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European Journal of Pharmacology 467 (2003) 163–168
www.elsevier.com/locate/ejphar

Estrogen alone or combined with medroxyprogesterone but not raloxifene reduce myocardial infarct size

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Received 31 January 2003; received in revised form 10 March 2003; accepted 14 March 2003

Abstract

We investigated whether estrogen protects the ischemic myocardium in oophorectomized female rabbits fed with a cholesterol-enriched diet, whether the addition of a progestin compound attenuates the beneficial effect of estrogen and whether raloxifene also limits myocardial necrosis. We treated 32 female oophorectomized hypercholesterolemic rabbits with (a) placebo ($N=8$, group I), (b) conjugated estrogens alone ($N=8$, group II), (c) conjugated estrogens combined continuously with medroxyprogesterone acetate ($N=8$, group III) and (d) raloxifene ($N=8$, group IV) all for 4 weeks. All rabbits underwent 30 min of ischemia and 120 min of reperfusion. Both infarct size (0.38 ± 0.08 and 0.45 ± 0.05 in groups II and III, respectively, vs. 0.78 ± 0.07 in group I, $P<0.005$) and infarct size/risk zone% (26.34 ± 4.18 and 35.01 ± 4.39 in groups II and III, respectively, vs. 52.18 ± 7.84 in group I, $P<0.05$) were significantly smaller in the estrogen treatment groups compared to placebo. No significant difference was observed between groups II and III. There was no significant difference between groups I and IV for infarct size (0.78 ± 0.07 vs. 0.69 ± 0.08, respectively) or for infarct size/risk zone% (52.18 ± 7.84 vs. 47.17 ± 4.3). Short-term estrogen protects ischemic myocardium in hypercholesterolemic oophorectomized female rabbits; this effect is not attenuated by the addition of a progestin compound. Raloxifene, however, does not decrease infarct size compared to placebo.

Keywords: Infarct size; Estrogen; Progestin; Raloxifene; Hypercholesterolemia

1. Introduction

Observations from more than 40 studies suggest a 35% to 50% reduction in risk of coronary heart disease for women using hormone replacement therapy compared to non-users (Grady et al., 1992; Stampfer and Golditz, 1991). However, three recent randomised clinical trials (Herrington et al., 2000a; Hulley et al., 1998; Writing Group for the Women's Health Initiative Investigators, 2002) evaluating the effect of postmenopausal hormone therapy on coronary disease did not confirm previous reports. A possible explanation for this discrepancy is an adverse effect of progesterone, although this is not supported by the data reported from the Nurses Health Study, where a similar reduction in risk was noted between women on unopposed estrogen and women on combined hormone therapy (Grodstein et al., 1996). Progestins may antagonize estrogen's beneficial effects on lipids, endothelial function and fibrinolysis as they seem to decrease high-density lipoprotein (HDL) cholesterol and nitric oxide availability and increase plasminogen antigen inhibitor-1 plasma levels (Guetta and Cannon, 1996). Raloxifene, a selective estrogen receptor modulator, with anti-estrogenic effects on breast and endometrium and therefore without some of hormone replacement therapy's long-term side-effects, appears an attractive alternative to estrogen replacement as it obviates the need for a progestin. In addition, raloxifene has no effect on c-reactive protein levels (Blum et al., 2000; Delmas et al., 1997; De Valk-de Roo et al., 1999), as opposed to estrogen (Cushman et al., 1999; Ridker et al., 1999; Walsh et al., 2001), and it is hypothesized that proinflammatory changes in atherosclerotic plaques may be one of the reasons for the absence of protection from coronary events in recent hormone replacement therapy trials. Furthermore, secondary analysis from the Multiple Outcomes of Raloxifene Evaluation (MORE)

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doi:10.1016/S0014-2999(03)01627-3

trial revealed that raloxifene significantly reduces the risk of cardiovascular events in women at high risk (Barrett-Connor et al., 2002).

We have previously shown that short-term estrogen alone reduces myocardial infarct size, in a dose-dependent manner, in oophorectomized female rabbits fed with a normal diet (Sbarouni et al., 1998). In the present study, we investigated whether estrogen protects ischemic myocardium in oophorectomized female rabbits fed with a cholesterol-enriched diet, whether the addition of a progestin compound attenuates the beneficial effect of estrogen and whether raloxifene can also limit myocardial necrosis.

