Lupus (1999) 8, 397–401
 0961-2033/99 $12.00
http://www.stockton-press.co.uk/lup

PAPER

Gender-related differences in vascular smooth muscle cell proliferation: implications for prevention of atherosclerosis

LA Fitzpatrick[1]*, M Ruan[1], J Anderson[1], T Moraghan[1] and V Miller[2]
[1]Department of Internal Medicine and [2]Department of Surgery, Mayo Clinic and Mayo Foundation, Rochester, MN, USA

Premenopausal women have a significant reduction in coronary artery disease compared to age-matched males. Little is known about the mechanism underlying this cardioprotective effect of estrogen. Contradictory evidence has been published and our lack of basic understanding of hormone interactions and bioavailability of different estrogens prevents definitive interpretation of these data. We demonstrate gender-specific effects in the proliferation of coronary artery vascular smooth muscle cells obtained from a sexually mature animal model. Vascular smooth muscle cells are an integral component of the atherosclerotic plaque, and inhibition of cell proliferation by estrogen may be one mechanism by which estrogen exerts its cardioprotective effect. Various types of estrogen may also have different mechanistic actions on the vascular system. No differences are demonstrated in overall estradiol binding in vascular smooth muscle cells obtained from male or female animals; however, differences in c-jun, c-fos and TIEG gene expression were gender related. Inhibition of vascular smooth muscle cell proliferation may have important implications in the prevention of atherosclerotic disease and these studies may provide evidence for the cardioprotective effect of estrogen.

Keywords: estrogen; athersclerosis; vascular biology

Introduction

Although estrogen is cardioprotective, little is known about the mechanisms underlying this positive effect. The protective effect of estrogen against the development of atherosclerosis has been demonstrated in animal models, and human epidemiologic studies strongly support a cardioprotective benefit from estrogen. Premenopausal women have a significant reduction in coronary artery disease (CAD) *vs* age-matched males and, after menopause, the mortality from CAD in women is similar to age-matched males.[1]

Studies of the relationships between physiological or pharmacological estrogen levels for protection from cardiac disease remain problematic. Observational studies in both men and women have not unequivocally demonstrated the link between endogenous estrogen levels and the risk of CAD.[2] Estimates of the relative risk of myocardial infarction in users of estrogen or estrogen–progesterone combination have varied widely. Many studies have presented relative risk ranging from 0.5–0.65. Contradictory literature has also been published (odds/ratio 0.96, 95% CI 0.66–1.40 in current users). Our lack of basic understanding of hormone interactions and bioavailability of different estrogens prevents definitive interpretation of these data. Studies have many inherent problems including different types of estrogen at different doses and different routes of administration that complicate data analysis.[3]

Many prospective studies have indicated that administration of oral estrogen is associated with a reduced relative risk for the development of coronary artery disease. The nurses health study, which followed 48 470 postmenopausal women, indicated that estrogen-treated women had half the risk of developing CAD compared with untreated women.[4] Case-control studies that use angiographically defined CAD in postmenopausal women found a greater than 50% reduction among estrogen users compared with non-users. Survival has also been documented to be improved in those women taking estrogen replacement therapy with angiographically proven disease. With the recent publication of the randomized trial of

*Correspondence: LA Fitzpatrick, Department of Internal Medicine, Mayo Clinic and Mayo Foundation, 200 First Street SW, Rochester, MN 55905, USA.

estrogen plus progestin for secondary prevention of coronary artery disease in postmenopausal women, the waters have become murkier regarding the role of estrogen in prevention of coronary artery disease. The primary outcome of this randomized, blinded placebo-controlled trial was the occurrence of non-fatal myocardial infarction or coronary heart disease death. There were no significant differences among groups in the primary outcome or in any of the secondary cardiovascular outcomes. The lack of overall effect occurred despite an 11% lower LDL cholesterol level and 10% higher HDL cholesterol level in the group treated with hormone replacement therapy compared with the placebo group.[5]

It is well established that estrogen has a favorable effect on serum lipids, with an increase in HDL cholesterol and a decrease in LDL cholesterol. However, this is felt to account for at most 30–50% of the cardiovascular protection in women on estrogen replacement therapy.[3] Recently, the effects of estrogen on plaque formation at the cellular level have indicated that estrogen has specific effects on vascular smooth muscle cells. Vascular smooth muscle cells synthesize extracellular matrix proteins that are components of the atherosclerotic plaque.[6] Vascular smooth muscle cells are present early in atherosclerosis and become the dominant cell type as the streak progresses to fibro fatty lesions.[7] Vascular smooth muscle cells (VSMCs) have estrogen receptors as demonstrated in animal and human studies. In this paper, we describe the effects of estrogen on cell proliferation and early oncogene expression in porcine vascular smooth muscle cells.

