ESTROGEN EFFECT ON ENDOTHELIAL NITRIC OXIDE (NO) PRODUCTION

by

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ABSTRACT

Pre-menopausal women have a much lower incidence of coronary heart disease than men, and the difference appears to be related to the estrogen circulating in women. Estrogen has two protective cardiovascular effects: one is on the blood lipid profile, and the other is a direct effect on the blood vessel wall and the generation of nitric oxide (NO). In this connection, the modulatory effects of chronic subcutaneous or oral estrogen, LY117018 and raloxifene, selective estrogen receptor modulators (SERMs), on the release of NO was studied in the rings of rat aorta. Treatment of ovariectomized rats with estrogen and LY117018 enhanced cholinergic, endothelium-dependent vasodilation of the aorta, and secondly, the inhibition of NO synthase (NOS) caused a greater enhancement of adrenergic vasoconstriction in estrogen, LY117018 and raloxifene-treated animals than those in male, ovariectomized progesterone plus estrogen-treated animals ($P < 0.05$). These effects occurred without changes in the sensitivity of smooth muscle cells to either NO donors, or to an adrenergic agonist. We, therefore, proposed that estrogen and SERMs exert their vasomotor effects primarily through enhancing endothelial-dependent vasodilation by increasing basal and stimulated release of NO.

In the next phase, the modulatory effects of chronic estrogenic treatment on the responses to cyclopiazonic acid (CPA), an sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, was studied in rings of rat aorta. In phenylephrine (PE, $2x10^{-6}$ M) pre-contracted rings with intact endothelium, CPA (10^{-7} to $3x10^{-5}$ M) produced endothelium-dependent relaxations in a concentration dependent manner. The CPA dilation as a percentage loss of PE tone was greater in aortic rings from female and estrogen -treated rats compared to those from male or ovariectomized rats ($P < 0.05$).

These relaxation responses of CPA were converted to contractions by pre-treatment with an inhibitor of NOS. There were no differences in CPA-induced contractions of aortas excised from either estrogen treated or untreated ovariectomized rats. These results demonstrate that CPA causes a greater endothelium-dependent dilation in estrogen-treated ovariectomized and control female rats.

Depletion of endoplasmic reticulum (ER) Ca^{2+} by CPA discharges Ca^{2+} from intracellular stores in endothelial cells which in turns triggers influx of Ca^{2+} from the extracellular space via receptor operated channels (ROCs)/ or store operated channels (SOCs) of the plasma membrane, and subsequently stimulates NOS. Although the passive Ca^{2+} leak, ROCs/ or SOCs are voltage independent, membrane potential (E_m) plays an important role in regulating Ca^{2+} entry. The next set of experiment was designed to investigate the role of E_m in the regulation of Ca^{2+} entry triggered by agonist/ or SERCA inhibitors. $[Ca^{2+}]_i$ was measured by fura-2/AM fluorescence imaging microscopy in freshly isolated rabbit aortic endothelial cells. No changes in $[Ca^{2+}]_i$ in response to PE (5 μM) was observed indicating PE (a selective agonist of α_1 receptor), contraction may be used as a test system for basal NO release. Acetylcholine (Ach; 10 μM) and CPA (10 μM) increased $[Ca^{2+}]_i$. The maintained $[Ca^{2+}]_i$ increase upon agonist or Ca^{2+} pump blocker application was blocked by tetraethylamonium (TEA; 3 mM), a K^+ channel blocker, indicating involvement of K^+ channel activity. ROCs were found to be responsible for the $[Ca^{2+}]_i$ increase, since SK&F96365 (50 μM), a ROC blocker greatly reduced the maintained $[Ca^{2+}]_i$ increase caused by Ach and CPA. When Ach and CPA were added together the induced Ca^{2+} plateau was less sensitive to TEA but could be abolished by a combination of TEA and the Cl^- channel inhibitor NPPB (50 μM). These

data suggested that maintenance of a polarized membrane potential by activity of K^+ and Cl^- channels is a requisite for Ca^{2+} influx through ROCs/ or SOCs and, therefore, for the synthesis/release of NO.

The possibility that enhanced Ca^{2+} stimulation of endothelial NOS contributes to estrogenic effects has not been previously investigated. The last phase of experiment was therefore designed to determine whether estrogen enhances NO release, at least in part, by raising $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by fura-2/AM fluorescence imaging microscopy in freshly isolated valvular endothelial cells taken from female and male rats. The basal level of $[Ca^{2+}]_i$ was significantly elevated in female valvular endothelial cells when compared to males ($P < 0.05$). Inhibition of SERCA with CPA (10 μM) caused a greater increase in the $[Ca^{2+}]_i$ in female than male endothelial cells. Removal of extracellular Ca^{2+} returned the $[Ca^{2+}]_i$ to the basal level in both female and male endothelium. The rate of $[Ca^{2+}]_i$ decline was significantly ($P < 0.05$) slower in female endothelial cells compared to males. There were no differences in the unstimulated rate of Mn^{2+} quenching between two groups.

In conclusion, these results indicate a novel mechanism for the protective action of estrogen in the blood vessels. It shows that a difference in Ca^{2+} homeostasis leading to greater basal $[Ca^{2+}]_i$ in female than male rats may be responsible for enhanced CPA endothelium-dependent vasodilation and NO secretion in female and estrogen-treated ovariectomized female rats, when compared to male or ovariectomized rats.

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Ach	acetylcholine
AM	acetoxymethyl ester
ANOVA	one-way analysis of variance
ATPasea	adenosine 5`-triphosphate
BHQ	dibenzohydroquinone
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular free calcium concentration
CAD	coronary artery disease
cGMP	cyclic guanosine 3`, 5`-monophosphate
CHD	coronary heart disease
CICR	Ca^{2+}-induced Ca^{2+} release
CIF	Ca^{2+} influx factor
CPA	cyclopiazonic acid
CRAC	calcium release-activated Ca^{2+} channels
CTX	charybdotoxin
CVD	cardiovascular disease
DiI	1,1'-diotadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMSO	dimethylsulfoxide
E_2	17 β-estradiol
ECS	extracellular space
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor

EGTA	ethylene glycol bis-(β-aminoethylether)N,N,N`,N`-tetraacetic acid
ER	endoplasmic reticulum
ERE	estrogen-response-element ERE
ERT	estrogen replacement therapy
HDL	high density lipoprotein
HEPES	hydroxyethylpiperazine ethansulphonic acid
HRT	hormone replacement therapy
IP_3	inositol 1,4,5-triphosphate
K_{ATP}	ATP-sensitive potassium channel
K_{ca}	calcium-dependent potassium channel
KD	kilodalton
LDL	low density lipoprotein
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NO	Nitric oxide
NOS	nitric oxide synthase
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
n-PSS	normal-physiological saline solution
PBS	phosphate buffered saline
PE	phenylephrine
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PSS	physiological saline solution

ROC	receptor opertaed channel
SBB	superficial Buffer Barrier
S.E.M	standard error of means
SERCA	sarco-endoplasmic reticulum Ca^{2+}-ATPase
SERM	selective estrogen receptor modulator
SNP	sodium nitroprusside
SOC	store operated channel
SR	sarcoplasmic reticulum
TEA	tetraethylamonium
VSMC	vascular smooth muscle cell

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Dedication

To ***Saeed***, my wonderful husband,

Arta, my lovely son

and ***Parveen***, my dedicated mom who believed in me at all times.

CHAPTER I. BACKGROUND

1.1. ESTROGEN AND VASCULAR REACTIVITY

1.1.1. Estrogen decreases cardiovascular disease in women

Cardiovascular disease is the leading cause of death in North American women, claiming more lives than cancer, diabetes, and accidents combined (Eaker *et al.,* 1993). While the overall mortality rate from coronary heart disease (CHD) is similar between men and women, the pathogenesis of the disease differs greatly between the sexes. During their reproductive years, women have a lower incidence of CHD compared to men of similar age (Castelli, 1988; Barret-Connor, 1994; Fig 1.1). For example, data obtained from 27 industrialized countries found the mortality rate due to ischemic heart disease to be six times higher in men than women at age forty (W.H.O., 1982). However, the risk of CHD increases dramatically in women after menopause (Barret-Connor & Bush, 1991). Of women ages 45-64 years, one in nine have cardiovascular disease and after age 65, the ratio increases to one in three (Sempos, 1993). Loss of endogenous estrogen associated with menopause contributes to the increased cardiovascular risk. Circulating plasma levels of 17 β-estradiol vary between 100 pmol and 1 nmol in women during their menstrual cycle (Genuth *et al.,* 1986). In late pregnancy estrogen levels may rise to 10 nmol/L and following menopause drop to less than 30 pmol/L (Lonning *et al.,* 1989). Conversely, in postmenopausal women taking estrogen replacement therapy (ERT), population-based studies indicate 50% fewer cardiovascular events (Stampfer *et al.,* 1991). These observations support an important role for ERT in the prevention of cardiovascular disease.

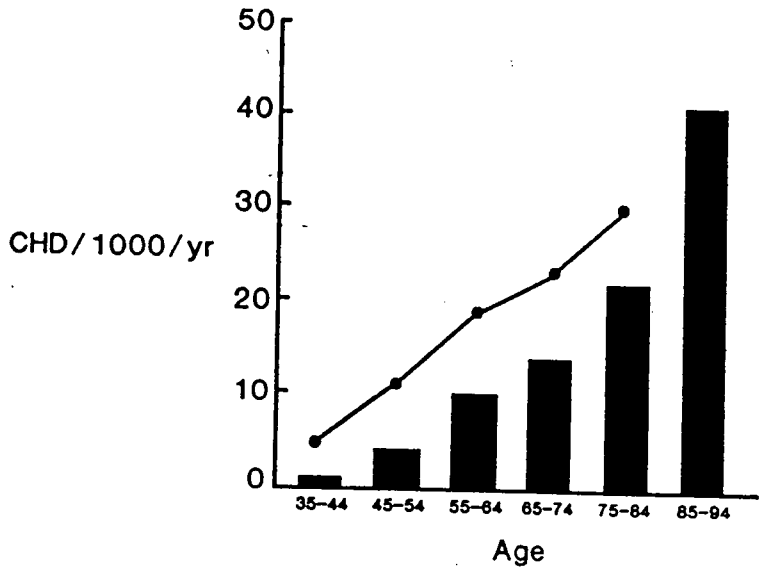

Fig.1.1. Annual rate of coronary heart disease (CHD) in men (indicated by line) and women (indicated by bars). (Adapted from the William & Castelli, 1988)

The concept of a "cardioprotective" effect of estrogen was initially derived from retrospective evidence available in case control studies. In most of these studies, ERT was associated with a decrease in the symptoms of coronary artery disease (CAD) (Rosenberg *et al.,* 1976; Adams *et al.,* 1981; Belchetz, 1994). This beneficial effect of estrogen is apparent despite small size and a duration of ERT of less than 2 yr. Prospective of ERT in postmenopausal women also have demonstrated a reduction in cardiovascular disease risk (Table 1.1). For example, one study following approximately 9000 women in a retirement community from 1981 to 1987 reported an age-adjusted death rate from myocardial infarction of 2.7/1000 for estrogen users compared to 4.5/1000 for nonusers (Henderson *et al.,* 1988). In another large study, of the 48,470 postmenopausal women participating in the study, those actively taking ERT had a 50% reduction in the risk of both fatal and non-fatal CAD compared to women who had never used supplement estrogen (Stampfer *et al.,* 1991).

Table 1.1

Relative risk of cardiovascular disease in post menopausal women receiving ERT.

Study	End point	Relative risk
Hammond *et al.*, (1979)	all CVD	0.33
Stampfer *et al.*, (1985)	all CVD	0.3
Bush *et al.*, (1987)	CVD death	0.34
Henderson *et al.*, (1988)	MI	0.54
McFarland *et al.*, (1989)	>70% LAD occlusion	0.5
Stampfer *et al.*, (1991)	Fatal & non fatal MI	0.5

MI, myocardial infarction; CVD, cardiovascular disease; LAD, left anterior descending coronary artery

1.1.2. Effect of estrogen on atherosclerosis

ERT reduces the development of atherosclerosis in ovariectomized rabbit following balloon injury and cardiac transplantation (Foegh *et al.*, 1987). Coronary artery atherosclerosis is more severe in cholesterol-fed ovariectomized cynomolgus monkeys compared to age-matched premenopausal monkeys (Adams *et al.*, 1985). ERT attenuates progression of atherosclerotic plaque in arteries of cholesterol-fed monkeys and rabbits (Williams *et al.*, 1990; Kushwaha & Hazzard, 1981). Indeed, low-density lipoprotein (LDL) uptake was less in coronary arteries of ovariectomized monkeys treated with estrogen than in hormone-deficient monkeys (Wagner *et al.*, 1991). Also, estrogen has been shown to reduce the susceptibility of LDL to oxidation (Maziere *et al.*, 1990).

1.1.3. Effect of Estrogen on lipids

The mechanism by which estrogen exerts its cardioprotective effect includes

modification of lipid profiles. When women enter menopause they develop a more atherogenic lipid profile that includes higher LDL cholesterol and lipoprotein levels and lower high density lipoprotein (HDL) cholesterol levels (Stevenson *et al.,* 1993). The lipid and lipoprotein profiles improve after ERT (Hong *et al.,* 1992) but still appear to account for only 25-50% of the observed cardiovascular risk reduction (Bush *et al.,* 1987). The presence of cardioprotective mechanisms of estrogen, which are unrelated to lipid lowering, are also evident in premenopausal women with heterozygous familial hypercholesterolemia (Hill *et al.,* 1991). These young women have higher LDL and total cholesterol levels than their age-matched male counterparts, but have significantly fewer manifestations of CAD (Hill *et al.,* 1991). Much of estrogen's beneficial cardiovascular effects may thus be mediated by its effect on vascular reactivity, especially by modifying the functional state of the endothelium.

1.1.4. Estrogen decreases vascular reactivity: Experimental models

The vascular endothelium of healthy individuals provides a protective layer that has anticoagulant and vasodilatory properties and inhibits vascular smooth muscle cell (VSMC) proliferation (Meredith *et al.,* 1993) at least in part through synthesis and release of nitric oxide (NO). NO may reduce vascular injury and retard atherogenesis by inhibiting monocyte chemotactic factors (Zeither *et al.,* 1993), monocyte adhesion (Bath *et al.,* 1991), platelet aggregation (Hogan *et al.,* 1988), and VSMC proliferation (Garg and Hassid, 1989).

Estrogen administration has been shown to improve endothelium-dependent vasodilation *in vivo* in non-human primates. Quantitative coronary angiography has been used to evaluate vasomotor responses to acetylcholine (Ach) of atherosclerotic coronary

arteries in ovariectomized monkeys who received ERT compared to those monkeys who had not received ERT. An attenuated vasoconstrictive response to Ach in the estrogen treated animal was indicative of improvement in endothelial function. Subsequent histologic sectioning of the coronary arteries revealed less atherosclerotic plaque in the coronary arteries from those monkeys who received ERT. Further, acute administration of estrogen improved endothelium-dependent vasodilation in atherosclerotic coronary arteries (Williams *et al.,* 1992). This acute effect of estrogen treatment could not result from a change in plaque size or HDL cholesterol levels. These investigators also studied the chronic effect of progesterone treatment on endothelial function in cholesterol-fed ovariectomized monkeys (William *et al,* 1994). In contrast to estrogen, progesterone alone did not improve endothelium-dependent vasodilation. When added to estrogen therapy, progesterone attenuated the favorable effects of estrogen on endothelium dependent vasodilation. Moreover, *in vitro* studies examining isometric tension development have also indicated enhanced endothelium-dependent relaxation in rabbit femoral arteries (Gisclard *et al.*, 1988) and rat thoracic aorta (Williams *et al.*, 1988) obtained from animals with elevated estrogen levels. Acute exposure of porcine left circumflex coronary arteries to estrogen also potentiated endothelium-dependent relaxations (Bell *et al.*, 1995). In ovariectomized female dogs treated with estrogen femoral artery endothelium-dependent relaxations are greatest among the estrogen-treated group (Miller & Vanhoutte, 1991). Treatment with estrogen for 16 weeks improved endothelium-dependent relaxation of coronary artery rings in hypercholestrolemic ovariectomized swine (Keaney *et al.,* 1994).