2. Methods

2.1. Animals and treatment

We performed transabdominal bilateral oophorectomy, under general anesthesia with ketamine (150 mg/kg intramuscularly), in 32 New Zealand white female rabbits, sexually mature (4 to 6 months of age) and weighing 3 to 3.5 kg. All oophorectomized animals were fed with a standard laboratory diet (25 g/day) enriched with 2 g of cholesterol (product USP23, Dolder) mixed with 6% corn-oil in every kilogram of food, for 8 weeks prior to the experiment. All animals were subsequently randomised into four groups: group I ($N=8$) was treated with intramuscular placebo injections (normal saline); group II ($N=8$) was treated with intramuscular conjugated estrogen, at a dose of 1 mg/day, once a day (Sbarouni et al., 1998); group III ($N=8$) was treated with intramuscular conjugated estrogen at a dose of 1 mg/day and intramuscular medroxyprogesterone acetate at a dose of 2.5 mg/day, once a day; and group IV ($N=8$) was treated with oral raloxifene at a dose of 180 mg/day. All four groups were treated for 4 weeks prior to the experiment. As conjugated estrogens, we used Premarin (Wyeth, France) in which 20 mg of conjugated estrogens is dissolved in 5 ml of sterile water containing 2% benzyl alcohol. The other two active treatments were progesterone (medroxyprogesterone acetate, Sigma, M 1629 diluted in pure ethanol) and raloxifene (Evista, Eli Lilly, Indianapolis, USA).

2.2. Surgical treatment

Eight weeks after oophorectomy, all rabbits were anesthetized with pentobarbital, intubated via a tracheal incision and mechanically ventilated with a respirator for small animals (MDI, Alabama, USA), at a rate of 35 respirations/min; catheters were inserted in the left jugular vein for fluid and top up anesthetic administration and the left carotid artery for systemic pressure monitoring throughout the experiment. The chest was opened via a left thoracotomy at the fourth intercostal space, and the pericardium was opened. The heart was exposed and a prominent branch of the left coronary artery was encircled with a silk suture, which was subsequently threaded through a small plastic tube and tightened in order to achieve coronary occlusion. Myocardial ischemia was confirmed by regional cyanosis of the myocardial surface and ST elevation on the electrocardiogram. Reperfusion was confirmed by restoration of colour (hyperemia) in the previously ischemic region. Heart rate and arterial pressure were monitored continuously on paper and measured at baseline, during occlusion and throughout reperfusion. Ten beats were averaged at each time. After reperfusion, the ends of the suture were cut short and tied so that a loose loop was left on the heart to mark the occlusion site. Following reperfusion, the rabbit was killed by pentobarbital overdose.

2.3. Infarct size determination

The heart was removed, mounted on a perfusion apparatus and flushed through the aorta with normal saline (20 ml/min at 20 °C) for 2 min. When all blood was washed out from the coronary tree, the suture was again tightened around the coronary artery at the previous site and Zn^{2+}–Cd^{2+} fluorescent particles (0.1%, 5 ml) were subsequently infused over a 3-min interval; this allowed the distinction between the normally perfused area (Zn^{2+}–Cd^{2+} uptake) and the risk zone (no Zn^{2+}–Cd^{2+} uptake). The heart was then frozen at −10 °C for 24 h and subsequently sliced into sections 3-mm thick, from apex to base; five to seven slices were used for each animal, the last slice being above the suture level and therefore normal. The total of risk and infarction areas was included in the slices under study. All slices were incubated in triphenyl tetrazolium chloride solution 1% at 37 °C for 20 min; the area not perfused with Zn^{2+}–Cd^{2+} particles and not stained with triphenyl tetrazolium chloride solution (triphenyl tetrazolium chloride solution-negative) was considered to be the infarct area; the area that was not perfused with Zn^{2+}–Cd^{2+} particles but was stained with triphenyl tetrazolium chloride solution (triphenyl tetrazolium chloride solution-positive) represented the risk zone. The slices were further kept in formaldehyde 10% for 24 h in order to delineate the infarct area more precisely. Consequently, all hearts were examined under ultraviolet light (366-nm wavelength) in order to distinguish the normally perfused area from the risk zone. The infarct, risk and normal areas were traced on an acetate sheet, and the traced areas were subsequently scanned with the Adobe Photoshop 6.0 and measured with the Scion Image program. The infarct and risk zones were expressed in cm^3, and the infarct-to-risk area ratio (I/R) was calculated as a percent.