Methods

Vascular smooth muscle cells from intact, sexually mature adult female and male pigs were isolated *via* explant technique from coronary arteries (left circumflex, left anterior descending and right). In cases where oophorectomy was performed, pigs were allowed to recover for one month prior to sacrifice. Cells were incubated in the phenol red-free media 199 with 10% fetal calf serum and were utilized between passages 3 and 9. The vascular smooth muscle cell (VSMC) phenotype was confirmed by immunocytochemical staining with alpha actin. VSMCs stained negative for fibroblast surface antigen.[8] Reagents were obtained from Sigma Chemicals, St Louis, MO, unless noted.

Thymidine incorporation

For thymidine incorporation to assess proliferation, cells were growth-arrested in serum-free media for 24 h prior to the addition of effector at varying concentrations. The effectors were added in 2% charcoal-striped serum for 24 h. Vehicle controls were included in all experiments.

Northern analysis

VSMCs were grown to confluency, incubated with media containing 10^{-7} M β-estradiol or an equivalent of volume of vehicle for varying times. The total RNA was isolated, resolved on a 1% agarose formaldehyde gel, and transferred to a nylon membrane by capillary diffusion. The blots were probed with ^{32}P-labeled c-fos, c-jun and TIEG (a TGF-β-inducible gene) cDNA. Probes were the generous gift of Dr T Spelsberg, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN.

Nuclear binding assay

Cultured VSMCs were homogenized and centrifuged. The nuclear pellet was resuspended in the presence of varying concentration of ^{3}H-17 β-estradiol for 30 min. Pellets were extracted overnight and radioactive estradiol concentration was determined by scintillation spectroscopy.

Statistics

One-way analysis of variance (ANOVA) was used to determine the differences among treatment versus vehicle-treated controls. The data are expressed as the mean $\pm$ the standard error of the mean of a minimum of four separate experiments performed on quadruplicate wells.

Results

Coronary artery smooth muscle cells obtained from sexually mature female animals

A significant inhibition ($P < 0.05$) of proliferation was observed in VSMC obtained from intact female animals treated with β-estradiol (β-E_2) at concentrations of $10^{-11} - 10^{-8}$ M compared to vehicle-treated controls (Figure 1). No inhibition was observed at superphysiologic concentrations of 10^{-7} M, and inhibition was most pronounced at physiologic

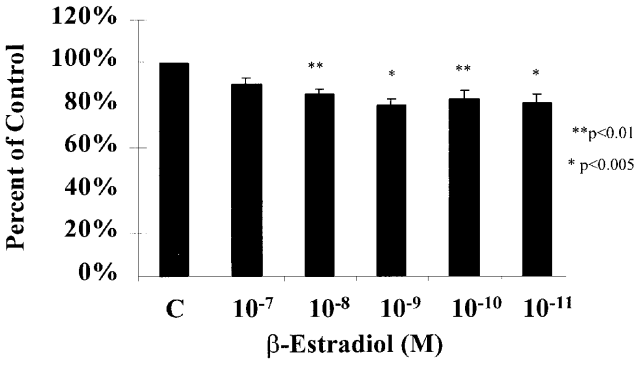

Figure 1 Dose response of β-estradiol on proliferation of porcine coronary vascular smooth muscle cells (VSMC) obtained from intact, sexually mature females as assessed by [^{3}H]-thymidine incorporation, corrected for protein. VSMC were treated with β-estradiol at concentrations of 10^{-11} to 10^{-7}M for 24 h. *$P < 0.01$; **$P < 0.005$.

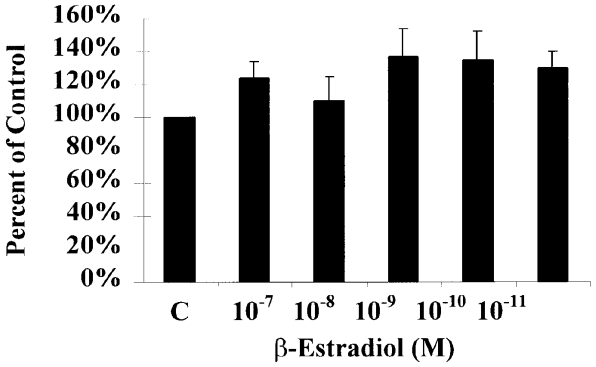

Figure 2 Dose response of β-estradiol on proliferation of porcine coronary vascular smooth muscle cells obtained from intact, sexually mature males as assessed by [^{3}H]-thymidine incorporation, corrected for protein. VSMC were treated with β-estradiol at concentrations of 10^{-11} to 10^{-7} for 24 h. No significant differences were noted.