1.1.5. Effect of estrogen on vascular reactivity in women

Estrogen improves endothelium-dependent relaxation to Ach of atherosclerotic epicardial coronary arteries and coronary resistance vessels of postmenopausal women (Reis *et al.,* 1994). The improved endothelial function results in a reduction of transient myocardial ischemia (Rosano *et al.,* 1994). Studies in both men and postmenopausal women with CAD have demonstrated that intracoronary administration of estrogen decreased the vasoconstrictor response to Ach in women but not in men (Collins *et al.,* 1995). The beneficial effects of estrogen on vascular reactivity are not confined to atherosclerotic coronary arteries. Estrogen compounds also improve endothelium-dependent vasodilation in nonatheromatous peripheral vessels. Studies of the effects of estrogen on forearm resistance vessels of healthy postmenopausal women show that acute administration of estrogen improves endothelium-dependent vasodilation (Gilligan *et al.,* 1994).

1.1.6. Direct actions of estrogen on VSMCs

A direct action of estrogen on vascular smooth muscles has also been reported by several *in vitro* studies. Harder & Coulson (1979) demonstrated that a high concentration of exogenous estrogen, diethylstilbestrol (1 μM or more) directly hyperpolarizes vascular smooth muscle by activating an outward K^+ current. This direct effect of micromolar estrogen appears to be a non-receptor mediated event and is possibly related to antagonism of voltage-dependent calcium channels located on VSMCs (Shan *et al.,* 1994).

1.1.7. Estrogen increases prostacyclin production

Estrogen has been shown to increase vasodilator prostaglandins (Miller *et al.,*

1988; Chang *et al.,* 1980), and decrease vasoconstrictor prostanoids (Gisclard *et al.,* 1988). *In vitro* production of prostacyclin is decreased in uterine arteries from post-menopausal compared to pre-menopausal women (Steinleitner *et al.,* 1989). Estrogen stimulates the production of prostacyclin in cell cultures from VSMCs and endothelial cells (Cheng *et al.,* 1980; Seillan *et al.,* 1983).

1.1.8. Estrogen increases release/production of NO

A number of reports indicate that NO production may play an important role in mediating the effects of estrogen on the vasculature. A positive correlation has been found between plasma 17 β-estradiol concentrations and levels of stable metabolites of NO (nitrite/nitrate) during follicular development in women (Rosseli *et al.*, 1994). Studies in a variety of experimental models have evaluated the effects of estrogen on endothelial NO production. Hayashi *et al* (1992) found that basal release of NO is greater in endothelium intact aortic rings excised from female rabbits than in those from either ovariectomized or male rabbits.

1.1.9. Endothelium-Derived Relaxing Factor-NO

1.1.9.1. History

In 1980, Furchgott & Zawadzki coined the term endothelium-derived relaxing factor (EDRF) for the labile factor, derived from endothelium, that was essential for the vasodilator action of Ach and other substances (Furchgott & Zawadzki, 1980; Furchgott 1984). Furchgott & Zawadzki postulated that EDRF was NO or a closely related derivated NO, acting in a similar way to the nitrovasodilator drug (Furchgott *et al.,* 1987; Ignarro *et al.,* 1987). Palmer *et al* (1987) subsequently confirmed this speculation. Since then, many stimuli were found to require the presence of the endothelium to produce

partial or complete relaxation of arteries, veins and microvessels. Among these are the calcium ionophore A23187, bradykinin, hypoxia, shear stress, and endogenous substances such as thrombin, adenosine-triphosphate (ATP), serotonin and other inflammatory factors (Furchgott, 1984;Moncada *et al.*, 1989; Luscher & Vanhoutte, 1990).

1.1.9.2. NO synthase

NO is synthesized form one of the guanidine nitrogens of L-arginine. The formation of NO and L-citrulline from L-arginine in mammalian cells is catalyzed by a family of isoenzymes, the so-called NO synthases (NOS) (Fig 1.2). Three different isoforms have been classified according to the calcium dependency of the enzyme and whether they are constitutively expressed or only to be found following cell stimulation with cytokines. The NOS isoforms present in certain neuronal cells (nNOS) and in endothelial cells (eNOS) are constitutive and bind calmodulin in a Ca^{2+}-dependent manner, and therefore can be activated by agonists which elevate the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$). A mainly Ca^{2+}-independent NOS isoform can be induced (iNOS) in a variety of cells, for example macrophages, mesangial cells and VSMCs, following exposure to cytokines and/or bacterial lipopolysaccaride (Nathan, 1992).

All of the NOS enzymes share a number of general characteristics; they require NADPH, flavin adenine dinucleotide, flavin mononucleotide and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) as cofactors (Nathan, 1992).

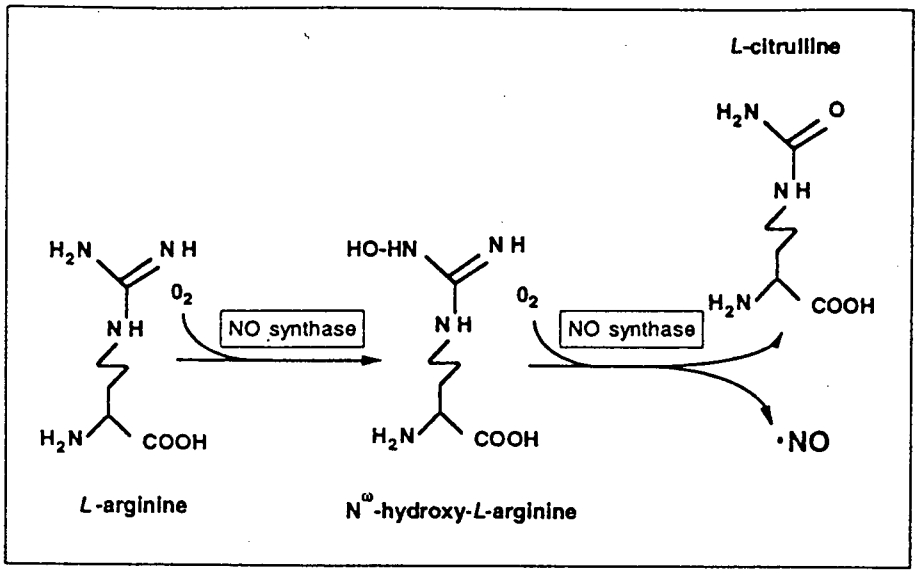

Fig.1.2. Pathway of nitric oxide (NO) biosynthesis from L-arginine by calcium-dependent and -independent NO synthase enzymes involves multiple steps with N-hydroxy-L-arginine as one intermediate (Adapted from Dusting, 1995).

1.1.9.3. Mechanism of action of NO in blood vessels

The primary target of NO in smooth muscle and platelets is the soluble guanylate cyclase (GC). Activation of GC leads to elevation of the intracellular level of cyclic 3', 5' guanosine monophosphate (cGMP) (Murad *et al.,* 1978; Ignarro *et al.,* 1981) which activates cGMP-dependent protein kinase (G-kinase) (Lincoln & Corbin, 1983). G-kinase is a serine/threonine kinase, which could potentially act at many phosphorylation sites. Several mechanisms have been proposed to account for the actions of G-kinase on vascular smooth muscle. Activity of plasmalemmal Ca^{2+}-ATPase pump (PMCA) appears to be selectively enhanced by G-kinase leading to extrusion of Ca^{2+} from the cytoplasm and muscle relaxation (Lincoln, 1989) (Fig. 1.3). Stimulation of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) may also occur through G-kinase phosphorylation of the regulatory protein phospholamban (Raeymekers *et al.,* 1988). Furthermore, a direct activation of calcium-dependent potassium (K_{Ca}) channels by NO and G-kinase has also been shown in canine coronary and bovine aortic smooth muscle cells (Taniguchi *et al.,*

1993; Archer *et al.*, 1994). The nitrovasodilators act in a similar way, being metabolized to NO in smooth muscle (Ignarro *et al.*, 1981). A different vascular relaxation mechanism has also been proposed, involving the release of endothelium-derived hyperpolarizing factor (EDHF), a factor that is distinct from NO and prostacyclin (Feletou & Vanhoutte, 1988; Taylor & Weston, 1989). In addition, both exogenous and endogenously produced NO themselves have been shown to activate charybdotoxin (CTX)-sensitive, K_{Ca} channels and induced hyperpolarization in vascular smooth muscle (Bolotina *et al.*, 1994; Archer *et al.*, 1994).

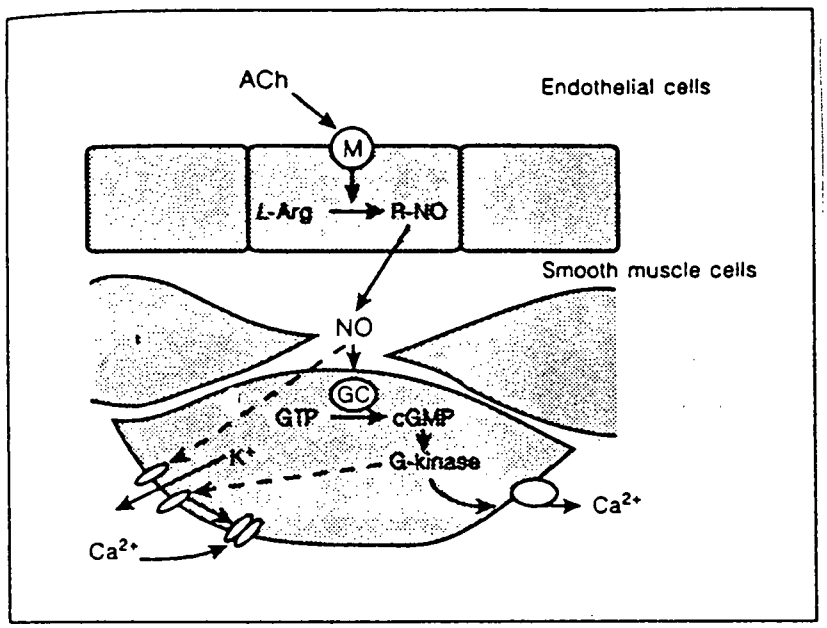

Fig.1.3. Mechanism of action of nitric oxide (NO) causing relaxation of vascular smooth muscle. Acetylcholine (Ach) also acts on the endothelial cells to activate NO synthase. The primary target of NO is the soluble guanylate cyclase (GC), leading to activation of a cGMP-dependent protein kinase (G-kinase) and subsequent extrusion of calcium, partly via a membrane pump. NO is also capable of hyperpolarizing the smooth muscle, probably by opening a K^+ channel, causing closure of voltage-operated calcium channels (Adapted from Dusting, 1995).

1.1.10. Mechanism of estrogen modulation of NO production

Increase in NO production can result from increased expression of functional enzyme as well as increased cofactors (intracellular calcium level, biopterin, calmodulin) or substrate (L-arginine transport) availability.

Endothelium-derived NO can be inactivated by superoxide anion to form peroxynitrite (Gryglewski *et al.,* 1986). Therefore, increased production of oxygen-derived free radicals may contribute to impairment of endothelium-dependent vasodilation that occurs in atherosclerosis (Harrison & O'Hara, 1995). One mechanism through which estrogen may restore endothelium-dependent relaxation is by acting as an antioxidant, thereby reducing the inactivation of NO. The antioxidant activity of estrogen may be related to the presence of the phenolic ring that is found in estrogen. Endothelium-dependent vasorelaxation is restored and LDL oxidation is reduced following estrogen administration to ovariectomized swine with dietary atherosclerosis (Keaney *et al.,* 1994). The susceptibility of LDL cholesterol to oxidation is also reduced in postmenopausal women receiving ERT (Sack *et al.,* 1994). Reduction of LDL oxidation is observed after acute administration of estrogen.

The dominant mechanism whereby estrogen exerts its long-term effects involves interactions between estrogen and classic estrogen receptor of target cell. The estrogen receptor is an intracellular receptor that is a member of large superfamily of nuclear receptors that function as ligand-activated transcription factors. Two highly conserved regions are observed in these receptors. One approximately the middle of the protein and known as the C domain, is involved in interaction with DNA. The other is in the carboxy-terminal region, the E/F domain that binds hormones (Fig 1.4). Binding of

estrogen to its receptor results in a conformational change in the receptor, which then acts as a transcriptional factor in activating genes that have estrogen-response-elements (ERE) in their promoters. The estrogen receptor also interact with DNA sites that do not contain ERE, for example, the AP1 site (which is bound by the transcription factors, c-Fos and c-Jun) (Paech *et al.,* 1997). It is also of interest that c-Fos and c-Jun expression have been associated with increased production of both endothelial (Eizirik *et al.,* 1993), and neuronal (Herdegen *et al.,* 1993, 1994) isozymes of constitutive NOS (eNOS and nNOS). The level of mRNA expression for both eNOS and nNOS was increased in skeletal muscle obtained from pregnant and estrogen treated guinea pigs (Weiner *et al.*, 1994). This estrogen-mediated increase in NOS in the vasculature could contribute to the cardioprotective effects of estrogen.

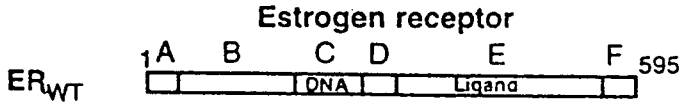

Fig.1.4. Schematic illustration of the wild-type estrogen receptors. Numbers indicate amino acid positions. Respective DNA and ligand binding domains are shown. (Adapted from Yang *et al.,* 1996)

1.1.11. The diversity of estrogen target tissues

Estrogen, acting via the estrogen receptor, plays important roles in regulating the growth, differentiation, and functioning of many reproductive tissues including the uterus, vagina, ovary, oviduct and mammary gland. In the uterus and mammary gland, estrogens increase proliferation and alter cell properties via, at least in part, the induction of growth factors and growth factors receptors (Katzenellenbogen *et al.,* 1979; Dickson and Lippman, 1987). Estrogens also have important sites of action in the pituitary,

hypothalamus, and specific brain regions, while exerting crucial actions as well on other tissues including bone, liver, and the cardiovascular system (Sarrel *et al.,* 1994; Kneifel & Katzenellenbogen, 1981; Toney & Katzenellenbogen, 1986). Thus these hormones exert their effects on diverse target tissues.

Reduction of ovarian function at menopause is, therefore, associated with a multitude of clinical symptoms that result from estrogen deficiency in various target organs such as bone, breast, skin, heart, the urogenital system and the central system. ERT (long-term treatment with estrogen) alleviates many of the symptoms of menopause and decreases the incidence of osteoporosis and heart disease; however, it is associated with an increased risk of breasts and endometrial cancer (Hammond, 1995). The challenge facing pharmacologists is to develop tissue selective agonists and/or antagonists that mimic the beneficial effects of estrogen but do not promote the growth of breast and uterine tissue.

1.1.12. Antiestrogens and selective estrogen receptor modulators

Several classes of compounds have been developed as antiestrogens. The structures of some estrogens and antiestrogens are shown in Figure 1.5. Tamoxifen (Fig. 1.5A) is an antiestrogen that is used in breast cancer chemotherapy and is believed to function as an antitumor agent by inhibiting the action of the estrogen receptor in breast tissue (Grainger & Metcalfe, 1996). Paradoxically, tamoxifen appears to function as an estrogen-like ligand in uterine tissue, and this tissue-specific estrogenic effect may explain the increased risk of uterine cancer that is observed during tamoxifen therapy (Kedar *et al.,* 1994). Chemical synthetic efforts have therefore yielded a variety of non-estrogenic compounds with varying degrees of tissue selectivity known as selective

estrogen receptor modulators or SERMs (Kauffman & Bryant, 1995). The benzothiophene, LY117018 and raloxifene (Fig. 1.5A) are examples of highly promising SERM. They have been reported to retain the antiestrogen properties of tamoxifen in breast tissue and to show minimal estrogen effects in the uterus; in addition, it has potentially beneficial estrogen-like effects in nonproductive tissue such as bone (Jones *et al.*, 1984; Black *et al.*, 1994; Sato *et al.*, 1996; Yang *et al.*, 1996; Yang *et al.*, 1996). One explanation for these tissue-specific actions of antiestrogens is that the ligand-bound estrogen receptor may have different transactivation properties when bound to different types of DNA enhancer elements.

