Estrogen Receptor Modulation:
Effects on Rat Aortic Endothelial Function

by

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ABSTRACT

Studies were performed to characterize the effects of chronic estrogen receptor activation on basal and stimulated nitric oxide release in the male and female ovariectomized Sprague-Dawley (SD) rat. To determine the effects of chronic treatment with oral LY117108, a selective estrogen receptor modulator, on NO release in the aortic endothelium of the ovariectomized rat, dilator responses to acetylcholine were obtained in phenylephrine-precontracted aorta, and phenylephrine concentration-response curves were generated both before and after pretreatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME). The level of ACh-induced relaxation is a measurement of the amount of receptor-mediated NO release. Since L-NAME is a nitric oxide synthase (NOS) inhibitor, the potentiation of PE contractions after L-NAME incubation is indicative of the amount of basal NO production in the tissue. Treatment with LY117018 caused an increase in both basal and stimulated NO release in the female ovariectomized SD rat. The potentiation of basal NO release increased with the time of LY117018 treatment, reaching a maximal level at three weeks and was not further augmented by longer treatment periods. The dose of LY117018 also affected the level of potentiation of basal

NO release. Maximal potentiation of basal NO release occurred with LY117018 doses between 1.0 and 5.0mg/kg/day, inclusive. With doses lower or higher than this range, the effect diminished. LY117018 potentiated stimulated NO release in a dose-dependent manner. This effect was not dependent on the duration of treatment. Chronic subcutaneous 17β-estradiol administration potentiated basal NO release in the male SD rat in a time- and dose-dependent manner, but showed no effect on stimulated NO release. 17β-estradiol increased the sensitivity of the aortic smooth muscle in the male rat to phenylephrine.

These results demonstrate that chronic administration of oral LY117018 enhanced basal and stimulated NO release in the ovariectomized rat in a time- and dose- dependent manner. Chronic administration of subcutaneous 17β-estradiol enhanced basal NO release in the male rat in a time- and dose-dependent manner. Because potentiation of NO release has been postulated to be one of the mechanisms underlying estrogen's cardioprotective effect, the results of this study have many potential clinical implications. The enhanced endothelial function caused by LY117018 or 17β-estradiol could be used to protect against many cardiovascular diseases in both men and women.

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INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death among women, accounting for close to 500 000 deaths in the United States annually (Figure 1). In the United States, 50% of deaths in women are due to atherosclerosis-related diseases, predominantly stroke and myocardial infarction (Castelli, 1988). Despite these overwhelming numbers, greater emphasis has generally been placed on CVD in men than in women. This may be due to the fact that, prior to menopause, women actually have a much lower incidence of CVD as compared to men of similar age. In men, the incidence of CVD increases progressively starting at the age of 35; in women, this increase in not seen on average until after the age of 55 (Figure 2) (Collins, 1996).

The risk of CVD in women increases with age. Before menopause, women are afflicted with half the probability as men of the same age group. As age increases, the risk of CVD accelerates sharply. After menopause, the rate of increase in the number of deaths from CVD is actually greater in women than in men (Castelli, 1988). Thus, women seem to have a protective factor that is lost after menopause. Since the greatest change that occurs at menopause is the decrease in estrogen levels, it has been postulated

that estrogen is the protective factor against CVD. Many epidemiological studies have supported this hypothesis.
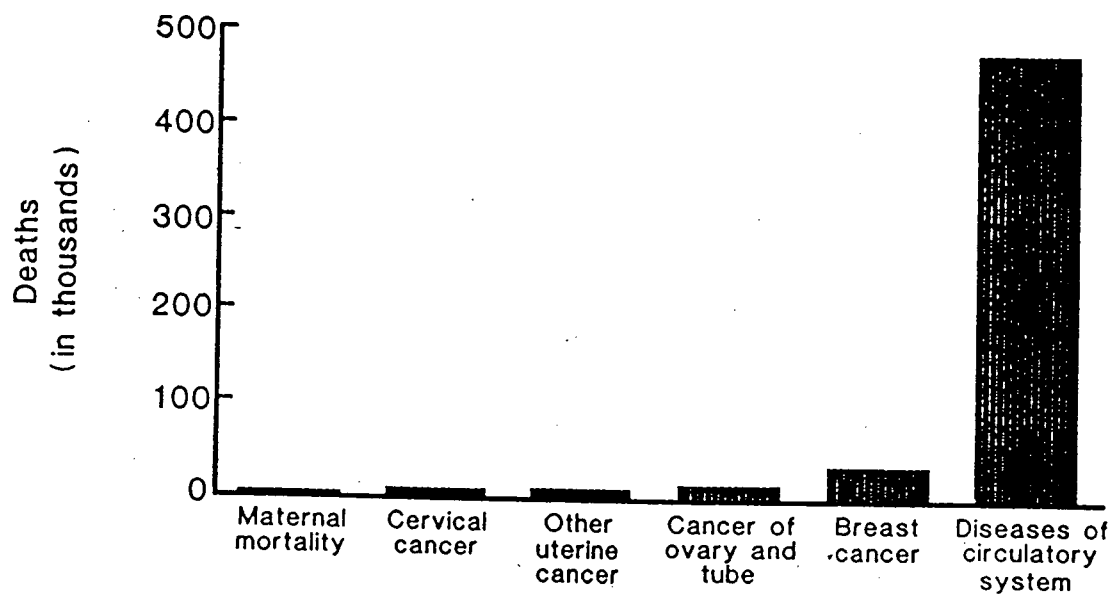
Figure 1: Deaths among women in the United States by selected causes, 1981. (Reproduced from American Journal of Obstetrics and Gynecology 1988; 158(6).)
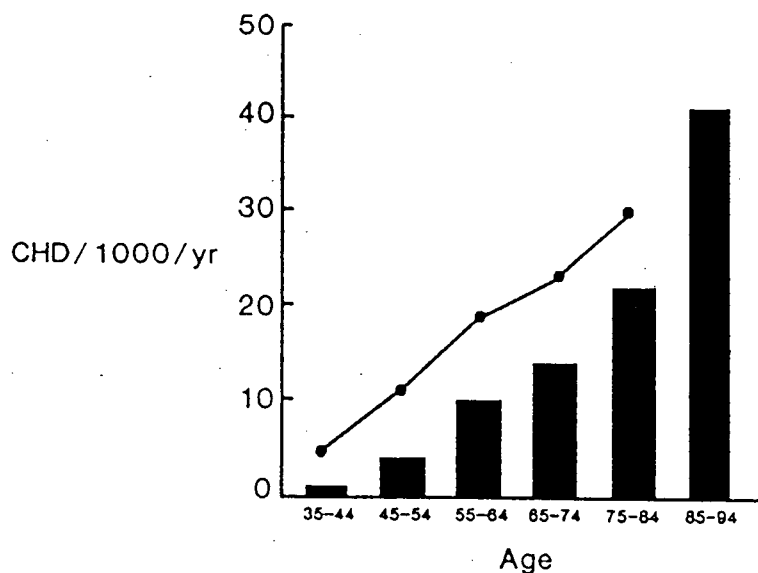
Figure 2: Annual rate of coronary heart disease (CHD) in men (indicated by line) and women (indicated by bars). (Reproduced from American Journal of Obstetrics and Gynecology 1988; 158(6).)

EPIDEMIOLOGICAL STUDIES

Although results from various epidemiological studies have not been completely consistent, a review of the literature finds overwhelming support for the hypothesis that estrogen exerts a cardioprotective effect in women (Stampfer and Colditz, 1991). For example, the results of a large prospective cohort study of 121 700 women in the United States show that women who had undergone bilateral oophorectomy and who had not taken estrogen after menopause had an increased risk of cardiovascular heart disease (CHD). After adjusting for age and smoking, the relative risk of CHD was 2.2 as compared to premenopausal women. The use of estrogen replacement therapy reduced the risk to 0.9 (Colditz *et al.*, 1987). The Nurses' Health Study Cohort, a study of 48 470 women, found that after adjusting for age and other cardiovascular risk factors, the relative risk of CVD in women taking estrogen was 0.56 (Stampfer *et al*, 1991).

In the Cardiovascular Health Study carried out by the Collaborative Research Group, postmenopausal estrogen use in older women was associated with favorable cardiovascular disease risk factor profiles and with lower measures of subclinical disease. The 2955 women who participated in this study were between the ages of 65 and 100 years. They included women who were taking estrogen at the time of the study, those who were past users and those who never took estrogen. The CVD risk factors that were measured included plasma lipid levels, glucose and insulin levels, and the waist/hip ratio. The subjects who were taking estrogen had a better lipid profile, with higher levels of HDL cholesterol and lower levels of LDL cholesterol. The fasting levels of insulin and glucose and the waist/hip ratio were lower in the estrogen users as was the incidence of

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subclinical diseases, such as carotid stenosis, carotid intimal thickening and ventricular hypertrophy (Table 1) (Manolio *et al.*, 1993).

Other studies have also shown a disparity in the risks of CVD in present and past users of estrogen. One study showed that current users of estrogen had a relative risk of coronary disease of 0.47, while that of past users was 0.62 (Henderson *et al.*, 1988). Another study reported a relative risk of 0.3 for current users and 0.7 for past users (Stampfer *et al.*, 1985). These results indicate that the continued presence of estrogen is important in maintaining its cardioprotective effect.

The results from these studies suggest that decreased levels of circulating estrogen may be an important component in the etiology of CVD. The mechanism underlying the cardioprotective effect is unclear; many factors may be involved, including estrogen effects on lipid profile and other risk factors, as well as the direct effects of estrogen on the vascular system.

Table 1: Comparison of Selected Risk Factors and Subclinical and Clinical Diseases in Present, Past and Never users of Estrogen

	Ever Use			
	Present Use	Past Use	Never Use	P, Ever vs Never *
Risk Factors				
HDL cholesterol, mg/dL	71	59	56	0.0001
LDL cholesterol, mg/dL	118	139	141	0.0001
Total cholesterol, mg/dL	218	226	225	0.002
Fasting insulin, μU/dL	13.8	3.99	17.9	0.0001
Fasting glucose, mg/dL	98	15.3	110	0.0001
Waist/hip ratio	0.876	0.893	0.894	0.003
Subclinical disease				
Common carotid thickness, mm	0.94	0.97	0.98	0.002
Carotid stenosis ≥ 1%, %	56	62	64	0.02

* "Ever vs never" indicates two-way comparison using ANCOVA adjusted for age for continous variables, comparing ever (past + present) vs never users.

ESTROGEN AND CARDIOVASCULAR RISK FACTORS

Atherosclerosis

Atherosclerosis is initiated when serum cholesterol, carried by low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL), filter through the endothelium of the arterial wall and enter the intimal layer. This is exacerbated by damage to the endothelium, which removes the barrier to the entrance of lipids into the arterial wall (Friedman and Byers, 1965). Some of the lipids become entrapped in the intima through interactions with intimal substances like glycosaminoglycans (GAGS). VLDL and LDL contain apoprotein B-100 (apo B-100), which is thought to be essential for the interactions with GAGS. Inside the intima, the lipoproteins undergo either of two types of modification: oxidation or derivatization. Macrophages in the arterial intima secrete superoxide as part of their phagocytic function. This superoxide causes oxidation and degradation of apo B-100, which in turn causes the lipoproteins to lose their integrity (Parthasarathy _et al._, 1986). Several types of derivatives can result from derivatization, including glycosylated apo B-100's and malonaldehyde-linked apo B-100's (Fogelman _et al._, 1980). These modified VLDL's and LDL's are then susceptible to engulfment by macrophages. (Goldstein _et al._, 1980)

According to the response-to-injury model, atherosclerosis is initiated by damage to the blood vessel wall. In an attempt to repair the damage, smooth muscle cells proliferate (Ross and Glomset, 1976) while macrophages take in excessive amounts of lipids (Ross, 1981). Lipid also fills the proliferating cells, giving them a foamy appearance (Ross and Glomset, 1976). These foam cells can be seen as yellow streaks in

the inner arterial lining. This early stage of atherogenesis is known as fatty streak formation. As the disease progresses, large amounts of lipid accumulate in the extracellular space. A fibrous cap develops as a barrier between the bloodstream and the lipid store. The lesion, now called an intermediate plaque, grows larger and thicker, eventually occluding the artery as an advanced lesion. Rupture of the plaque can lead to thrombus formation, which in turn can cause clinical manifestations such as myocardial infarction and stroke. (Grundy, 1990; Wild, 1996)

Lipid Profiles

Serum cholesterol level is a major risk factor for CVD. Results from the Framingham Heart Study showed a positive correlation between cholesterol levels and CVD risk, with a 1 % increase in the total serum cholesterol level corresponding to a 2 % increase in coronary heart disease incidence (Figure 3) (Newman *et al.*, 1986). The Multiple Risk Factor Intervention Trial (MRFIT), a follow-up survey of 361 662 men, found a strong, positive curvilinear relationship between cholesterol levels at initial screening and subsequent CHD mortality (Kannel *et al.*, 1986).

Lipids are highly insoluble in aqueous solutions. They must therefore be carried in the bloodstream bound with one or more members of a group of at least 12 specific proteins called apolipoproteins (apoproteins). These complexes of lipids and apoproteins are called lipoproteins. Some lipids, such as triglycerides, are more polar than others such as phospholipids. Cholesterol is an alcohol and is slightly water-soluble. Most of the cholesterol in the body is in the esterified form; that is, it possesses a fatty acid ester
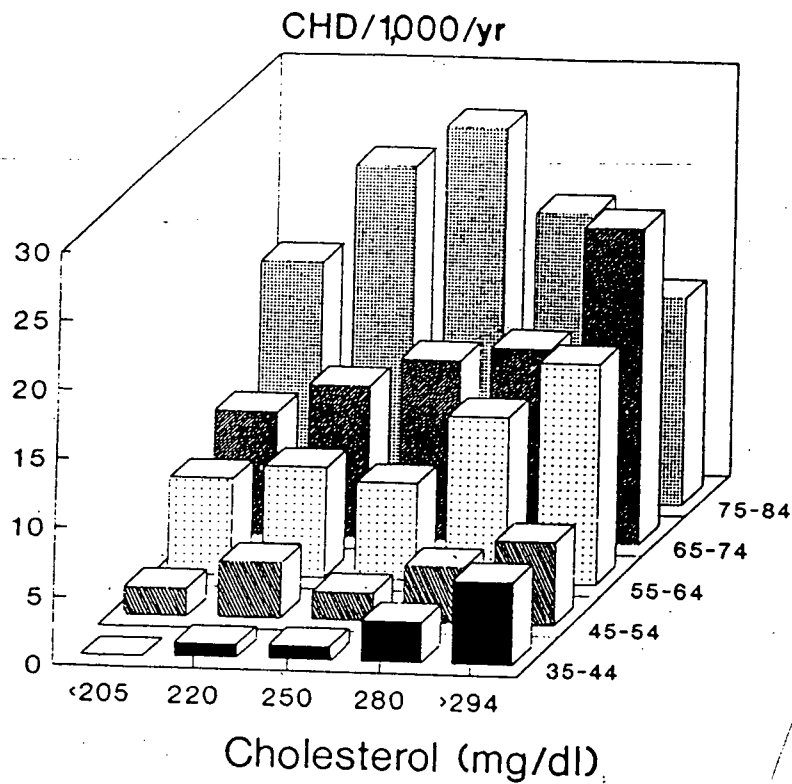
Figure 3: Annual rate of coronary heart disease (CHD) in women in relation to serum cholesterol level. (Reproduced from American Journal of Obstetrics and Gynecology 1988; 158(6).)

bond to the steroid ring structure. The addition of this chain decreases the solubility of the compound. Plasma lipoproteins consist of a hydrophobic core containing nonpolar lipids and a surface coat containing hydrophilic lipids and apolipoproteins (Figure 4) (Swartz, 1992).

All lipoproteins contain cholesterol, triglycerides and phospholipids. The density of the particles varies widely and depends on the percentage composition of the lipid and protein components (Table 2) (Swartz, 1992). Serum levels of these three types of particles seem to exert the greatest influence on the risk of CVD. They are as follows: very-low-density lipoprotein (VLDL), which primarily carries triglycerides; low-density lipoprotein (LDL), which consists mainly of cholesterol; and high-density lipoprotein (HDL), which in involved in transport of cholesterol to the liver for excretion via the bile duct. Both VLDL and LDL accumulate in the serum and increase the risk of CVD. HDL allows excretion of cholesterol and thus reduces the risk of CVD (Grundy, 1990).

The liver synthesizes triglycerides both as an energy source for peripheral tissues and for storage in adipose tissues. VLDLs carry triglycerides to the peripheral tissues where they undergo stepwise degradation. During this process, the triglycerides are stripped out of the VDLDs by lipase enzymes found on the inside surface of the capillary endothelium. Some of the remnants are taken up and catabolized by the liver; the remainder undergoes further remodeling to form LDLs (Swartz, 1992).

LDLs, carrying mostly cholesterol, are taken up by peripheral cells and hepatocytes through a high-affinity pathway involving the $apoB_{100}$ receptor. The cholesterol is then used for membrane and steroid hormone synthesis. Other cells, such
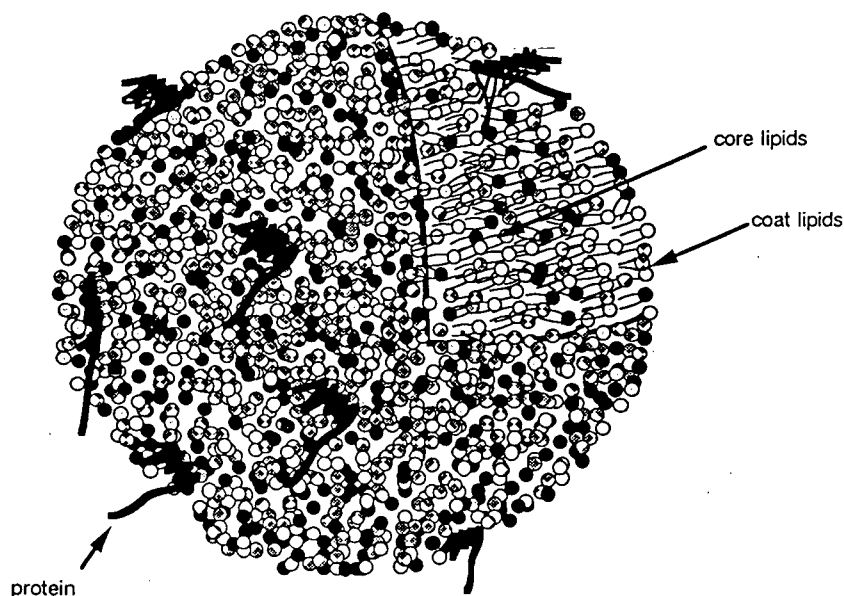
Figure 4: Schematic diagram of a lipoprotein structure. Cutaway view of the lipoprotein reveals protein and polar lipids forming a surface coat that covers a core of nonpolar lipids. (Reproduced from Hormone Replacement Therapy 1992.)

Table 2: Composition of Human Plasma Lipoproteins. (Reproduced from Hormone Replacement Therapy 1992.)

Lipoprotein	Density Range (g/ml)	% Composition (by weight) *				Apolipoproteins
		Prot	Chol	Trig	PL	
Chylomicrons	<1.006	1-1	1-4	86-94	3-8	C, B, AI
VLDL	<1.006	5-10	16-22	55-65	12-18	B, C, E
LDL	1.006-1.063	20-24	40-50	8-12	20-25	B
HDL	1.063-1.21	45-50	17-22	3-6	20-30	AI, AII, C, E

* Prot = protein; Chol = cholesterol; Trig = triglycerides; PL = phospholipids

as macrophages can also catabolize LDLs. Estrogens lower plasma LDL-C levels by increasing the number of $apoB_{100}$ receptors in tissues, especially the liver. This increases LDL catabolism and clearance from the plasma (Figure 5) (Letterie *et al.*, 1988).

LDL can filter through the vascular endothelium and accumulate in the intimal layer of blood vessels. Superoxide secreted by macrophages can oxidize the entrapped LDL. These oxidized LDL particles have many atherogenic properties (Table 3) (Steinbrecher, 1991)).

Both the liver and the small intestine synthesize nascent HDL, disc-shaped particles that are precursors for HDL. Excess cholesterol in the peripheral tissues is accumulated into the center of the nascent HDL, forming a lipid core. As the amount of cholesterol increases, the disc-shaped particles take on a more spherical shape. The smallest spherical form of HDL is named HDL_3. HDL_3 continues to accumulate cholesterol, forming HDL_2 particles. These latter particles can then be taken up by the liver and catabolized by hepatic lipase (Eisenberg, 1984). Thus, HDL is important in removing excess cholesterol from the body. Estrogen can increase the hepatic secretion of apoAI, a major protein of HDL, and thereby increase plasma levels of HDL. Higher levels of plasma HDL is thought to be beneficial in reducing the risks of CVD (Figure 6) (Gordon *et al.*, 1977). Estrogen also inhibits hepatic lipase, an enzyme which converts HDL_2 to HDL_3 (Letterie *et al.*, 1988). This increases HDL_2 level, which is important because HDL_2 has been most strongly associated with a decreased risk of coronary atherosclerosis (Nabulsi *et al.*, 1993).
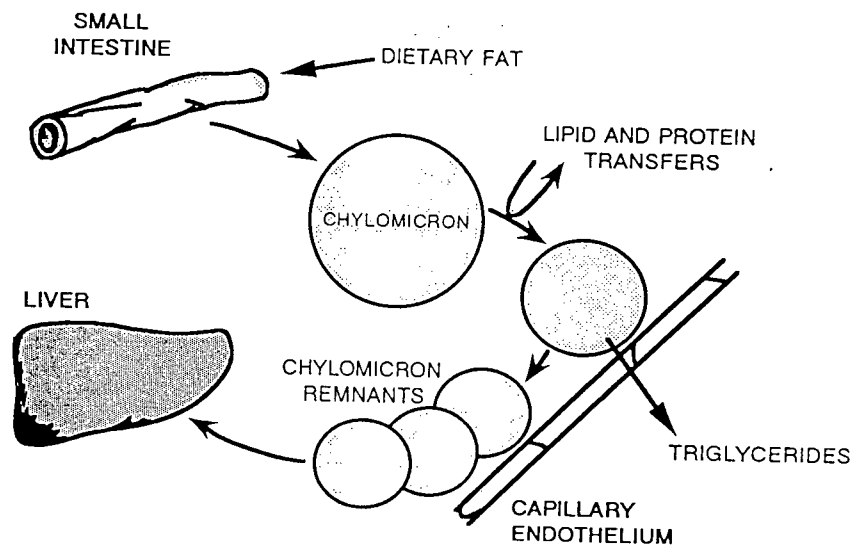
Figure 5: Overview of VLDL and LDL metabolism. VLDLs carry triglycerides to the peripheral tissues. The VLDL remnants are either taken up by the liver or converted to LDLs. LDLs interact with high-affinity binding sites on peripheral cells and on hepatocytes where they are taken up and metabolized. LDLs can also be catabolized by other cells such as macrophages. (Reproduced from Hormone Replacement Therapy 1992.)