2.4. Statistical analysis

The data are expressed as means ± S.E.M. Infarct size (I), risk zone (R) and infarct size/risk zone ratio (I/R%) were compared using an analysis of variance (ANOVA) as these

Table 1
Body weight (BW), heart weight (HW) and infarct size/risk zone ratio (I/R%) data

	BW (kg)	HW (g)	R (cm^3)
Group I ($N=6$)	2.98 ± 0.05	8.6 ± 0.21	1.58 ± 0.15
Group II ($N=7$)	3.03 ± 0.03	8.7 ± 0.10	1.41 ± 0.15
Group III ($N=8$)	2.93 ± 0.03	8.4 ± 0.09	1.31 ± 0.09
Group IV ($N=7$)	3.02 ± 0.04	8.8 ± 0.13	1.45 ± 0.05

Results are expressed as means ± S.E.M.
Group I: placebo; group II: estrogen; group III: estrogen plus progesterone; group IV: raloxifene.

data were normally distributed; when a significant difference was found ($P<0.05$), the Fisher's Least Square Differences (LSD) test was further applied for post-hoc analysis to compare differences between groups. Similarly, in order to compare the heart rate, the systolic, diastolic and mean blood pressure and the rate-pressure product at baseline, 20 min after coronary occlusion and 1 h after the start of reperfusion, between groups (I, II, III and IV), we used the analysis of variance (ANOVA). Heart rate, systolic, diastolic and mean blood pressure and rate-pressure product at the same time points, in each of the four groups, throughout the study, were analyzed with repeated measures analysis of variance. A P value <0.05 was considered to be statistically significant. All analyses were performed with the commercially available statistical program, SPSS v.8.

3. Results

3.1. Mortality

From the 32 oophorectomized rabbits, 4 died, 2 in group I, 1 in group II and 1 in group IV, all dying of ventricular fibrillation during the experiment. Thus, the data are reported for 28 animals: 6 in group I, 7 in group II, 8 in group III and 7 in group IV. Body weights and heart weights were similar for the four groups (Table 1).

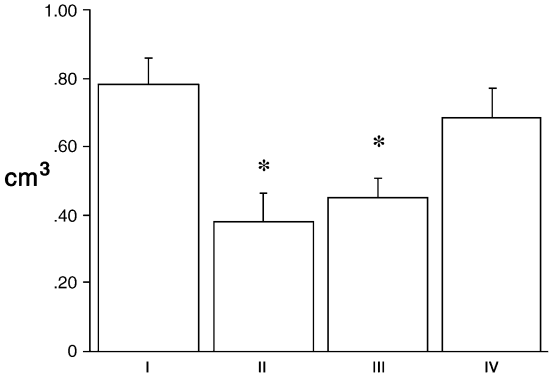

Fig. 1. The infarct size in the four groups. Results are expressed as means ± S.E.M. Group I: placebo; group II: estrogen; group III: estrogen plus progesterone; group IV: raloxifene. *$P<0.05$ vs. group I and group IV.

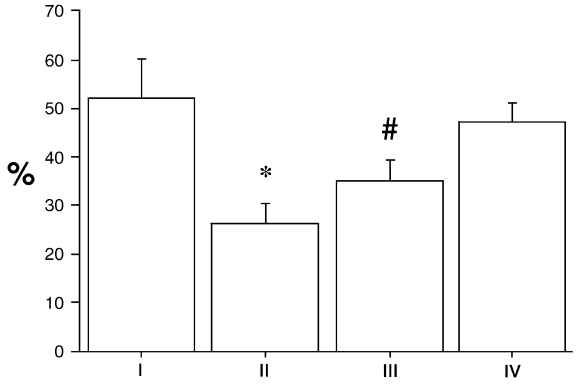

Fig. 2. The infarct size/risk zone ratio in the four groups. Results are expressed as means ± S.E.M. Group I: placebo; group II: estrogen; group III: estrogen plus progesterone; group IV: raloxifene. *$P<0.05$ vs. group I and group IV, #$P<0.05$ vs. group I.

3.2. Infarct size

The risk zone (R) did not differ significantly between groups I, II, III and IV ($P=0.47$). The infarct size (I) ($P=0.0035$ for ANOVA) was significantly smaller in groups II and III compared to both group I and IV (I vs. II $P=0.0016$, I vs. III $P=0.0063$, II vs. IV $P=0.0081$, III vs. IV $P=0.0317$) and no significant difference was noted between groups II and III or between groups I and IV (II vs. III $P=0.48$ and I vs. IV $P=0.43$). The infarct size-to-risk zone ratio (I/R%) ($P=0.0081$ for ANOVA) was significantly smaller in groups II and III compared to that in group I ($P=0.0028$ and $P=0.0281$, respectively); it was also smaller in group II compared to group IV ($P=0.0085$),

