

Endothelium-independent relaxation of rabbit coronary artery by 17β -oestradiol *in vitro*

Canwen Jiang, Philip M. Sarrel, David C. Lindsay, Philip A. Poole-Wilson & ¹Peter Collins

Department of Cardiac Medicine, National Heart and Lung Institute, Dovehouse Street, University of London, London SW3 6LY

1 We assessed the relaxant effect of 17β -oestradiol (10^{-7} , 10^{-6} and 10^{-5} M) on rabbit isolated coronary arteries precontracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M), high extracellular potassium (30 mM) and Bay K 8644 (10^{-6} M) plus high extracellular potassium (15 mM) by measuring isometric tension. 17β -Oestradiol (10^{-6} and 10^{-5} M) induced significant relaxation in coronary arteries from male and female rabbits. No differences were seen between arteries with or without endothelium. There were also no differences between coronary arteries isolated from male and female rabbits.

2 Inhibitors of endothelium-derived relaxing factor and vasodilator prostanoids, namely, reduced haemoglobin, N^{ω} -nitro-L-arginine methyl ester and indomethacin, did not affect the relaxation induced by 17β -oestradiol in endothelium-intact coronary arteries.

3 Methylene blue, an inhibitor of guanylate cyclase, did not affect the coronary artery relaxation induced by 17β -oestradiol.

4 The calcium concentration-dependent contraction curve in potassium-depolarization medium was shifted to the right by 17β -oestradiol (10^{-6} and 10^{-5} M) in the rabbit coronary artery and rat aorta. The $-\log EC_{50}$ s of calcium in control and after incubation with 17β -oestradiol (10^{-6} and 10^{-5} M) were 3.7 ± 0.09 , 3.1 ± 0.10 and 2.8 ± 0.08 respectively in rabbit coronary arteries and 3.8 ± 0.11 , 3.3 ± 0.14 and 2.9 ± 0.15 in rat aorta.

5 The results indicate that 17β -oestradiol induces rabbit coronary artery relaxation by an endothelium-independent mechanism *in vitro*. A calcium antagonistic property may be involved in the mechanism of rabbit coronary arterial relaxation by 17β -oestradiol.

Keywords: 17β -Oestradiol; coronary relaxation; vascular endothelium; calcium antagonism; vasodilator prostanoid

Introduction

Oestrogen replacement therapy in postmenopausal women reduces the risk of cardiovascular and cerebrovascular disease (Godsland *et al.*, 1987). Recently, large-scale epidemiological studies indicate that postmenopausal women who receive oestrogens appear to have one-third to one-half the cardiovascular mortality rate and one-half the cerebrovascular mortality rate when compared to untreated women (Colditz *et al.*, 1987; Bush *et al.*, 1987). These findings are supported by the evidence that oestrogen affects cholesterol metabolism and deposition, inhibiting atherosclerotic plaque formation in the liver and arterial walls (Sarrel, 1990), and by angiographic studies that show a protective effect of postmenopausal oestrogens on coronary occlusion (Gruchow *et al.*, 1988; Sullivan *et al.*, 1988). However, analyses of lipid changes in postmenopausal women taking oestrogens show they do not fully account for the cardiovascular protective effects (Adams *et al.*, 1990). It is important to study other mechanisms by which oestrogens may provide cardiovascular benefits to women. Another important mechanism may be the modulation of coronary vasomotion.

17β -Oestradiol increases cardiac output and arterial flow velocity, but decreases vascular resistance, systolic and diastolic blood pressure (Magness & Rosenfeld, 1989). 17β -Oestradiol causes coronary artery vasodilatation in isolated perfused hearts of rabbits (Raddino *et al.*, 1986) and increases myocardial perfusion in ewes (Magness & Rosenfeld, 1989). Recent reports demonstrate that 17β -oestradiol treatment modulates responses to acetylcholine in coronary arteries in cynomolgus monkeys (Williams *et al.*, 1990) and femoral arteries in rabbits (Miller *et al.*, 1988), suggesting an endothelium-dependent mechanism *in vivo* in the long term. It has also been reported that oestrogen decreases calcium entry into the vascular smooth muscle cell of pig uterine arteries

(Stice *et al.*, 1987a,b). However, the effect of 17β -oestradiol on the coronary artery and its mechanism of action is not well understood.