Table 3: Potentially Atherogenic Properties of Oxidized Low-Density Lipoprotein (Reproduced from Obstetrics and Gynecology, 47(2).)

Cytotoxic to endothelial cells and smooth-muscle cells
Causes cholesterol accumulation in macrophages
Chemotactic for macrophages
Inhibits macrophage migration
Increases adhesiveness of monocytes and endothelial cells
Alters production of growth factors and inflammatory mediators
Alters prostaglandin production
Promotes platelet aggregation
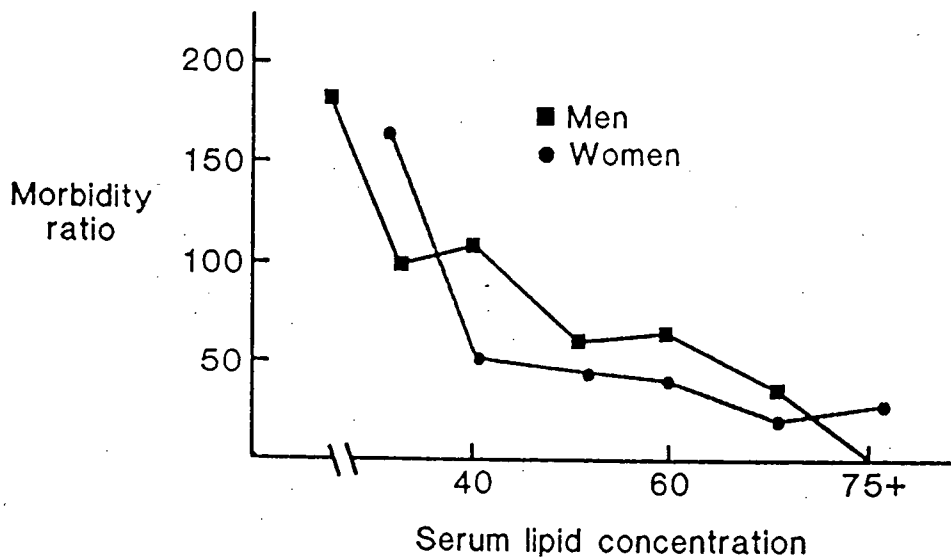
Figure 6: Risk of coronary heart disease in relation to HDL levels in men and women. (From the Framingham Heart Study) (Reproduced from American Journal of Obstetrics and Gynecology 1988; 158(6).)

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The beneficial effects of estrogen on lipid profile account for 30-50% of estrogen's cardioprotective effect. Before menopause, women generally have a more favorable lipid profile than men of similar age; that is, they have lower plasma levels of LDL and VLDL cholesterol, and higher levels of HDL cholesterol, especially HDL_2 (Wilson et al., 1988). With the onset of menopause, lowered levels of estrogen cause decreased catabolism of LDL and decreased production of HDL (Walsh et al., 1991). The increased LDL transports more cholesterol to the walls of arteries, while decreased HDL allows less cholesterol to be drawn away from the walls. This type of lipid profile has been strongly associated with increased incidence of coronary atherosclerosis (Kannel et al., 1971).

Using isotope-labeled cholesterol, it has been shown that women receiving estrogens can catabolize LDL and produce HDL to a greater degree than those receiving placebo. The administration of unopposed estrogen at a dosage of 0.625mg/day decreased LDL cholesterol by 12-19% and increased HDL cholesterol by 9-13% (Sarrel, 1990).

Increased levels of lipoprotein(a) have also been implicated in CVD. Lipoprotein (a) (Lp(a)) is a complex composed of a LDL particle joined by a disulfide bond to a plasminogen analogue. The plasminogen analogue acts as an antagonist in the reaction that converts plasminogen to plasmin. Plasmin is a proteolytic enzyme that breaks down the fibrin polymer found in clots into soluble fragments. Thus, Lp(a) may be atherogenic in two ways: it carries cholesterol, and it interferes with normal clot lysis (Heinrich et al., 1991). Lp(a) is a predictor of CVD risk in both men and women, but there is no evidence

indicating that lowering Lp(a) levels will decrease the risk. Estrogen has been found to reduce Lp(a) levels (Lobo *et al.*, 1992).

Vascular Effects

Abnormal coronary vasoactivity has been implicated in the generation of myocardial ischemia. Estrogen seems to possess beneficial effects on vascular tone and reactivity. Acute administration of sublingual 17β-estradiol in postmenopausal women caused an increase in blood flow and a decrease in vascular resistance in the forearm, while causing no change in mean arterial pressure (Volteranni *et al.*, 1995). In patients with atherosclerotic coronary vascular disease, administration of acetylcholine induced constriction in the coronary arteries and caused a decrease in blood flow. In healthy coronary arteries, acetylcholine induces relaxation. Intravenous infusion of 17β-estradiol reversed this effect, allowing dilation of the blood vessels and increased blood flow (Collins and Sarrel, 1995). Treatment with 17β-estradiol for 22 weeks in normotensive postmenopausal women caused a decrease in the pulsatility index of the carotid artery, which is a measure of impedence to blood flow (Ganger *et al.*, 1991).

Many possible mechanisms have been proposed to explain the vascular effects of estrogen, including modulation of autonomic neurotransmission, direct effects on smooth muscle cells and alterations in endothelial function.

Modulation of Autonomic Neurotransmision

Cardiovascular function is controlled by both the sympathetic and parasympathetic nervous systems. Abnormalities in the autonomic nervous system may increase the risk of CVD and trigger clinical events.

Over 90% of sudden cardiac deaths are due to ventricular tachyarrhythmias. In studies with anaesthetised rats subjected to coronary artery occlusion, male rats showed a higher incidence of ventricular tachycardia and fibrillation than female rats (Siegmund *et al.*, 1979). Of the subjects in the Framingham study, there was a lower number of women who died of sudden cardiac death (Kannel and Schatzkin, 1985).

There is much evidence to suggest that sympathetic hyperactivity triggers cardiac arrhythmias and that augmented parasympathetic tone exerts protective, antifibrillatory effects (Schwartz *et al.*, 1992; Hohnloser *et al.*, 1994). Thus, it is likely that estrogen modulation of autonomic function plays a role in reducing the risk of sudden cardiac death in women.

Sympathetic Nervous System

When subjected to stress, men experienced a greater increase in plasma concentrations of noradrenaline (NA) than women, although basal levels of NA are not different (Lenders *et al.*, 1987). Administration of clonidine, a α_2-adrenoceptor agonist, decreased plasma NA more in premenopausal women than men (-70% compared to –35%) (Del Rio *et al.*, 1993). Postmenopausal women had both an increased basal level of plasma NA and a greater stress-induced increase compared to premenopausal women. These differences were abolished, however, by 6 weeks treatment with estrogen

(Lindheim *et al.*, 1992). A single physiological dose of 17β-estradiol reduced the sympathetic response in men during mental stress, who showed decreased heart rates and systolic blood pressure (Del Rio *et al.*, 1994).

Parasympathetic Nervous System

Estrogen promotes cholinergic activity by increasing production of acetylcholine. The activity of choline acetyltransferase and the level of acetylcholine are higher in females than males (Kaufman *et al.*, 1988). These decrease after ovariectomy (Egozi *et al.*, 1982), and increase again following estrogen treatment (Kaufman *et al.*, 1988). Estrogen also enhances the high-affinity uptake of choline (O'Malley *et al.*, 1987).

Effects On Vascular Smooth Muscle

High concentrations of estrogen (10^{-7}-10^{-5} M) can induce relaxation in isolated rabbit coronary arteries denuded of endothelium. N^{ω}-nitro-L-arginine (L-NAME, 10^{-4} M), an inhibitor of endothelium-derived relaxing factor (EDRF) production did not affect 17β-estradiol-induced relaxation in rabbit coronary arteries, nor did methylene blue (10^{-5}M), an inhibitor of guanosine cyclase. These results indicate that the 17β-estradiol-induced relaxation was independent of EDRF (Jiang *et al.*, 1991).

In vitro, supraphysiological concentrations (0.1 – 10 μM) of 17β-estradiol were found to attenuate agonist-induced contractions in various smooth muscle types. 17β-estradiol reversibly inhibited the development of high K^+- or agonist (phenylephrine)-induced contractions in the endothelium-denuded rat femoral artery and portal vein smooth muscle male (Kitazawa, 1997). It also caused relaxation in the high K^+- or

phenylephrine- precontracted vessels (Kitazawa, 1997). There was no difference in the dose-response curve of 17β-estradiol-induced relaxation between femoral arteries taken from male and female rats, suggesting that there are no gender differences in the sensitivity to the acute response to estrogen. This indicates that no differences exist in the smooth muscle cells between the female and the male rats with regard to their responses to acute application of estrogen (Kitazawa, 1997). The acute inhibitory effects of 17β-estradiol on vascular smooth muscle have been suggested to be due to the inhibition of voltage-dependent Ca^{2+} channels though a pertussis toxin-sensitive GTP-binding protein (Ogata, 1996).

Estrogen was also found to relax isolated human coronary arteries. Atherosclerosis-free male and female human epicardial arteries were removed from patients undergoing heart, or combined heart and lung, transplantation. 17β-estradiol was found to cause relaxation of the arteries precontracted with U46619, a thromboxane A_2 analog. This relaxation was greater in vessels from female patients. There were no significant differences in the response between arteries with or without endothelium. Neither N^Gmonomethyl-L-arginine (a nitric oxide synthase inhibitor) nor indomethacin (a cyclo-oxygenase inhibitor) affected the estrogen-induced relaxation. These results show that not only does 17β-estradiol induce relaxation in human coronary arteries, but that the sensitivity of the vessels to 17β-estradiol is greatly affected by gender (Chester *et al.*, 1995). Gender did not affect 17β-estradiol-induced relaxation in endothelium-denuded rat femoral artery (Kitazawa, 1997). These seemingly contracting results may be due to the use of different animals and tissues.

Effects on Vascular Endothelium

Coronary vasomotor tone is regulated primarily by the arterial endothelial cells, a monolayer of cells lining the inner surface of the entire circulatory system. The endothelium produces a number of substances that affect vascular tone, including vasodilators such as endothelium-derived relaxing factor (EDRF) and prostaglandin, and vasoconstrictors such as endothelin. EDRF is one of the most important vasoactive molecules released by the endothelium (Collins, 1996).

Furchgott and Zawadzki first described EDRF in 1980. Acetylcholine stimulation of the endothelium induced the release an unstable substance that caused relaxation of isolated aortic rings (Furchgott and Zawadski, 1980). This substance, termed EDRF, was later identified as nitric oxide (NO) (Palmer *et al.*, 1987). NO is synthesized in a nitric oxide synthase (NOS)-catalyzed reaction that converts L-arginine into L-citrulline (Palmer *et al.*, 1988). This is a complex oxidation-reduction reaction, requiring oxygen and NADPH, calmodulin, as well as other cofactors (FAD, FMN, heme, and tetrahydrobiopterin) (Marletta, 1994; Nathan and Xie, 1994).

Under normal physiological conditions, NO is released from the endothelium upon NOS activation by receptors (Mombouli and Vanhoutte, 1992) or by shear stress on the blood vessel wall (Uematsu *et al.*, 1995). NO diffuses freely through the endothelium to inhibit platelet aggregation and adhesion (Radomski *et al.*, 1987), and to cause vasorelaxation (Figure 7). NO has also been shown to inhibit smooth muscle proliferation (Dubey and Overbeck, 1994). Intimal lesions were created by intravascular balloon injury in rat carotid arteries *in vivo*. This caused damage to the endothelium and loss of NOS activity. Gene therapy with eNOS (endothelial NOS) restored NOS

expression and NO release in the injured vessel. There was also a significant reduction in neointimal proliferation as compared with control (Von Der Leyen *et al.*, 1995). Thus, it is very likely that endothelial dysfunction, with the consequent reduction in NO release, plays a part in atheroma formation.
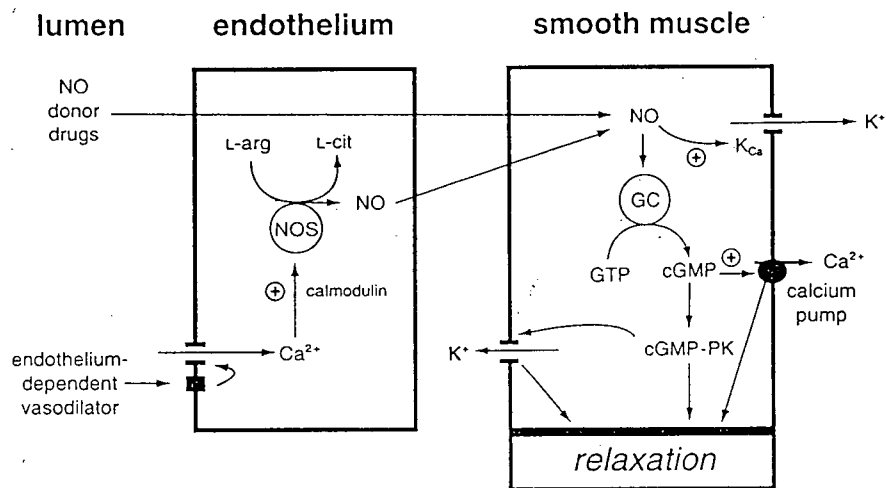
Figure 7: A simplified schematic diagram showing the mechanism of action of endothelium-dependent vasodilators and NO donor drugs on vascular smooth muscle. L-arg = L-arginine; L-cit = L-citrulline; GC = guanylate cyclase; cGMP = guanosine 3'5' cyclic monophosphate; cGMP-PK = cGMP-dependent protein kinase; GTP = guanosine triphosphate; NOS = nitric oxide synthase. (Reproduced from Trends in Pharmacological Sciences 1996; 16.)

A positive correlation between plasma 17β-estradiol levels and levels of stable metabolites of NO (nitrate/nitrite) was found in women (Roselli, 1994). Estrogen treatment in both ovariectomized monkeys (Williams *et al.*, 1994a) and postmenopausal women (Gilligan *et al.*, 1994) enhanced endothelium-dependent coronary artery vasodilation. *In vitro* studies of rabbit femoral arteries (Gisclard *et al.*, 1988) and rat thoracic aorta (Williams *et al.*, 1988) from animals with elevated estrogen levels have also revealed enhanced endothelium-dependent vasorelaxation. This effect was also seen with acute exposure of porcine left circumflex coronary arteries to estrogen (Bell *et al.*, 1995).

Estrogen appears to enhance the basal release of NO. Inhibition of basal NO release produced a greater increase in tension in partially contracted aortic segment from female rabbits compared with male or ovariectomized animals. There were no significant differences in relaxation in response to acetylcholine, indicating that the stimulated release of NO is unaffected by estrogen (Hayashi *et al.*, 1992). Ovariectomized female rats treated chronically (five weeks) with 17β-estradiol showed increases in both the basal and the agonist-stimulated NO release as compared with control rats (Rahimian *et al.*, 1997a).

Direct Antiatherogenic Effects

Estrogen also has direct effects on the vascular wall that may contribute to its antiatherogenic effects. Estrogens are antioxidants and can inhibit the oxidation of LDL (Keaney *et al.*, 1994). Other antiatherogenic effects include inhibition of lipoprotein-induced smooth-muscle proliferation and foam cell formation (Cheng *et al.*, 1991).

ESTROGEN REPLACEMENT THERAPY

Although estrogen replacement therapy (ERT) has been shown to be beneficial for both its bone preserving and its cardioprotective effects, it has also been linked to several adverse effects. These include the changes estrogen induces in reproductive organs, producing uterine and breast cancers.

Prevention of Osteoporosis

For women entering menopause, the lifetime risk of suffering a hip fracture is about 15%, which is the combined risk of breast, uterine, and ovarian cancer (Cummings, 1987). Natural estrogen exerts a protective effect on bone by inhibiting bone resorption. The role of estrogen-deprivation in osteoporosis was established in several studies. Oophorectomy resulted in a significant reduction in bone mass (Richelson *et al.*, 1984). Estrogen therapy following oophorectomy prevented this bone loss (Lindsay *et al.*, 1977). Estrogen therapy is useful even in patients with well-established osteoporosis (Lindsay and Tohme, 1990).

Endometrial Cancer

Studies have shown that women with intact uteri who receive ERT have an increased incidence of developing endometrial cancer (Mack *et al.*, 1976; Jick *et al.*, 1979). This risk can persist for at least 5 years after discontinuation, especially in cases of long-term exposure (Brinton *et al.*, 1993). This prompted physicians to prescribe progestins along with estrogen for hormone replacement therapy. Studies show that this

combination is effective in protecting against endometrial cancer (Gambrell *et al.* 1979; Persson *et al.* 1989). The addition of progestin not only protects against the development of this type of cancer, but it can also reverse established endometrial hyperplasia to normal endometrium (Whitehead *et al.*, 1977). The risk of developing endometrial cancer in women undergoing combined progestin-estrogen therapy is less than in those taking unopposed estrogen and also in those not receiving hormone therapy. In a prospective study of more than 5500 women followed for 9 years, the incidence of endometrial cancer was 391, 49 and 246 per 100,000 for women using estrogen alone, using combination estrogen and progestin, and using no hormone, respectively (Gambrell *et al.*, 1986).

Breast Cancer

Breast cancer is the most common cancer among women and is the second leading cause of death in women in the United States (American Cancer Society, 1992). Much research has been done on the effects of ERT on the risk of breast cancer; despite this, there is still much controversy about this issue.

The Cancer and Sex Hormone (CASH) Study of the Centers for Disease Control and Prevention (CDC) reported no increased risk of breast cancer with postmenopausal estrogen use. There was also no relation between risk and the duration of estrogen treatment up to 20 years or longer (Wingo *et al.*, 1987).

The Nurses' Health Study followed 69,000 postmenopausal women for 16 years. This study revealed that present users of estrogen who had been on estrogen therapy for more than 5 years had an increased risk (1.46) of developing breast cancer. Women who

have taken estrogen for less than 5 years had a risk of 0.99, while past users had a risk of 0.80. In addition, the study found that a combined estrogen-progestin treatment did not reduce the risk of developing breast cancer (Colditz *et al.*, 1995).

In one case-control study, the results indicated that women who had used low doses of estrogen (<1.25mg/day) had a relative risk of 0.8 of developing breast cancer. Those using high doses (>1.25mg/day) had a relative risk of 1.2 (Kaufman *et al.*, 1984). This indicates an increased risk of developing breast with higher doses of estrogen. Most women now use low doses of estrogen (0.625mg/day) (Kaufman *et al.*, 1984).

The effect of estrogen on the risk of breast cancer is still unknown. Thus, many women are reluctant to use HRT, fearing an increased risk of breast cancer. However, one must keep in mind that the deaths arising from CVD far outweigh those arising from all cancers combined (Castelli, 1988).

Selective Estrogen Receptor Modulators (SERM)

Despite the fact that the effect of estrogen on the risk of breast cancer remains to be established, many women are reluctant to use HRT. Besides the effects that estrogen may have on breast cancer, HRT also has a number of other side effects. These include changes in vaginal bleeding patterns, breast tenderness, gastrointestinal symptoms (nausea, abdominal cramps), central nervous system effects (headaches, depression), weight changes, edema, changes in libido and fatigue (Gambrell *et al.*, 1989). Compliance has thus been a significant consideration with HRT (Ravnikar, 1987).

New non-estrogenic compounds are presently being investigated. These compounds, known as selective estrogen receptor modulators (SERMS), have varying

degrees of selectivity on estrogen receptors in different tissues (Kauffman and Bryant, 1995). The most selective of these are found to have the beneficial effects of estrogen on the skeletal and cardiovascular system, but to lack the adverse effects on mammary and uterine tissue. The benzothiophene LY117018 has been shown to lower serum total cholesterol and triglyceride levels and to decrease bone resorption in ovariectomized animals (Bryant *et al.*, 1995; Kauffman *et al.*, 1997). LY117018 does not have any estrogenic activity in the rat uterus (Jones *et al.*, 1984). It also antagonizes estrogen binding to the estrogen receptor (Black *et al.*, 1983) and inhibits estrogen-induced proliferation of cultured MCF-7 cells from human mammary tumor (Sato *et al.*, 1995; Wakeling *et al.*, 1985).

Estrogen, a steroid hormone, exerts its genomic effects using a pathway mediated by intracellular receptors (Korach, 1994). These receptors are generally believed to be located predominantly in or near the cell nucleus (King and Greene, 1984), with a fraction of the receptors shuttling between the cytoplasm and the nucleus (Dauvois *et al.*, 1993). The binding of estrogen to the cytoplasmic receptor is thought to cause translocation to the nucleus (Dauvois *et al.*, 1993). Prior to estrogen binding, the receptor is transiently bound to heat shock proteins (Schuh *et al.*, 1985), which may be responsible for the stability of the receptor. *In vitro* experiments show that the heat shock proteins dissociate upon estrogen binding (Catelli *et al.*, 1990). The receptor is then phosphorylated, causing a conformational change in its structure (Arnold *et al.*, 1994). This allows dimerization of the receptors (Kumar and Chambon, 1988) and exposes two zinc "fingers", cysteine-rich regions capable of binding to zinc (Parker, 1991). These zinc "fingers" promote the binding of the complex to DNA sequences known as estrogen

27

response elements (Parker and Bakker, 1991). Once bound, the estrogen-receptor complex promotes the transcription of nearby genes (Webster *et al.*, 1988).