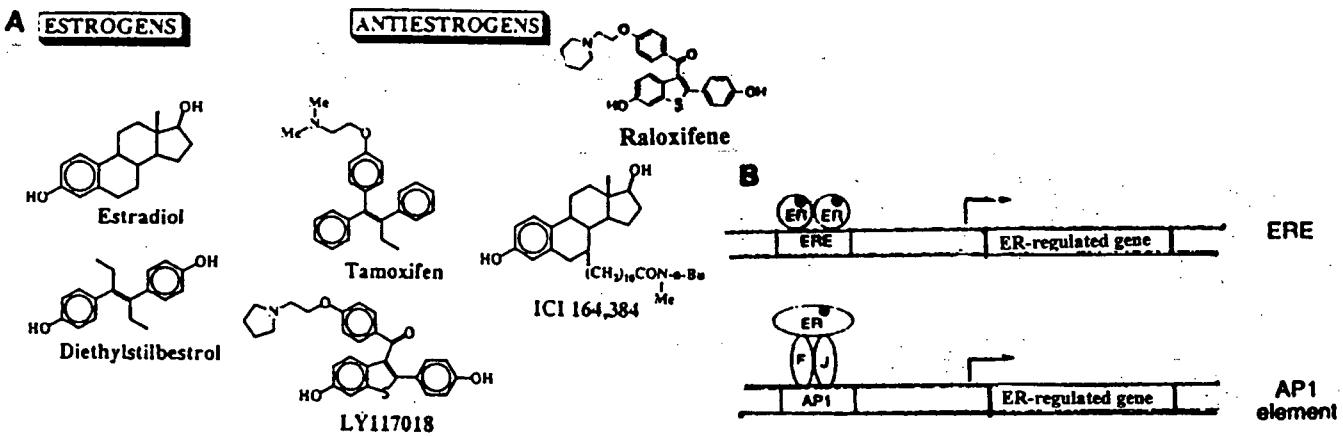

Fig.1.5. **(A)** Structures of several estrogenic and antiestrogenic ligands for the estrogen receptor. The antiestrogens include the nonestroidal compounds tamoxifen, LY117018, and raloxifene and the steroidal antiestrogen ICI164,384. Bu, butyl; Me, methyl. **(B)** Models of estrogen receptor (ER) action at a classical estrogen ER element (ERE) and an ER-dependent AP1 response element. The filled circles represent the ligand bound to the ER. The AP1 proteins Jun and Fos are labeled J and F, respectively. (Adapted from Paech *et al.*, 1997).

The classical ERE is composed of two inverted hexanucleotide repeats, and ligand-bound estrogen receptor binds to the ERE as a homodimer (Fig 1.5B). The estrogen receptor also mediates gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors c-Fos and c-Jun for transcriptional activation (Fig 1.5B) (Umayahara *et al.,* 1994). In transactivation experiments, tamoxifen inhibits the transcription of genes that are regulated by a classic ERE, but like the natural estrogen hormone 17 β-estradiol (E_2) (Fig 1.5A), tamoxifen activates the transcription of genes that are under the control of an AP1 element (Webb *et al.,* 1995). At the end of 1995, a second estrogen receptor (β) was cloned from a rat prostate cDNA library (Kuiper *et al.,* 1996), and subsequently, the human (Mosselman *et al.,* 1996) and mouse (Tremblay *et al.,* 1997) homologues were cloned. The first identified estrogen receptor has been renamed estrogen receptor α (Kuiper *et al.,* 1996). Both the beneficial and the unwanted effects of estrogen are mediated by estrogen receptors α and β which have unique tissue distributions and different affinities for estrogenic agonists and antagonists (Kuiper *et al.,* 1996). The existence of two estrogen receptors, therefore, presents another potential source of tissue-specific estrogen regulation. Paech *et al* (1997) recently reported that estrogen receptors α and β respond differently to certain ligands at an AP1 element. In the presence of estradiol, estrogen receptor α activates transcription from the AP1 site whereas estrogen receptor β inhibits the transcription. On the contrary, in the presence of SERMs such as raloxifene, estrogen receptor β stimulates transcription from the AP1 site whereas estrogen receptor α prevents it. Therefore, the discovery of selective estrogen receptors α and β agonist and antagonist will greatly facilitate the

search for tissue-specific modulators of estrogen.

1.2. VASCULAR ENDOTHELIUM

1.2.1. The structure of endothelial cells

The endothelium constitutes of a monolayer of cells lining the luminal surface of the blood vessels (vascular endothelial cells) and heart cavities (endocardial endothelial cells). The endothelial cell has a nucleus. The cytoplasm contains a variable number of Weibel-Palade bodies (cytoplasmic inclusion bodies composed of longitudinally located tubules), endoplasmic reticulum (ER), mitochondria, Golgi apparatus, vesicles and bundles of intermediate filaments. The plasmalemmal membrane contains caveoli which are 50-100 nm membrane domains of various flat or invaginated morphology, increasing the surface area and functioning in the transcytosis of certain blood-borne macromolecules (Lisanti *et al.,* 1994). The ER, a major intracellular Ca^{2+} store, is a highly convoluted meshwork of interconnected membranous tubules or flattened cisternae partly decorated with ribosomes. The ER constitutes more than half of the total membrane of the endothelial cell and extends throughout the cytoplasm (Albert *et al.,* 1989). The ER membrane mediates rapid exchange of Ca^{2+} between the ER lumen and the cytoplasm. Although there is no direct histological support for the apposition of the plasmalemma and the ER in endothelial cells, a bridging structure has been observed between these two compartments in other types of cells such as VSMCs (Somlyo, 1985). It is therefore likely that these junctional regions may be present in endothelial cells that separate the cytoplasm leaflets of the plasmalemma and the adjacent ER, with the "superficial ER" located in the cell periphery and the "deep or central ER" located in the deeper cytoplasm.

The continuous endothelial monolayer has intact basal lamina and gap junctions between endothelial cells. However, gap junctions between endothelial cells and smooth muscle cells, the so-called myoendothelial junctions, are absent in endocardial endothelial cells. The junctional area in the endocardial cells is either a simple structure with straight intracellular clefts between two adjacent cells, or a complex organization with considerable overlapping between peripheral cell parts and membrane interdigitations. At some points there may be some close contacts such as tight junctions (Andries & Brutsaert, 1991; Laskey *et al.,* 1994).

1.2.2. Endothelial cell sources

Several endothelial cell types from different sources can be used for endothelial studies. Freshly isolated, cultured or intact endothelial cells are mainly used.

1.2.2.1. Isolated endothelial cells

Endothelial cells are freshly isolated from the vasculature and dispersed on a coverslip for study. There are several ways of harvesting cells from vasculature:

a) Mechanical harvesting: Endothelial cells on the surface of vessels can be mechanically isolated by scraping with a scalpel or peeling off the luminal surface of a vessel. The endothelial sheets collected in this way preserve the original polarity. However, initial isolates are not suitable for experiments requiring large numbers of uniform endothelial cells and have some inherent disadvantages such as contamination by smooth muscle cells. This method is not suitable for the study of endothelial cells from small vessels.

b) Proteolytic enzyme digestion: Due to the possibility of contamination by other cell types such as smooth muscle cells and fibroblast cells, researchers attempted to isolate endothelial cells from blood vessels by perfusing vessels with proteolytic enzymes

(trypsin, collagenase, papain, etc.), making it possible to isolate relatively greater populations of endothelial cells. The enzymes, especially collagenase, selectively digest the subendothelial basement membrane, leaving the internal elastic lamina intact (Majno, 1970). This method markedly increases the yield of endothelial cells, decreases the contamination by smooth muscle cells or fibroblast cells and supports replication of endothelial cells.

c) Microcarrier beads: Isolation of endothelial cells from small vessels can be achieved by perfusion with cold solution and microcarrier beads without using proteolytic enzymes (Ryan *et al.*, 1982). Cold solution can cause endothelial cells to round up and detach from the vessel wall. The loosened cells can be collected by perfusion. Microbeads in the perfusion solution provide a large surface area for endothelial adhesion and the yield of cells is enhanced. Cells can be removed from beads by vortexing.

1.2.2.2. Cultured endothelial cells

The number of cells collected by isolation procedures is not sufficient for some studies. The endothelial cells can be subsequently seeded onto culture flasks containing tissue culture medium to proliferate. Monolayer growth appears to be a fundamental characteristic of vascular endothelium. After transferring to a new flask, cells can then be replicated for more passages.

1.2.2.3. Intact endothelial cells

To approximate the physiology of the intact vasculature, an intact endothelial preparation is used as a preparation that has not been exposed to any enzyme or culture medium. This preparation has the least mechanical damage.

1.2.3. Identification of endothelial cells

The vessel source for endothelial cells also contain smooth muscle cells, fibroblast, etc. It is therefore important to characterize the endothelial cells among these cells. The following is a list of the main markers that are frequently used for the positive identification of endothelial cells (Zetter, 1984): microscopy, non-thrombogenic surface, factor VIII/ von willebrand (VIII/vWF) antigen, Weibel-palade bodies, angiotensin converting enzyme (ACE), production of prostacyclin (PGI_2), and uptake of acetylated low density lipoprotein (Ac-LDL),

1.2.3.1. Uptake of Ac-LDL

The presence of Ac-LDL uptake is often used as the positive identification of endothelial cells. Cells are incubated in growth media containing Ac-LDL labeled with the fluorescent probe 1,1'-diotadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). The uptake of fluorescent DiI-Ac-LDL by cells can be visualized on an epifluorescence microscope equipped with rhodamine fluorescence filters. All endothelial cells incorporate DiI-Ac-LDL in localized regions. A negative control is provided by contractile smooth muscle cells and fibroblasts.

1.2.4. Endothelial ion channels

The endothelial cell is an interesting example of a multifunctional cell type. Endothelial cells form an ideal surface for blood flow; they prevent blood clotting but can also trigger it in response to various signals, and thus can exert thrombolytic as well as thrombogenic activity. As antigen-presenting cells, they are involved in immune responses. Changes in their contractile state and their ability to modulate cell-cell contact control the permeability of the blood-tissue interface. Furthermore, they help to adjust the vessel diameter to hemodynamic needs. These multiple functions are mediated by the

production and release of a variety of vasoactive agents. These substances include NO, EDHF, various prostaglandins, endothelin, natriuretic peptide, substance P, ATP, growth factors, steroids, and even large proteins such as receptors and proteins involved in the blood clotting cascade (Inagami *et al.,* 1995; Nilius & Casteels, 1996). Endothelial cells respond not only to humoral substances, which bind to receptors on their luminal and abluminal surface, but also to mechanical forces such as changes in flow rate (shear stress) or blood pressure (biaxial tensile stress) (Davies & Tripathi, 1993; Malek & Izumo, 1994). It is well documented that production and release of most of these agents is initiated by Ca^{2+}-dependent mechanisms. Ion channels activated by agonists and/or mechanical factors provide influx pathways for Ca^{2+} (Nilius, 1991). The membrane potential which is mainly controlled by K^+, Cl^-, and possibly nonselective cation channels, is an important regulator of intra- and intercellular signal transduction in various vascular functions, especially by modulating the driving force for transmembrane Ca^{2+} fluxes.

1.2.5. Regulation of $[Ca^{2+}]_i$

Regulation of endothelial $[Ca^{2+}]_i$ is composed of activating mechanisms which supply Ca^{2+} to the cytoplasm and homeostatic mechanisms which remove cytoplasmic Ca^{2+} after stimulation. The activating mechanisms include Ca^{2+} entry from extracellular space (ECS) and Ca^{2+} release from the intracellular stores.

1.2.5.1. Ca^{2+} entry pathways

a) Ca^{2+} "leak": Under physiological conditions a passive non-regulated Ca^{2+} leak across the plasmalemma, driven by the electrochemical gradient for Ca^{2+} (E_m-E_{Ca}, E_m: membrane potential; E_{Ca}: the equilibrium potential for Ca^{2+}), is present and increases

$[Ca^{2+}]_i$ in endothelial cells (Johns *et al.,* 1987; Schilling, 1989; Demirel *et al.,* 1993). Depolarization reduces the driving force for Ca^{2+} entry through this leak pathway by reducing the electrochemical gradient. The nature of the leak pathway in endothelial cells is not known but it may play an important physiological role in the basal release of NO and other vasoactive mediators, thus regulating vascular tone.

b) Non-selective receptor operated cation channels (ROCs): The existence of ROCs permeable to Ca^{2+} has been demonstrated in endothelial cells (Bregestovski *et al.,* 1988; Nilius, 1990). The binding of an agonist to its receptor leads to an enhanced Ca^{2+} influx (Whorton *et al.,* 1984; Johns *et al.,* 1987; Lodge *et al.,* 1988). Agonists such as Ach, histamine, bradykinin, ATP, serotonin, substance P, and endothelin 1 activate ROCs. The observation that apparently similar ion channels are activated by different agonists suggests a convergence of the intracellular messenger cascade between receptor activation and channel opening. Application of SERCA inhibitors CPA, thapsigargin, dibenzohydroquinone (BHQ), and also intracellular application inositol 1,4,5, triphosphate (IP_3), which releases Ca^{2+} from intracellular stores, activate ROCs. Activation of these channels has been correlated with store depletion (Inazu *et al.,* 1994; Pasyk *et al.,* 1995). The Ca^{2+} entry blocker SK&F96365 inhibits ROCs in endothelial cells, but only in a narrow concentration range (Schwarz *et al.,* 1994).

c) Stretch-activated Ca^{2+} channels (SACs): Mechanosensitive (stretch-activated) ion channels in endothelial cells may serve as transducers for detecting changes in blood pressure or flow (shear stress) (Lansman *et al.,* 1987; Popp & Gogelein, 1992). Mechanosensitive ion channels could change endothelial cell membrane potential, and thus the driving force for passive Ca^{2+} entry. This may be a possible mechanism by

which the vascular endothelium in intact vessels regulates smooth muscle tone in response to hemodynamic stimuli (Rubanyi *et al.,* 1990).

d) Na^+-Ca^{2+} exchange: The Na^+-Ca^{2+} exchanger functions reversibly so that Ca^{2+} can be transported in either direction (inwardly or outwardly) across the plasmalemma in exchange for Na^+, depending on the electrochemical gradient of Na^+ and Ca^{2+} across the membrane (Blaustein, 1977). Variation of the intracellular or extracellular Na^+ concentration ($[Na^+]_i$ or $[Na^+]_o$, respectively) thus affects the level of $[Ca^{2+}]_i$. The net Ca^{2+} movement $J_{Ca(Na/Ca)}$ mediated by the exchanger is determined by the E_m, the reversal potential of the exchanger ($E_{Na/Ca}$) and the kinetic parameter (k) that controls the rate of exchange: $J_{Ca(Na/Ca)} = k\ (E_m - E_{Na/Ca})$. The stoichiometry for the exchanger has been shown to be $3Na^+$: $1Ca^{2+}$. Thus the reversal potential for the Na^+-Ca^{2+} exchange is $E_{Na/Ca} = 3E_{Na} - 2E_{Ca}$, where the equilibrium potential for Na^+: $E_{Na} = (RT/F)\ \ln\ ([Na^+]_o/[Na^+]_i)$, and the equilibrium potential for Ca^{2+}: $E_{Ca}=(RT/2F)\ \ln\ ([Ca^{2+}]_o/[Ca^{2+}]_i)$. R, T, and F are the gas constant (1.987 $calK^{-1}\ mol^{-1}$), absolute temperature (273.16+T[°Celsius]) and Faraday's number ($9.648 \times 10^4 C\ mol^{-1}$), respectively. If $E_{Na/Ca}$ is lower than the E_m, the exchanger *in vivo* will operate in the Ca^{2+} influx mode (inwardly); if the $E_{Na/Ca}$ is higher than E_m, the exchanger will operate in the Ca^{2+} efflux mode (outwardly) (Blaustein, 1984). The existence of the Na^+-Ca^{2+} exchanger in the endothelium have been demonstrated in cultured bovine pulmonary and aortic endothelial cells (Sago *et al.,* 1991; Hansen *et al.,* 1991). Data obtained from intact endothelium of rabbit cardiac valve also support a role for Na^+-Ca^{2+} exchange (Li & van Breemen, 1995).