The purpose of this study was to investigate the possible relaxant effect of 17β -oestradiol on the coronary artery *in vitro* and the role of endothelial modulation and calcium antagonism in this relaxation by use of rabbit coronary arterial smooth muscle preparations.

Methods

Animals and tissues

Adult male or non-pregnant female New Zealand white rabbits (2.5–3 kg) were killed by an overdose of pentobarbitone (60 mg kg^{-1}) and heparin ($150 \text{ units kg}^{-1}$). The heart was removed and epicardial coronary arteries were dissected free of connective tissue. Male Wistar rats (5–6 weeks old) were killed by pentobarbitone (30 mg kg^{-1}) and the thoracic descending aortae were removed and dissected free of connective tissue. Arterial rings were prepared and in alternate rings, the endothelium was removed by gentle rubbing with a wooden probe. Each ring (2–3 mm length) was suspended horizontally between two stainless steel parallel hooks for the measurement of isometric tension in individual organ baths containing 7 ml modified Krebs solution at 37°C , bubbled with 95% O_2 and 5% CO_2 . The composition of modified Krebs solution was as follows (mM): NaCl 118.3, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, K_2PO_4 1.2 and glucose 11.1.

Relaxant effect of 17β -oestradiol on precontracted coronary arteries

Coronary arterial rings with or without endothelium from male and female rabbits were allowed to stabilize for 90 min under a resting tension of 1 g before the rings were contracted

¹ Author for correspondence.

with potassium (K^+ , 30 mM), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 3×10^{-6} M) or Bay K 8644 (Bay K, 10^{-6} M) plus K^+ (15 mM). 17β -Oestradiol (10^{-7} , 10^{-6} and 10^{-5} M) or equivalent ethanol solvent (1:2000) was added 7 min after the addition of constrictor agents. In another group of coronary arteries from male or female rabbits, testosterone (dissolved in ethanol, 10^{-7} , 10^{-6} , 10^{-5} , 3×10^{-5} and 10^{-4} M) was added 7 min after the addition of constrictor agents.

Effect of reduced haemoglobin, N^{ω} -nitro-L-arginine methyl ester and indomethacin on relaxation induced by 17β -oestradiol

Reduced haemoglobin was prepared as previously described and is an inhibitor of endothelium-dependent vasodilatation mediated by endothelium-derived relaxing factor (EDRF) (Martin *et al.*, 1985). N^{ω} -nitro-L-arginine methyl ester (L-NAME) is an inhibitor of EDRF synthesis from L-arginine in vascular endothelial cells (Rees *et al.*, 1990). Reduced haemoglobin (10^{-5} M) or L-NAME (10^{-4} M) was added to precontracted coronary arterial rings with endothelium. Indomethacin was dissolved by sonication in a Na_2CO_3 solution. Indomethacin (10^{-5} M), an inhibitor of prostanoic acid synthesis, was incubated with endothelium-intact rings for 20 min before they were precontracted with the agonists. 17β -Oestradiol (10^{-6} and 10^{-5} M) was subsequently added 7 min after the addition of constrictor agents. In addition, effects of indomethacin (10^{-5} M) incubation on relaxation induced by arachidonic acid (3×10^{-5} M) were assessed in endothelium-intact tissues.

Effect of methylene blue on relaxation induced by 17β -oestradiol

To determine the possible involvement of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the relaxation induced by 17β -oestradiol, coronary artery rings without endothelium were incubated with methylene blue (10^{-5} M) for 20 min before being contracted with $PGF_{2\alpha}$ (3×10^{-6} M). 17β -Oestradiol (10^{-6} and 10^{-5} M) was subsequently added.