The activated estrogen receptor has been suggested to be capable of initiating different molecular pathways to cause gene activation. This hypothesis was conjured when it was discovered that the estrogen antagonist tamoxifen appeared to have contradicting effects in breast and uterine tissues. Tamoxifen, a commonly used drug to treat or prevent breast cancer, blocks estrogen's cancer-promoting effects in breast tissue (Yang *et al.*, 1996), but mimics them in uterine tissue (Sato *et al.*, 1996), where it increases the risk of uterine cancer. LY117018 can imitate estrogen in its cardioprotective effects (Rahimian *et al.*, 1997a), but it blocks estrogen's uterotropic effects (Black *et al.*, 1983). LY117018 also inhibited estrogen-induced cell proliferation in cultured ES-1 cells, a cell line isolated from human breast cancer MCF-7 cells (Uchiumi, 1991).

Raloxifene, a close analogue of LY117018, was found to antagonize estrogen action in the breast (Jordan, 1995) while mimicking it in the bone (Frolik *et al.*, 1996). The effects of raloxifene in bone seem to be exerted through a new pathway. *In vivo*, both estrogen and raloxifene activate the gene that encodes for transforming growth factor-β3 (TGF-β3), thought to be important in bone preservation. In cultured cells, however, only raloxifene was able to activate this gene (Yang *et al.*, 1996).

The responses to estrogen, raloxifene, LY117018 and tamoxifen start when the lipid-based molecules diffuse through the cell's plasma membrane. They bind the estrogen receptor and the complex translocates into the nucleus. The estrogen-receptor complex binds to a DNA sequence called the estrogen response element (ERE) to

activate a set of target genes (Parker and Bakker, 1991; Webster *et al.*, 1988). Raloxifene does not bind to the ERE to activate the TGF-β3 gene; when the DNA-binding region of the estrogen receptor was removed, the raloxifene-receptor complex still activated the gene. The TGF-β3 gene contains a region called the raloxifene response element that is necessary for activation by raloxifene. The binding of the raloxifene-receptor complex to the gene was also found to require a yet unidentified adapter protein, as activation of the gene only occurred in the presence of cellular extracts. 17-Epiestriol, a metabolite of estrogen, also activates the gene in this fashion. This explains why estrogen can activate TGF-β3 production in living animals but not in cultured cells which do not produce 17-epiestriol (Yang *et al.*, 1996). Because LY117018 and raloxifene are very similar in structure, LY117018 is likely to promote gene transcription through the same mechanism.

Estrogen binds to another response element called the AP1 site in uterine tissue. Both estrogen and tamoxifen act through this site to cause cell proliferation. Neither raloxifene nor LY117018 act on this site, thus explaining why they do not possess the cancer-promoting effects of estrogen and tamoxifen in the uterus. Instead, they act as competitive antagonists at the estrogen receptor binding site, thereby inhibiting estrogen-induced cell proliferation (Pennisi, 1996).

ESTROGEN EFFECTS IN MALES

The effect of estrogen in men was examined in male to female transsexuals. Estrogen (ethinyl estradiol or conjugated equine estrogen) was prescribed for the males for purpose of feminization. The transsexuals showed flow-mediated vasodilatation in the brachial artery that was comparable to that in premenopausal women and greater than that in men. The transsexuals had a higher level of HDL-C and a lower level of LDL-C as compared with males. These levels were similar to those found in the premenopausal women. However, the transsexuals had a substantially higher TG level as compared with either age-matched men or premenopausal women. Thus, estrogen appears to have a beneficial effect on both lipid profiles and vascular function in males. Unfortunately, it was not possible to measure the level of serum estrogen, because the radioimmunoassay kit used for 17β-estradiol is not accurate for measuring ethinyl estradiol and conjugated equine estrogen. Also, the transsexuals had a lower level of testosterone than the males for two reasons: 1. Some of the transsexuals had had gender reassignment surgery (involving bilateral orchidectomy), and 2. Estrogen treatment suppressed the production of testosterone. Thus, it was not possible to be certain about the relative contribution of the increase in estrogen levels and the decrease in testosterone levels in producing the observed changes in vascular function (New, 1997).

HYPOTHESIS

Estrogen-receptor activation by chronic treatment with LY117018 or 17β-estradiol potentiates nitric oxide (NO) release in a time- and dose-dependent manner in both male and female Sprague-Dawley (SD) rats.

OBJECTIVES

1. To determine if chronic treatment with LY117018 in female ovariectomized S.D. rats enhances basal or stimulated NO release in a time-dependent manner.

2. To determine if chronic treatment with LY117018 in female ovariectomized S.D. rats enhances basal or stimulated NO release in a dose-dependent manner.

3. To determine if chronic treatment with LY117018 or 17β-estradiol in male S.D. rats enhances basal or stimulated NO release.

SPECIFIC AIMS

1. The basal NO release will be characterized by measuring the L-NAME potentiation of phenylephrine contractions in isolated aortic rings from rats treated with LY117018 or 17β-estradiol.

2. The stimulated NO release will be characterized by determining the amount of acetylcholine-relaxation in phenylephrine-precontracted aortic rings from rats treated with LY117018 or 17β-estradiol.

3. The time-dependent effects of LY117018 on basal and stimulated NO release will be determined by treatment of ovariectomized SD rats for increasing time periods.

4. The dose-dependent effects of LY117018 on basal and stimulated NO release will be determined by treatment of ovariectomized SD rats using increasing doses of LY117018.

5. The effects of chronic 17β-estradiol and LY117018 treatment in the male rat on basal and stimulated NO release will be characterized.

MATERIALS & METHODS

LY117018 EFFECTS ON AORTIC FUNCTION IN THE FEMALE OVARIECTOMIZED RAT

LY117018 Time-Dependent Effects

Fifty mature cycling female Sprague-Dawley rats (250-300g) were used in this study (Charles River Laboratories, Quebec, Canada). Ovariectomy was performed on the rats at Charles River Laboratories. Rats were shipped seven days after the surgery to allow for their recovery. Ten days after ovariectomy, the animals were divided into ten groups of five rats each. These were treated with either placebo (vehicle only) or with LY117018 (1.0mg/kg) in vehicle for treatment periods that ranged from one to five weeks (Table 4). LY117018 was dissolved in a hydroxypropyl-β-cyclodextrin vehicle. Daily oral administration of the drug or vehicle was performed by gavage.

Table 4: Protocol for determining the time-dependent effects of LY117018 on endothelial function in ovariectomized rats. Rats were orally treated with either 1.0mg/kg LY117108 in hydroxypropyl-β-cyclodextrin vehicle (Treated) or vehicle only (Control) for treatment periods ranging between 1 to 5 weeks, inclusive. Each group consisted of 5 female ovariectomized Sprague-Dawley rats.

Treatment Period (Weeks)	Number of rats	
	Control	Treated
1	5	5
2	5	5
3	5	5
4	5	5
5	5	5

LY117018 Dose-Dependent Effects

Mature cycling female ovariectomized Sprague-Dawley rats (250-300g) were used in this study (Charles River Laboratories, Quebec, Canada). Ten days after ovariectomy, the fifty rats were divided into ten groups. Group 1 was treated with 1.0 mL/kg water. Groups 2 to 10 were treated with LY117018 (dissolved in hydroxypropyl-β-cyclodextrin vehicle) using doses ranging from 0 to 6.0 mg/kg (Table 5). The drugs were administered daily by oral gavage and the treatment period was three weeks.

Table 5: Protocol for determining the dose-dependent effects of LY117018 on endothelial function in ovariectomized rats. Rats were treated for three weeks with water, vehicle (hydroxypropyl-β-cyclodextrin) only, or varying doses of LY117018 in vehicle. Each group consisted of 5 female ovariectomized Sprague-Dawley rats.

Group	Type of treatment	Dose of LY117018
1	water	0
2	vehicle	0
3	LY	0.20
4	LY	0.50
5	LY	0.75
6	LY	1.0
7	LY	1.25
8	LY	3.0
9	LY	5.0
10	LY	6.0

LY117018 AND 17β-ESTRADIOL EFFECTS ON AORTIC FUNCTION IN THE MALE RAT

Effects of Oral Administration of LY117018 and Estrogen in the Male Rat

Fifteen male Sprague-Dawley rats (250-300g) were assigned to three treatment groups. Each group was treated for three weeks with one of the following: placebo (hydroxypropyl-β-cyclodextrin vehicle), 1.0mg/kg LY117018 in hydroxypropyl-β–cyclodextrin vehicle, or 0.1mg/kg 17β-estradiol in hydroxypropyl-β-cyclodextrin vehicle. The rats were treated daily with oral administration via gavage.

Dose-Dependent Effects of Estrogen in the Male Rat

Eighteen male Sprague-Dawley rats (250–300g) were assigned to three different treatment groups, each receiving a different dose of 17β-estradiol. An estrogen pellet was implanted subcutaneously on the back of each rat, where it remained for five weeks until the rats were sacrificed. The pellets were constructed to release 0.5mg, 1.5mg or 5.0mg of 17β-estradiol over 60 days.

EXPERIMENTAL PROTOCOL

Preparation of Aortic Rings

At the end of each treatment period, rats were sacrificed by a lethal dose of pentobarbital (65mg/kg, i.p.) after an intravenous injection of heparin (4000units/kg, i.p.). Blood was extracted from the abdomenal vena cavae and frozen at -70°C for later analysis of plasma 17β-estradiol levels. The rats were exsanguinated by cutting through both carotid arteries. The thoracic aorta was dissected and placed into ice-cold modified Krebs' buffer of composition 119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 0.023 mM EDTA, 11.1 mM D-glucose and 1.6 mM $CaCl_2$. The fatty tissue and connective tissue surrounding the aorta was removed and the aorta was cut into 2-4 mm long rings. The rings of aorta were hung horizontally between two stainless steel hooks. The bottom hook was anchored while the top hook was connected to a force transducer for measurement of isometric tension. The tissues were bathed in individual baths in 5 ml Krebs' buffer at 37°C and aerated with 95% O_2/ 5% CO_2. The rings were allowed to equilibrate for 45 min under a resting tension of 1 g to allow development of a stable basal tone. The tissues were then stimulated with 80mM K^+ until stable, reproducible contractions were obtained.

Response to Acetylcholine

The rings of aorta were first precontracted with phenylephrine (2μM), to give roughly 80% of the maximal effect (EC_{80}). Dilation responses to acetylcholine (10^{-5} M)

were then obtained. The tissues were washed with Krebs' buffer for 30 min to allow the return to basal tone.

Potentiation of Phenylephrine Contractions by L-NAME

A cumulative concentration-response curve to phenylephrine (PE, 10^{-8} to 10^{-5} M) was obtained. The rings were then washed with modified Krebs' buffer for roughly 30 minutes until tension returned to basal levels. The tissues were then incubated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 200μM) for 30 minutes. The concentration-response curve to PE was then repeated. Contraction was measured as a percentage of the maximum tension elicited by PE before L-NAME incubation.

CHEMICAL REAGENTS AND DRUGS

Acetylcholine chloride (ACh) acts on muscarinic receptors to stimulate nitric oxide production from the vascular endothelium. L-NAME is an inhibitor of nitric oxide synthase. L-phenylephrine hydrochloride (PE) is an adrenergic drug that causes contraction in vascular smooth muscle. The above mentioned drugs were purchased from Sigma Chemical Co. (St. Louis, MO, US). An aqueous solution of hydroxypropyl-β-cyclodextrin was used both as the placebo and as a vehicle for LY117018 and 17β-estradiol. The compound was obtained from Aldrich Chemical Co. (Milwaukee, WI, US). LY117018 is a benzothiophene and a selective estrogen receptor modulator (Figure 8). This compound was obtained from Eli Lilly Co. The 17β-estradiol pellets were purchased from Innovative Research of America (Toledo, OH, US) (Figure 8).

DATA ANALYSIS

Values are expressed as means $\pm$ standard error of means. The Student's t-test for unpaired values, the one-way analysis of variance (ANOVA) (repeated measures where appropriate) and multiple comparison (Dunnett's test) were used to identify differences among the groups. A probability value of less than 0.05 ($P<0.05$) was considered to be significant.

A
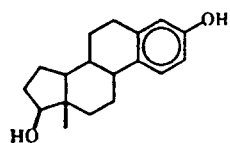
B
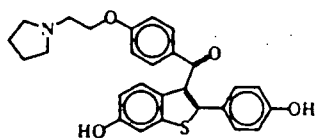
Figure 8: Structure of 17β-estradiol (A) and LY117018 (B). (Reproduced from Biology of Reproduction 1996; 53.)

RESULTS

LY117018 EFFECTS ON AORTIC FUNCTION IN THE FEMALE OVARIECTOMIZED RAT

Basal Nitric Oxide Synthesis and Release

Chronic treatment with oral LY117018 augmented basal NO release in the ovariectomized rat. This was indicated by the greater L-NAME potentiation of PE-induced contraction in the treated rats as compared with controls (Figures 9-13). L-NAME-induced contractions were also greater in treated than control animals (Figure 14). However, this difference was not statistically significant (P<0.05, Student's t-test).

Receptor-Mediated Release of NO

Chronic treatment of ovariectomized rats with LY117018 enhanced endothelium-dependent relaxation to ACh (10^{-5} M) in PE-precontracted aortic rings (Figures 15-19). This enhancement of ACh-induced relaxation was statistically significant (P<0.05, Student's t-test) with LY117018 treatment for 2, 3 and 5 weeks.

A.
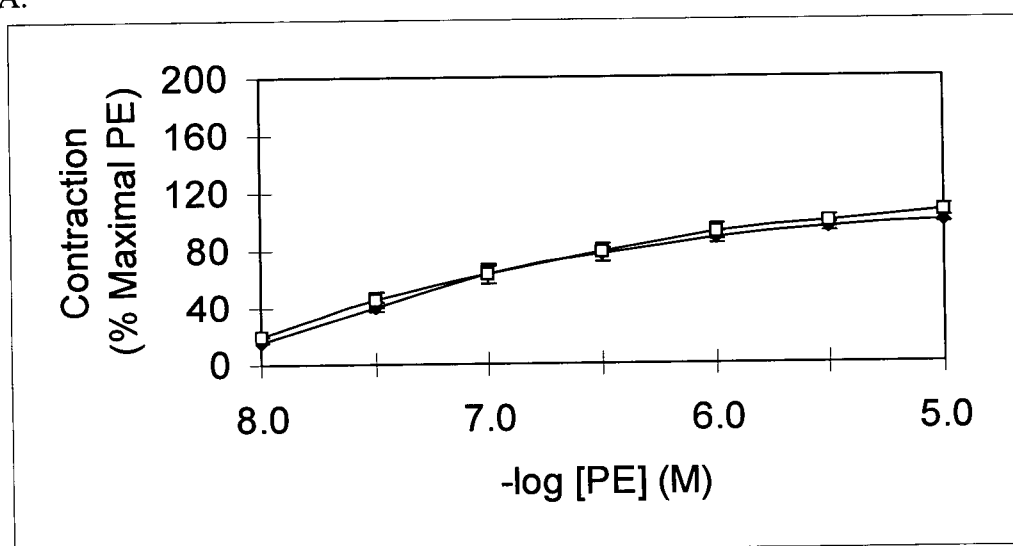
B.

Figure 9: Concentration-response curves to PE in the thoracic aorta of ovariectomized rats treated with (A) placebo or with (B) LY117018 for 1 week both before (♦) and after (□) L-NAME pretreatment (200µM). Results are expressed as a percentage of the maximum response to PE (10µM) before L-NAME treatment. The results represent the mean ± S.E.M. of twenty tissues from five rats. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).

A.

B.

Figure 10: Concentration-response curves to PE in the thoracic aorta of ovariectomized rats treated with (A) placebo or with (B) LY117018 for 2 weeks both before (♦) and after (□) L-NAME pretreatment (200µM). Results are expressed as a percentage of the maximum response to PE (10µM) before L-NAME treatment. The results represent the mean ± S.E.M. of twenty tissues from five rats. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).