e) Voltage gated Ca^{2+} channels (VGCs): Although several reports have provided evidence for the existence of voltage gated ion channels in endothelial cells (Rubanyi *et al.,* 1985;

Singer & Peach, 1982; Bossu *et al.,* 1992), it is generally accepted that they are non-excitable and that voltage gated channels are functionally not important (Furchgot, 1983; Jacob *et al.,* 1988; Olesen *et al.,* 1988; Sturek *et al.,* 1991). In the case of studies that argue against the presence of VGC, the role of E_m in regulating Ca^{2+} entry is simply to determine the driving force for Ca^{2+} entry (E_m-E_{Ca}). Therefore, depolarization decreases and hyperpolarization increases $[Ca^{2+}]_i$ in endothelial cells.

f) K^+ channels: K^+ channels play an important role in the regulation of the endothelial cell membrane potential. Membrane depolarization of endothelial cells by elevating extracellular K^+ reduces agonist-stimulated Ca^{2+} influx in endothelial cells (Adams *et al.,* 1989; Laskey *et al.,* 1990, Luckhoff & Busse, 1990). There are at least three types of K^+ channels present in endothelial cells: 1) an inwardly rectifying K^+ channel (IRK) activated by membrane hyperpolarization (Johns *et al.,* 1987; Takeda *et al.,* 1987) or shear stress (Olesen *et al.,* 1988). Tetraethylammonium (TEA) and tetrabutylammonium (TBA) block this channel (Inazu *et al., 1994b*); 2) calcium-activated K^+ channels (K_{Ca} channels) activated by membrane depolarization or a rise in $[Ca^{2+}]_i$ (Sauve *et al.,* 1988; Rusko *et al.,* 1992). They are blocked by TEA, charybdotoxin (CTX), d-tubocurarine (Rusko *et al.,* 1992); 3) an ATP-sensitive K^+ channel (K_{ATP}) (Janigro *et al.,* 1992) activated by micromolar concentration of the K^+ channel opener levcromakalim and also by shear stress. It is blocked by an increase in intracellular ATP, glibenclamide, extracellular Ca^{2+} and TEA.

g) Ca^{2+}-permeable channels: Changes in $[Ca^{2+}]_i$ induced by agonists such as Ach, ATP, bradykinin, substance P consist of an initial fast peak due to release of Ca^{2+} from IP_3-sensitive intracellular stores followed by a sustained rise due to Ca^{2+} entry. The increase

in $[Ca^{2+}]_i$ activates K_{Ca} current, which modulates the driving force for Ca^{2+} influx. The long lasting plateau, as well as the K_{Ca} current, disappears in Ca^{2+}-free extracellular solution. The relation between store depletion and activation of Ca^{2+} influx pathway is not clear yet. Compounds such as BHQ and CPA, which deplete intracellular Ca^{2+} stores without affecting IP_3 production, activate a Ca^{2+} influx in endothelial cells (Dolor *et al.,* 1992; Schilling *et al.,* 1992), but the link with store depletion has not been unequivocally demonstrated.

Several intracellular messengers, such as a low-molecular weight, nonpeptide Ca^{2+} influx factor (CIF) (Randriamampita & Tsien, 1993) and cGMP (Bahnson *et al.,* 1993), have been proposed to be responsible for signaling the degree of store filling to the plasma membrane. In endothelial cells, this signaling may be mediated by 5,6-epoxyeicosatrienoic acid (5,6-EET), an arachidonic acid metabolite synthesized by a P_{450} mono-oxygenase located in the ER membrane and activated by a decrease of intraluminal Ca^{2+} (Graier *et al.,* 1995; Berridge, 1995). Two low molecular weight tyrosine kinases (42 and 44 KD MAP-kinases) (Fleming *et al.,* 1995) and another tyrosine kinase (MAP-kinase, integrine-associated Ca^{2+} entry) (Schwartz *et al.,* 1993), as well as protein kinase A (PKA) (Graier *et al.,* 1993) and protein kinase C (PKC) (Murphy *et al.,* 1994), have been reported to modulate Ca^{2+} influx. Ni^{2+}, La^{3+}, heparin, SK&F96365, the tyrosine kinase inhibitor genistein, P_{450}-inhibitors, and surprisingly, also the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) inhibit this influx (Dolor *et al.,* 1992, Fleming *et al.,* 1995; Gerick *et al.,* 1994 ,Graier *et al.,* 1995; Schilling *et al.,* 1992; Vaca & Kunze, 1994).

This Ca^{2+} influx pathway may be extremely important for several endothelial cell

functions such as the synthesis and release of vasoactive substances, e.g. NO, PGI_2, and gene expression (Inagami *et al.,* 1995, Nilius & Casteels, 1996; Resnick & Gimbrone, 1995). The putative channels gated by store depletion have been called CRAC (calcium release activated Ca^{2+} channels) or more general SOC (store-operated Ca^{2+} channels) (Clapham, 1995; Berridge, 1995).

1.2.5.2. Ca^{2+} release pathways

a) IP_3-mediated Ca^{2+} release from ER: The intracellular IP_3 level is enhanced upon agonist stimulation and is correlated with $[Ca^{2+}]_i$ increase (Lambert *et al.,* 1986). Agonist occupation of a receptor coupled G-protein leads to the activation of phospholipase C (PLC) and production of IP_3 (second messenger) which in turn triggers the release of Ca^{2+} from an intracellular store. IP_3 binds to specific receptors to open Ca^{2+}-permeable channels on ER membranes to rapidly release Ca^{2+} (Derian & Moskowitz, 1986). This messenger is effective in releasing ER Ca^{2+} with high affinity (K_d = 1 μM), but has no effect on the mitochondrial Ca^{2+} (Freay *et al.,* 1989).

b) Ca^{2+}-induced Ca^{2+} release (CICR): CICR may be involved in propagating Ca^{2+} release initiated by IP_3 (Berridge & Gallione, 1988). In this model, IP_3 releases Ca^{2+} from an IP_3-sensitive store to give an initial Ca^{2+} signal which can then act as a "primer" to drive a CICR process from IP_3-sensitive pools to further elevate $[Ca^{2+}]_i$. The idea of CICR was first proposed by Fabiato (1985), but to date there has been sparse evidence for this model (Lipscombe *et al.,* 1988).

c) Ca^{2+} release from mitochondria: The mitochondrion represents another organelle capable of accumulating Ca^{2+}. A significant involvement of the mitochondria in Ca^{2+} homeostasis is generally believed only to occur when the cytosolic $[Ca^{2+}]_i$ rises to levels

so high that they might become ultimately dangerous for cell life (Meldolesi *et al.,* 1990). For example, injury may alter the permeability of the plasma membrane permitting an excessive influx of calcium into the cell. If this occurs, the mitochondrial uptake system becomes activated, leading to the storage of large amounts of precipitated calcium phosphate in the matrix. If the injury heals, mitochondria release the stored calcium slowly, at a rate that is compatible with the exporting ability of the plasma membrane systems. Thus, they protect the cell against a calcium overload.

1.2.5.3. Ca^{2+} extrusion pathways

The resting free $[Ca^{2+}]_i$ in all cells so far investigated is close to 100 nM. This is at least 10000 fold lower than the external $[Ca^{2+}]_o$, implying that there is a large chemical gradient for Ca^{2+} across the plasma membrane. Because the interior of the cell is negatively charged relative to the outside, there is, in addition, an electrical gradient that favors Ca^{2+} movement into the cell. The Ca^{2+} extrusion mechanism of the plasma membrane continuously extrudes Ca^{2+} that is leaking into the cell and keeps the resting $[Ca^{2+}]_i$ low. In endothelial cells, Ca^{2+} removal from the cytoplasm is mediated by Ca^{2+} extrusion towards the ECS and Ca^{2+} uptake into intracellular stores.

a) PMCA: The PMCA appears to be primarily responsible for Ca^{2+} extrusion towards the ECS (Hagiwara *et al.,* 1983). Blockade of this pump would, therefore, raise $[Ca^{2+}]_i$.

b) Na^+-Ca^{2+} exchange on the plasmalemmal membrane: The Na^+-Ca^{2+} exchanger contributes to Ca^{2+} extrusion. There are observations that strongly suggesting the presence of the Na^+-Ca^{2+} exchanger in the endothelium. It was reported that if cultured endothelial cells were first Na^+-loaded with the Na^+ ionophore monensin and then exposed to physiological saline solution with the external Na^+ substituted by Li^+, a large

transient increase in $[Ca^{2+}]_i$ ensued (Sago *et al.,* 1991). This observation clearly established the presence of Na^+-Ca^{2+} exchange in cultured endothelial cells. A similar conclusion was obtained by decreasing the Na^+ gradient of the endothelial cells through either Na^+-Ca^{2+} pump inhibition (ouabain), or reversing the Na^+ gradient through Na^+ substitution; both mechanisms increased $[Ca^{2+}]_i$ in intact endothelial cells of rabbit cardiac valve (Li & van Breemen, 1995).

1.2.5.4. Ca^{2+} sequestration into the ER

a) SERCA: The SERCA pumps cytoplasmic Ca^{2+} into the ER, therefore, decreasing $[Ca^{2+}]_i$. The Ca^{2+} transport mechanisms on the plasmalemma and ER work in an integrated manner to maintain the Ca^{2+} homeostasis at rest and under stimulated condition (Adams *et al.,* 1993).

1.2.6. $[Ca^{2+}]_i$ measurement with fluorescent dyes

It has been recognized that many cellular processes are mediated by changes in $[Ca^{2+}]_i$ (Cheung *et al.,* 1986). However, an adequate evaluation of the role of $[Ca^{2+}]_i$ requires quantitative measurement of this ion. The most popular method for measuring $[Ca^{2+}]_i$ uses fluorescent dye probes such as quin-2, fura-2, indo-1, and fluo-3 (Grynkiewicz *et al.,* 1985; Tsien, 1989). Fluorescent indicators have much faster response times than Ca^{2+}-sensitive microelectrodes (Marban *et al.,* 1980) and can be loaded into cells without disruption of the plasma membrane. Table 1.2 shows properties of fluorescent indicators that are commonly used. Fura-2 has been widely used because it has several properties that are advantageous compared with those of other Ca^{2+} indicators (Tsien *et al.,* 1982). Fura-2 has a higher quantum yield and an improved selectivity for Ca^{2+}, and is more resistant to photobleaching when compared to its predecessor quin-2

(Grynkiewicz *et al.*, 1985).

Table. 1.2. Properties of fluorescent indicators of Ca^{2+}

Dye	Excitation Wavelength (nm)	Emission Wavelength (nm)	Apparent K_d for Ca^{2+} (nM)
Fura-2	340 & 380	500	224
Indo-1	340	405 & 485	250
Fluo-3	500	530	400

The 30-fold increase in fluorescence intensity makes it possible to decrease intracellular dye loading and buffering of $[Ca^{2+}]_i$. Most importantly, the peak excitation wavelength changes when fura-2 binds Ca^{2+}. As shown in figure 1.6, there is a marked shift in the excitation spectrum; the signal with 340-nm excitation increases with Ca^{2+} saturation, while the 380-nm signal decreases.

As a consequence, measurement of the fluorescence ratio at two excitation wavelengths can be used to obtain an estimate of $[Ca^{2+}]_i$ that is independent of cytosolic dye concentration, cell thickness, and excitation light intensity. Fura-2 and other fluorescent Ca^{2+} dyes are available in the acetoxymethyl (AM) esters which are hydrolyzed by intracellular esterases, trapping the Ca^{2+}-sensitive free acid inside the cell (Tsien, 1981). This technique makes dye loading simple; cells are incubated with the AM ester of the dye for some time and subsequently washed with dye-free buffer.

$[Ca^{2+}]_i$ can be calculated according to the following equation (Grynkiewicz *et al.*, 1985): $[Ca^{2+}]_i = K_d . b . [(R-R_{min})/(R_{max}-R)]$ where K_d is the dissociation constant of Ca^{2+}-fura 2 complex; R is the above-mentioned fluorescence ratio (F_{340}/F_{380}); R_{min} and R_{max} are

the ratios measured by the addition of the Ca^{2+} ionophore ionomycin (10 μM) to Ca^{2+}-free [with 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)] solution and Ca^{2+}-replete (2 mM $CaCl_2$) solution, respectively; and b is the ratio of the 380-nm signals in Ca^{2+}-free and Ca^{2+}-replete solution. This equation has to be used with three assumptions: 1) only the deesterified form of the dye is present inside cells; 2) the only source of fluorescence in cells or in the calibration solutions is fura-2; and 3) fura-2 behaves in cells as it does in the calibration solution (Grynkiewicz *et al.*, 1985).

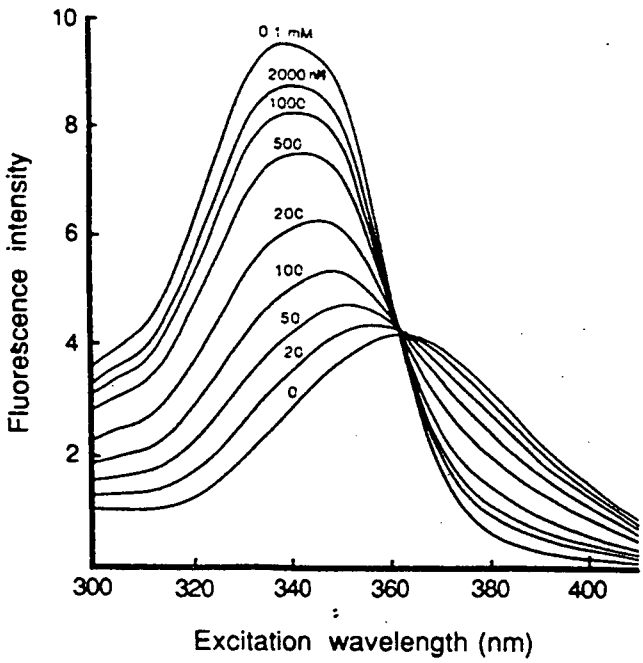

Fig.1.6. Excitation spectra of fura-2. This shows a typical set of excitation scans obtained under ionic conditions appropriate for mammalian cytoplasm as fura-2 is titrated through a series of increasing Ca^{2+} concentrations. The excitation maxima shift toward 340-nm as Ca^{2+} increases. (Adapted from Hayashi & Haruo, 1994)

1.2.7. Hypotheses for regulated Ca^{2+} entry

There are three main hypotheses regarding the regulation of Ca^{2+} entry, which are not mutually exclusive.

1.2.7.1. The "Superficial Buffer Barrier (SBB)" hypothesis

The "SBB" hypothesis has been proposed first by van Breemen (van Breemen, 1977). In essence the "SBB" hypothesis states: 1) Ca^{2+}, which enters the cell across the plasmalemma, is in part pumped into the ER before it exerts its biological function. The peripherally located ER thus functions as a barrier for Ca^{2+} diffusion into the deeper cytoplasm. The Ca^{2+}-ATPase on the superficial ER membrane (SERCA) essentially contributes to the process by pumping Ca^{2+} into the ER. In this fashion the superficial ER would play a specialized Ca^{2+} entry buffer function. Inhibition of the ER buffering of Ca^{2+} entry is thus postulated to raise $[Ca^{2+}]_i$. 2) The influx of Ca^{2+} across the plasmalemma combined with its removal from the cytoplasm by the ER creates a Ca^{2+} gradient in the peripheral cytoplasm. In other words, in the resting state, $[Ca^{2+}]_i$ in the peripheral cytoplasm is higher than in the deeper part of the cell. 3) Ca^{2+} accumulation by the SERCA contributes to Ca^{2+} extrusion from the cell due to vectorial release of ER Ca^{2+} towards a restricted space in the inner surface of the plasmalemma, from where it is extruded by the Na^{+}-Ca^{2+} exchanger and the PMCA. A pictorial model of this hypothesis as originally proposed (van Breemen, 1977& 1986 & 1995) is illustrated in figure 1.7.