Table 2
Hemodynamic data

	I ($N=6$)	II ($N=7$)	III ($N=8$)	IV ($N=7$)
HRB	272 ± 7	281 ± 6	266 ± 5	277 ± 8
HRI	270 ± 8	264 ± 6	256 ± 5	261 ± 8
HRR	258 ± 9	246 ± 4	244 ± 8	244 ± 11
SBP B	104 ± 2	99 ± 4	91 ± 4	121 ± 8
SBP I	91 ± 3	87 ± 3	89 ± 7	94 ± 2
SBP R	84 ± 3	79 ± 2	89 ± 7	89 ± 3
DBP B	71 ± 2	79 ± 7	81 ± 7	
DBP I	74 ± 2	54 ± 4	66 ± 8	74 ± 5
DBP R	50 ± 5	53 ± 2	51 ± 6	64 ± 3
MBP B	203 ± 5	195 ± 6	220 ± 17	230 ± 17
MBP I	173 ± 4	160 ± 6	178 ± 15	193 ± 7
MBP R	151 ± 10	149 ± 5	157 ± 15	173 ± 6
RPP B	28 300 ± 942	27 929 ± 1198	30 621 ± 1957	33 800 ± 2641
RPP I	24 533 ± 1096	23 036 ± 909	23 179 ± 1155	24 686 ± 1032
RPP R	21 775 ± 1186	19 386 ± 1105	21 629 ± 1992	21 714 ± 1386

Results are expressed as means ± S.E.M.
$P<0.01$ for B, I, R in all four groups (I, II, III, IV) and for all five parameters (HR, SBP, DBP, MBP, RPP).
Group I: placebo; group II: estrogen; group III: estrogen plus progesterone; group IV: raloxifene.
HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; RPP: rate pressure product; B = baseline; I = ischemia; R = reperfusion.

whereas it did not differ between groups III and IV ($P=0.964$); groups II and III were similar ($P=0.22$), as were groups I and IV ($P=0.51$) (Table 1, Figs. 1 and 2).

3.3. Hemodynamics

The hemodynamic parameters, heart rate, systolic, diastolic and mean blood pressure and the rate-pressure product, were similar among all four groups at all time points throughout the study, at baseline, during ischemia and reperfusion. When these parameters were examined separately for each group, at baseline, during ischemia and during reperfusion, a significant difference was found in all four groups ($P<0.01$) with heart rate, blood pressure (systolic, diastolic and mean) and the rate-pressure product being lower during ischemia and reperfusion, presumably due to a combination of ischemic dysfunction, anesthetic administration and temperature reduction (Table 2).

4. Discussion

We found that estrogen limited myocardial necrosis in hypercholesterolemic oophorectomized female rabbits, medroxyprogesterone did not attenuate this protection and raloxifene had no effect. According to reports from recently completed randomised trials, however, combined estrogen/progesterone replacement therapy does not reduce cardiovascular risk in either primary or secondary prevention (Hulley et al., 1998; Grady et al., 1992; Writing Group for the Women's Health Initiative Investigators, 2002). Conversely, there is evidence that postmenopausal women, already on hormone replacement therapy, have lower mortality when they sustain a myocardial infarction, compared to non-users (Shlipak et al., 2001) which is consistent with our findings.

4.1. Infarct size and estrogen—previous studies

Estrogen, both 17β-estradiol and conjugated estrogens, acutely and short-term, decrease infarct size and the incidence of ventricular arrhythmias in various normocholesterolemic animal models of both sexes in most studies (Hale et al., 1996; Kim et al., 1996; Mc Hugh et al., 1995; Node et al., 1997; Sbarouni et al., 1998), but not in all (Smith et al., 2000). This protective effect has been attributed to increased nitric oxide production, Ca^{2+}-dependent K^{+} channel activation or antioxidant activity. Whether this protection occurs in hypercholesterolemic animals had not been investigated. Hypercholesterolemia is associated with increased oxygen-derived free radical production in the vasculature and could therefore be responsible for the augmented nitric oxide degradation (Ohara et al., 1993); estrogen may counteract these unfavourable biological effects of hypercholesterolemia since it promotes nitric oxide synthesis and release (Hayashi et al., 1992; Weiner et al., 1994) and acts as a free radical scavenger (Keaney et al., 1994; Swaery et al., 1997). We indeed found that conjugated estrogens protect the ischemic myocardium in hypercholesterolemic animals.