A.
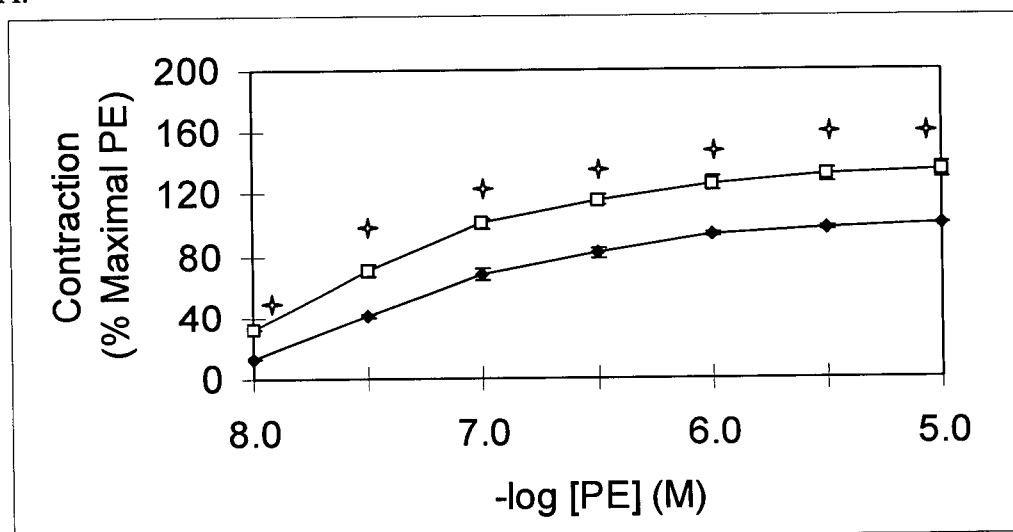
B.
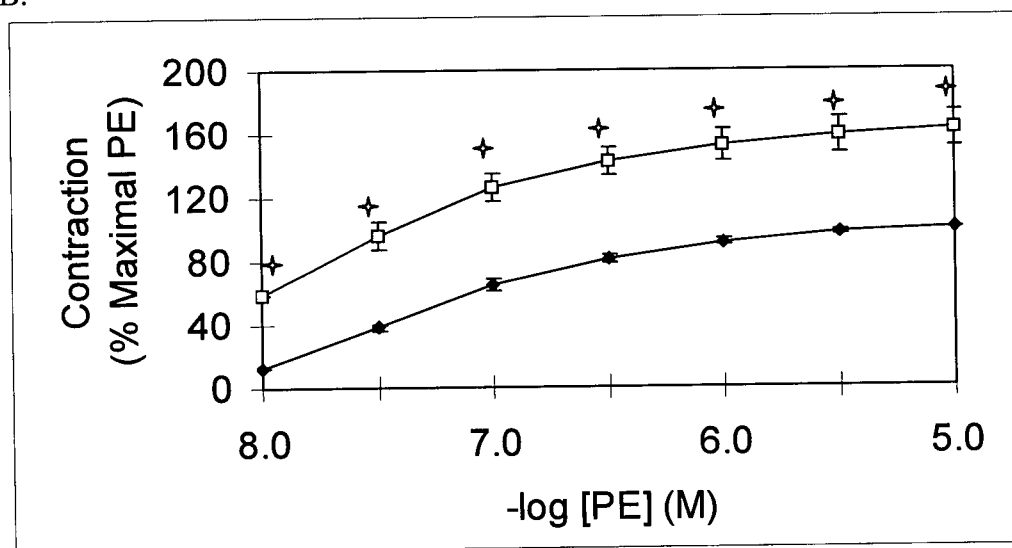
Figure 11: Concentration-response curves to PE in the thoracic aorta of ovariectomized rats treated with (A) placebo or with (B) LY117018 for 3 weeks both before (♦) and after (□) L-NAME pretreatment (200μM). Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of twenty tissues from five rats. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
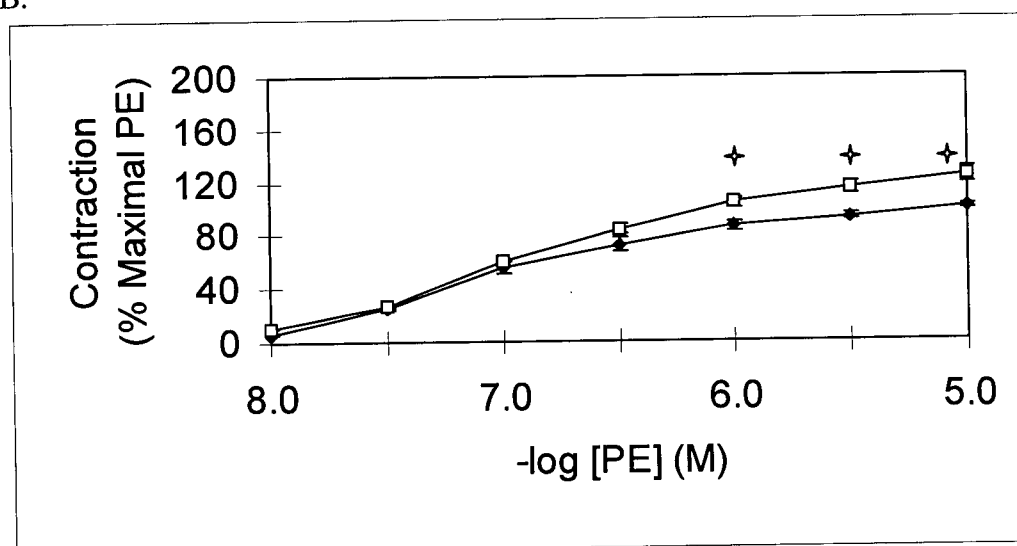
A.
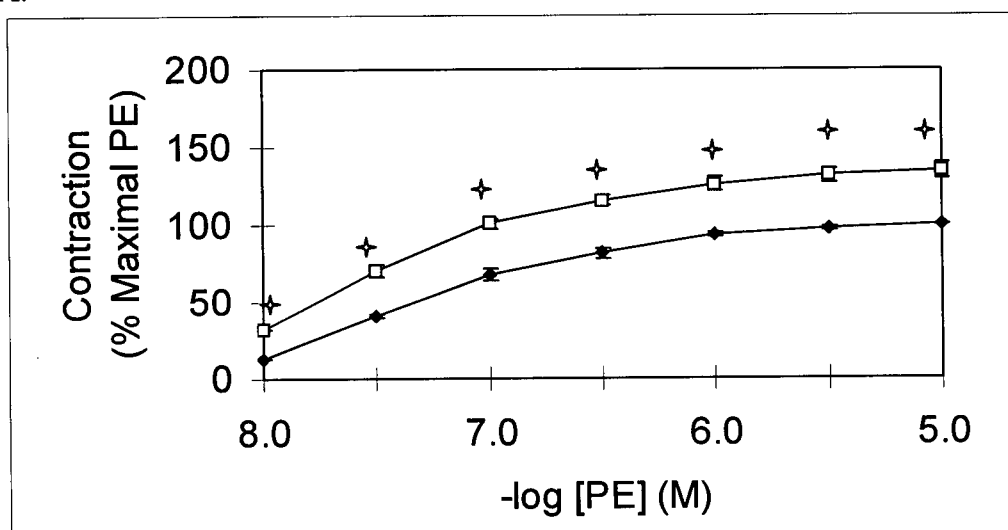
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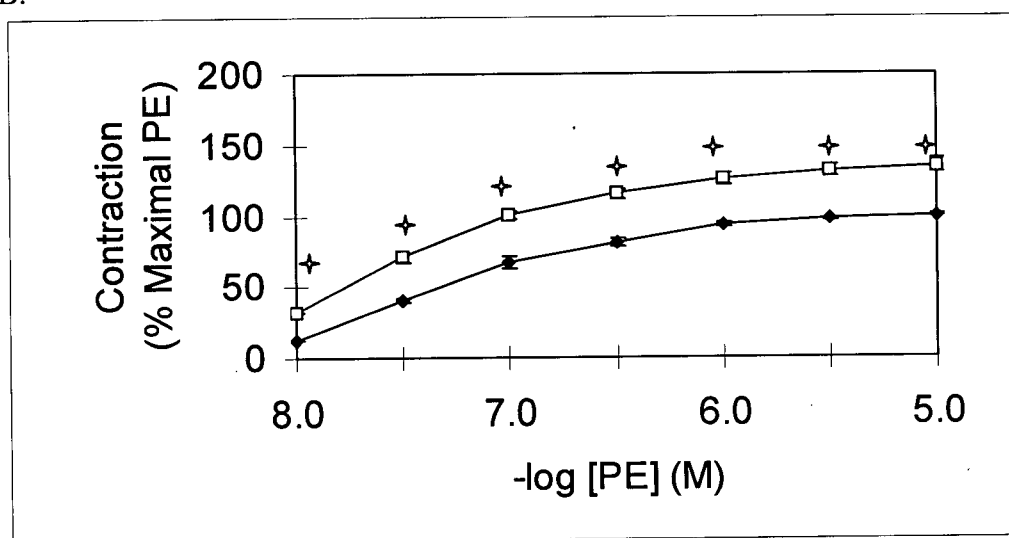
Figure 12: Concentration-response curves to PE in the thoracic aorta of ovariectomized rats treated with (A) placebo or with (B) LY117018 for 4 weeks both before (♦) and after (□) L-NAME pretreatment (200μM). Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of twenty tissues from five rats. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
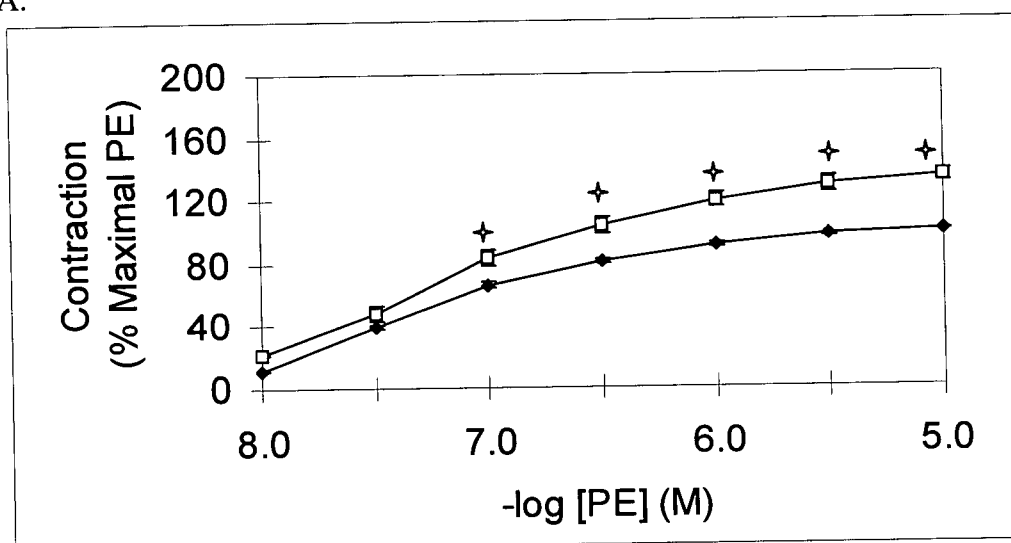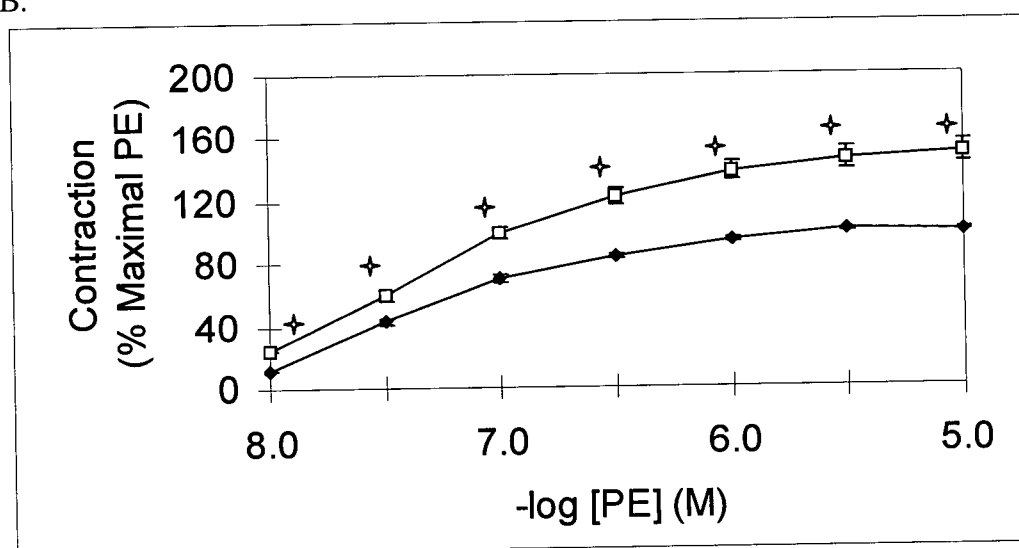
A.
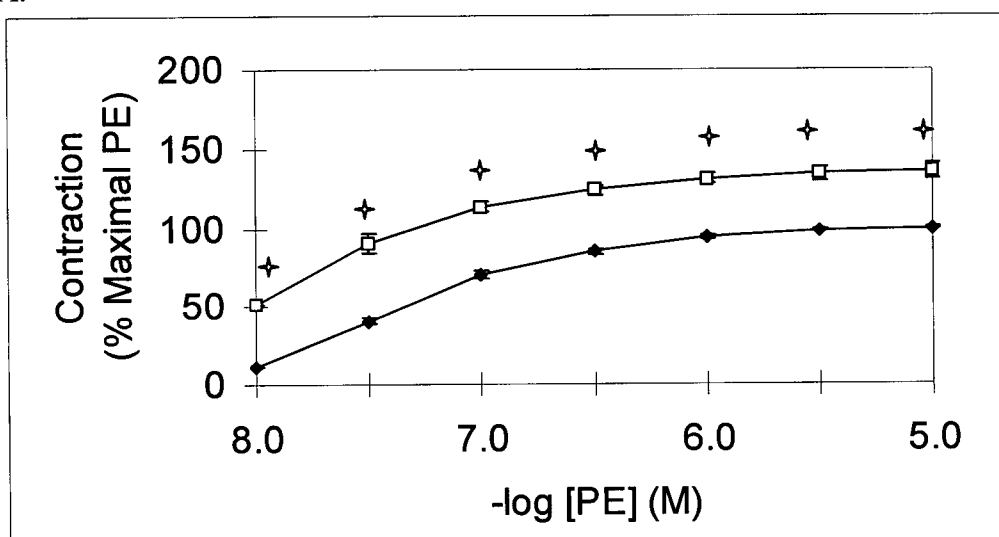
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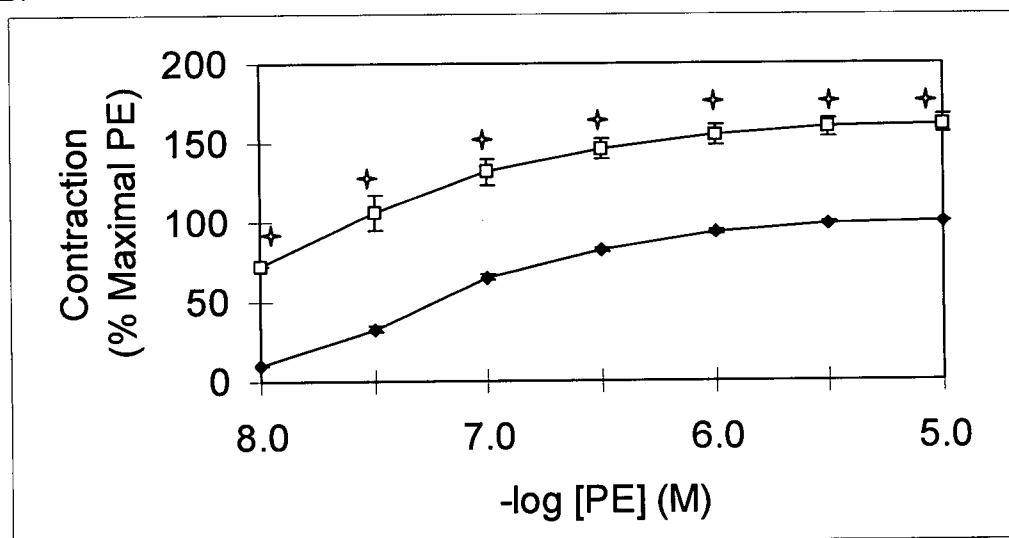
Figure 13: Concentration-response curves to PE in the thoracic aorta of ovariectomized rats treated with (A) placebo or with (B) LY117018 for 5 weeks both before (◆) and after (□) L-NAME pretreatment (200μM). Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of twenty tissues from five rats. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
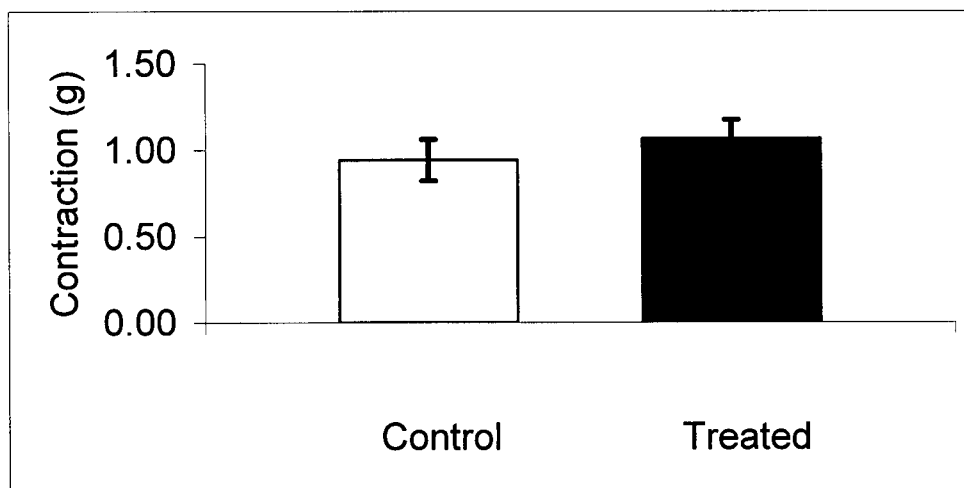
Figure 14: L-NAME-induced contraction in control and LY117018-treated (1.0mg/kg for five weeks) ovariectomized Sprague-Dawley rats. The results represent the mean ± S.E.M. of twenty tissues from five rats.
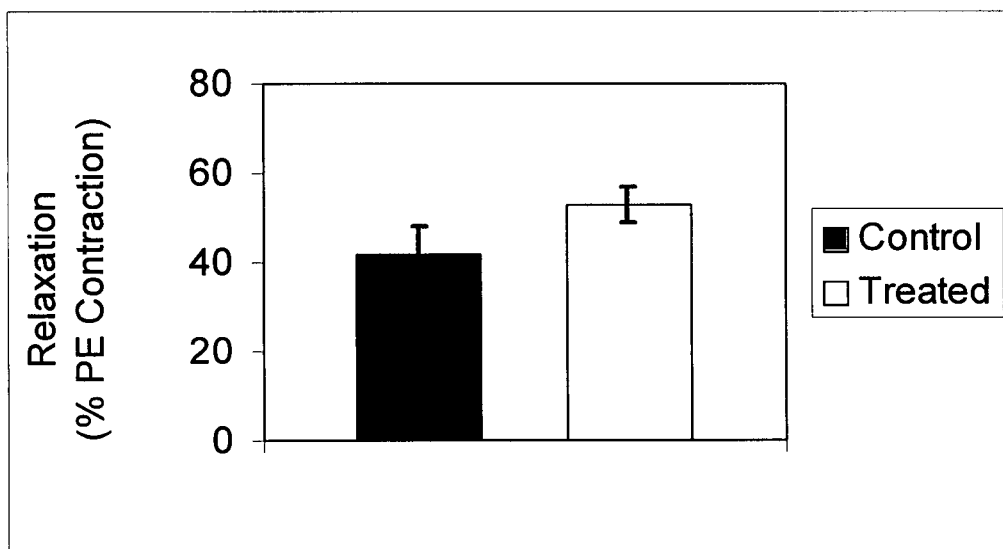
Figure 15: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day for one week) and control ovariectomized Sprague-Dawley rats. The data are expressed as percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of twenty tissues from five rats in each group.
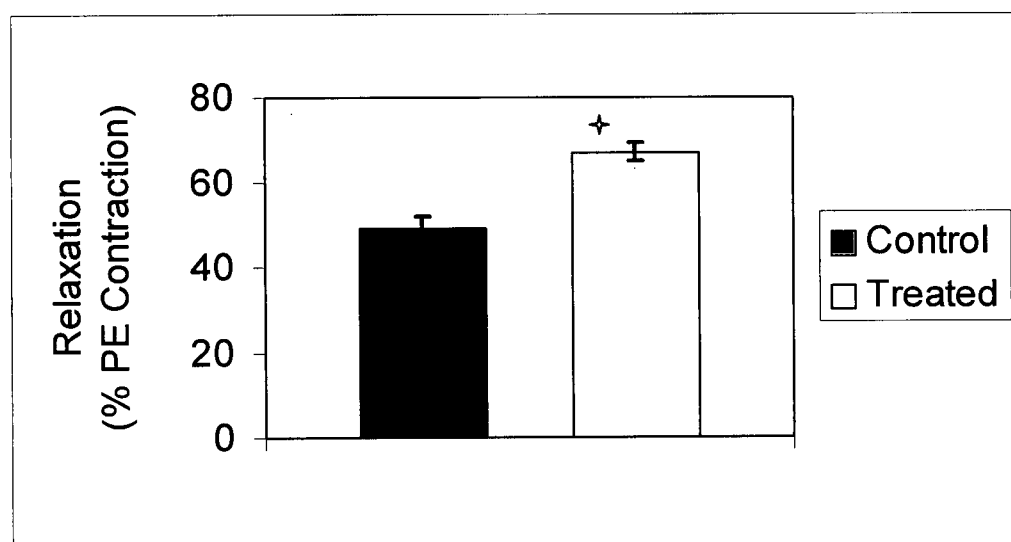
Figure 16: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day for two weeks) and control ovariectomized Sprague-Dawley rats. The data are expressed as a percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of of twenty tissues from five rats in each group. ◇ Significantly different (P<0.05) from the control group by two-sample t-test.
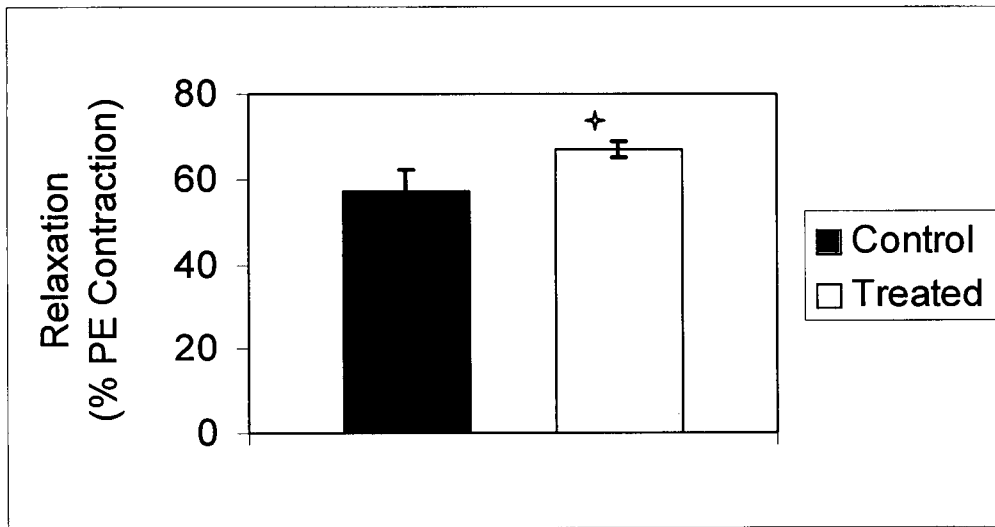
Figure 17: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day for three weeks) and control ovariectomized Sprague-Dawley rats. The data are expressed as a percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of of twenty tissues from five rats in each group. ◇ Significantly different (P<0.05) from the control group by two-sample t-test.
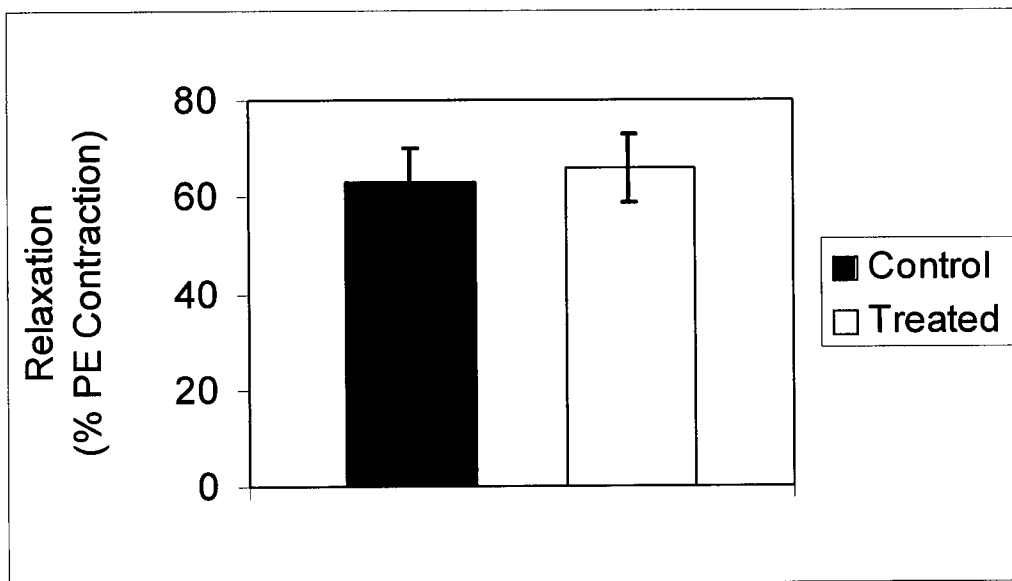
Figure 18: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day for four weeks) and control ovariectomized Sprague-Dawley rats. The data are expressed as a percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of twenty tissues from five rats in each group.
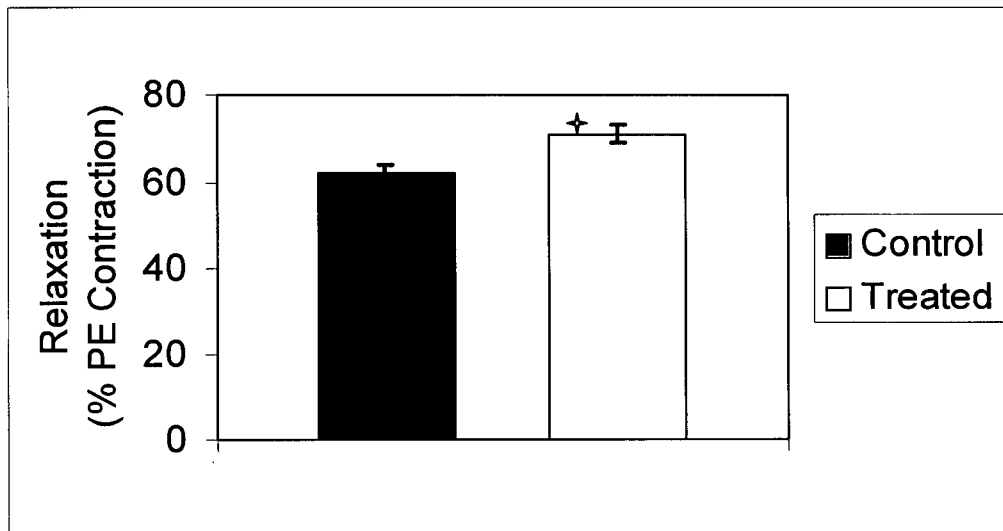
Figure 19: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day for five weeks) and control ovariectomized Sprague-Dawley rats. The data are expressed as a percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of twenty tissues from five rats in each group. ◇ Significantly different (P<0.05) from the control group by two-sample t-test.

Smooth Muscle Contractility

Neither the PE-induced (after L-NAME; Figure 20) nor the high K^+-induced (Figure 21) contractions differed between the treated and control groups. Thus, the contractility of the smooth muscle was not affected by LY117018 treatment. The sensitivity of α-adrenoceptors was not significantly affected by chronic LY117018 treatment either before or after inhibition of NOS. This was indicated by the similarities in PE EC_{50} values in the aortae from LY117018-treated and untreated ovariectomized rats (Figure 22). The PE contractions normalized to the maximal high K^+ contractions did not differ among groups (Figure 23).