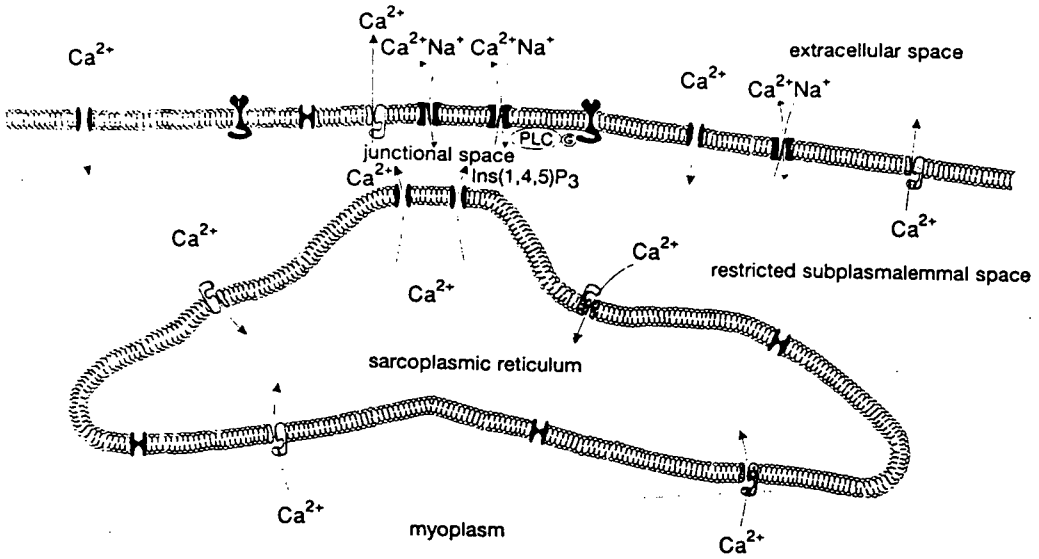

Fig. 1.7. Schematic representation of the superficial buffer barrier (SBB). Ca^{2+} entry through the basal Ca^{2+} leak and ligand-, voltage- or stretch-gated channels is partially sequestrated by the superficial endoplasmic reticulum (ER) from a restricted subplasmalemmal space by the ER Ca^{2+}-ATPase (SERCA). The superficial ER functions as a Ca^{2+} buffer barrier. Mobilization of ER Ca^{2+} will short circuit the SBB and enhance the flow of Ca^{2+} into the deeper cytoplasm. G, G protein; PLC, phospholipase C.(Adapted from van Breemen *et al.,* 1995).

1.2.7.2. The " Capacitative Ca^{2+} entry" hypothesis

Casteels & Droogmans (1981), Cauvin & van Breemen (1985), Putney (1986), and Bourreau *et al* (1991) have proposed a direct Ca^{2+} pathway between the ECS and the ER, which bypasses the SERCA. The essence of this hypothesis is that the depletion of the ER by any mechanism (e.g., IP_3 generation, SERCA blockade) signals the opening of a pathway on the plasmalemma and thus increases Ca^{2+} entry from the ECS into the cytoplasm. The notion of a direct coupling between the ER and plasmalemma was subsequently retracted and the generation of an unknown messenger from the depleted ER to open cell membrane Ca^{2+} channels was proposed (Putney, 1990; Randriamampita & Tsien, 1993). A pictorial model of this hypothesis is illustrated in figure 1.8.

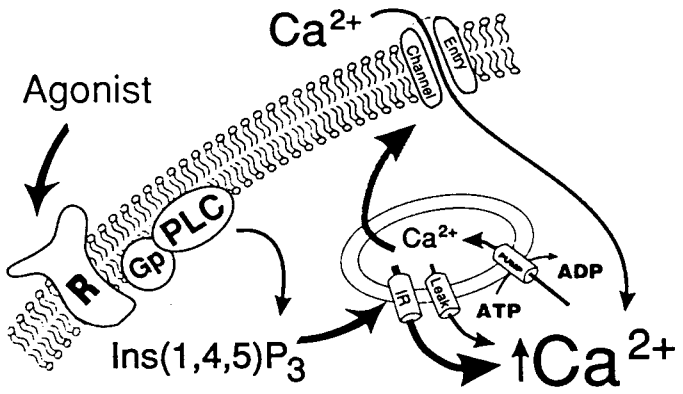

Fig.1.8. Schematic representation of the " capacitative Ca^{2+} entry" hypothesis: Ca^{2+}-release-activated Ca^{2+} channels (CRAC). Agonists activate surface membrane receptor (R) which in turn activate a phospholipase C (PLC); in many instances, a guanine nucleotide-dependent regulatory protein (Gp) is involved in coupling receptor to PLC. This leads to the production of (1,4,5) IP_3, which in turn activates the release of Ca^{2+} via an IP_3 receptor-channel (IR). The release of Ca^{2+} is faster that the rate at which Ca^{2+} is returned by the associated Ca^{2+}-ATPase or PUMP, leading to depletion of the Ca^{2+} content of this internal organelle. The depletion of Ca^{2+} store causes, by unknown mechanism (large gray arrow), the opening of plasma membrane (Entry channel) for Ca^{2+}. (Adapted from Putney & Bird, 1993).

1.2.7.3. Receptor-mediated Ca^{2+} entry

A direct coupling of receptors to plasmalemmal membrane channels mediated by one or more specific G proteins has been proposed for Ca^{2+} entry (Fasolato *et al.*, 1988, 1994; Graier *et al.*, 1991). This type of G protein involved in Ca^{2+} influx regulation is different from the G proteins responsible for the coupling of receptors to PLC (Komori & Bolton, 1990). In addition, Ca^{2+} could also enter through channels that are controlled by second messengers generated as a result of agonist-receptor binding (Penner *et al.*, 1993).

1.3. VASCULAR SMOOTH MUSCLE

Vascular smooth muscle controls vasoconstriction and vasodilation and therefore plays an important role in regulating circulation. Ca^{2+} may be regarded as the main regulator of smooth muscle cells. Ca^{2+} enters the cell from the extracellular space or is released from the sarcoplasmic reticulum (SR) into the cytoplasm, and is subsequently

extruded from the cell into the extracellular space or removed by uptake into the SR from the cytoplasm.

1.3.1. The structure of VSMCs

Smooth muscle cells are spindle- or branch-shaped cells approximately 100-500 μm long and 2-6 μm in diameter (Somlyo, 1986). They are embedded in extracellular connective tissues that constitute between 10-60% of the tissue volume (Gabella, 1979). The plasmalemma forms rows of small invagination (caveolae) which increase the surface area by about 75% . The SR occupies about 1.5-7.5% of the total smooth muscle cell volume. The SR appears as two groups: one distributes underneath the plasmalemma which is called superficial or peripheral SR, and the other is located in the deep cytoplasm which is called deep or central SR. Myofilaments, which compose the contractile apparatus, are located throughout the smooth muscle cells.

1.3.2. Contractile apparatus

There are three types of myofilaments in smooth muscle cells: thick (myosin), thin (actin), and intermediate.

a) Myosin: The thick filaments are about 2.2 μm in length and have cross-bridges with a periodicity of 14.3 nm. The molecular weight of smooth muscle myosin molecules is 470 kilodalton (KD). They have two globular heads joined to a 150 nm long tail. Two light chains (20 and 17 KD) are associated with each head.

b) Actin: Filamentous actin is a two-stranded helix made up of actin monomers of molecular weight 42.5 KD and binds tropomyosin which lies in the groove on either side of the actin filament.

c) Intermediate filaments and dense bodies: Intermediate filaments (10 nm) are not

directly involved in the contractile process. Instead they form a cytoskeleton linking the dense bodies throughout the cell. Dense bodies are scattered throughout the cytoplasm of smooth muscle cells and are also bound to the inner leaflet of the plasmalemma and save as points of attachment of the thin filaments.

1.3.3. Regulation of contraction

Filo *et al* (1965) first described Ca^{2+} activation in the skinned smooth muscle cells. Both tension and the initial contraction rate increased from 10^{-7} M with increasing $[Ca^{2+}]_i$ and then saturated at concentrations slightly in excess of 10^{-6} M. Ca^{2+} combines with calmodulin to form a complex that converts the enzyme myosine light chain kinase to its active form. The latter phosphorylates the myosin light chains, thereby initiating the interaction of myosin with actin. Dephosphorylation of myosin by a phosphatase promotes relaxation.

1.3.4. Regulation of $[Ca^{2+}]_i$ in VSMCs

Two integrated membrane system are involved in control of smooth muscle $[Ca^{2+}]_i$, the plasmalemma and SR. Plasmalemma Ca^{2+} permeability is regulated by the Ca^{2+} leak (van Breemen *et al.,* 1972), the voltage gated Ca^{2+} channels (VGC) (Bean *et al.* 1986), ROC (van Breemen & Saida, 1989), the Na^+/Ca^{2+} exchanger (Matlib, 1992), and the plasmalemma Ca^{2+}-ATPase (Wuytack *et al,* 1984). On the SR membrane, there are the Ca^{2+} leak (van Breemen & Saida, 1989), IP_3-sensitive Ca^{2+} channels (Somlyo and Franzini-Armstrong, 1985), Ca^{2+} sensitive channels (CICR) (Saida & van Breemen, 1984) and SR Ca^{2+}-ATPase (Raeymaekers *et al.*, 1990).

1.3.4.1. Ca^{2+} entry pathways

Membrane depolarization opens the VGC and thus induces Ca^{2+} entry from the

extracellular space. Agonists activate the ROC and also facilitate the opening of VGC (Nelson *et al.*, 1988). The binding of agonists to the receptors in addition leads to IP_3 production. For example, stimulation of α_1 receptors by phenylephrine leads to the activation of a G_q coupling protein. The α subunit of this G protein activates the effector, PLC, which leads to the release of IP_3 and DAG from PIP_2. IP_3 activates the IP_3-sensitive Ca^{2+} channels on the SR and causes Ca^{2+} release from the SR. Ca^{2+} may then activate Ca^{2+}-dependent protein kinases, which in turn phosphorylate their substrates.

Under physiological conditions while both the extracellular Ca^{2+} concentration and the Ca^{2+} concentration inside the SR are much higher than $[Ca^{2+}]_i$, the plasmalemma and SR Ca^{2+} leak pathways also contribute to the increase in $[Ca^{2+}]_i$.

1.3.4.2. Ca^{2+} extrusion pathways

Both the plasmalemma Ca^{2+}-ATPase and the Na^+/ Ca^{2+} exchanger are involved in the Ca^{2+} extrusion process under normal circumstances. Since Na^+/ Ca^{2+} exchanger can transport Ca^{2+} in both directions, it may also contribute to the Ca^{2+} influx. The SR Ca^{2+}-ATPase (Chen & van Breemen, 1992) mediates Ca^{2+} accumulation by the SR.

In smooth muscle cells all these Ca^{2+} transport mechanisms on the plasmalemma and the SR work together to maintain Ca^{2+} homeostasis at rest and respond to activation.

1.4. STATEMENT OF PROBLEMS

1.4.1. The originality of endothelial cells from different sources

A few qualifying statements concerning the animal model are warranted: The rabbit was chosen as the animal model in the study of regulation of Ca^{2+} entry by membrane potential because (a) it does exhibit estrogen stimulation on NO production (Hayashi *et al.*, 1992) and (b) we have an isolated rabbit aortic endothelial cell

preparation in our laboratory (Wang *et al.*, 1995a & 1995b).

The rat was not chosen because of the difficulties in isolating endothelial cells from its aorta. In particular, enzymatic dissociation of aortic endothelial cells from smaller species such as the rat has proven extremely difficult (McGuire & Orkin, 1987). Our preliminary experiments have confirmed that these cells are resistant to release in a viable condition from the vascular wall by enzymatic treatment. In the study of estrogen effect on the basal $[Ca^{2+}]_i$, rat cardiac valvular endothelial cells were used in part because of the ease of isolation and selective fura-2 loading. Moreover, in our laboratory experiments using cardiac valves demonstrated that it is possible to obtain new insight into $[Ca^{2+}]_i$ regulation in valvular endothelium (Li & van Breemen, 1996). As a continuation of endocardium, the cardiac valvular endothelium shares at least some common functions with those of vascular endothelium. Endocardial endothelium contains the enzyme eNOS and releases NO in response to vasodilators (Ku *et al.*, 1990), and has been shown to modulate inotropic responses of subjacent myocardium (Muelemans & Brutasert, 1991). However, in addition to the practicality of studying endothelial cells of cardiac valves, the preparation is of considerable medical relevance. Valves are exposed to intermittent turbulent blood flow that can provoke platelet adhesion and lesion formation. The study of valvular endothelial cells, therefore, has significance for both vascular and cardiac research.

1.5. STUDIES (HYPOTHESIS, AIMS AND RATIONALE)

Our hypothesis was that the estrogen enhances eNOS activity at least in part, by increasing the Ca^{2+} level in endothelial cells. In this regard, the following studies were performed:

1.5.1. Study of the effects of estrogen and SERMs on NO release in rat aorta

During their reproductive years, women have a lower incidence of CHD compared to men of similar age (Castelli, 1988; Barret-Connor, 1994). However, women experience a dramatic increase in the incidence of CHD with the onset of menopause. Some reports show that estrogen replacement therapy in post-menopausal women reduces mortality due to cardiovascular disease (Barret-Connor and Bush, 1991; Stampfer and Colditz, 1991). The cardioprotective effect is in part related to the action of estrogen on blood lipid profiles and resultant inhibition of atherosclerotic coronary stenosis (Barrett-Connor and Bush, 1991). Although it is clear that estrogen-mediated changes in total serum cholesterol are important factors in delineating the cardioprotective effects of estrogen, there is evidence suggesting that estrogen have effects that are independent of its lipoprotein effects. A number of reports indicate NO production may play an important role in mediating the effects of estrogen on the vasculature. A positive correlation has been found between plasma 17 β-estradiol concentrations and levels of stable metabolites of NO during follicular development in women (Rosseli *et al.*, 1994). Consistent with a role for NO, endothelium-dependent coronary artery vasodilation is enhanced by estrogen treatment in ovariectomized monkeys (Williams *et al.*, 1994) and postmenopausal women (Gilligan *et al.*, 1994). Acute exposure of porcine left circumflex coronary arteries to estrogen also potentiated endothelium-dependent relaxations (Bell *et al.*, 1995).

However, there are also some studies reporting that chronic estrogenic treatment has no effect on receptor-mediated release of NO. Hayashi *et al* (1992) found no significant difference in the relaxant responses to Ach in aortic rings from male, female,

or ovariectomized rabbits. Similar findings were reported by Miller & Vanhoutte (1990). A direct action of estrogen on vascular smooth muscles has also been reported by several *in vitro* studies (Harder & Coulson, 1979; Jiang *et al.*, 1991; Ravi *et al.*, 1994). The mechanism of estrogen-mediated relaxation is thus controversial.

Aim: In view of these conflicting reports, our first objective here was to compare NO-dependent responses in intact aortic rings from sham-operated, estrogen-treated and untreated ovariectomized and male rats both in the basal state and after stimulation by Ach, an endothelium-dependent vasodilator.

Although estrogen replacement therapy is both cardioprotective and bone-preserving in postmenopausal women, it is accompanied by liabilities related to reproductive organs, including an elevated risk of breast and uterine cancers (Kauffman & Bryant, 1995). Chemical synthetic efforts have yielded a variety of non-estrogenic compounds with varying degrees of tissue selectivity known as SERM (Kauffman & Bryant, 1995). The most selective of these compounds preserve the beneficial properties of estrogen in the cardiovascular and skeletal systems and minimize or eliminate estrogenicity in mammary and uterine tissue. Such compounds have considerable therapeutic potential in women's health. The benzothiophenes, LY117018 and LY139481 (raloxifene), are examples of highly promising SERMs. Like estrogen, LY117018 and raloxifene have been demonstrated to lower serum total cholesterol and triglyceride concentrations and preserve bone against resorption in ovariectomized animals (Kauffman *et al.*, 1997; Bryant *et al.*, 1995). Unlike estrogen, SERMs are nearly devoid of estrogenic activity in rat uterus (Jones *et al.*, 1984; Black *et al.*, 1994). Furthermore, LY117018 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 (Wakeling *et al.*, 1984; Sato *et al.*, 1995).