Table 1 Effect of estrone and equilin sulfate on proliferation of VSMCs from mature male and female animals

	Control	*Estrone 10^{-9}M*	*Equilin 10^{-9}M*
Male	100	104.4 ± 10.7	105.5 ± 10.3
Female	100	78.4 ± 6.8*	100.5 ± 3.9

$P < 0.05$ compared to control. VSMCs were isolated *via* explant and cultured. After the growth arrest with serum-free media, estrone or equilin were added for 24 h in 2% charcoal-striped fetal calf serum. Proliferation was assessed by thymidine incorporation corrected for cell protein content. Data are expressed as mean ± S.E.M and were normalized to vehicle-containing controls.

concentrations of β-estradiol (10^{-11} and 10^{-9} M, $P < 0.005$). In all experiments, the inactive form of estradiol, α-estradiol, was added and no difference in proliferation compared to controls was noted (data not shown).[8]

Estrone-inhibited proliferation in VSMCs obtained from female animals at concentrations of 10^{-6}–10^{-10} M ($P < 0.05$) compared to vehicle controls (Table 1). Inhibition of cell proliferation was also pronounced at physiologic concentrations of estrone. Equilin sulfate was added to VSMCs obtained from coronary arteries from female animals. Inhibition of proliferation was only noted at 10^{-10} M equilin.

Coronary vascular smooth muscle cells obtained from sexually mature male animals

In contrast to coronary VSMCs obtained from female animals, no effect on proliferation in VSMC from intact male animals was noted in the presence of β-estradiol, estrone or equilin (Figure 2). Consistent with the inactive form of estradiol, there were no observed effects on cellular proliferation in VSMCs obtained from male animals exposed to α-estradiol.

Nuclear binding assay

Scatchard analysis of the radioactivity level of bound, [^{3}H]-17 β-estradiol in VSMCs from gonadally intact female and male pigs was performed. High-affinity estrogen receptors were present in the nuclei of VSMCs from both female and male animals. There were no significant differences between the Kd or the Bmax for VSMC from male *vs* female animals (Table 2).

Northern analysis

Northern analysis indicated that β-estradiol-induced TIEG mRNA levels in VSMCs obtained from intact female animals. Levels were highest at 30 min. No induction of TIEG mRNA was noted in VSMC obtained from male animals. Differential effects of

Table 2 Nuclear estrogen receptors in porcine VSMC

	Kd (nM)	*Bmax (fmol/μgDNA)*
VSMC from females	5.42	1.97
VSMC from males	3.86	1.13

VSMCs were isolated by explant technique from mature, gonadally intact male or female pigs. Nuclear extracts were incubated with ^{3}H-17 β-estradiol. Scatchard analysis revealed no statistical differences in binding affinity (Kd) or number of receptors (Bmax).

c-fos and c-jun expression were also noted in response to exposure to β-estradiol. The protooncogene c-fos was upregulated in the presence of β-estradiol in VSMCs from female animals; no differences were noted in VSMCs from male pigs. In contrast, c-jun expression was downregulated in response to β-estradiol in VSMCs from female animals. No response was noted in VSMCs from male animals.

Discussion

Gender-related differences in the incidence of coronary artery disease may be inherent to the VSMC environment. VSMC are important cellular components of the atherosclerotic lesion and express estrogen receptors. Inhibition of VSMC proliferation by estrogen could be a mechanism by which estrogen prevents the development of atherosclerosis. To test this hypothesis, we compared VSMC obtained from sexually mature, gonadally intact male and female animals. To date, most studies with the porcine model had been performed in sexually immature animals making comparisons of gender differences difficult.

Significant inhibition of proliferation of coronary VSMC obtained from female animals by β-estradiol was noted. Other studies have documented inhibition of proliferation by β-E_2 on human saphenous vein VSMC and pig coronary VSMC derived from segments of the left anterior descending artery. However, the gender status of these studies was either not mentioned nor clearly defined. We found no effect of β-E_2 on proliferation in coronary artery VSMC obtained from sexually intact mature males. One previously published study indicated that β-E_2 inhibited aortic VSMC proliferation from male and female rats. However, in this study the concentrations of estradiol used were superphysiologic (10^{-7}–10^{-5}M). Steroid hormones have cytotoxic effects when administered *in vitro* at high concentrations and may account for the inhibition described in this study.