LY117018 Time-Dependent Effects

To determine the time-dependent effects of chronic LY117108 treatment on basal NO release in the aortic endothelium, PE concentration-response curves were generated both before and after pretreatment with L-NAME. Since L-NAME is a NOS inhibitor, the potentiation of PE contractions after L-NAME incubation is indicative of the amount of basal NO production in the tissue. For each treatment period (1 to 5 weeks), the amount of potentiation was greater in treated rats than in controls. Treatment with LY117018 increased basal NO release; thus, L-NAME potentiation of PE contractions was greater in treated than in control rats. This effect increased with increasing time of LY117018 treatment reaching a maximum level at three weeks and was not further augmented by longer treatment periods (Figure 24). The LY117018-induced increase in basal NO was statistically significant after three and five weeks of treatment ($P<0.05$, Student's t-test).
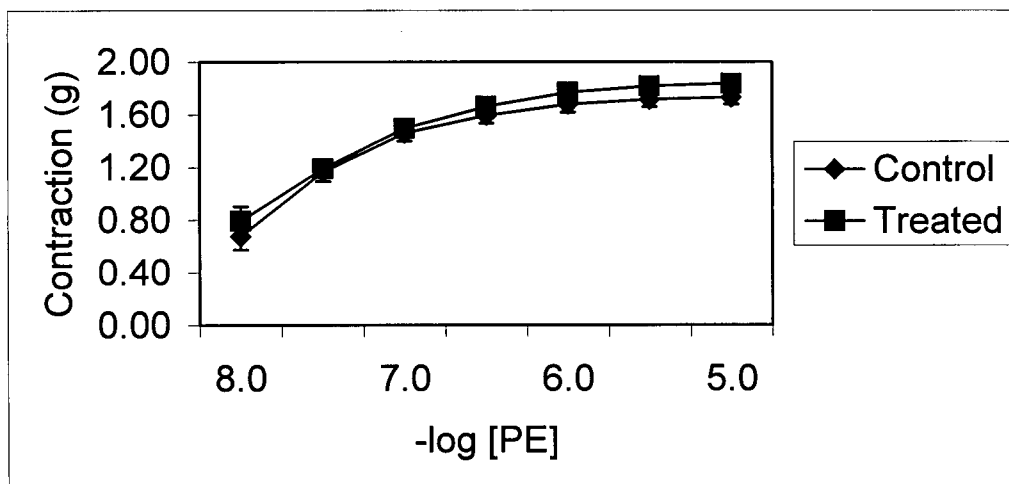
Figure 20: Concentration-response curves to phenylephrine in the thoracic aorta of control and LY117018-treated (1.0mg/kg for five weeks) ovariectomized Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The results are shown as the mean ± S.E.M. of twenty tissues from five rats in each group.
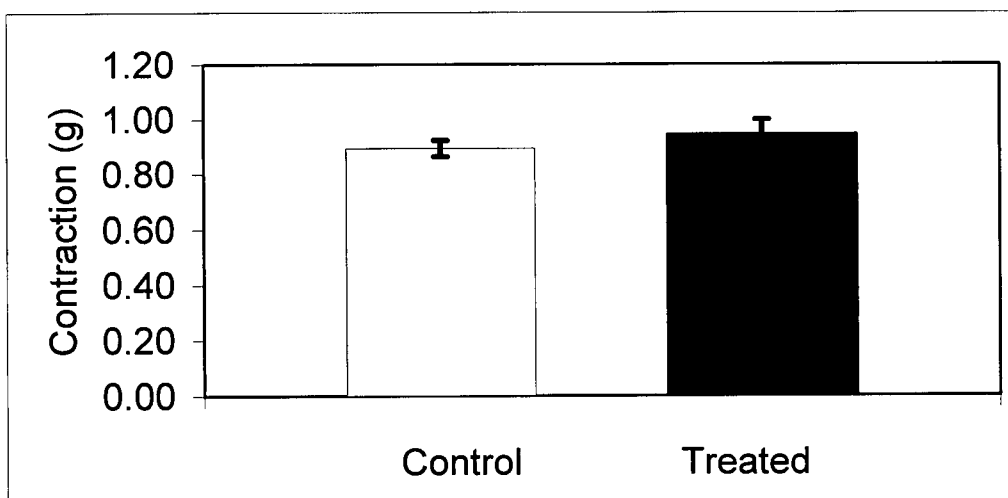
Figure 21: High K^+-induced (80mM) contractions in the thoracic aorta of control and LY117018-treated (1.0mg/kg for five weeks) ovariectomized Sprague-Dawley rats. The results are shown as the mean ± S.E.M. of twenty tissues from five rats in each group.

A.
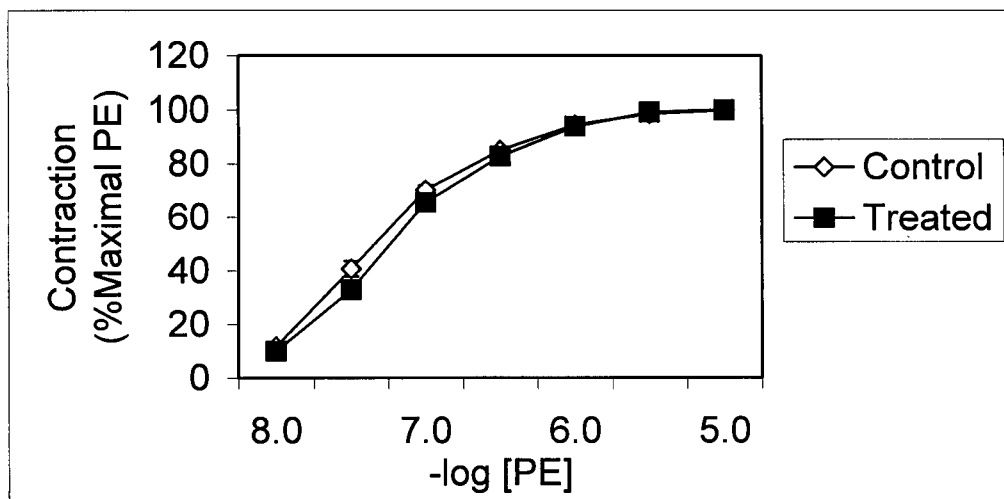
B.
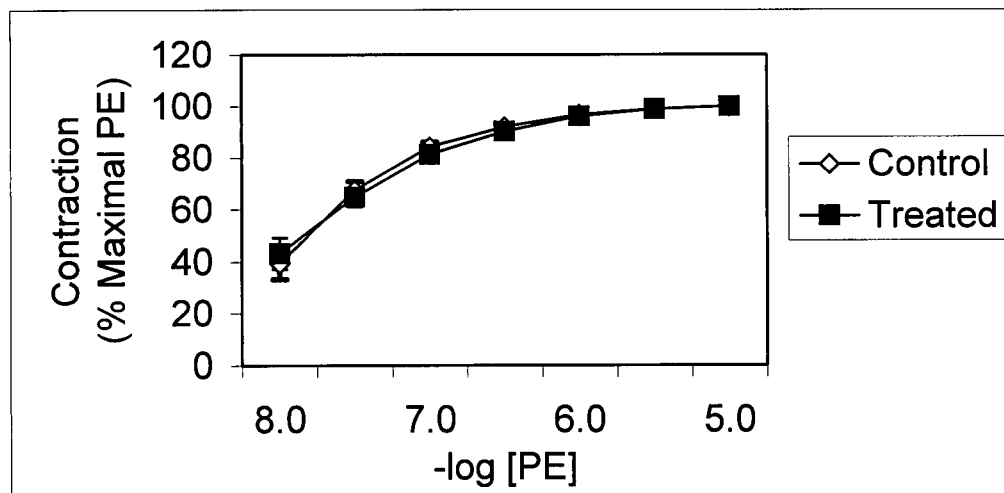
Figure 22: Concentration-response curves to phenylephrine in the thoracic aorta of control and LY117018-treated (1.0mg/kg/day for five weeks) ovariectomized Sprague-Dawley rats. The contractions were generated A) before and B) after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to PE (10μM) after L-NAME. The results are shown as the mean ± S.E.M. of twenty tissues from five rats in each group. The sensitivity to PE was not significantly different (P>0.05, ANOVA) between the groups of tissues.
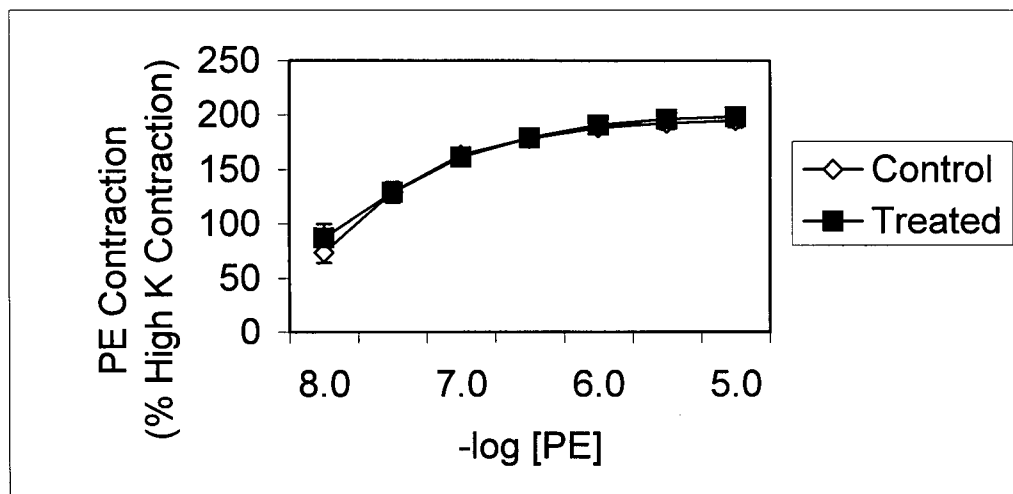
Figure 23: Concentration-response curves to phenylephrine in the thoracic aorta of control and LY117018-treated (1.0mg/kg for five weeks) ovariectomized Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to high K^+ (80mM). The results are shown as the mean ± S.E.M. of twenty tissues from five rats in each group.
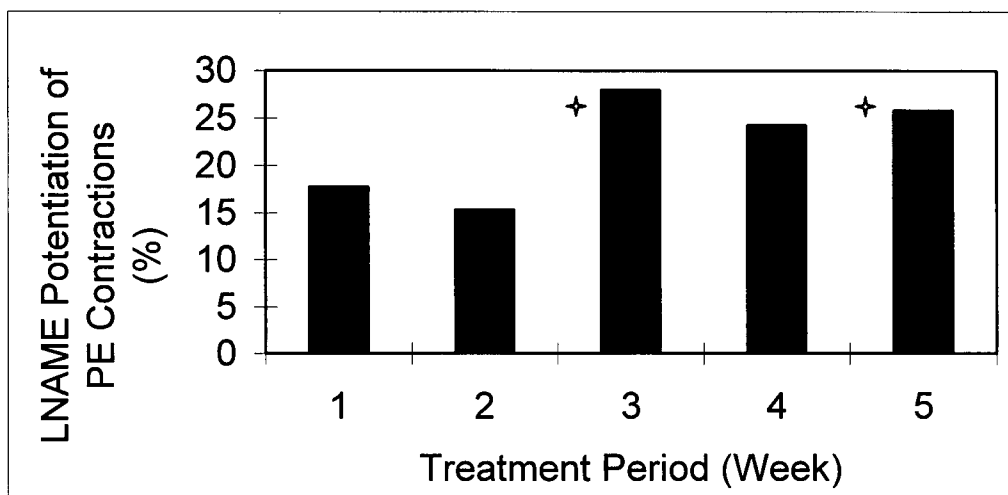
Figure 24: LY117018 time-dependent effects on the L-NAME potentiation of the maximal response to PE (10μM). Each column represents that the difference in L-NAME potentiation between treated and control rats. ◇ Significantly different (P<0.05) from the control group (of the same treatment period) by ANOVA and multiple comparison.

LY117018 potentiated ACh-induced relaxations in PE-precontracted aortic rings for all treatment periods. This increase of ACh-induced relaxation was significant after two, three and five weeks of LY117018 treatment. The effect of LY117018 on ACh relaxation did not vary with length of treatment (Figure 25).

LY117018 Dose-Dependent Effects

L-NAME potentiation of PE contraction was also used as an indicator of the dose-dependent effects of LY117018. NO production in the rats treated with water or with LY117018 doses between 0.25 to 0.75 mg/kg for a period of three weeks were not statistically different from control. Between 0.75 mg/ kg and 1.0 mg/kg, however, there was a sharp increase in the response to LY117018. At 1.0 mg/kg, the effect reached a maximum, a level at which it remained for LY117018 doses of 3.0 and 5.0 mg/kg. With 6.0mg/kg of LY117018, the effects of LY117018 on NO production diminished (Figure 26).

LY117018 potentiated ACh-induced relaxations in PE-precontracted aortic rings in a dose-dependent manner (Figure 27). This increase of ACh-induced relaxation was significant using LY117018 doses of 3.0 and 6.0mg/kg.
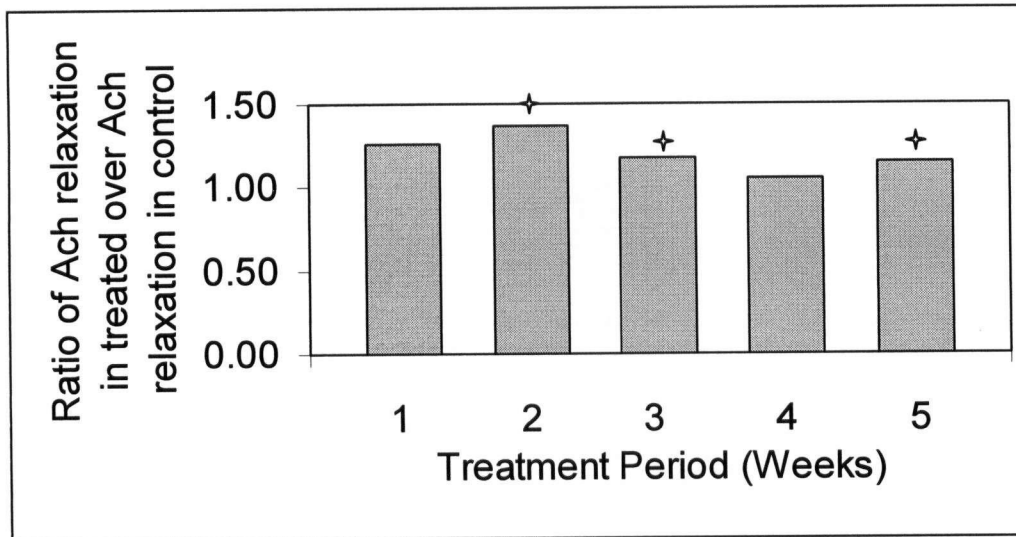
Figure 25: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (1.0mg/kg/day) ovariectomized Sprague-Dawley rats compared to that from control for varying treatment periods. The data are expressed as a ratio of ACh relaxation in treated animals over ACh relaxation in control animals. ✧ Significantly different (P<0.05) from the control group (of the same treatment period) by ANOVA and multiple comparison.
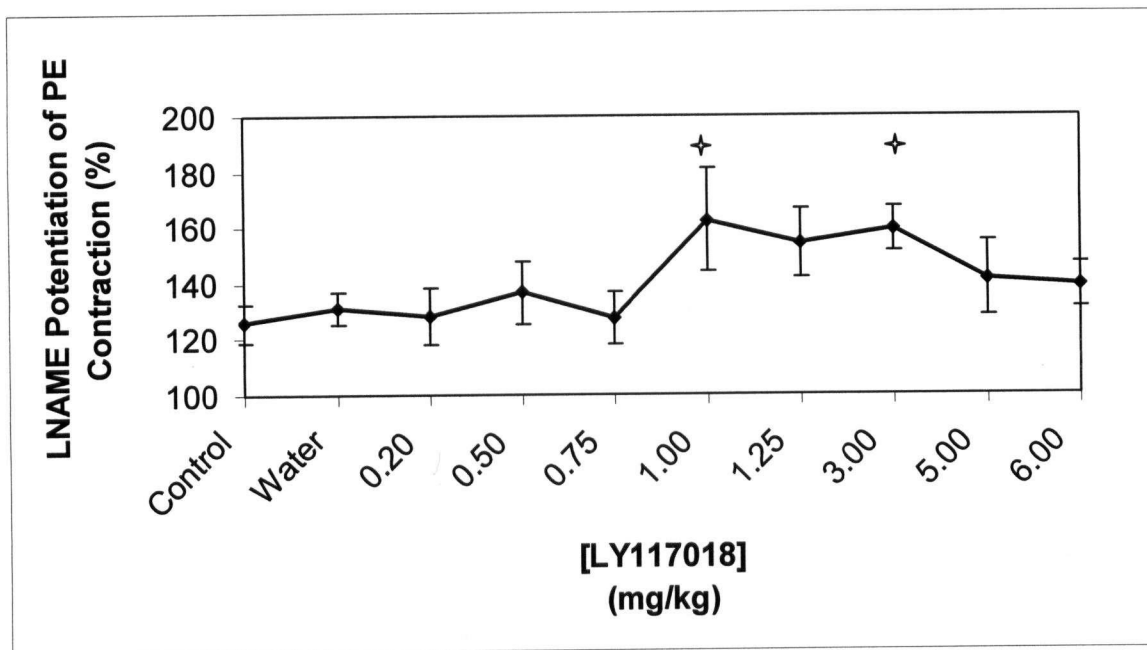
Figure 26: LY117018 dose-dependent effects on the L-NAME potentiation of the maximal response to PE (20μM). Each point represents the maximal contraction elicited by PE after L-NAME pretreatment normalized to the maximal PE contraction before L-NAME. ✧ Significantly different (P<0.05) from the control group by ANOVA and multiple comparisons.

Figure 27: Acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings from LY117018-treated (0.2 – 6.0 mg/kg/day for three weeks), water-treated (WA), and vehicle-treated (VE) ovariectomized Sprague-Dawley rats. The data are expressed as the percentage of inhibition of PE (10^{-6} M) contraction. ◇ Significantly different (P<0.05) from the control group by ANOVA.

LY117018 AND 17β-ESTRADIOL EFFECTS ON AORTIC FUNCTION IN THE MALE RAT

Nitric Oxide Synthesis and Release

The effects of LY117018 and estrogen on NO production in the thoracic aorta of male rats were then investigated. The addition of L-NAME potentiated the PE contraction of all groups (Figures 28-30). Neither LY117018 (1.0mg/kg) nor 17β-estradiol (0.1mg/kg) treatment for three weeks caused any significant differences in the level of basal (Figure 31) or ACh-stimulated NO release (Figure 32).

Subcutaneous implantation of 17β-estradiol pellets (0.5, 1.5 and 5.0mg 17β-estradiol/pellet) for five weeks caused a dose-dependent increase in NO basal release in the male rat (Figures 33-36). 17β-Estradiol treatment did not enhance ACh-induced relaxation in the male rat aorta (Figure 37).