Aim: Since the cardiovascular effects of LY117018 and raloxifene have not been investigated yet, our second objective here was to compare the effects of estrogen, LY117018 and raloxifene on modulation of arterial function due to its effects on endothelial NO synthesis/release.

1.5.2. Study of the effect of estrogen on CPA-mediated endothelium-dependent vasodilation in rat aorta

Biosynthesis of NO is correlated to the $[Ca^{2+}]_i$ in the cytoplasm of endothelial cells (Johns *et al.*, 1987; Schmidt *et al.*, 1989). In endothelial cells, agonist-induced increases in $[Ca^{2+}]_i$ are due to a combination of Ca^{2+} influx from the extracellular pool and the release of intracellular stored Ca^{2+} (Schilling *et al.*, 1992; Dolor *et al.*, 1992). Inhibitors of the SERCA, such as CPA, discharge Ca^{2+} from the ER by inhibiting Ca^{2+} uptake (Seidler *et al.*, 1989). Depletion of ER Ca^{2+} subsequently activates Ca^{2+} influx (Zhang *et al.*, 1994). In vascular smooth muscle, CPA, raises resting tension and induces Ca^{2+}-dependent contraction (Deng & Kwan, 1991), possibly by inhibiting Ca^{2+} uptake into SR and subsequently depleting stored Ca^{2+}.

It has been reported that CPA induces an endothelium-dependent relaxation and cGMP production in the rat aorta. These effects were inhibited by inhibitors of NOS and calmodulin and by removal of Ca^{2+}, suggesting that Ca^{2+}-dependent production of NO is involved in this vasodilation (Moritoki *et al.*, 1994; Zheng *et al.*, 1994). NO activates soluble GC to produce cGMP leading to relaxation of vascular smooth muscle (Moritoki *et al.*, 1994).

Aim: We recently reported that chronic estrogen treatment increases the release/production of NO in rat aorta (Rahimian *et al.,* 1997a). The present study was, therefore, carried out to determine whether estrogen modulates CPA-induced relaxation in rat aorta.

1.5.3. Study of regulation of Ach- and CPA-induced Ca^{2+} entry by membrane potential in rabbit aortic endothelial cells

In endothelial cells, depletion of ER Ca^{2+} by agonists or SERCA inhibitors (CPA) subsequently activates Ca^{2+} influx by opening SOCs (Zhang *et al.,* 1994). Although the SOCs that allow Ca^{2+} influx into the endothelial cells are voltage independent, membrane potential plays an important role in regulating Ca^{2+} entry. The membrane potential determines the electrochemical gradient that provides the driving force of Ca^{2+} influx. K^+ channels play an important role in the regulation of the endothelial cell membrane potential. It has been suggested that endothelium-dependent vasodilators cause an increase in cytoplasmic Ca^{2+}, which subsequently leads to the opening K_{Ca} channels in endothelial cells. Pasyk *et al* (1995) observed that release of Ca^{2+} by CPA, leads to the initiation or enhancement of an outward K^+ current in cultured bovine pulmonary endothelial cells. Beside the K^+ current, chloride (Cl^-) currents may also influence the membrane potential in the endothelial cells. Nillius *et al* (1997) reported the presence of Ca^{2+} dependent and/or volume regulated Cl^- channels in several types of vascular endothelial cells. Recently some reports suggested that in several preparations the Cl^- conductance might have modulatory effects on Ca^{2+} influx. In mesangial cells, for example, the removal of Cl^- from the extracellular space caused immediate abolition of Ca^{2+} entry (Kremer *et al.,* 1995).

Aim: The objective of our research was to elucidate the contribution of K^+ and Cl^- channels in response to Ach and CPA-induced Ca^{2+} entry in freshly isolated rabbit aortic endothelial cells.

1.5.4. Study of the effect of estrogen on the basal $[Ca^{2+}]_i$ in rat valvular endothelial cells

We recently reported a gender-based difference in the endothelium-dependent CPA vasodilation as well as in the basal release/ or production of NO in the rat aorta (Rahimian *et al.,* 1997b, 1997a). The aim of the present study was to elucidate one of the possible mechanisms whereby estrogen treatment enhances endothelial NO release. NO, a potent vasodilator (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987; Ignarro *et al.,* 1987) is produced in vascular endothelial cells by the constitutive enzyme NOS (Palmer *et al.*, 1988). $[Ca^{2+}]_i$, a cofactor in the activity of eNOS, plays a key role in regulation of the synthesis of endothelial NO (Johns *et al.,* 1987; Schmidt *et al.,* 1989). Weiner *et al* (1994) reported that estrogen treatment and pregnancy in the guinea pig increases the activity of Ca^{2+}-dependent NOS in uterine artery and some other organs as well as the levels of mRNA expression of the eNOS in skeletal muscle.

Aim: The possibility that enhanced Ca^{2+} stimulation of eNOS contributes to estrogenic effects has not been previously investigated. This study was, therefore, carried out to determine whether estrogen enhances NO release, at least in part by raising $[Ca^{2+}]_i$ in rat valvular endothelial cells.

CHAPTER II. METHODS AND MATERIALS

2.1. CONTRACTILE STUDIES

2.1.1. Experimental design

2.1.1.1. Study of the effects of estrogen and LY117018 on NO release in rat aorta

Experiments were performed to evaluate the effects of chronic subcutaneous or oral estrogen and LY117018, a SERM, on the release of NO in rings of rat aorta studied under isometric conditions. Two types of treatment protocol were used. The rats were implanted with a subcutaneous pellet delivery system for 21 days. However, since orally administered estrogen is of greater clinical interest, we also chose to administer estrogen via this route. An additional group was dosed orally with LY117018.

Implantation group: Sixteen (12 ovariectomized and 4 sham-operated) female and five male Sprague-Dawley rats weighing 275-300 g, were purchased from Charles River (Quebec, Canada). Using a 10-gauge trochar, a pellet was implanted subcutaneously at the back of the neck of rats where it remained until sacrifice 21 days later. Rats were assigned to five treatment groups (at least two or three aortic segments were taken from each animal). Group 1 was sham-operated, placebo-treated (sham); group 2 was ovariectomized, placebo-treated (ovex); group 3 was ovariectomized, 17 β-estradiol (0.5 mg/pellet)-treated (ovex + E_2); group 4 was ovariectomized, progesterone (15 mg/pellet) + 17 β-estradiol (0.5 mg/pellet)-treated (ovex + PG/E_2), and Group 5 was male rats (male).

Oral group: Twenty (15 ovariectomized and 5 sham-operated) female Sprague-Dawley rats weighing 275-300 g were assigned to four treatment groups. All groups received oral administration of drug or vehicle via gavage for 35 days. Group 1 was

ovariectomized, dosed with vehicle (hydroxypropyl-β-cyclodextrin); group 2 was ovariectomized, dosed with 17 α-ethinyl estradiol-treated (0.1 mg kg^{-1}/day) (ovex + 17 α-E_2); group 3 was ovariectomized, dosed with LY117018 (1 mg kg^{-1}/day) (ovex + LY) and group 4 was sham-operated, dosed with vehicle (sham).

2.1.1.2. Study of the effects of estrogen and raloxifene on basal NO release in rat aorta

Experiments were performed to evaluate the effects of chronic oral estrogen and raloxifene, a SERM, on the basal release of NO in rings of rat aorta studied under isometric conditions. Twenty-five (19 ovariectomized and 6 sham-operated) female Sprague-Dawley rats weighing 275-300 g were assigned to four treatment groups. Based on pharmacokinetic data on LY117018 (Tam *et al.,* unpublished), the period of oral treatment was chosen to be shorter compared to the orally group in study 2.1. All groups received oral administration of drug or vehicle via gavage for 21 days. Group 1 was ovariectomized, dosed with vehicle (hydroxypropyl-β-cyclodextrin)(ovex); group 2 was ovariectomized, dosed with 17 α-ethinyl estradiol-treated (0.1 mg kg^{-1}/day) (ovex + E_2); group 3 was ovariectomized, dosed with raloxifene (1 mg kg^{-1}/day)(ovex + Ralox) and group 4 was sham-operated, dosed with vehicle (sham).

2.1.1.3. Study of the effect of estrogen on CPA-mediated endothelium-dependent vasodilation in rat aorta

Experiments were performed to evaluate the modulatory effects of chronic estrogen treatment on the responses to CPA, an ER Ca^{2+}-ATPase inhibitor, in rat aorta. Fifteen (10 ovariectomized and 5 control) female and 5 male Sprague-Dawley rats weighing 275-300 g were purchased from Charles River (Quebec, Canada). Rats were

assigned to four treatment groups (at least two or three aortic segments were taken from each animal). Group 1 was female rats (female); group 2 was male rats (male). Using a 10-gauge trochar, a pellet was implanted subcutaneously at the back of the neck of rats (groups 3 and 4) where it remained until sacrifice 21 days later. Group 3 was ovariectomized, placebo treated (ovex); group 4 was ovariectomized, 17 β-estradiol treated (0.5 mg/pellet) (ovex + E_2).

2.1.2. Experimental procedures

2.1.2.1. Measurement of arterial contraction in rat aorta

a. Preparation of tissue

The rats were killed on day 21 or 35 with pentobarbital (65 mg kg^{-1}, i.p) after an intravenous injection of heparin. On the day of sacrifice, blood samples were collected from the vena cava and the plasma fraction was frozen (-70°C) for later analysis of 17 β-estradiol levels. The thoracic aorta was removed and placed in ice-cold modified Krebs solution containing (in mmol/L): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$, 1.17; $NaHCO_3$, 24.9; EDTA, 0.023; $CaCl_2$, 1.6; Glucose, 11.1. The aorta was cleared of fatty tissue and adhering connective tissue before being cut into rings 2- to 4-mm in length. Rings of aorta were suspended horizontally between two stainless steel hooks for measurement of isometric tension in individual organ baths containing 5 ml Krebs solution at 37° C, bubbled with 95% O_2 and 5% CO_2. Rings were equilibrated for 45 minutes under a resting tension of 1 g, to allow development of a stable basal tone and reproducible evoked contractile responses. Stimulation of rings with 80 mM K^+ was repeated every 15 minutes 2-3 times until responses were stable.

b. Responses to Ach

Rings of aorta were contracted with phenylephrine (PE, $2x10^{-6}$ M), which represented a concentration that produced 80% of maximal effect (EC_{80}). Dilator-response curves were obtained by the addition of increasing concentrations of Ach (10^{-8} to 10^{-5} M). Tissues were washed with Krebs solution for 30 minutes to allow relaxation to basal tone. Figure 2.1 shows a typical tracing of a concentration-response curve to Ach (10^{-8} to 10^{-5} M) in PE precontracted aortic rings from the control group of rats. Relaxation is expressed as the percent decrease from maximum PE-induced tension.

c. Contractile effect of PE

A concentration-response curve to PE was obtained by adding increasing concentrations of PE (10^{-8} to 10^{-5} M). The rings were then washed with Krebs solution for 30 minutes and N^{ω}-nitro-L-arginine methyl ester (L-NAME; $2x10^{-4}$ M), an inhibitor of endothelium derived NOS (Rees *et al.,* 1989), was added for 30 minutes. This concentration of L-NAME was based on studies by others (Hayashi *et al.*, 1992; Zheng *et al.*, 1994; Paredes-Carbajal *et al.*, 1995). The concentration-response curves to PE (10^{-9} to 10^{-5} M) were then repeated. Figure 2.1 shows a typical tracing of a concentration-response curve to PE (10^{-8} to 10^{-5} M) before and after pre-treatment with L-NAME ($2x\ 10^{-4}$ M) in aortic rings from the control group of rats. Contraction was measured as the percent increase from maximum PE-induced tension.

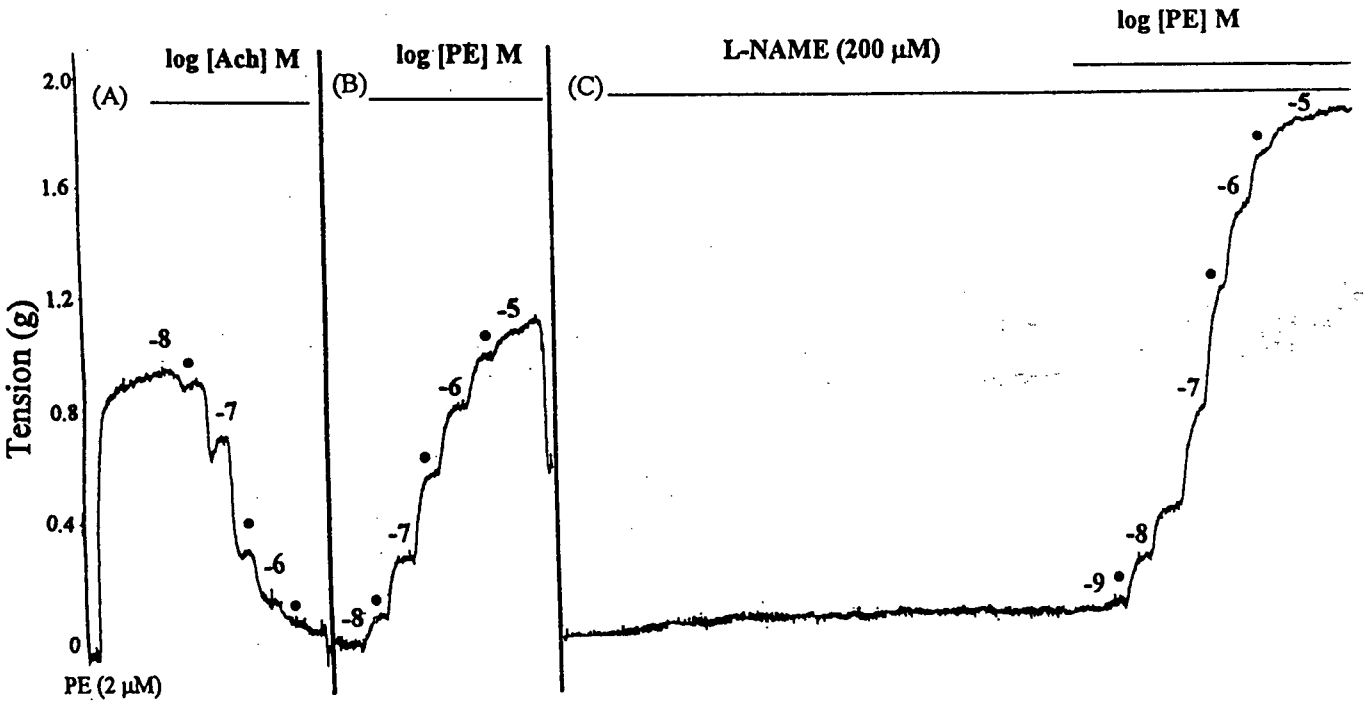

Fig. 2.1. Representative traces of rat aortic rings showing: **A)** Relaxation-response curve induced by acetylcholine (Ach, 10^{-8} to 10^{-5} M) in rings precontracted with phenylephrine (PE, $2x10^{-6}$ M). **B)** Contraction-response curve to PE (10^{-8} to 10^{-5} M) in the same ring. **C)** Contraction-response curve to PE (10^{-9} to 10^{-5} M), after pretreatment of the same ring with L-NAME ($2x10^{-4}$ M; 30 min).

d. Relaxing effect of sodium nitroprusside

Concentration-response curves to sodium nitroprusside (SNP), an endothelium-independent vasodilator agent, (10^{-9} to 10^{-6} M) were made in aortic rings precontracted with PE $2x10^{-6}$ M, before and after pretreatment with L-NAME ($2x10^{-4}$ M; 30 min).

e. Concentration-response curves to CPA

Rings of aorta were precontracted with PE ($2x10^{-6}$ M), which represented as EC_{80}. Dilator concentration-response curves were obtained by adding increasing concentrations of CPA (10^{-7} to 3 x 10^{-5} M). The rings were then washed with Krebs solution for 30 minutes, to allow relaxation to basal tone, and L-NAME ($2x10^{-4}$ M) were added for 30 minutes. The same concentration range of CPA (10^{-7} to $3x10^{-5}$ M) was then added to PE ($2x10^{-6}$ M)-precontracted rings. Relaxation is expressed as the percent decrease in tension below that elicited by PE pretreatment. Contraction was measured as the percent increase from maximum PE-induced tension.

