M.W., Rosas, G.O., Cappola, T.P., Kobeissi, Z.A., Hobal, I.A., Lemmon, C.A., Burnett, A.L., O'Rurke, B., Rodriguez, E.R., Huang, P.L., Lima, J.A.C., Berkowitz, D.E., Hare, J.M., 2002. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 416, 337–340.

Barrett-Connor, E., 1997. Sex differences in coronary heart disease: why are women so superior? The 1995 Ancel Keys Lecture. Circulation 95, 252–264.

Barrett-Connor, E., Cox, D.A., Anderson, P.X., 1999. The potential of SERMs for reducing the risk of coronary heart disease. Trends Endocrinol. Metab. 10, 320–325.

Barrett-Connor, E., Grady, D., Sashegyi, A., Anderson, P.W., Cox, D.A., Hoszowski, K., Rautaharju, P., Harper, K.D., 2002. Raloxifene and cardiovascular events in osteoporotic postmenopausal women. Four-year results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized clinical trial. JAMA 287, 847–857.

Brosnihan, K.B., Weddle, D., Anthony, M.S., Heise, C., Li, P., Ferrario, C.M., 1997. Effects of chronic hormone replacement on the renin–angiotensin system in cynomolgus monkeys. J. Hypertens. 15, 719–726.

Chambliss, K.L., Shaul, P.W., 2002. Estrogen modulation of endothelial nitric oxide synthase. Endocr. Rev. 23, 665–686.

Clarkson, T.B., Cline, J.M., Williams, J.K., Anthony, M.S., 1997. Gonadal hormone substitutes: effects on cardiovascular system. Osteoporos. Int. 1 (Suppl. 1), S43–S51.

Furberg, C.D., Vittinghoff, E., Davidson, M., Herrington, D.M., Simon, J.A., Wenger, N.K., Hulley, S., 2002. Subgroup interactions in the heart and estrogen/progestin replacement study. Circulation 105, 917–922.

Grodstein, F., Manson, J.E., Colditz, G.A., Willet, W.C., Speizer, F.E., Stampfer, M.J., 2000. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. Ann. Intern. Med. 133, 933–944.

Grohé, C., Kahlert, S., Löbbert, K., Vetter, H., 1998. Expression of oestrogen receptor α and β in rat heart: role of local oestrogen synthesis. J. Endocrinol. 156, R1–R7.

Humphrey, L.L., Chan, B.K.S., Sox, H.C., 2002. Postmenopausal hormone replacement therapy and the primary prevention of cardiovascular disease. Ann. Intern. Med. 137, 273–284.

Kauffman, R.F., Bean, J.S., Fahey, K.J., Cullinan, G.J., Cox, D.A., Bensch, W.R., 2000. Raloxifene and estrogen inhibit neointimal thickening after balloon injury in the carotid artery of male and ovariectomized female rats. J. Cardiovasc. Pharmacol. 36, 459–465.

Manson, J.E., Hsia, J., Johnson, K.C., Rossouw, J.E., Assaf, A.R., Lasser, N.L., Trevisan, M., Black, H.R., Heckbert, S.R., Detrano, R., Strickland, O.L., Won, N.D., Crouse, J.R., Stein, E., Cushman, M., 2003. Estrogen plus progestin and the risk of coronary heart disease. N. Engl. J. Med. 349, 523–534.

Moncada, S., Higgs, E.A., 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J. 9, 1319–1330.

Mori, T., Ishigai, Y., Fukuzawa, A., Chiba, K., Shibano, T., 1995. Pharmacological profile of semotiadil fumarate, a novel calcium antagonist, in rat experimental angina model. Br. J. Pharmacol. 116, 1668–1672.

Morishita, T., Tsutsui, M., Shimokawa, H., Horiuchi, M., Tanimoto, A., Suda, O., Tasaki, H., Huang, P.L., Sasaguri, Y., Yanagihara, N., Nakashima, Y., 2002. Vasculoprotective roles of neuronal nitric oxide synthase. FASEB J. 16, 1994–1996.

Morschl, É., Bretus, I., Nemcsik, J., László, F., Pávó, I., 2000. Estrogen-mediated up-regulation of the Ca-dependent constitutive nitric oxide synthase in the rat aorta and heart. Life Sci. 68, 49–55.