Smooth Muscle Contractility

Neither 17β-estradiol nor LY117018 treatment altered high K^+-induced contraction (Figure 38) or maximal PE-induced contraction in the male rat aorta (Figure 39). Increasing the dose of 17β-estradiol did not significantly alter the maximal contractions to high K^+ (Figures 40, 41) or PE (Figure 42). Chronic treatment with LY117018 or 17β-estradiol increased the sensitivity of α-adrenoceptors both before and after inhibition of NOS (Figure 43). The PE EC_{50} values in the aortae from LY117018-treated and 17β-estradiol-treated rats were significantly lower than controls. The PE-contraction/high K^+-contraction ratio was not significantly different between groups at
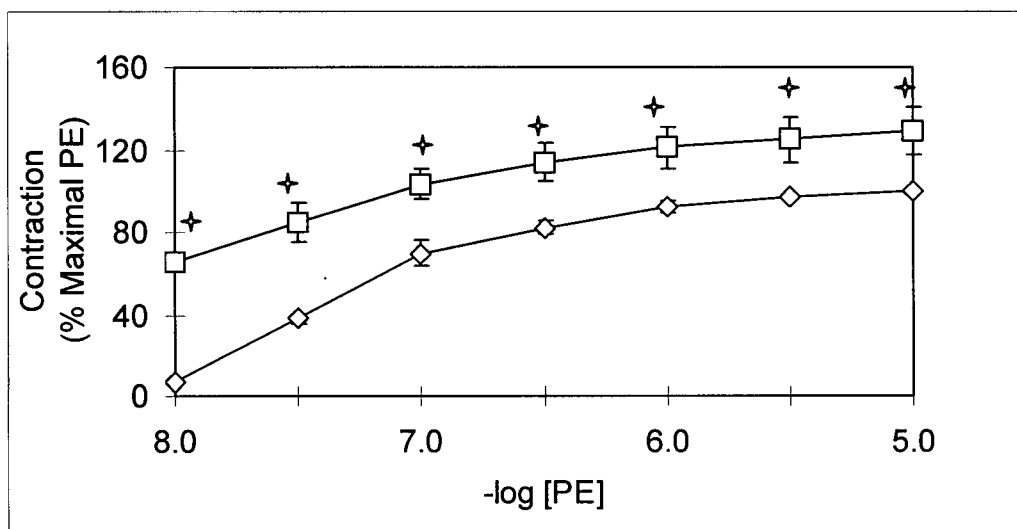
Figure 28: The concentration-response curves to PE in the thoracic aorta before (◊) and after (□) L-NAME treatment in male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ◊ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
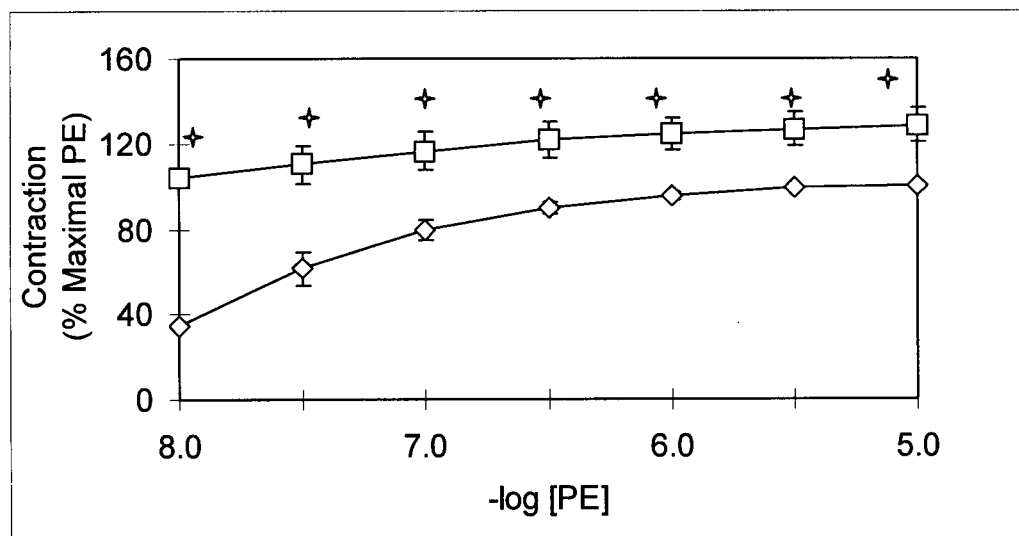
Figure 29: Effect of estrogen on basal NO production in the male rat. The graph shows the concentration-response curves to PE in the thoracic aorta before (◊) and after (□) L-NAME treatment in 17β-estradiol-treated (0.1mg/kg/day for three weeks) male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ◊ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
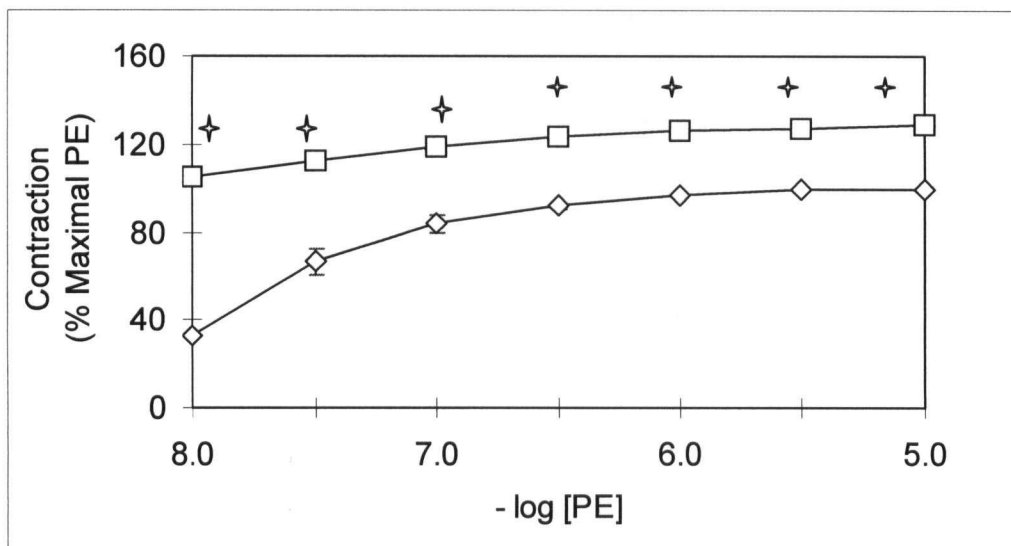
Figure 30: Effect LY117018 on basal NO production in the male rat. The graph shows the concentration response curves to PE in the thoracic aorta before (◊) and after (□) L-NAME treatment in LY117018-treated (1.0mg/kg/day for three weeks) male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ◇ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
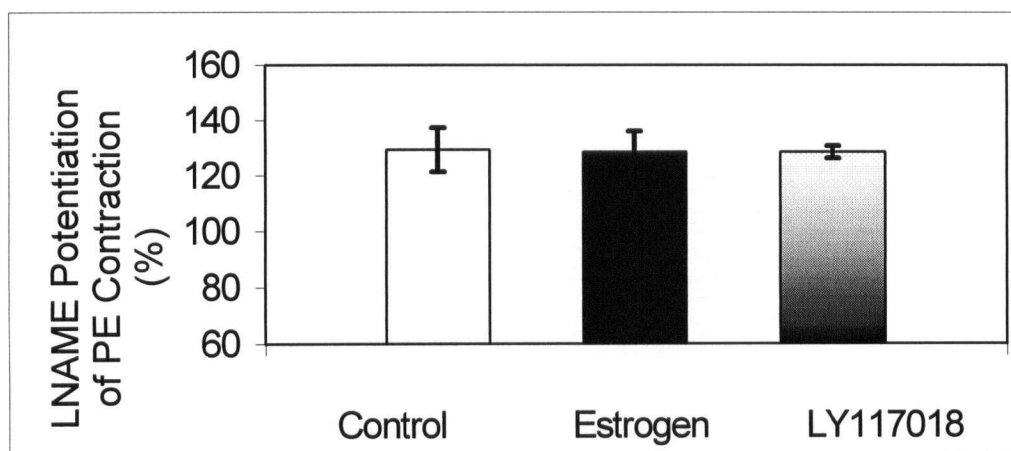
Figure 31: Effect of estrogen and LY117018 on basal NO production in the male rat. Each bar represents the L-NAME (200μM) potentiation of phenylephrine-induced (20μM) contraction in control, estrogen-treated (0.1mg/kg/day for 3 weeks) or LY117018-treated (1.0mg/kg/day for 3 weeks) male Sprague-Dawley rats. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group.

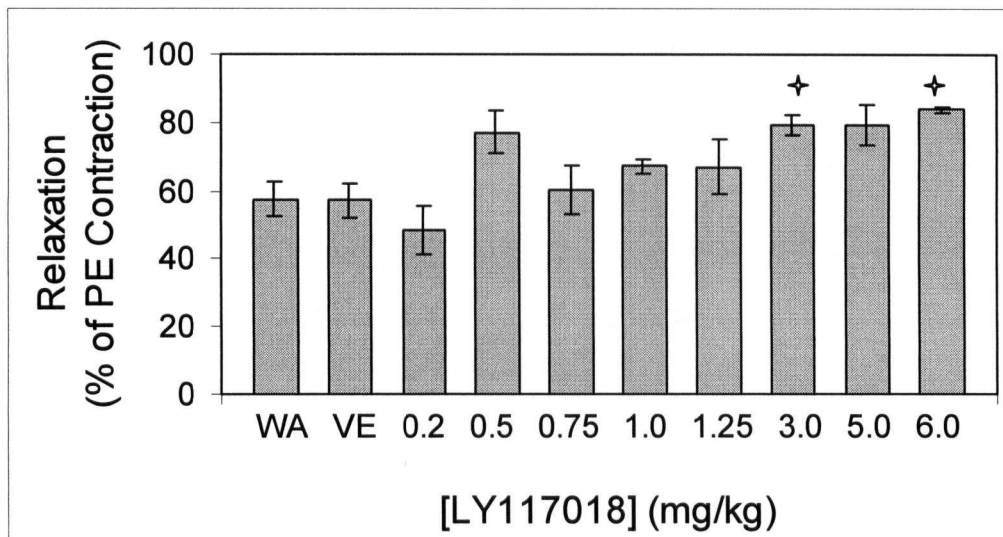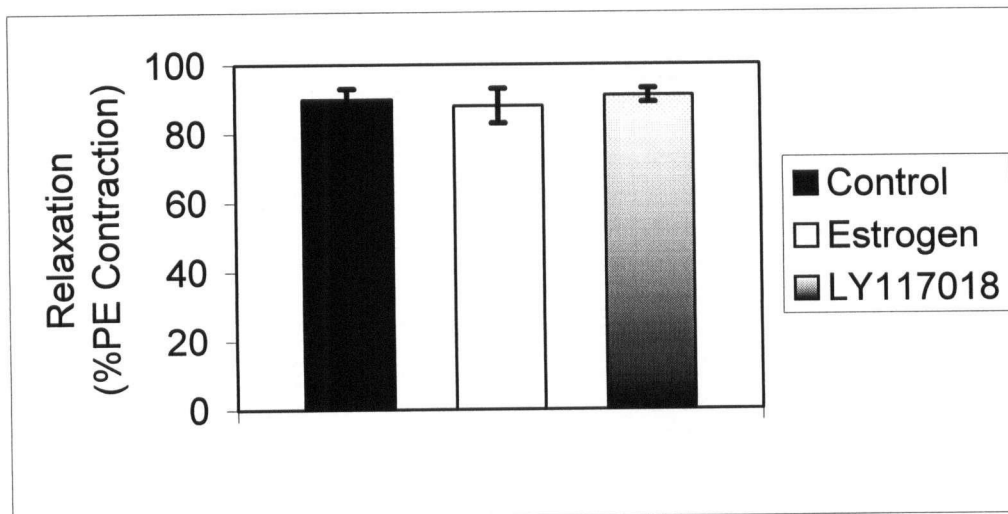Figure 32: Effects of LY117018 and estrogen on ACh-induced relaxation. Data on acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2µM) thoracic aortic rings were obtained from LY117018-treated (1.0mg/kg/day for three weeks), 17β-estradiol-treated (0.1mg/kg/day for three weeks) and control male Sprague-Dawley rats. The data are expressed as the percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group.
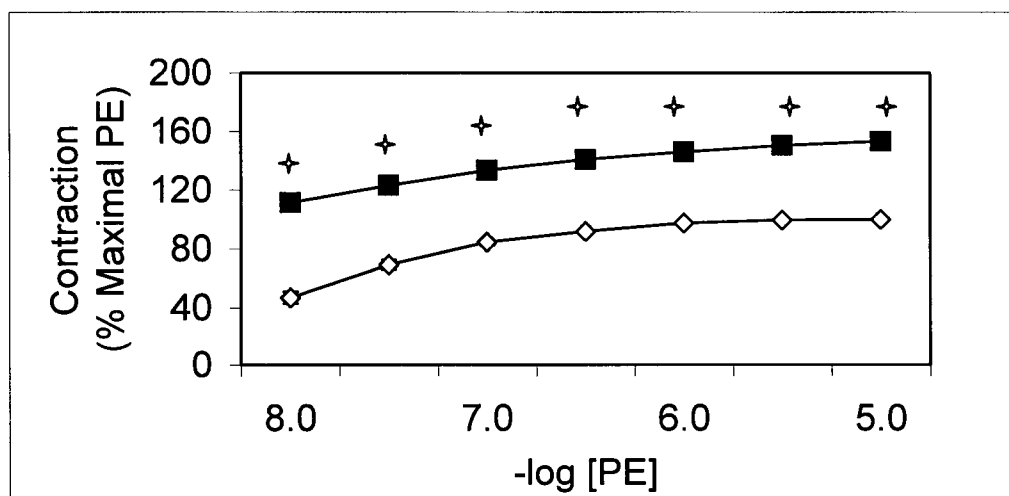
Figure 33: Effect of estrogen on basal NO production in the male rat. The graph shows the concentration-response curves to PE in the thoracic aorta before (◊) and after (■) L-NAME treatment in 17β-estradiol-treated (0.5mg released over five weeks) male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ✧ The upward shift in the curve after L-NAME is significant (P<0.05, ANOVA).
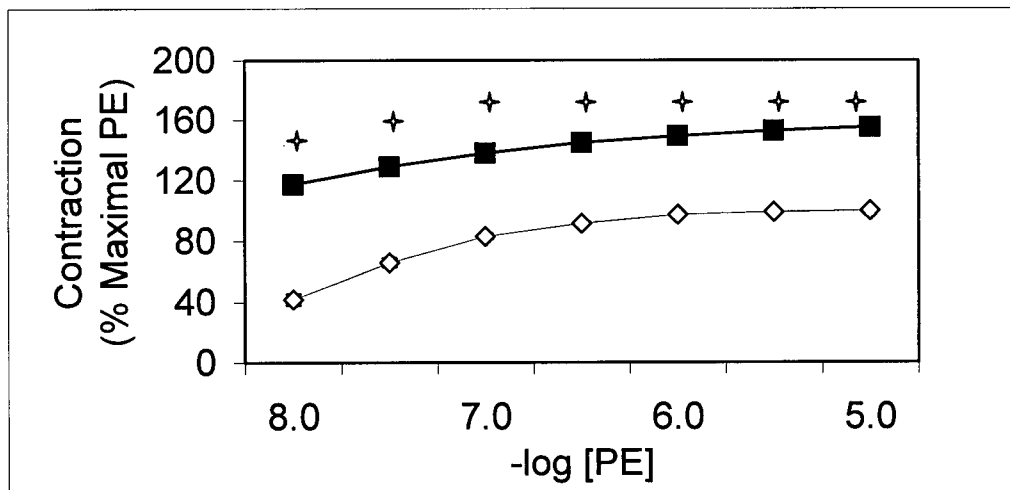
Figure 34: Effect of estrogen on basal NO production in the male rat. The graph shows the concentration-response curves to PE in the thoracic aorta before (◊) and after (■) L-NAME treatment in 17β-estradiol-treated (1.5mg released over five weeks) male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ✧ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
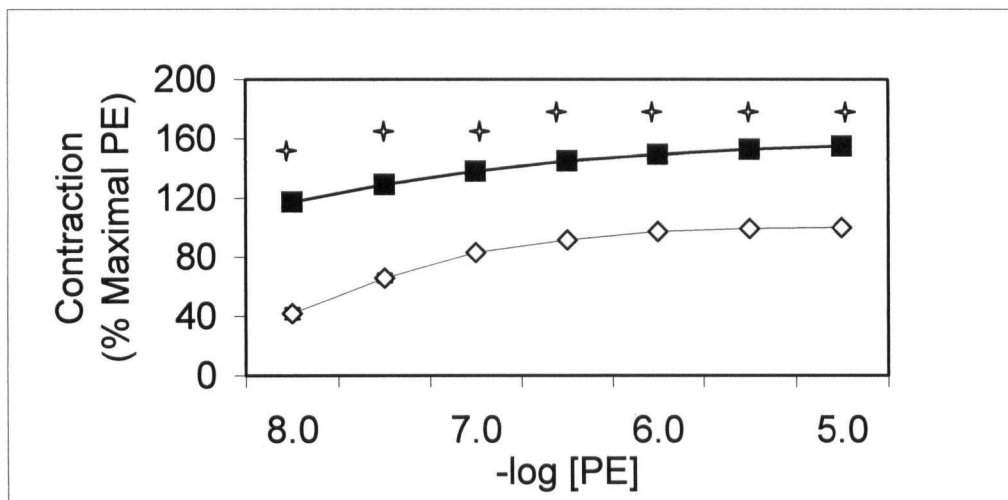
Figure 35: Effect of estrogen on basal NO production in the male rat. The graph shows the concentration-response curves to PE in the thoracic aorta before (◊) and after (■) L-NAME treatment in 17β-estradiol-treated (5.0mg released over five weeks) male Sprague-Dawley rats. Results are expressed as a percentage of the maximum response to PE (10μM) before L-NAME treatment. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group. ✧ The upward shift in the curve after L-NAME is significant (P<0.05, repeated measures ANOVA).
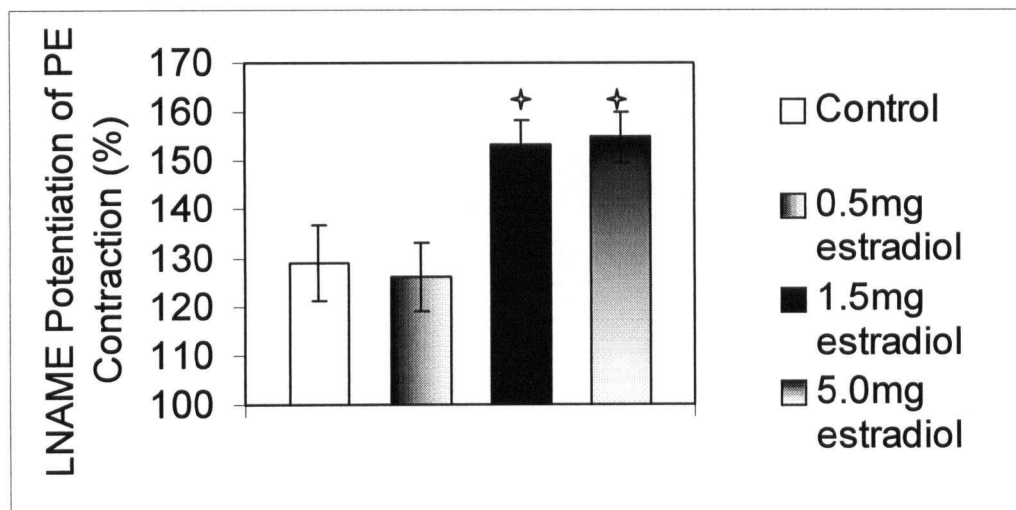
Figure 36: Estrogen dose-dependent effects on the L-NAME potentiation of the maximal response to phenylephrine (10μM). Each point represents the maximal contraction elicited by PE after L-NAME (shown as percentage of maximal PE (10μM) contraction before L-NAME pretreatment). Data are mean ± S.E.M. of fifteen tissues from five rats in each group. ✧ Significantly different (P <0.05) from the control group by ANOVA and multiple comparison.
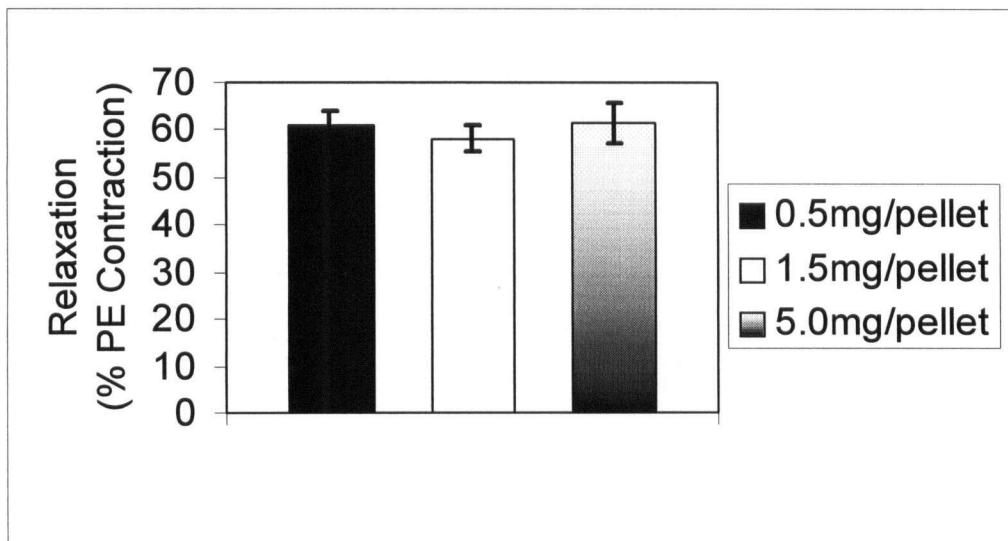
Figure 37: Dose-dependent effects of estrogen on ACh-induced relaxation. Data on acetylcholine-induced (10^{-5}M) relaxation in phenylephrine-precontracted (2μM) thoracic aortic rings were obtained from 17β-estradiol-treated (0.5, 1.5 and 5.0mg released over five weeks) male Sprague-Dawley rats. The data are expressed as the percentage inhibition of the phenylephrine-induced contraction. The results represent the mean ± S.E.M. of fifteen tissues from five rats in each group.
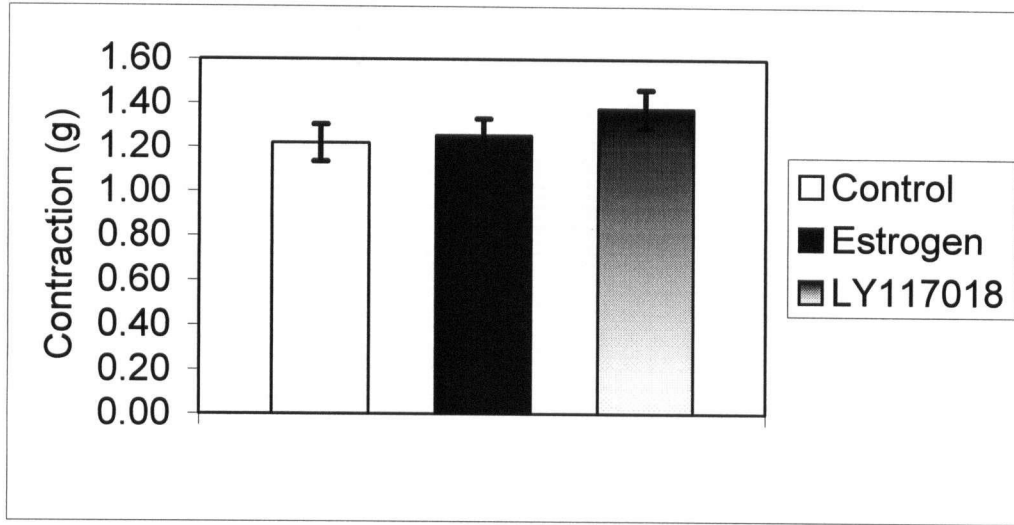
Figure 38: High K$^+$-induced (80mM) contractions in the thoracic aorta of control, 17β-estradiol (0.1mg/kg/day for three weeks) and LY117018-treated (1.0mg/kg/day for three weeks) male Sprague-Dawley rats. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.
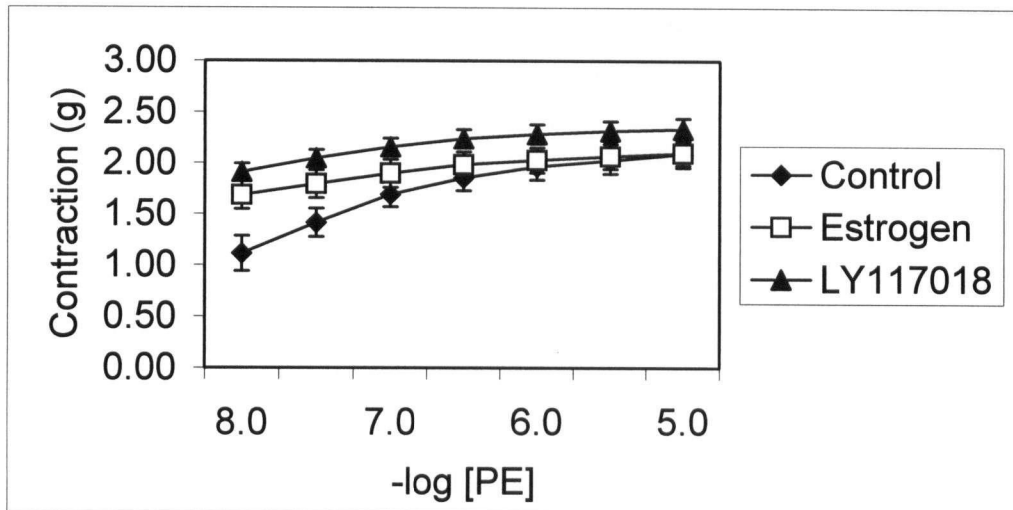
Figure 39: Concentration-response curves to phenylephrine in the thoracic aorta of control, 17β-estradiol (0.1mg/kg/day for three weeks) and LY117018-treated (1.0mg/kg/day for three weeks) male Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.
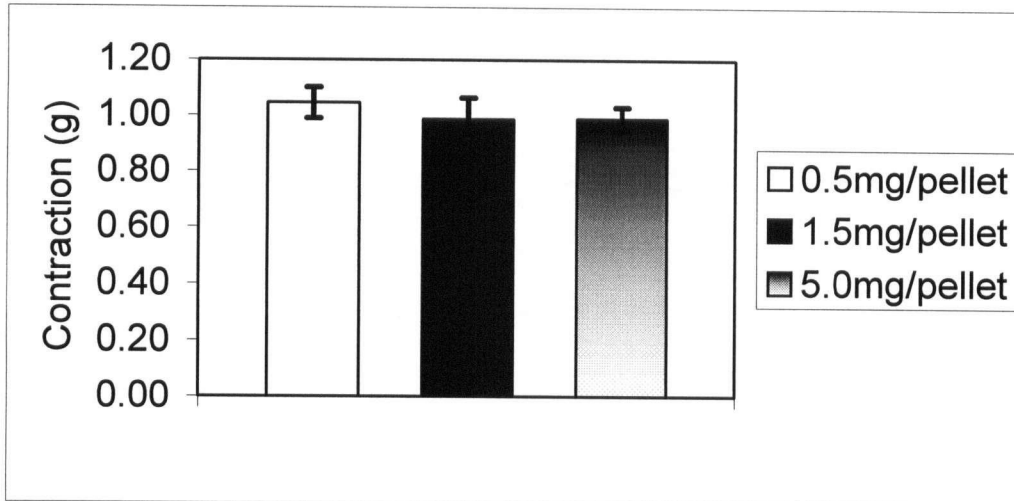
Figure 40: High K$^+$-induced (80mM) contractions in the thoracic aorta of 17β-estradiol-treated (0.5, 1.5 and 5.0 mg pellets released over five weeks) male Sprague-Dawley rats. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.
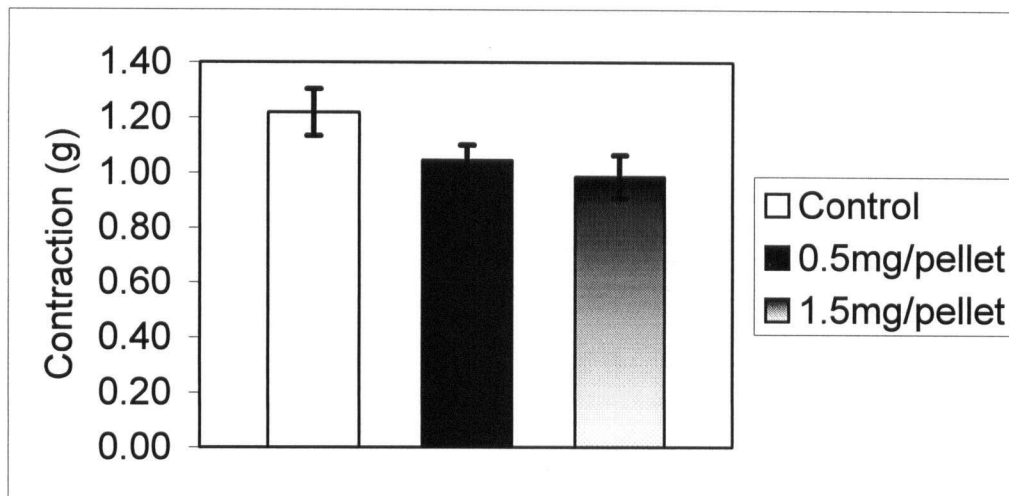
Figure 41: High K$^+$-induced (80mM) contractions in the thoracic aorta of control and 17β-estradiol-treated (0.5 and 1.5 mg pellets released over five weeks) male Sprague-Dawley rats. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.
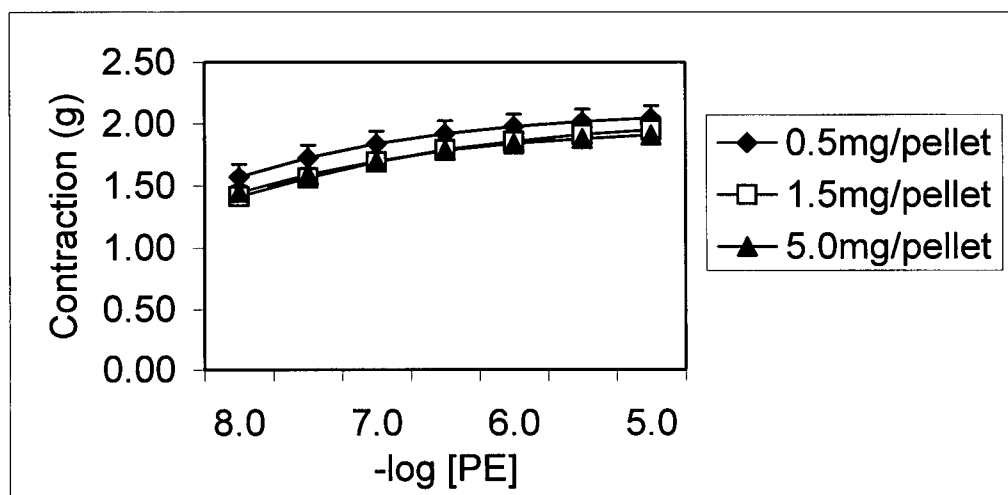
Figure 42: Concentration-response curves to phenylephrine in the thoracic aorta of 17β-estradiol-treated (0.5, 1.5 and 5.0 mg released over five weeks) male Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.