2.1.2.2. Radioimmunoassay for estradiol measurement

Plasma concentrations of 17 β-estradiol were measured by using an ^{125}I radioimmunoassay kit. Briefly, 1 ml of the ^{125}I-estradiol was added to assay tubes containing 100 μL of plasma or standard solution. They were incubated at 37° C for 90 minutes and the content of tubes was aspirated or decanted. The supernatant was counted for ^{125}I in a gamma counter. A standard curve was used to estimate the 17 β-estradiol concentration of each sample.

2.1.3. Chemical reagents and drugs

Ach, L-PE hydrochloride, SNP, L-NAME, 17 α-ethinyl estradiol were obtained from Sigma Chemical Co (St. louis, MO, USA). CPA was purchased from Research

Biochemicals International (Natick, MA, USA). Hydroxypropyl-β-cyclodextrin was purchased from Aldrich Chemical Co (Milvaukee, WI, USA). LY117018 and Raloxifene were obtained from Eli Lilly Co. (Indianapolis, IN, USA). 17-β estradiol (0.5 mg/pellet), progesterone (15 mg/pellet) and placebo pellets were purchased from Innovative Research of America (Toledo, OH, USA) and designed to release 17 β-estradiol and progesterone over a 21 day period. ^{125}I radioimmunoassay kit was purchased from ICN Biomedical Inc. (Carson, CA, USA). All drugs were prepared as aqueous solutions except CPA, which were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and diluted before use. DMSO at the applied concentrations had no effect.

2.1.4. Data analysis

Values are expressed as means ± standard error of means (S.E.M). Comparisons of means were made by using the Student' t-test for unpaired values; when more than two groups were compared, one-way analysis of variance (ANOVA) and Newman-Keuls test for multiple comparison were used to identify differences among groups. A probability value of less than 5% ($P < 0.05$) was considered significant. Sensitivity is expressed as negative log molar concentration required for 50% of maximal relaxation or contraction (EC_{50}) determined.

2.2. MEASUREMENT OF $[Ca^{2+}]_i$

2.2.1. Experimental design

2.2.1.1. Study of regulation of Ach- and CPA-induced Ca^{2+} entry by membrane potential in rabbit aortic endothelial cells

Experiments were performed to elucidate the contribution of K^+ and Cl^- channels in response to Ach- and CPA-induced Ca^{2+} entry in freshly isolated rabbit aortic

endothelial cells.

2.2.1.2. Study of the effect of estrogen on the basal $[Ca^{2+}]_i$ in rat valvular endothelial cells.

Experiments were performed to determine whether estrogen enhances NO release, at least in part, by increasing $[Ca^{2+}]_i$ in rat valvular endothelial cells. Eighteen (ten female and eight male) Sprague-Dawley rats weighing 275-300 g, purchased from Charles River (Quebec, Canada), assigned to two groups. Group 1 was female rats; group 2 was male rats.

2.2.2 Experimental procedures

a. Isolation of rabbit aortic endothelial cells

Endothelial cells were isolated freshly from the adult New Zealand White rabbit aorta. The rabbit (2.5-3 kg) was killed using CO_2 and the thoracic aorta was excised and placed in normal-physiological saline solution (n-PSS) containing (in mmol/L): NaCl, 126; KCl, 5; $MgCl_2$, 1.2; *D*-glucose, 11; HEPES, 10; $CaCl_2$, 1 (pH 7.4). After careful removal of the surrounding fat and connective tissue the aorta was placed into a test tube containing Ca^{2+} free PSS (containing in mmol/L: NaCl, 126; KCl, 5; $MgCl_2$, 1.2; *D*-glucose, 11; HEPES, 10 (pH 7.4)) with 0.1 mg/ml collagenase, 0.1% elastase, 1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin (BSA). After 35 minutes of enzyme treatment at 37°C in a water bath (no stirring was involved), the endothelial cells were dispersed by trituration using a Pasteur pipette. These cells were then seeded on a glass coverslip precoated with poly-*D*-lysine and kept in an incubator at 37°C until transferring to the experimental perfusion chamber. The final preparation consists of single cells and small clusters of cells, which maintain their typical tile-like morphology.

b. Isolation of rat valvular endothelial cells

The rats were killed by CO_2 asphyxiation and their hearts were rapidly excised and placed in n-PSS containing (in mmol/L): NaCl, 126; KCl, 5; $MgCl_2$, 1.2; *D*-glucose, 11; HEPES, 10; $CaCl_2$, 1 (pH 7.4). The apex of the ventricle was then cut away to facilitate removal of blood. Both the aortic and pulmonary arteries were opened with a longitudinal incision at their respective attachments to the left and right ventricles. The aortic and pulmonary valves (each has 3 leaflets) were dissected free and were placed in nominally Ca^{2+}-free PSS containing 1 mg/ml Collagenase, 0.5 mg/ml elastase, 1 mg/ml trypsin inhibitor and 1 mg/ml BSA. After 40 minutes of enzyme digestion at 37°C in a water bath bath (no stirring was involved), the endothelial cells were dispersed by trituration using a Pasteur pipette. These cells were then seeded on a glass coverslip precoated with poly-*D*-lysine and kept in an incubator at 37°C until transferring to the experimental perfusion chamber. The final preparation consisted of small clusters of 3-15 cells, which maintained typical tile-like morphology.

c. DiI-Ac-LDL uptake

The endothelial nature has been confirmed using Ac-LDL labeled with the fluorescent probe, DiI, (DiI-Ac-LDL) uptake assay. Briefly, the coverslips containing cells were incubated with 3 ml of 10 μg/ml DiI-Ac-LDL at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) for 4 h. The medium was then removed and the cells were washed with probe-free medium for 10 minutes, rinsed with phosphate buffered saline (PBS) and then fixed with 10% buffered formaline phosphate for 5 minutes. Coverslips were inverted over a drop of 10% PBS in glycerol prior to reviewing. Cells were then examined with a Zeiss photomicroscope II using a 25 x Zeiss PlanNeofluor objective. DiI

was visualized using the standard rhodamine excitation/emission filter combinations. Photomicrographs were recorded on Kodak Tri-X pan film with an exposure setting of ASA 1600.

d. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ of the isolated endothelial cells was measured using a fluorescent imaging system. The composition of this imaging fluorescence microscopy is shown in figure 2.2. The light of a high-density Xenon lamp passes through a light filter to provide a light wavelength of high spectral purity. The filter wheel contains 340-nm, 380-nm, and 360-nm wavelength filters (bandwidth 10-nm) of ultraviolet light. Qualitative observations of morphology simultaneously with fluorescence recording was made possible by inserting onto the side port of the microscope a dichroic mirror that reflected the alternating excitation light onto the lower surface of the cells at the bottom of chamber. From the cells, the light was then reflected to a mirror and passed through an 510-nm wavelength (bandwidth 40-nm) cut off filter before acquisition by an ICCD camera (an intensified charge-coupled device; model 4093G, 4810 series, San Diego, CA), permitting observation of the cell on a video monitor during fura-2 signal recording. Fluorescence signals can be recorded as digital image data using a Sun Sparc1 + Workstation and Inovision acquisition and analysis software (Inovision, Research Triangle park, NC), which was controlled by a Data Translation frame grabber (DT 2861) housed in a PC 80286 computer.

Cells were loaded with 1 μM membrane-permeable fura-2 AM ester (1 mM stock in DMSO) in n-PSS for 30 minutes at room temperature. After a 10 minute recovery in dye-free solution, the fura-2 loaded coverslip was transferred to the stage of an inverted

microscope (Nikon, Diaphot) with a 20 x quartz objective.

A syringe was situated above the coverslip to infuse the cells with warmed experimental solutions. A total volume of 5 ml was used to change the solution. Total volume of the chamber was kept at ~ 0.5 ml, which was constantly maintained by using a vacuum suction at the surface of the fluid.

Autofluorescence of unloaded cells was minimal and background images at 340-nm and 380-nm were obtained from a region of the chamber away from cells. Pairs of the fluorescence ratio signals were collected every 10 seconds at alternating 340-nm and 380- nm excitation wavelength (F_{340}/F_{380}) and plotted as background-subtracted ratio cell versus time on line during the experimental procedure. $[Ca^{2+}]_i$ can be calculated using the equation of Grynkiewicz *et al* (1985), as $[Ca^{2+}]_i = K_d . b . [(R-R_{min})/(R_{max}-R)]$ where K_d is the dissociation constant of Ca^{2+}-fura 2 complex; R is the above-mentioned fluorescence ratio (F_{340}/F_{380}); R_{min} and R_{max} are the ratios measured by the addition of the Ca^{2+} ionophore ionomycin (10 μM) to Ca^{2+}-free (with 10 mM EGTA) solution and Ca^{2+}-replete (2 mM $CaCl_2$) solution, respectively; and b is the ratio of the 380 nm signals in Ca^{2+}-free and Ca^{2+}-replete solution.

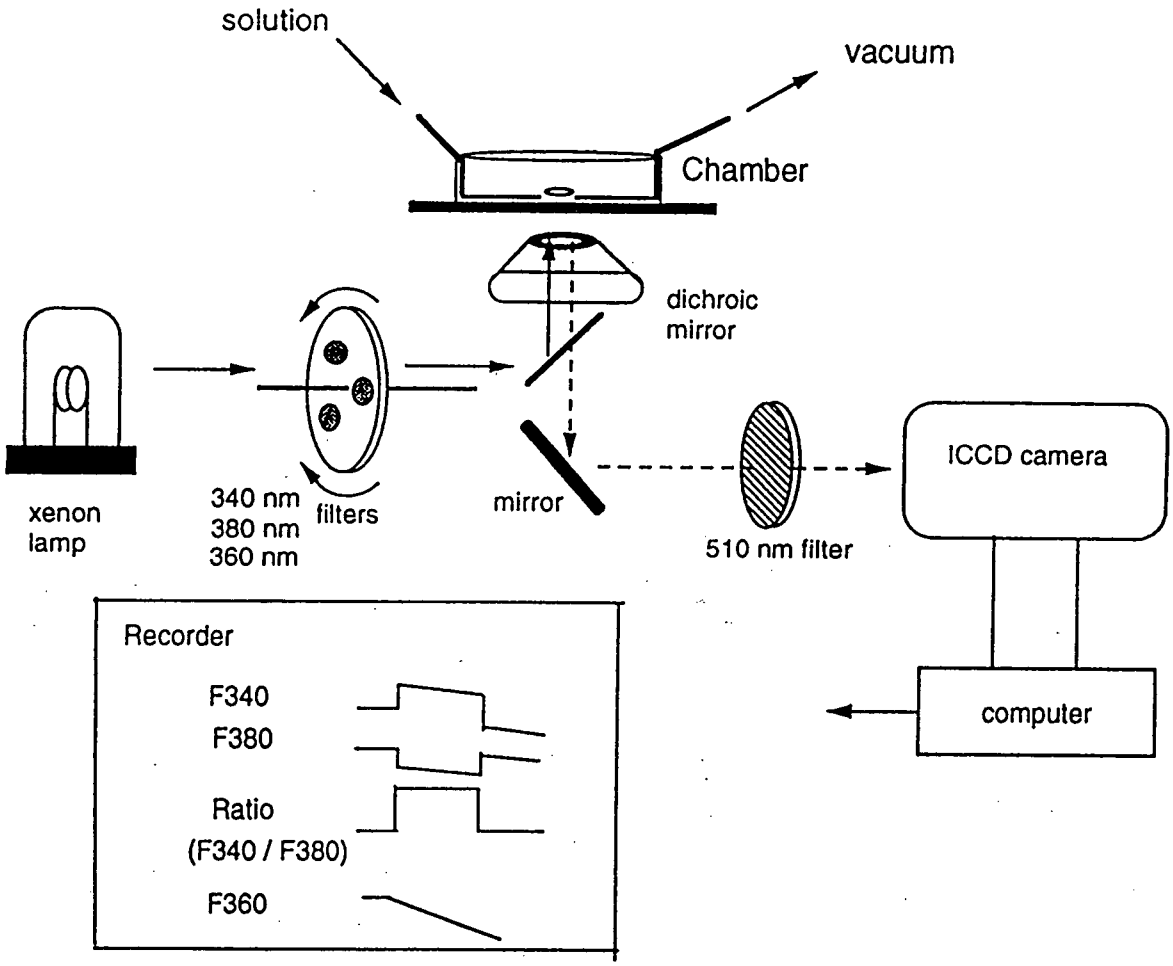

Fig.2.2. Schematic illustration of a fluorescence microscope. Isolated cells are superfused in an experimental chamber, which is mounted on the stage of an inverted microscope equipped with a Nikon Fluo x 20 objective. The cells are illuminated by ultraviolet (UV) light via an epifluorescence illuminator from a 300-W xenon lamp equipped with an interference filter. Fluorescence images are obtained with an ICCD camera, with the output digitized by an image-processing computer. Images of fura-2 fluorescence at 510-nm emission are obtained with 340- and 380-nm excitation wavelengths. Images of fluorescence ratios are then obtained by dividing the 340-nm image after background subtraction by the 380-nm image after background subtraction.

Background-subtracted fluorescence intensity ratio signals ($F_{340/F380}$) were reported in the rabbit aortic endothelial cell study as a relative indication of $[Ca^{2+}]_i$. The ratio (F_{340}/F_{380}) range was between 0.70-1.80.

In rat valvular study, using a K_d=242 nM, R_{min}= 0.149, R_{max}= 1.782, and b= 1.195, we estimated the resting Ca^{2+} level and peak response caused by CPA in the presence and absence of extracellular Ca^{2+}.

e. Mn^{2+}-Quenching

At the isosbestic wavelength of 360-nm, the fura-2 fluorescence intensity (FI) is not influenced by $[Ca^{2+}]_i$ changes (Chen and van Breemen, 1993). Mn^{2+} has been shown to quench fura-2 after binding to the dye (Grynkiewicz *et al.*, 1985). Both Mn^{2+} and Ca^{2+} share common entry pathways in the plasmalemma (Merrit *et al.*, 1989). The rate of Mn^{2+} entry measured by the slope of fura 2 fluorescence quenching trace at 360-nm in the presence of Mn^{2+} is therefore regarded as an index of divalent cation influx. Experiments were carried out by adding 150 μM $MnCl_2$ in Ca^{2+}-free medium that excludes Ca^{2+} competition for the divalent cation entry pathway and enhances the observed fluorescence quenching resulting from Mn^{2+} entry. Following measurement of basal Mn^{2+} entry, 10 μM ionomycin and 150 μM $MnCl_2$ was added to reveal maximum quenching.

2.2.3. Chemical reagents and drugs

CPA, NPPB, and SK&F96365 were purchased from Research Biochemicals International (Natick, MA, USA). Collagenase, elastase, trypsin inhibitor, BSA, PBS, TEA, manganese chloride, ionomycin and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO, USA). EGTA was obtained from Fisher Scientific (Fair Lawn, NJ,

USA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Mediatech, Inc. (Herndon, Virginia, USA). Dil-Ac-LDL was purchased from Biomedical technologies Inc. (Stoughton, MA, USA). All drugs were prepared as aqueous solutions except CPA, which was dissolved in DMSO as stock solutions and diluted before use. DMSO at the applied concentrations had no effect.

2.2.4. Data analysis

Agonists or a combination of different agonists were included in the applicable bathing solution for the different protocols. The traces are representative of similar responses obtained in at least four to five preparations. Where applicable, values are expressed as means ± S.E.M. Comparisons of means were made by using the Student' t-test. A probability value of less than 5% was considered significant. Chemicals and/or drugs were applied as indicated by the horizontal bars or arrows in each figure.

A nonlinear, biexponential curve fitting equation (Jandel Scientific-SigmaPlot) of the form, $f = ae^{-bx} + ce^{-dx}$ where f is $[Ca^{2+}]_i$, a and c are compartment sizes, x is time in seconds, and b and d are the fast and slow rate constant, respectively, was used to estimate the rate of $[Ca^{2+}]_i$ decline (Nazer & van Breemen, 1997).