Differential effects of estrone, equilin and β-estradiol were noted. In VSMC obtained from intact female animals, estrone and β-estradiol significantly inhibited cell proliferation. Estrone was a more potent inhibitor than β-estradiol. Surprisingly, equilin sulfate had no effect on cells obtained from the coronary arteries of female animals. In contrast, equilin and β-estradiol did not alter proliferation of VSMC obtained from male animals. Differences in the pharmacokinetics and bioavailability of various estrogens are well recognized.[9] Intersubject differences result in varying responses and therapeutic equivalence among estradiol, estrone and equilin (or a combination of these compounds) is difficult to assess. Often, differences in pharmacokinetics are thought to be responsible for variations noted in the effect of estrogens on target tissue. In this study, we indicate that gender differences provide an additional variable in the response of the target tissue to estrogen. Scatchard analysis of nuclear binding confirmed high affinity functional estrogen receptors in coronary VSMCs from both female and male animals. No statistically significant differences were noted in Bmax or Kd, suggesting that the binding affinity and number of estrogen receptors are not significantly different among these two genders. It is possible that differences in the α versus β isoforms of the estrogen receptor may be noted between genders but our preliminary data with Western analysis in vascular smooth muscle cells do not support this hypothesis.

To further evaluate one of the possible non-genomic mechanisms by which estrogen could exert gender-related differences, Northern analysis for early gene expression was performed. TIEG is a TGF-β-inducible gene derived from a human osteoblast cell line. β-estradiol upregulated TIEG gene expression in VSMC from female pigs in comparison to VSMC obtained from male pigs. In addition, c-fos gene expression was noted in VSMC from female animals when stimulated by β-estradiol but not in male animals. A slight inhibition of c-jun expression by β-estradiol was observed in coronary VSMCs from female animals. These early genes suggest that this may be one mechanism whereby activation of the estrogen receptor by estradiol exerts gender differences at this target tissue.

The cardioprotective effect of various estrogens are of great interest and several responsible mechanisms have been proposed. Inhibition of proliferation of VSMC obtained from female animals is one mechanism by which estrone and estradiol exert their cardioprotective effects. In addition, estrogen suppresses the synthesis of collagen and causes a marked reduction in vascular resistance. In studies in humans and animals, blood flow was increased by the administration of estrogen.[10] At a cellular level, estrogen can act as a calcium antagonist in VSMCs.[11] In another clinical study, equilin exhibited a higher antioxidant potency as determined by fatty acids and steroid oxidation, compared to estrone and β-estradiol.

We conclude that gender-specific effects are found in proliferation in coronary VSMCs obtained from sexually mature pigs. VSMCs are an integral component of the atherosclerotic plaque. Inhibition of cell proliferation may be one mechanism by which estrogen exerts its cardioprotective effect, resulting in gender-related differences in coronary artery disease. Varying types of estrogen may have different

mechanistic actions on the vascular system. As there are no differences in estradiol binding, non-genomic mechanisms may be responsible for this anti-proliferative effect. Therapeutic strategies directed towards inhibition of VSMC proliferation may have important implications in the prevention of atherosclerosis.

References

1 Fitzpatrick LA. Editorial: Coronary artery disease in women-An equal opportunity killer. *J Clin Endocrinol Metab* 1998; **83**: 719–720.
2 Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 1991; **20**: 47–63.
3 Barrett-Connor E. Sex differences in coronary heart disease. *Circulation* 1997; **95**: 252–264.
4 Grodstein, F, Stampfer MJ, Colditz GA. Postmenopausal hormone therapy and mortality. *N Engl J Med* 1997; **336**: 1769–1775.
5 Hulley S, Grady D, Bush T *et al*. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA* 1998; **280**: 605–613.
6 Severson AR, Ingram RT, Fitzpatrick LA. Matrix proteins associated with bone calcification are present in human vascular smooth muscle cells grown in vitro. *In Vitro Cell Dev Biol* 1995; **31**: 853–857.
7 Raines EW, Ross R. Smooth muscle cells and the pathogenesis of the lesion of atherosclerosis. *Br Heart J* 1993; **69** (Suppl): S30–S37
8 Moraghan T, Antoniucci DM, Grenert JP *et al*. Differential response in cell proliferation to beta estradiol in coronary artery vascular smooth muscle cells obtained from mature female versus male animals. *Endocrinology* 1996; **137**: 5174–5177.
9 Troy SM, Hicks DR, Parker VD *et al*. Differences in pharmacokinetics and comparative bioavailability between Premarin and Estratab in healthy postmenopausal women. *Curr Ther Res* 1994; **55**: 359–372.
10 Gilligan DM, Badar DM, Panza JA *et al*. Acute vascular effects of estrogen in postmenopausal women. *Circulation* 1994; **90**: 786–791.
11 Mueck AO, Seeger H, Lippert TH. Calcium antagonistic effect of natural and synthetic estrogens. Investigations on a nongenomic mechanism of direct vascular action. *Int J Clin Pharmacol Ther* 1996; **34**: 424–426.