A.
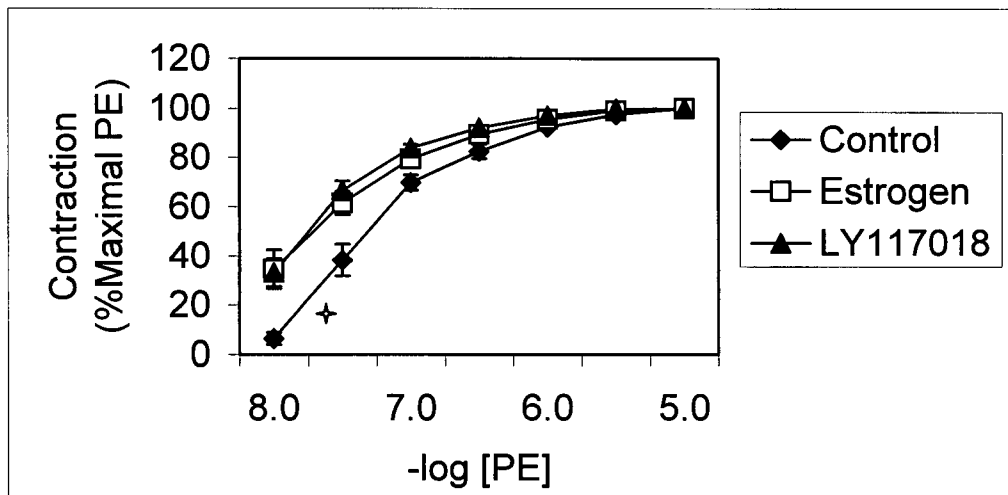
B.
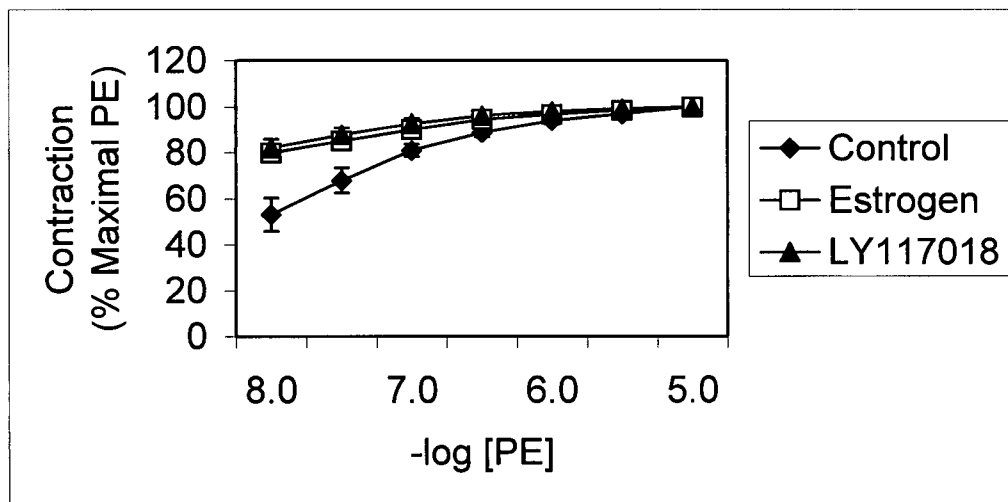
Figure 43: Concentration-response curves to phenylephrine in the thoracic aorta of control, 17β-estradiol-treated (0.1mg/kg/day for three weeks) and LY117018-treated (1.0mg/kg/day for three weeks) male Sprague-Dawley rats. The contractions were generated A) before and B) after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to PE (10μM) after L-NAME. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group. The EC_{50} values (for PE contraction after L-NAME) for the 17β-estradiol-treated, LY117018-treated and control tissues were 0.0205±0.0056, 0.0160±0.0035 and 0.0597 ±0.0117μM, respectively. ◇ The EC_{50} values for the treated groups were significantly different from that of controls (P<0.05, ANOVA).

high concentrations of PE. At low concentrations (10^{-8} and $10^{-7.5}$ M) of PE, this ratio was lower in control than either LY117018- or 17β-estradiol-treated animals (Figure 44). Estrogen's effect on smooth muscle sensitivity to PE was significant after three weeks of 17β-estradiol treatment. Increasing the dose of 17β-estradiol did not further enhance the α-adrenoceptor sensitivity (Figure 45) nor alter the PE contraction/high K^+ contraction ratio (Figure 46). A summary of the results is shown in Table 6.
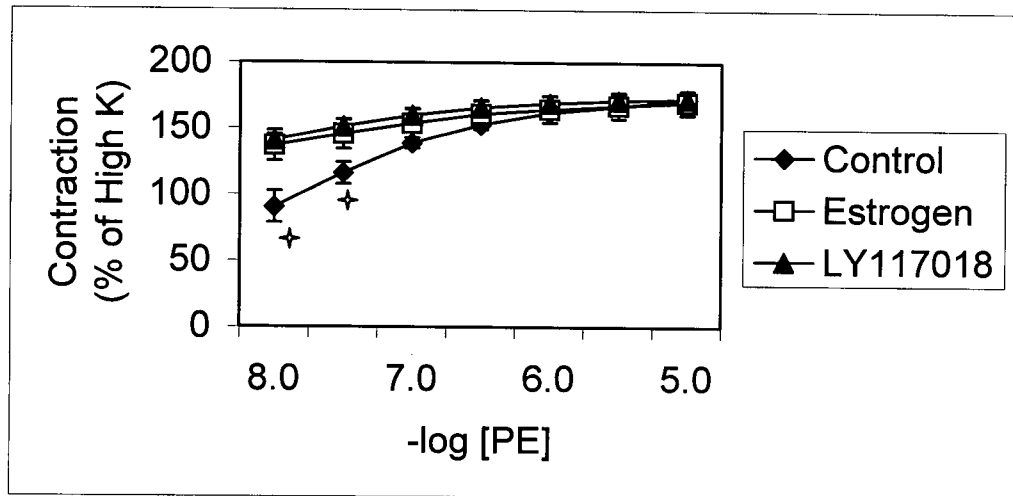
Figure 44: Concentration-response curves to phenylephrine in the thoracic aorta of control 17β-estradiol-treated (0.1mg/kg/day for three weeks) and LY117018-treated (1.0mg/kg/day for three weeks) male Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to high K^+ (80mM). The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.. ◇ Significantly different (P<0.05) from 17β-estradiol- or LY117018-treated by ANOVA and multiple comparisons.

A.
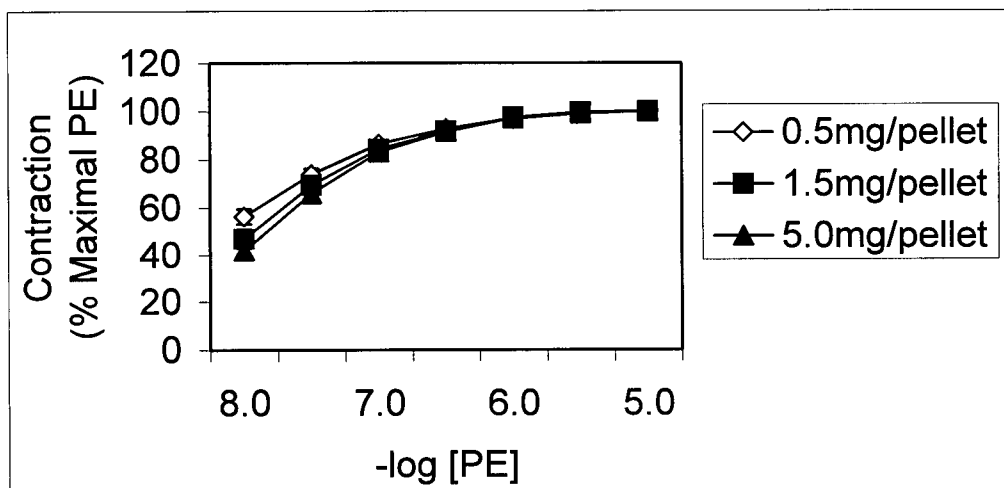
B.
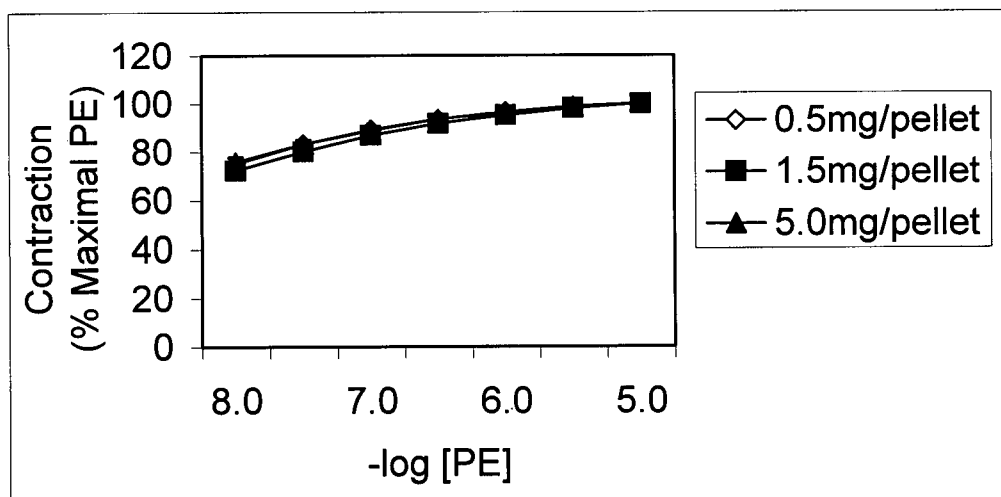
Figure 45: Concentration-response curves to phenylephrine in the thoracic aorta from 17β-estradiol-treated (0.5, 1.5 or 5.0mg release over five weeks) male Sprague-Dawley rats. The contractions were generated A) before and B) after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to PE (10μM) after L-NAME. The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.
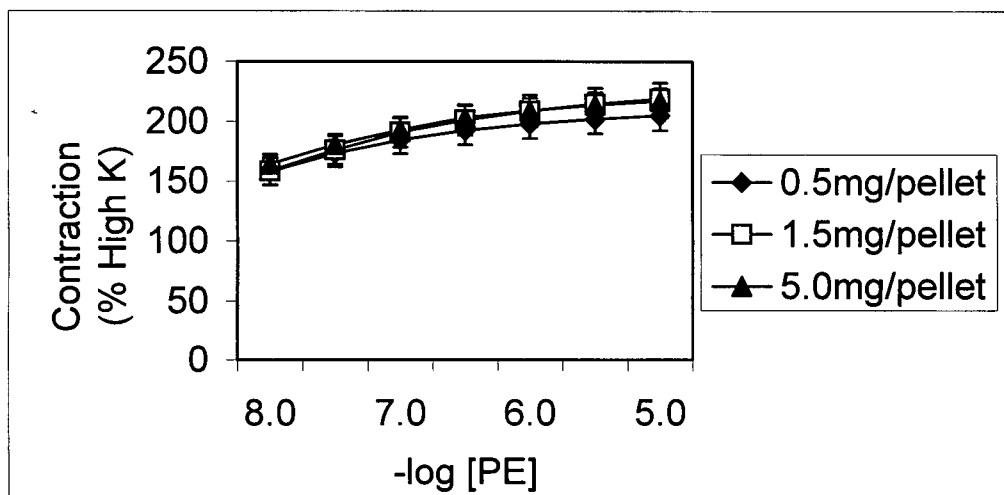
Figure 46: Concentration-response curves to phenylephrine in the thoracic aorta of 17β-estradiol-treated (0.5, 1.5 or 5.0mg release over five weeks) male Sprague-Dawley rats. The contractions were generated after pretreatment with L-NAME. The responses are normalized to the maximal contractile responses to high K^+ (80mM). The results are shown as the mean ± S.E.M. of 15 tissues from five rats in each group.

Table 6: Effects of estrogen receptor activation on rat aortic function.

	↑ Basal NO Release		↑ Stimulated NO Release		↑ PE Sensitivity	
	Time[1]	Dose[2]	Time	Dose	Time	Dose
Female Ovariectomized	≥3 weeks	1-3mg/kg LY[3]	≥2 weeks	≥1mg/kg LY	NA	NA
Male	5 weeks	≥1.5 mg pellet E2[4]	NA[5]	NA	3 weeks	≥0.5mg pellet E2

[1] Time = time required for significant effect; [2] Dose = dose required for significant effect; [3] LY=LY117018; [4] E2=17β-estradiol; [5] NA=non-applicable (no effects were found)

DISCUSSION

The results of this study demonstrate that chronic treatment with LY117018, a selective estrogen receptor modulator, enhances basal NO release in the rat aorta in a time- and dose-dependent manner. The maximal effect on basal NO production occurred with treatment with LY117018 for three weeks or longer. At doses less than 1.0mg/kg, the effect of LY117018 increased with increasing dose. The maximal effect occurred between the doses 1.0 and 3.0mg/kg. At doses higher than 3.0mg/kg, the effect on NO release diminished. LY117018 treatment for three weeks enhanced receptor-mediated NO release in the ovariectomized female rat in a dose-dependent manner. Statistically significant differences in ACh-induced relaxation between control and treated rats were seen with LY117018 doses of 3.0 and 6.0 mg/kg.

LY117018 (1.0mg/kg) treatment for three weeks showed no effect on NO production in the male rat. Treatment with 0.1mg/kg 17β-estradiol for three weeks, previously shown to enhance NO production in the female ovariectomized rat (Rahimian *et al*, 1997a), did not increase basal NO release in male rats. Subcutaneous implantation of pellets containing 1.5 mg or 5.0 mg 17β-estradiol for five weeks was necessary to increase basal NO production in male rats. Neither LY117018 (1.0mg/kg for three

weeeks) nor 17β-estradiol (0.1mg/kg for three weeks, or 0.5, 1.5 or 5.0 mg released over five weeks) enhanced receptor-mediated NO release in the male rat.

LY117018 EFFECTS ON AORTIC FUNCTION IN THE FEMALE OVARIECTOMIZED RAT

Basal Nitric Oxide Synthesis and Release

The results of the present study demonstrate that chronic oral treatment with LY117018 enhanced basal NO release in ovariectomized rats. Inhibition of NOS with L-NAME (200μM) caused a greater potentiation of adrenergic vasoconstriction in LY117018-treated animals than in control animals. This indicates that chronic LY117018 treatment enhances basal NO release from the aortic endothelium in the ovariectomized rat, in agreement with previous findings. Both LY117018 (1.0mg/kg) and 17β-estradiol (0.1mg/kg) treatment in ovariectomized rats promoted basal NO production in the aortic endothelium (Rahimian *et al.*, 1997a). Oophorectomy of female rabbits diminished basal release of aortic nitric oxide (Hayashi *et al.*, 1992). Hormone-replacement therapy using 17β-estradiol and norethisterone acetate increased circulating nitric oxide levels in postmenopausal women (Rosselli, 1995).

Receptor-Mediated Release of NO

Chronic treatment of ovariectomized rats with LY11018 enhanced endothelium-dependent relaxation to ACh (10^{-5} M) in PE-precontracted aortic rings. This enhancement of ACh-induced relaxation was statistically significant ($P<0.05$, Student's t-test) with LY117018 treatment for 2, 3 and 5 weeks. ACh activates muscarinic receptors in the endothelium, inducing the synthesis and release of NO (Furchgott and Zawadzki, 1980). In a previous study, chronic treatment with LY117018 (1.0mg/kg) or 17β-estradiol (0.1mg/kg) in female ovariectomized rats enhanced ACh-induced (10^{-6} and 10^{-5}M) dilation (Rahimian et al., 1997a). Results from other studies also demonstrated that chronic estrogen-receptor activation enhanced ACh dilation. Endothelium-dependent vasodilation was enhanced by estrogen treatment in the coronary artery of ovariectomized monkeys (Williams et al., 1990). Chronic treatment with 17β-estradiol increased ACh-induced dilations in isolated rabbit femoral arteries (Gisclard et al., 1988). ACh-induced relaxation in guinea-pig uterine arteries was greater during pregnancy when estrogen levels were much higher than normal (Weiner et al., 1989).

The increased ACh relaxation in LY117018-treated rats may be due to increased sensitivity of the aortic smooth muscle cells to NO, or to an increased receptor-mediated release of NO. The effects of 17β-estradiol and of LY117018 on vasodilation to sodium nitroprusside (SNP) were investigated previously (Rahimian et al., 1997a). SNP is a NO donor and causes endothelium-independent relaxation in smooth muscle. The sensitivity of the smooth muscle to SNP did not differ among the LY117018-treated, 17β-estradiol-treated or placebo-treated rats. The sensitivity of resistance mesenteric arteries to SNP was similar in estrogen-treated and control ovariectomized rats (Meyer et al., 1997). This

indicates that smooth muscle sensitivity to NO does not change in response to chronic estrogen-receptor activation. Thus, the increased ACh-induced relaxation seen with LY117018 treatment is likely to be due to enhanced NO receptor-mediated release. This may result from alterations in the number of ACh receptors. Chronic estrogen treatment was found to increase muscarinic receptor density in the rabbit uterus (Levin *et al.*, 1980).