4.2. Progesterone effects

Progestins may offset estrogens' benefits. Some animal studies have shown that progesterone diminishes the endothelium-dependent relaxation induced by estrogens, both in vivo and in vitro (Miller and Vanhoutte, 1991; Williams et al., 1994), attenuates the estrogen-mediated inhibition of neointima formation after arterial balloon injury (Levine et al., 1996; Oparil et al., 1997) and antagonizes the reducing effect of estrogen on the extent of atherosclerosis (Adams et al., 1997; Hanke et al., 1996). Other studies, however, have yielded contradictory results, with the addition of progesterone having no effect on coronary vasomotion (Miyagawa et al., 1997), attenuation of cholesterol aortic accumulation (Haarbo et al., 1991) and inhibition of atherosclerosis induced by estradiol (Adams et al., 1990). These differing results may imply that the effects of progestins depend on whether they are natural or synthetic, their potency, dose, route (oral or parenteral) and pattern (continuous or sequential) of administration. In the present study, we found that medroxyprogesterone acetate administered parenterally and continuously does not abolish the protection of ischemic myocardium induced by conjugated estrogens. The dose we used was in the mid-range; both lower and higher doses have been administered in previous studies (Adams et al., 1990; Levine et al., 1996; Oparil et al., 1997; Williams et al., 1994).

4.3. Raloxifene

Raloxifene reduces total and low-density lipoprotein (LDL) cholesterol as well as Lp(a) but has no effect on HDL and triglycerides (Delmas et al., 1997; De Valk-de Roo et al., 1999; Walsh et al., 1998; Herrington et al., 2000b). Raloxifene also decreases fibrinogen but has no effect on plasminogen activator inhibitor-1 levels (Herrington et al., 2000b; Walsh et al., 1998). In addition, it has antioxidant activity in vitro (Zuckerman and Bryan, 1996) and promotes endothelium-dependent vasodilation both in experimental animal (Figtree et al., 1999) and in human studies (Herrington et al., 2000b; Saitta et al., 2001). It also lowers plasma adhesion molecules (De Valk-de Roo et al., 1999; Herrington et al., 2000b) and, unlike estrogen, does not increase c-reactive protein levels (Blum et al., 2000; De Valk-de Roo et al., 1999; Walsh et al., 2000). Raloxifene therefore shares some but not all of estrogen's beneficial effects; it does not increase HDL levels nor does it decrease plasminogen activator inhibitor-1 levels. Whether raloxifene can limit the atherosclerotic process in animal models remains controversial (Bjarnason et al., 1997, 2001; Clarkson et al., 1998; Holm et al., 1997). In a previous study, raloxifene had no effect on plaque size in the coronary arteries, despite a

significant reduction in LDL cholesterol plasma levels. Estrogen therapy, however, resulted in a 70% reduction in atherosclerotic lesions in the same study (Clarkson et al., 1998). We observed no protection regarding infarct size in hypercholesterolemic oophorectomized rabbits pretreated with raloxifene, at a dose that is adequate to plasma levels (Bjarnason et al., 1997, 2001; Clarkson et al., 1998; Kaufman et al., 1997; Lundeen et al., 1997). It is possible that raloxifene lacks an estrogen agonistic effect in the coronary arteries, at least in these models. Whether raloxifene exerts agonist/antagonist effects in various tissues may be associated with the existence of two different DNA response elements that are activated when estrogen and estrogen-like compounds bind to the estrogen receptors; when raloxifene is bound to the estrogen receptor, a different response element is recognized than when estradiol is bound, and it is possible that the estrogen receptors in the coronary arteries are unable to activate the raloxifene response element (Yang et al., 1996).

4.4. Limitations

We have not performed histology to document the induction of atherosclerosis in the coronary vascular bed with the cholesterol-enriched diet we used; however, this particular diet, administered to male rabbits for 8 weeks, has been shown by our group to cause atherogenesis in the ostia and the epicardial parts of the coronary vessels (Kremastinos et al., 2000). We have compared the effect of the various treatments on infarct size compared to placebo, but we have not investigated the possible mechanisms responsible for these effects; this could be the aim of a subsequent study. We have not measured hormone plasma levels, although the doses selected are based on a hard body of evidence from previous studies.

4.5. Conclusions

We have shown that both estrogen alone as well as estrogen plus medroxyprogesterone reduce myocardial infarct size in oophorectomized hypercholesterolemic female rabbits compared to placebo. Progesterone does not seem to attenuate the protection achieved with estrogen. Raloxifene, however, does not limit ischemic myocardial injury compared to placebo.

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