Ogita, H., Node, K., Asanuma, H., Sanada, S., Liao, Y., Takashima, S., Asakura, M., Mori, H., Shinozaki, Y., Hori, M., Kitakaze, M., 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.

Pávó, I., László, F., Morschl, É., Nemcsik, J., Berkó, A., Cox, D.A., László, F.A., 2000. Raloxifene, an oestrogen-receptor modulator, prevents decreased constitutive nitric oxide and vasoconstriction in ovariectomized rats. Eur. J. Pharmacol. 410, 101–104.

Rahimian, R., Laher, I., Dube, G., van Breemen, C., 1997. Estrogen and selective estrogen receptor modulator LY117018 enhance release of nitric oxide in rat aorta. J. Pharmacol. Exp. Ther. 283, 116–122.

Rahimian, R., Dubé, G.P., Toma, W., Dos Santos, N., McManus, B.M., van Breemen, C., 2002. Raloxifene enhances nitric oxide release in rat aorta via increasing endothelial nitric oxide synthase. Eur. J. Pharmacol. 434, 141–149.

Reckelhoff, J.F., 2001. Gender differences in the regulation of blood pressure. Hypertension 37, 1199–1208.

Riggs, B.L., Hartmann, L.C., 2003. Selective estrogen-receptor modulators—mechanisms of action and application to clinical practice. N. Engl. J. Med. 348, 618–629.

Rosano, G.M., Sarrel, P.M., Poole-Wilson, P.A., Collins, P., 1993. Beneficial effect of oestrogen on exercise-induced myocardial ischaemia in women with coronary artery disease. Lancet 342, 133–136.

Rosenfeld, C.R., Chen, C., Roy, T., Liu, X.-T., 2003. Estrogen selectively up-regulates eNOS and nNOS in reproductive arteries by transcriptional mechanisms. J. Soc. Gynecol. Investig. 10, 205–215.

Sack, M.N., Rader, D.J., Cannon III, R.O., 1994. Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women. Lancet 343, 269–270.

Salter, M., Knowles, R.G., Moncada, S., 1991. Widespread tissue distribution, species distribution and changes in activity of Ca(2+)-dependent and Ca(2+)-independent nitric oxide synthases. FEBS Lett. 291, 145–149.

Schmidt, H.H.H.W., Gagne, G.D., Nakane, M., Pollock, J.S., Miller, M.F., Murad, F., 1993. Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase and novel paraneural functions for nitrinergic signal transduction. J. Histochem. Cytochem. 40, 1439–1456.

Schwarz, P., Diem, R., Dun, N.J., Forstermann, U., 1995. Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat heart sympathetic nerves. Circ. Res. 77, 841–848.

Simoncini, T., Varone, G., Fornari, L., Mannella, P., Luisi, M., Labrie, F., Genazzani, A.R., 2002. Genomic and nongenomic mechanisms of nitric oxide synthesis induction in human endothelial cells by a fourth-generation selective estrogen receptor modulator. Endocrinology 143, 2052–2061.

Takahashi, K., Ohmichi, M., Yoshida, M., Hisamoto, K., Mabuchi, S., Arimoto-Ishida, E., Mori, A., Tsutsumi, S., Tasaka, K., Murata, Y., Kurachi, H., 2003. Both estrogen and raloxifene cause G1 arrest of vascular smooth muscle cells. J. Endocrinol. 178, 319–329.

Wang, X., Abdel-Rahman, A.A., 2002. Estrogen modulation of eNOS activity and its association with caveolin-3 and calmodulin in rat hearts. Am. J. Physiol., Heart Circ. 282, H2309–H2315.

Wassmann, S., Laufs, U., Stamenkovic, D., Linz, W., Stach, J.P., Ahlbory, K., Rösen, R., Böhm, M., Nickenig, G., 2002. Raloxifene improves endothelial dysfunction in hypertension by reduced oxidative stress and enhanced nitric oxide production. Circulation 105, 2083–2091.

Weiner, C.P., Lizasoain, I., Baylis, S.A., Knowles, R.G., Charles, I.G., Moncada, S., 1994. Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc. Natl. Acad. Sci. U. S. A. 91, 5212–5216.

Zoma, W.D., Baker, R.S., Clark, K.E., 2000. Coronary and uterine vascular responses to raloxifene in the sheep. Am. J. Obstet. Gynecol. 182, 521–528.