Smooth Muscle Contractility

Neither the PE-induced (after L-NAME) nor the high K^+-induced contractions differed between the treated and control groups. Thus, the contractility of the smooth muscle was not affected by LY117018 treatment. The sensitivity of α-adrenoceptors was not significantly affected by chronic LY117018 treatment either before or after inhibition of NOS. This was indicated by the similarities in PE EC_{50} values in the aortae from LY117018-treated and untreated ovariectomized rats. These results are supported by a previous study in which 17β-estradiol and LY117018-treated ovariectomized rats did not show increased sensitivity to PE as compared with control (Rahimian *et al.*, 1997a).

Receptor agonists cause a larger increase in force than depolarizing agents for the same level of $[Ca^{2+}]_i$. This is called the Ca^{2+}-sensitizing effect of agonists. Agonists such as PE increase the Ca^{2+} sensitivity of the contractile elements in smooth muscle (Bradley and Morgan, 1987). The ratio of PE-induced (10^{-5} M) tension over high K^+-induced (80mM) tension can be used to investigate the Ca^{2+}-sensitizing effect in the smooth muscle cells of LY117018-treated and control rats. If LY117108 treatment enhanced the Ca^{2+}-sensitization ability of PE, the ratio (PE contraction/ high K^+

contraction) would be greater in treated than control animals. This ratio was found to be the same in LY117018-treated and control rats in this study. Thus, LY117018 treatment does not affect the Ca^{2+}-sensitizing effect of PE.

Time-Dependent Effects of LY117018 on NO Release

In this study, chronic LY117018 treatment caused a time-dependent increase in basal NO release. This slow up-regulation of NO production by estrogen receptor activation was also seen in other studies using cultured endothelial cells. In the cultured bovine aortic endothelial cells (BAECs), estrogen upregulation of NOS activity was significant after 8 hours incubation with 17β-estradiol. This effect reached a maximum after 16 hours and reached a plateau at submaximal levels after 24 hours (Hayashi, 1995). In cultured fetal pulmonary endothelial cells (PAECs), maximal upregulation of NOS activity was evident after 48 hours and persisted for at least 96 hours (MacRitchie, 1997).

The time required for upregulation of NO release suggest that the effect is not dependent on the acute actions of LY117018. It is more likely that the effect is due to LY117018 initiation of gene transcription. This would result in various phenotypic changes that may include increased eNOS expression or variations in calcium homeostasis.

LY117018 treatment (1.0mg/kg) enhanced ACh-induced relaxation in PE-precontracted aortic rings. This increase in relaxation was significantly higher in animals treated with LY117018 for two, three and five weeks ($P<0.05$, Student's t-test). With treatment for one or four weeks, ACh relaxation was greater, but this difference was statistically insignificant ($P>0.05$, Student's t-test). The LY117018 enhancement of

receptor-mediated NO release did not show any relationship with length of treatment.

Dose-Dependent Effects of LY117018 on NO Release

Chronic LY117018 treatment caused a dose-dependent increase in basal NO release. This effect increased with increasing dose and was maximal at 1.0mg/kg. At doses greater than 3.0m/kg, the effect diminished. These results are supported by previous studies using intact tissues and cultured endothelial cells.

Ovariectomized guinea pigs also showed a dose-dependent response to 17β-estradiol. The animals were given 21-day continuous-release pellets containing either 0.25, 0.5, 1.5, or 7.5 mg 17β-estradiol placed subcutaneously in the abdomen. NOS activity was enhanced in the coronary arteries from the animals receiving 0.25 mg and 0.5 mg but not those receiving 1.5 mg and 7.5 mg 17β-estradiol. Thus, enzyme activity was stimulated at some 17β-estradiol levels, but was inhibited as 17β-estradiol levels increased. NOS activity in the guinea pig myocardium and the amount of eNOS mRNA in skeletal muscle also increased with low-dose 17β-estradiol (0.25 and 0.5 mg) and decreased at doses greater than 0.5 mg 17β-estradiol. The same dose-dependent effect was found for levels of eNOS mRNA in the guinea pig forebrain (Thompson *et al.*, 1997).

In human umblical vein endothelial cell (HUVEC) and bovine aortic endothelial cell (BAEC) cultures, incubation with 17β-estradiol at concentrations of $10^{-12} - 10^{-8}$ M significantly enhanced NO production. There appeared to be a dose-dependent increase in eNOS activity, with maximal activity at 10^{-10} M 17β-estradiol. At higher concentrations, this effect diminished, eventually reaching control levels with 10^{-6} M of

17β-estradiol (Hayashi *et al.*, 1995).

Not all of estrogen's effects exhibit this biphasic pattern. In the present study, LY117018 treatment increased ACh-induced relaxation in PE-precontracted tissues. This effect increased with increasing dose of LY117018. This enhancement of ACh-induced relaxation effect was seen with all doses of LY117018 except at 0.2mg/kg. The difference in receptor-mediated NO between treated and control rats was significant with LY117018 doses of 3.0 and 6.0 mg/kg ($P<0.05$, ANOVA).

Other studies also found that high doses of 17β-estradiol potentiated NO release. In cultured human aortic endothelial cells (HAECs), the threshold for estrogen effects on NO levels occurred at 200 pg/ml and reached a plateau 20 000 pg/ml (Hishikawa *et al.*, 1995). Both low-dose (100μg/kg) and high-dose (600μg/kg) estradiol given to monkeys with intact ovaries as weekly intramuscular injections for 6 weeks decreased systemic vascular resistance (Williams *et al.*, 1994). A 5-day time course of a high-dose estradiol supplementation (500μg/kg/d) in guinea pigs with intact ovaries increased myocardial NOS activity (Lizasoain *et al.*, 1997).

High levels of plasma estrogen leads a high degree of occupancy of estrogen receptors. This may cause down-regulation of estrogen-receptor function through conformational changes other than those that occur with normal receptor activation. These conformational changes may mask the DNA-binding region of the protein. Two forms of the nuclear estrogen receptor (ligand-bound form) were found in the mouse uterus (Korach *et al.*, 1988). This may explain the paradoxical effect of high doses of LY117018 on NO release. Low doses of estrogen enhanced cell proliferation and thymidine uptake in MCF-7 cells (a line of human breast cancer cells). High doses of

17β-estradiol (>10^{-7} M) inhibited cell proliferation and thymidine uptake. Inhibition of cell proliferation was also seen with high doses of 17α-estradiol. This inhibition was unaffected by addition of tamoxifen, an estrogen antagonist (Lippman *et al.*, 1976). These results suggest that high doses of estrogen may exert non-specific effects. It is thus possible that high doses of LY117018 exerted non-specific effects on the aortic endothelium. This is a possible explanation for the bell-shaped dose-response curve exhibited by LY117018 acting on basal NO release.

LY117018 AND 17β-ESTRADIOL EFFECTS ON AORTIC FUNCTION IN THE MALE RAT

Basal Nitric Oxide Synthesis and Release

To determine if 17β-estradiol and LY117018 can exert similar effects on cardiovascular function in males as in females, male Sprague-Dawley rats were treated with either drug for three weeks. Oral treatment with doses of 17β-estradiol and LY117018 (0.1 mg/kg and 1.0 mg/kg, respectively) shown to cause maximal effects in female rats did not enhance NO basal release in male rats. 17β-estradiol (0.1mg/kg) was previously found to enhance NO basal in the female ovariectomized rat (Rahimian *et al.*, 1997a).

To determine if 17β-estradiol exerts time- and dose-dependent effects on basal NO release in male rats, animals were treated using varying doses of 17β-estradiol for

five weeks. Subcutaneous implantation of 17β-estradiol pellets (0.5, 1.5 and 5.0mg 17β-estradiol/pellet) for five weeks caused a dose-dependent increase in NO basal release in the male rat. The pellets containing 1.5mg of 17β-estradiol are constructed to give plasma levels (20-100pg/mL) similar to those obtained with oral administration of 0.1mg/kg 17β-estradiol. Thus, a longer treatment period appears to be required for enhancement of endothelial function in the male rat.

Estrogen receptor distribution has been reported to differ in tissues from male and female animals. Priming with 17β-estradiol increased estrogen binding in the male rabbit aorta, but had no effect on estrogen binding in the female rabbit aorta (Tamaya, 1993). Binding of radiolabeled 17β-estradiol was higher in coronary arteries from sexually mature female pigs than those from castrated males (Vargas, 1993). Thus, it is possible that the male rats in the present study had a lower initial number of estrogen receptors. Treatment with 17β-estradiol may have enhanced the expression of estrogen receptors. As the number of receptors increased, 17β-estradiol treatment allowed up-regulation of NO basal release.

The route of administration may also affect estrogen's actions on the endothelium. Estrogens taken orally are subjected to intestinal absorption and hepatic metabolism before they enter into the bloodstream (Lobo, 1987). The liver modulates the production of sex hormone-binding globulin (SHBG), a major serum protein that specifically binds estrogens and controls their bioavailability (Stumpf *et al.*, 1981). Subcutaneous implantation of 17β-estradiol pellets allows for direct absorption into the circulation and continous release at a relatively constant rate (Lobo *et al.*, 1980). With the oral route, 17β-estradiol is administered as a bolus dose.

Receptor-Mediated Release of NO

Neither LY117018 nor 17β-estradiol treatment enhanced ACh-induced relaxation in the male rat aorta. This was not expected, because there was a significant increase in basal NO release with 17β-estradiol treatment in male rats. 17β-Estradiol treatment of ovariectomized rats was found to enhance receptor-mediated release in the present study. Also, previous experiments have reported that estrogen augmented ACh-induced relaxation in the female rat aorta (Cheng *et al.*, 1994; Rahimian *et al.*, 1997a) and female rabbit femoral artery (Gisclard *et al.*, 1988). The disparity in these results may reflect differences in the way male and female animals respond to estrogen. Other studies reported that chronic estrogen treatment did not affect receptor-mediated NO release. Chronic 17β-estradiol treatment did not alter ACh-induced relaxation in mesenteric arteries of oophorectomized rats (Meyer *et al.*, 1997). Estrogen augmented endothelium-dependent relaxation to acetylcholine in rat aortic rings precontracted with high K^+ but not in rings precontracted with PE (Vedernikov *et al.*, 1997). Estrogen enhanced basal but not ACh-stimulated NO release from the endothelium of the rabbit (Hayashi *et al.*, 1992) and rat aorta (Kauser and Rubanyi, 1994).

Estrogen may modulate basal eNOS activity through a different mechanism than its modulation of agonist-stimulated NO synthesis. Estrogen has been suggested to cause changes in calcium homeostasis in endothelial cells. 17β-Estradiol increased $[Ca^{2+}]_i$ in cultured bovine pulmonary artery endothelial cells through an estrogen-receptor dependent mechanism. This moderate increase in $[Ca^{2+}]_i$ enhanced eNOS basal activity (Lantin-Hermoso *et al.*, 1997). The larger increase in $[Ca^{2+}]_i$ in response to ACh would mask the more moderate $[Ca^{2+}]_i$ increase produced by estrogen. Thus, the estrogen-

induced changes in calcium homeostasis would result in an increase of basal, but not stimulated NO release.

Smooth Muscle Contractility

Chronic LY117018 and 17β-estradiol treatment increased the sensitivity of α-adrenoceptors both before and after inhibition of NOS. The PE EC_{50} values in the aortae from LY117018-treated and 17β-estradiol-treated rats were significantly lower than control. Estrogen potentiated the sensitivity of female rat tail artery (Lydrup and Nilsson, 1996) and aorta (Cheng and Gruetter, 1992) to norepinephrine. Estrogen treatment also enhanced catecholamine sensitivity in male rat mesenteric artery (Colucci *et al.*, 1982). In radioligand-binding studies on rat mesenteric artery, estrogen was found to increase the affinity of α-adrenergic receptors (Colucci *et al.*, 1992). The estrogen-induced increase in α-adrenergic receptor affinity could account for the increase in PE sensitivity reported here. Because LY117018 and 17β-estradiol had no effect on maximal PE contraction, it is unlikely that the properties of the cross-bridge interaction or the structure of the contractile system were affected, or that the number of smooth muscle cells was increased.

Estrogen's effect on smooth muscle sensitivity to PE was significant after three weeks treatment with 17β-estradiol. Increasing the dose of 17β-estradiol did not further enhance the α-adrenoceptor sensitivity or alter the PE contraction/high K^+ contraction ratio. The effects of estrogen on basal NO release were not noticeable after three weeks treatment, but were significant after five weeks treatment with 17β-estradiol. Thus, less time was necessary for estrogen to exert its effects on smooth muscle cells than on

endothelium cells in the male rat aorta. In the ovariectomized rat, treatment with 17β-estradiol did not alter PE sensitivity. This gender-specific effect on smooth muscle PE sensitivity may be due to differential distribution of estrogen receptors. Estrogen-induced endothelium-dependent relaxation was greater in female than male rat tail artery (McNeill *et al.*, 1996) and aorta (Tran *et al.*, 1997). This suggests that female endothelial cells may have a higher density of estrogen receptors than male. Another possible explanation for these gender differences is that estrogen may alter vascular function in the male rat through indirect mechanisms. Estrogen suppressed testosterone production in male to female transsexuals (New *et al.*, 1997). Androgen deprivation following orchiectomy has been associated with enhanced NO release in adult men (Herman *et al.*, 1996).

UP-REGULATION OF NO RELEASE: POSSIBLE MECHANISMS

Nitric oxide is produced in the endothelium in a reaction that converts L-arginine to L-citrulline (Palmer *et al.*, 1988). This reaction is catalyzed by the constitutive enzyme endothelial nitric oxide synthase (eNOS) (Figure 47) (Lamas *et al.*, 1992). NO activates guanylate cyclase, increasing cyclic guanosine monophosphate (cGMP) and thereby stimulating cGMP-dependent protein kinases (Ignarro *et al.*, 1987). This results in increased activity of calcium-activated potassium (K_{Ca}) channels in the smooth muscle cells (Archer *et al.*, 1994), causing membrane hyperpolarization and inactivation of voltage-dependent Ca^{2+} channels (Nelson and Quayle, 1995). The subsequent decrease in intracellular calcium concentration ($[Ca^{2+}]_i$) leads to vasodilation.

Many hypotheses have been proposed to explain the up-regulation of NO release by estrogen. The increase in NO production may be due to an increased amount of functional eNOS caused by up-regulation of eNOS mRNA and protein, or enhanced function of the enzyme (such as an increase in Ca^{2+} sensitivity) through post-transcriptional modification. Increased levels of the substrate or cofactors (such as $[Ca^{2+}]_i$, calmodulin and biopterin) can also account for the increase in NO release. Decreased degradation of eNOS mRNA, eNOS protein or the NO molecule itself may also lead to greater basal levels of NO.

Studies have indicated that the up-regulation of NOS activity by estrogen is dependent on the presence of a functional ER. Incubation of human umblical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) with physiological concentrations of 17β-estradiol (10^{-12}-10^{-8} M) significantly enhanced NOS activity. The addition of estrogen receptor antagonists tamoxifen and ICI182780 reduced this effect (Hayashi *et al.*, 1995). In fetal pulmonary artery endothelium cells (PAECs), the up-regulation of NOS activity by 17β-estradiol was fully inhibited by addition of ICI182780, an estrogen-receptor antagonist (MacRitchie *et al.*, 1997).

17β-Estradiol enhanced NO production in PAECs, an effect abolished by addition of ICI182780. When Western and Southern blot analyses were performed, both eNOS protein and eNOS mRNA expression were found to have increased in these cells (MacRitchie *et al.*, 1997). These results imply that estrogen can increase transcription and translation of the gene encoding for eNOS. NOS activity in both control and estrogen-treated fetal PAECs was fully inhibited by calcium chelation (MacRitchie *et al.*, 1997). Thus, the increase in NOS activity is related to up-regulation of the calcium-
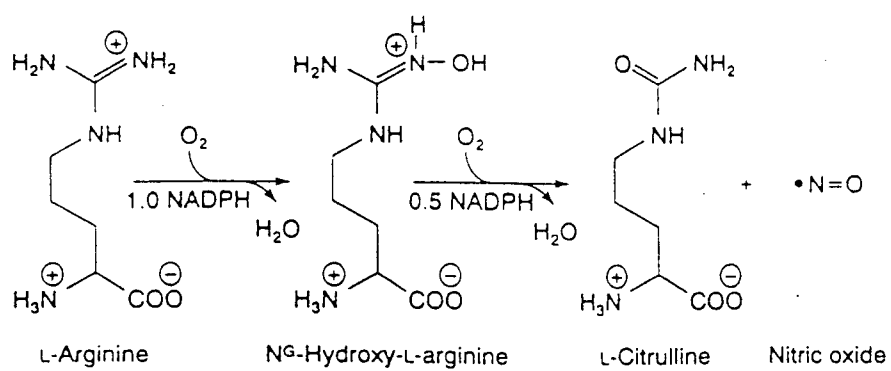
Figure 47: The nitric oxide synthase reaction. (Reproduced from TIBS 1997; 22.)

dependent eNOS and not the calcium-independent iNOS. Also, NOS enzymatic activity was determined in cell lysates in the presence of excess quantities of the required substates and cofactors. Thus, the increase in activity represents an increase in the quantity of eNOS enzyme. This was confirmed by the increase in eNOS protein as detected by immunoblot analysis. (MacRitchie et al., 1997)

Estrogen may up-regulate eNOS transcription indirectly by inhibiting the production of certain cytokines. Studies have revealed that cytokines such as TNFα down-regulate eNOS through destabilization of the eNOS mRNA (Yoshizumi et al., 1993). Increases in NO production can also occur if estrogen increases the availability of eNOS cofactors though enhancement of synthesis or intracellular transport. Estrogen has also been shown to increase the synthesis of calmodulin, an important cofactor required for the Ca^{2+}-dependent activation of NOS, in rabbit myometrium (Matsui et al., 1983). Estrogen may also be involved in post-transcriptional regulation of eNOS. Localization of eNOS is mediated by specific post-translational modifications, such as myristoylation and palmitoylation, which target the enzyme to the plasmalemmal caveolae. The localization of eNOS in the caveolae is important for its activation (Liu et al.,1995; Shaul et al., 1996). Phosphorylation of eNOS, shown to occur after bradykinin activation (Michel et al., 1993) and shear stress (Ayajiki et al., 1996), can increase its translocation to the caveolae (Michel et al., 1993) or its calcium sensitivity. This would enhance eNOS activity. All these processes are potential targets for estrogen.

Estrogen may also enhance NO release through alterations in calcium homeostasis. Cyclopiazonic acid (CPA)-induced dilation of phenylephrine-precontracted aortae from intact female and 17β-estradiol-treated ovariectomized Sprague-Dawley rats

were found to be enhanced as compared to that in the male or ovariectomized rats (Rahimian *et al.*, 1997b). CPA is an endoplasmic reticulum Ca^{2+}-ATPase inhibitor (Seidler *et al.*, 1989). Inhibition of the Ca^{2+} pump causes an increase in intracellular $[Ca^{2+}]$ in the endothelial cells, resulting in release of NO and relaxation of the nearby smooth muscle cells (Luo *et al.*, 1993).

Whole-cell membrane current recordings of freshly isolated rabbit aortic endothelial cells under voltage clamp showed that 17β-estradiol (1-30μM) enhanced the activity of the large Ca^{2+}-activated K^+ channels. Fura-2 measurements showed an increase in intracellular Ca^{2+} concentrations. Thus, estrogen may hyperpolarize the endothelial cell, increasing the electrochemical gradient for Ca^{2+} entry (Rusko *et al.*, 1995). This would enhance the CPA-induced $[Ca^{2+}]_i$ increase and NO release in the female and the 17β-estradiol-treated rats.

POTENTIAL SIDE-EFFECTS OF SERMS

A 2-year clinical trial of raloxifene in post-menopausal women showed that raloxifene increased bone mineral density, decreased bone turnover, lowered serum levels of total and LDL cholesterol (Delmas *et al.*, 1997). There was no evidence of endometrial growth (Delmas *et al.*, 1997). Thus, raloxifene may be useful for preventing osteoporosis and atherosclerosis without causing uterine cancer. However, the long-term effects of raloxifene are still unknown; other side effects might result from the antiestrogenic effects in reproductive tissues. Also, because raloxifene is not very potent, high concentrations must be used to exert any therapeutic effect. Thus, raloxifene may

compete with endogenous, dietary or pharmaceutical agents for its elimination pathways, glucuronidation or oxidation. The major metabolites of raloxifene are glucuronides, but its phenolic groups could be further metabolized into catechol compounds. Long-term treatment with raloxifene could induce increased expression of P450 enzymes, resulting in increased production of toxic catechols (Gustafsson, 1998).

FUTURE EXPERIMENTS

It would be interesting to determine the time- and dose-dependent increases in NO release in response to 17β-estradiol treatment in both the female ovariectomized and male castrated rats. This would give a better understanding of the difference in the kinetics of estrogen in the different sexes. Furthermore, more investigation needs to be placed on LY117018 effects on endothelial function in the male rats. It is highly possible that even though the treatment period and doses used to enhance NO basal release in the female is insufficient in the male, longer treatment with higher doses will cause enhanced endothelial function in the male rat.

The effects of other sex hormones may also contribute to the health of the endothelium. For example, testosterone has been shown to have vascular effects and was found to improve impaired coronary artery ACh responses in both post-menopausal females and males with atherosclerosis (Hutchison, 1997). Thus, the vascular effects of different sex hormones may be better understood with more *in vitro* experiments.

CONCLUSION

LY117018 caused a time- and dose-dependent increase in basal nitric oxide release in the aorta of the female ovariectomized Sprague-Dawley rat. The greatest effect occurred with treatment periods greater or equal to 3 weeks and with doses of LY117018 between 1.0 and 3.0 mg/kg (inclusive). Male Sprague-Dawley rats treated orally with 0.1 mg/kg 17β-estradiol or with 1.0 mg/kg LY117018 for three weeks did not show enhanced NO release. Subcutaneous treatment of male rats for five weeks with 17β-estradiol caused a dose-dependent increase in NO basal release.

LY117018 caused time- and dose-dependent enhancement of endothelial function in the female rat. This finding is important scientifically in helping to elucidate the mechanism underlying estrogen's antiatherogenic effects and clinically in determining the proper protocol for administration of estrogen and SERMs. Estrogen exerted beneficial effects on endothelial function in the male rat. This suggests that estrogen or SERM treatment could have beneficial effects on the cardiovascular system in men as well as women.

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