Calcium antagonistic effect of 17β -oestradiol on coronary artery and rat aorta

Rabbit coronary artery and rat aortic rings without endothelium were incubated in calcium-free solution containing 0.5 mM EGTA for 10 min. Calcium concentration-dependent contraction curves were then performed in K^+ -depolarization medium ($K^+ = 100$ mM). The rings were readjusted in modified Krebs solution for 20 min before being incubated in calcium-free solution containing 0.5 mM EGTA for another 10 min. These rings were preincubated with 17β -oestradiol or solvent 30 min before the calcium concentration-dependent contraction curves were obtained.

Drugs

The following drugs were used: 17β -oestradiol (dissolved in ethanol, Sigma); indomethacin (Sigma); L-NAME (Sigma); testosterone (Sigma); arachidonic acid (Sigma); Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer UK); prostaglandin $F_{2\alpha}$ (Sigma); pentobarbitone (Sigma); haemoglobin (Sigma); methylene blue (Sigma). All drugs were Analar grade.

Statistics

All results are expressed as mean \pm s.e.mean. Relaxation was expressed as percentage relaxation of contraction induced by agonists and K^+ . The results were analysed with Student's *t* test for paired and unpaired observations. Each group was compared to the time-matched ethanol solvent control. A

probability level of less than 0.05 was considered significant; *n* indicates the number of animals.

Results

Relaxant effect of 17β -oestradiol on precontracted coronary arteries

K^+ (30 mM), $PGF_{2\alpha}$ (3×10^{-6} M) or Bay K (10^{-6} M plus 15 mM K^+) induced comparable contractile responses in rings with and without endothelium (0.8 ± 0.1 , 0.7 ± 0.2 and 0.9 ± 0.1 g; $P > 0.05$). 17β -Oestradiol (10^{-7} M) had no effect on the contraction evoked by these agents. However, 10^{-6} M and 10^{-5} M 17β -oestradiol induced significant concentration-related relaxation of contracted rings with or without endothelium from male or female rabbits (compared to time-matched ethanol solvent controls, all $P < 0.01$). There were no differences between arteries from male and female rabbits (all $P > 0.05$, $n = 8$ in male group and $n = 6$ in female group). There were no differences between groups with and without endothelium in K^+ precontracted rings ($P > 0.05$, Figure 1). No differences were seen between endothelium-denuded rings precontracted with the 3 agents ($P > 0.05$, $n = 8$ in each group). In another group of rabbits (male = 5, female = 2) testosterone (10^{-7} , 10^{-6} and 10^{-5} M) did not induce significant relaxation (all $P > 0.05$ in comparison with time-matched ethanol controls) in either endothelium-intact or -denuded coronary arteries precontracted with three constrictor agents. Higher doses of testosterone (3×10^{-5} M and 10^{-4} M) caused a slight relaxation in endothelium-intact and -denuded rings precontracted with K^+ (30 mM) by $14 \pm 1.8\%$ and $16 \pm 2.6\%$; $19 \pm 2.1\%$ and $18 \pm 3.4\%$ respectively (compared with time-matched ethanol controls, all $P < 0.05$). Similar results were obtained in endothelium-denuded rings precontracted with $PGF_{2\alpha}$ (3×10^{-6} M) or Bay K (10^{-6} M plus 15 mM K^+).

Effect of reduced haemoglobin, L-NAME and indomethacin on 17β -oestradiol-induced relaxation

Reduced haemoglobin (10^{-5} M) and L-NAME (10^{-4} M) did not inhibit the relaxation induced by 17β -oestradiol (10^{-6} and 10^{-5} M) in rings with endothelium ($P > 0.05$, Figure 2). Indomethacin (10^{-5} M) significantly reduced the transient relaxation induced by arachidonic acid (3×10^{-5} M) by $93 \pm 2.1\%$ ($n = 5$) in endothelium-intact coronary arteries precontracted

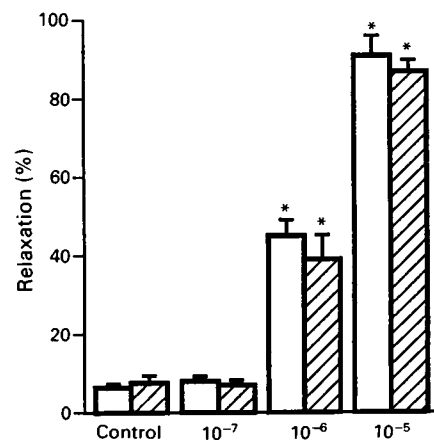


Figure 1 The relaxant effect of 17β -oestradiol (10^{-7} , 10^{-6} and 10^{-5} M; 10^{-7} , 10^{-6} and 10^{-5} respectively) on rabbit coronary arteries with and without endothelium (open and hatched columns respectively) precontracted by K^+ (30 mM). Data are expressed as percentage relaxation of contraction induced by K^+ (mean with s.e.mean shown by vertical bars, $n = 8$ in each group). Control = time-matched ethanol solvent controls.

Significant differences in comparison with corresponding controls * $P < 0.01$.

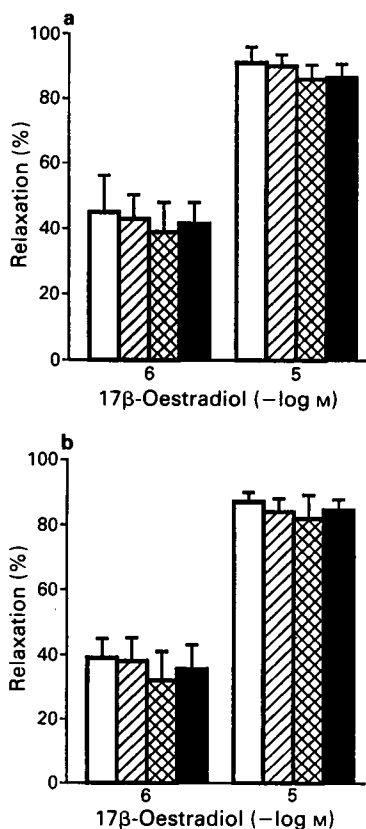


Figure 2 The effect of reduced haemoglobin (10^{-5} M, hatched columns), indomethacin (10^{-5} M, cross-hatched columns) and N^o-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M, solid columns) on the relaxation induced by 17 β -oestradiol (10^{-6} and 10^{-5} M, open columns) in rabbit coronary artery with endothelium, precontracted by K⁺ (30 mM, a) or prostaglandin F_{2 α} (PGF_{2 α} , 3×10^{-6} M, b). Data are expressed as a percentage relaxation of contraction induced by K⁺ or PGF_{2 α} (mean with s.e.mean shown by vertical bars, $n = 8$ in each group).

with K⁺ (30 mM). However, the same dose of indomethacin did not affect the relaxation induced by 17 β -oestradiol (10^{-6} and 10^{-5} M) in rings with endothelium ($P < 0.05$, Figure 2).

Effect of methylene blue on 17 β -oestradiol induced relaxation

Incubation with the guanylate cyclase inhibitor methylene blue (10^{-5} M), for 20 min before contraction with PGF_{2 α} (3×10^{-6} M) had no effect on relaxation induced by 17 β -oestradiol (10^{-6} and 10^{-5} M) in rings without endothelium. Relaxation induced by 17 β -oestradiol (10^{-6} and 10^{-5} M) was $34 \pm 4\%$ and $84 \pm 3\%$ in the presence of methylene blue, and $35 \pm 10\%$ and $88 \pm 4\%$ in controls ($P > 0.05$).

Calcium antagonistic effects on rabbit coronary artery and rat aorta

The calcium concentration-dependent contraction curves in K⁺-depolarization medium were shifted to the right after incubation with 17 β -oestradiol (10^{-6} and 10^{-5} M) in rabbit coronary arterial and rat aortic rings, without endothelium. Maximal contraction was also reduced by $19 \pm 1\%$ and $36 \pm 2\%$ in rabbit coronary arteries and by $12 \pm 1\%$ and $29 \pm 1\%$ in rat aorta respectively. The $-\log EC_{50}$ s of calcium in control and after incubation with 17 β -oestradiol (10^{-6} and 10^{-5} M) were 3.7 ± 0.09 , 3.1 ± 0.10 and 2.8 ± 0.08 in rabbit coronary arterial rings (Figure 3). Similar results were obtained from rat aorta preparations ($n = 6$) and the $-\log EC_{50}$ s of calcium in control and after incubation with 17 β -oestradiol (10^{-6} and 10^{-5} M) were 3.8 ± 0.11 , 3.3 ± 0.14 and 2.9 ± 0.15 respectively.

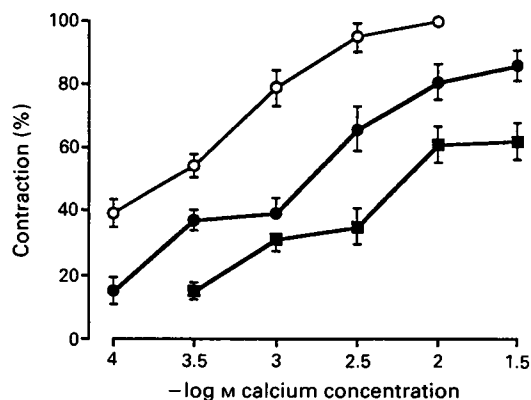


Figure 3 The effect of 17 β -oestradiol (10^{-6} and 10^{-5} M) on the calcium concentration-dependent contraction curves in rabbit coronary arteries without endothelium: (○) control; (●) 10^{-6} M 17 β -oestradiol; (■), 10^{-5} M 17 β -oestradiol. Data are expressed as percentage of maximal contraction induced by calcium in controls (mean with s.e.mean shown by vertical bars, $n = 6$ in each group).

Discussion

We have demonstrated that 17 β -oestradiol (10^{-6} M and 10^{-5} M) induces relaxation in precontracted rabbit coronary arteries with or without endothelium. No difference was seen between arteries from male and female rabbits. Reduced haemoglobin, L-NAME, indomethacin and methylene blue did not affect this relaxation. 17 β -Oestradiol shifted the calcium concentration-dependent contraction curve to the right, in high K⁺ (100 mM) solution, in rabbit coronary and rat aortic preparations. In addition, testosterone up to a dose of 10^{-5} M did not induce any relaxation in similar preparations.

Oestrogen receptors are present in both vascular and cardiac tissues of male and female rats (Stumpf *et al.*, 1977). Administration of oestrogen has been shown to induce haemodynamic changes in male human subjects after a transsexual operation (Slater *et al.*, 1986). However, it is well known that oestradiol has different effects on bioenergetic enzymes in the female and male. In the present study, we have demonstrated that 17 β -oestradiol induces an identical relaxation in coronary arteries from female and male rabbits. Therefore, we suggest that the acute relaxant effect of 17 β -oestradiol in the rabbit coronary artery *in vitro* is independent of sex. The other possible sex-related effects of this steroid hormone on vascular tissues are not excluded.

The heart is also a target for androgens in female monkeys (McGill *et al.*, 1980), although their effects on coronary vascular tissue are not well understood. In the present study, however, testosterone, which is the most active androgen, up to a concentration of 10^{-5} M, did not cause any relaxation in coronary arteries from either male or female rabbits, whilst 3×10^{-5} M and 10^{-4} M testosterone induced a small relaxation. We conclude that the acute relaxant effect of 17 β -oestradiol is not a property shared by testosterone in our preparations.

Recently, Gisclard *et al.* (1988) reported that femoral arteries from rabbits treated with 17 β -oestradiol show an enhanced endothelium-dependent relaxation to acetylcholine. Infusion of acetylcholine in ovariectomized guinea-pigs caused relaxation only after treatment with oestrogen (Bell, 1973; Bell & Coffey, 1982). Furthermore, the infusion of acetylcholine into atherosclerotic coronary arteries of ovariectomized, hypercholesterolaemic cynomolgus monkeys caused contraction while a comparison group of 17 β -oestradiol-treated, animals showed a relaxation response to acetylcholine (Williams *et al.*, 1990). Acetylcholine induces endothelium-dependent vascular relaxation mediated by the release of EDRF (Furchgott & Zawadzki, 1980). These studies may therefore indicate that 17 β -oestradiol is affecting vascular tone by an EDRF-dependent mechanism. However, we have demonstrated that 17 β -oestradiol induced an equal degree of

relaxation in rabbit coronary arteries with and without endothelium. Haemoglobin, a well-known inhibitor of EDRF activity (Martin *et al.*, 1985), did not affect 17β -oestradiol-induced relaxation in endothelium-intact coronary arteries. L-NAME, a novel inhibitor of EDRF synthesis (Rees *et al.*, 1990), markedly inhibited acetylcholine-induced endothelium-dependent relaxation in rabbit coronary artery preparations (data not shown). However, the same dose of L-NAME did not affect the relaxation by 17β -oestradiol. Methylene blue, an inhibitor of EDRF-induced increase of cyclic GMP (Martin *et al.*, 1985), also had no effect on relaxation induced by 17β -oestradiol. Our results suggest that the *in vitro* acute relaxation of rabbit coronary arteries by 17β -oestradiol is independent of EDRF. The effect of 17β -oestradiol on EDRF-mediated relaxation *in vivo* over a long time course may still occur.

The possibility that relaxation of smooth muscle by 17β -oestradiol may be via prostacyclin release is suggested by the increased production of a prostacyclin metabolite in the lower uterine artery of postmenopausal hypoestrogenic women induced by administration of 17β -oestradiol (Steinleitner *et al.*, 1989). Increased prostacyclin production activity in rat aortic smooth muscle cells in culture with 17β -oestradiol stimulation has also been reported (Chang *et al.*, 1980). However, indomethacin does not affect 17β -oestradiol-induced enhancement of endothelium-dependent vasorelaxation and the authors concluded that a major role of prostanoid-induced vasorelaxation was unlikely (Gisclard *et al.*, 1988). Indomethacin inhibits synthesis of prostaglandins (Ferreira *et al.*, 1971; 1973). In the present study, indomethacin markedly inhibited the transient relaxation induced by arachidonic acid which has been shown to induce prostanoid-like action via transformation to prostaglandins in various tissues (De Mey *et al.*, 1982). However, indomethacin did not affect 17β -oestradiol-induced relaxation in endothelium-intact coronary arteries. These results indicate the release of vasodilator prostanoids is not involved in 17β -oestradiol-induced coronary relaxation *in vitro*.

Oestrogens have calcium antagonistic properties in uterine arteries. Stice *et al.* (1987a,b) reported that oestradiol decreases calcium entry into uterine vascular smooth muscle cells. Downing *et al.* (1988) observed that the inhibitory effect of nifedipine, a calcium entry blocker, was enhanced by oestradiol in the rat uterine artery. Sandahl *et al.* (1978) reported in the rat that nifedipine, a potential-sensitive calcium channel blocker, caused an increase in uterine arterial flow. However, this was not the case in rats pretreated with 17β -oestradiol possibly suggesting they were already maximally dilated by 17β -oestradiol. Calcium antagonistic properties of 17β -oestradiol have also been reported in isolated smooth muscle cells from uterine arteries and veins (McCalden, 1975; Takayama, 1986). Potential-sensitive calcium channels are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. The uterine arterial smooth muscle tone of gilts is reduced in oestrous when endogenous oestrogen is increased, suggesting that oestrogen may reduce intracellular calcium via a potential-operated calcium channel mechanism (Ford *et al.*, 1984). Bay K is a specific potential-sensitive calcium channel agonist (Brown *et al.*, 1984). In the present study, we found that 17β -oestradiol induced relaxation of coronary arterial contraction evoked by

Bay K. This may be evidence that 17β -oestradiol has an inhibitory effect on potential-sensitive calcium channel activation. We also demonstrated that 17β -oestradiol induced relaxation of contraction evoked by $PGF_{2\alpha}$, an agonist of receptor-operated calcium channels, in the presence of extracellular calcium. These results indicate that 17β -oestradiol has similar relaxant effects on contraction induced by activation of both receptor-operated and potential-operated calcium channels. Furthermore, incubation with 17β -oestradiol shifted the calcium concentration-dependent contraction curves to the right in high K^+ -depolarization medium in rabbit coronary arteries and rat aorta without endothelium. The curves shifted by incubation with different 17β -oestradiol concentrations were parallel, in rabbit coronary arteries and rat aorta, although the maximal contractions were also affected. These results suggest that 17β -oestradiol may have a partial calcium antagonistic property. This calcium antagonistic property could be one of the mechanisms of 17β -oestradiol-induced endothelium-independent relaxation in rabbit isolated coronary arterial preparations. Animal studies have shown that calcium-channel blockers such as nifedipine can alter the progression of atherosclerosis in animals fed a cholesterol-rich diet (Henry & Bentley, 1981). Recent clinical studies demonstrate the beneficial effect of nifedipine (Lichtlen *et al.*, 1990) and nicardipine (Water *et al.*, 1990) on the development of early atheromatous lesions seen at coronary angiography. Similar results have been obtained in cynomolgus monkeys (Williams *et al.*, 1990) where oestrogens inhibit the progression of atherosclerosis in the coronary arteries of these animals. If 17β -oestradiol does have a calcium antagonistic effect in man then its protective effect in atherosclerotic disease may also involve this mechanism.

The risk of coronary heart disease is lower in premenopausal women compared with men of the same age (Godsland *et al.*, 1987). Sex hormone withdrawal associated with the menopause substantially increases the incidence of myocardial infarction in postmenopausal women (Kannel *et al.*, 1976). Oestrogen replacement therapy in postmenopausal women reduces the cardiovascular mortality rate (Matthews *et al.*, 1989). The beneficial effects of oestrogen on menopausal symptoms (Campbell & Whitehead, 1977) and skeletal health (Ettinger *et al.*, 1985) have been increasingly recognised. However, the beneficial effects of oestrogen on the cardiovascular system would now appear to be the most important (Ettinger, 1988).

In conclusion, we have demonstrated that 17β -oestradiol induces endothelium-independent relaxation in rabbit isolated coronary artery preparations. 17β -Oestradiol-induced relaxation may play an important role in the regulation of coronary tone and this may be one of the explanations as to why oestrogen replacement therapy reduces the risk of coronary heart disease in postmenopausal women. 17β -Oestradiol has effects on contraction evoked by activation of both receptor- and potential-operated calcium channels. 17β -Oestradiol may also have calcium antagonistic properties. If 17β -oestradiol does have a calcium antagonistic effect in the human then its protective effect in atherosclerotic disease may also involve this mechanism. Many studies have demonstrated the effect of calcium antagonists on animal experimental atheroma causing regression. Some such studies emphasise the requirement for long term therapy.

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