CHAPTER III. RESULTS

3.1. STUDY OF THE EFFECTS OF ESTROGEN AND LY117018 ON NO RELEASE IN RAT AORTA

3.1.1. Effect of estrogen treatment on plasma estradiol level

Estrogen treatment significantly increased the concentrations of plasma 17 β-estradiol in ovariectomized rats (Table 3.1). 17 β-estradiol concentrations were significantly ($P < 0.01$, ANOVA) lower in male and ovariectomized rats compared with that in female and estrogen-treated ovariectomized rats. The ranges of plasma concentrations of estradiol reported in the literature for estrogen-treated and untreated ovariectomized rats are 27 ± 3.3 to 180 ± 17.5 pg/ml and 12 ± 4.7 to 21 ± 2.4 pg/ml, respectively (Cheng *et al.,* 1994, Hayashi *et al.,* 1992). In agreement with Ferrer *et al* (1996), the body weights of rats treated with estrogen was significantly ($P < 0.01$) lower than that of non-treated rats at the time of death (339 ± 3.5 g *vs.* 389 ± 6.6 g).

TABLE 3.1

Mean plasma concentrations of 17 β-estradiol (E_2, pg/ml) in the various group of rats.

Animal	17 β-estradiol (pg/ml)
Female	67.01 ± 5.50
Ovariectomized	11.76 ± 1.05*
Ovariectomized + E_2	54.96 ± 1.71
Male	35.86 ± 3.96*

Each value (± S.E.M) represents a mean of four to five animals.
* Value is significantly less ($P < 0.01$, ANOVA) compared with that in intact female and 17 β-estradiol-treated ovariectomized rats.

3.1.2. Effect of estrogen treatment on relaxation responses to Ach

Relaxation to Ach was used to examine the effect of estrogen treatment on receptor-mediated endothelium-dependent release of NO (Fig. 3.1). In the parenteral treatment study, no significant differences in response to low concentration of Ach (10^{-8} to 10^{-6} M) occurred between aortic rings from male, sham operated, and ovariectomized rats receiving progesterone plus estrogen, estrogen and LY117018. However, aortic rings from sham and ovariectomized rats receiving estrogen relaxed more ($P < 0.05$) to Ach (10^{-6} to 10^{-5} M) compared to those from ovariectomized, progesterone plus estrogen-treated, and male rats (Fig. 3.1A). Aortic rings from LY117018- treated rats in the orally treated group also relaxed more ($P < 0.05$) to Ach (10^{-6} to 10^{-5} M) compared to ovariectomized rats (Fig 3.1B). These results have been summarized in figure 3.2. This figure shows the differences in the maximum dilator responses to Ach (10^{-5} M), in the various groups of rats. Only the LY117018-treated ovariectomized rats are from the orally treated category in this figure.

3.1.3. Effect of L-NAME on contraction induced by PE

To examine whether estrogen affects endothelial NO production, concentration response curves to PE were generated in rings of aorta before and after pretreatment with L-NAME, a NOS inhibitor. Significant changes as a result of L-NAME pre-treatment would reveal effects of basal NO production on contraction. In figures 3.3 and 3.4, we show that incubation of the aortic ring segments with L-NAME ($2x10^{-4}$ M) resulted in a significant potentiation of the contractile responses to PE in all groups of aortae taken from orally category (Fig. 3.3) as well as five groups of aorta taken from implanted-

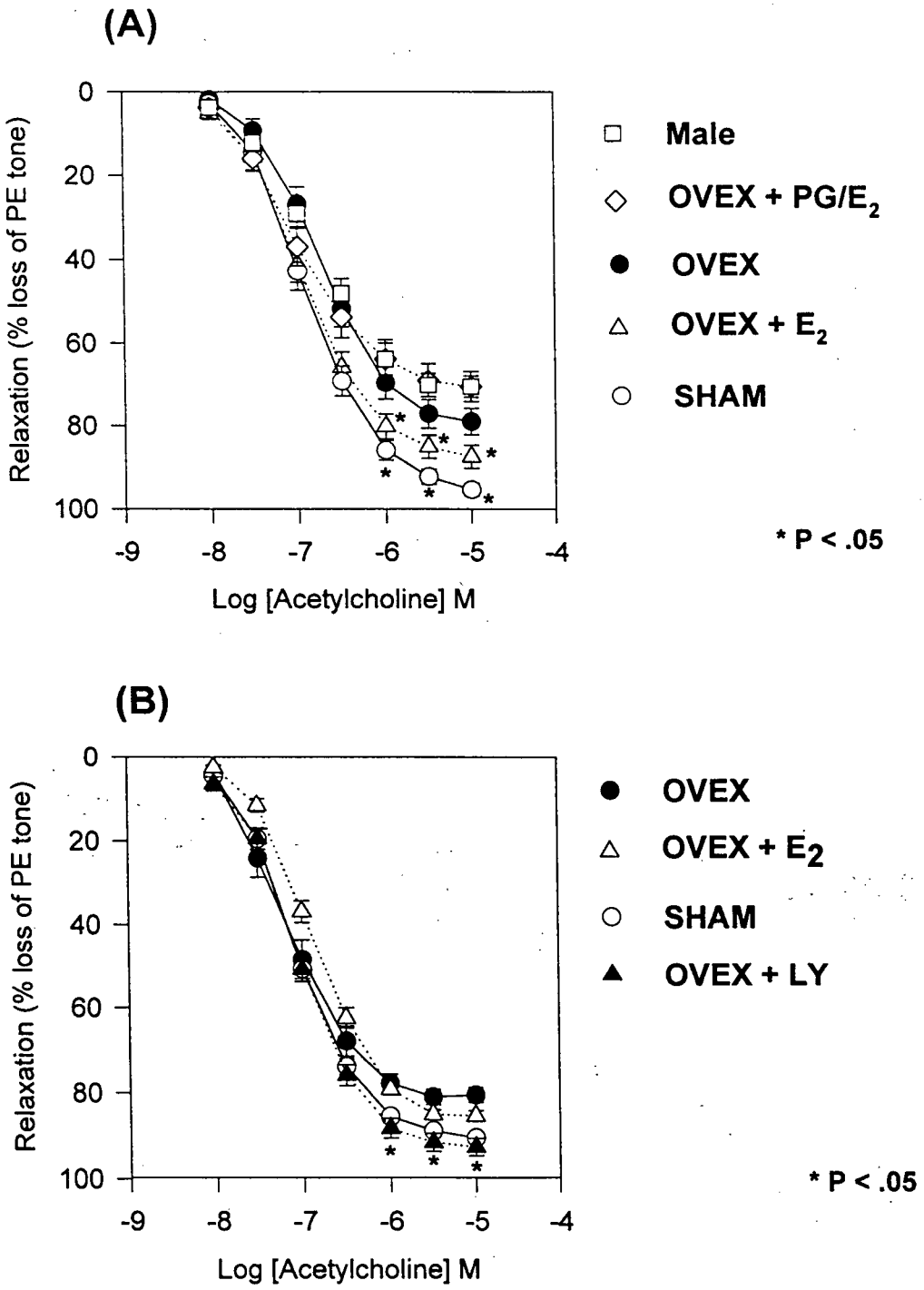

Fig. 3.1. Effect of chronic estrogen treatment of ovariectomized rats, **(A)** implanted groups **(B)** orally treated groups, on the relaxation-response to cumulative concentrations of acetylcholine (Ach) in intact aortic rings precontracted with phenylephrine (PE, $2x10^{-6}$ M). Relaxation to Ach is expressed as a percentage of PE ($2x10^{-6}$ M) maximum contraction. Points are shown as means ± S.E.M. of 4-5 rats per group.

* Denotes that relaxations of sham, 17 β-estradiol (E_2) and LY117018-treated rats are significantly different from those of the ovariectomized, progesterone plus 17 β-estradiol (PG/E_2)-treated and male rats ($P < 0.05$, ANOVA).

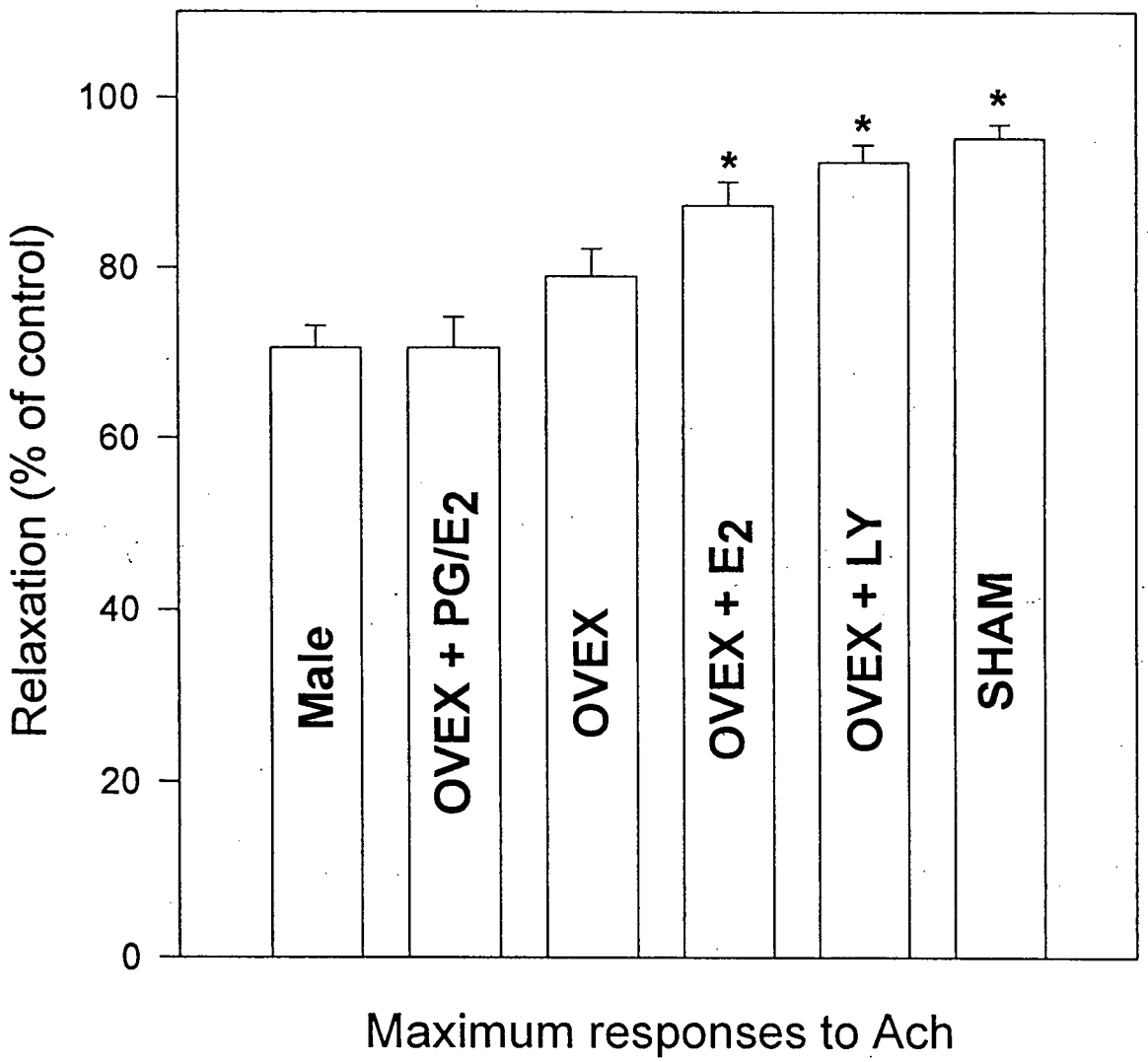

Fig. 3.2. Comparison of maximum dilator-responses to acetylcholine (Ach, 10^{-5} M) in the rat aortic rings. Data are means ± S.E.M. of 4-5 rats per group. Only the LY117018-treated ovariectomized rats are from the orally treated category.
* Significantly different ($P < 0.05$) from ovariectomized (OVEX) group by ANOVA and multiple comparison.

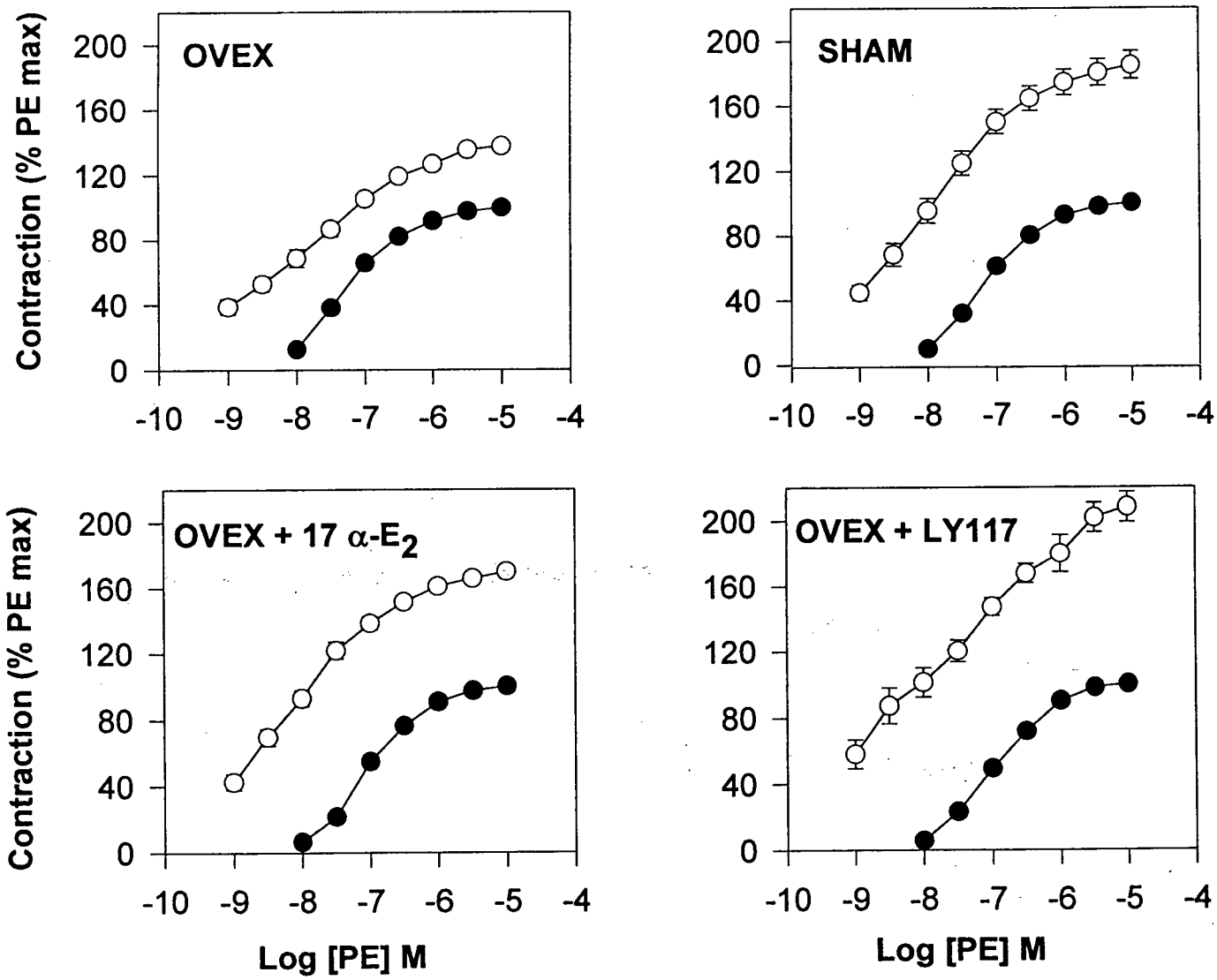

Fig. 3.3. Concentration-response curves for phenylephrine (PE) in thoracic aorta of sham-operated, treated and untreated ovariectomized rats (orally category), in the absence (•) or presence (O) of L-NAME ($2x10^{-4}$ M).
Results are expressed as a percent of the control maximal response to PE (10^{-5} M) obtained in the absence of L-NAME. The upward shift in the curves induced by L-NAME is significant ($P < 0.05$, ANOVA) in all groups. The results are shown as the mean ± S.E.M. of 4-5 rats per group.

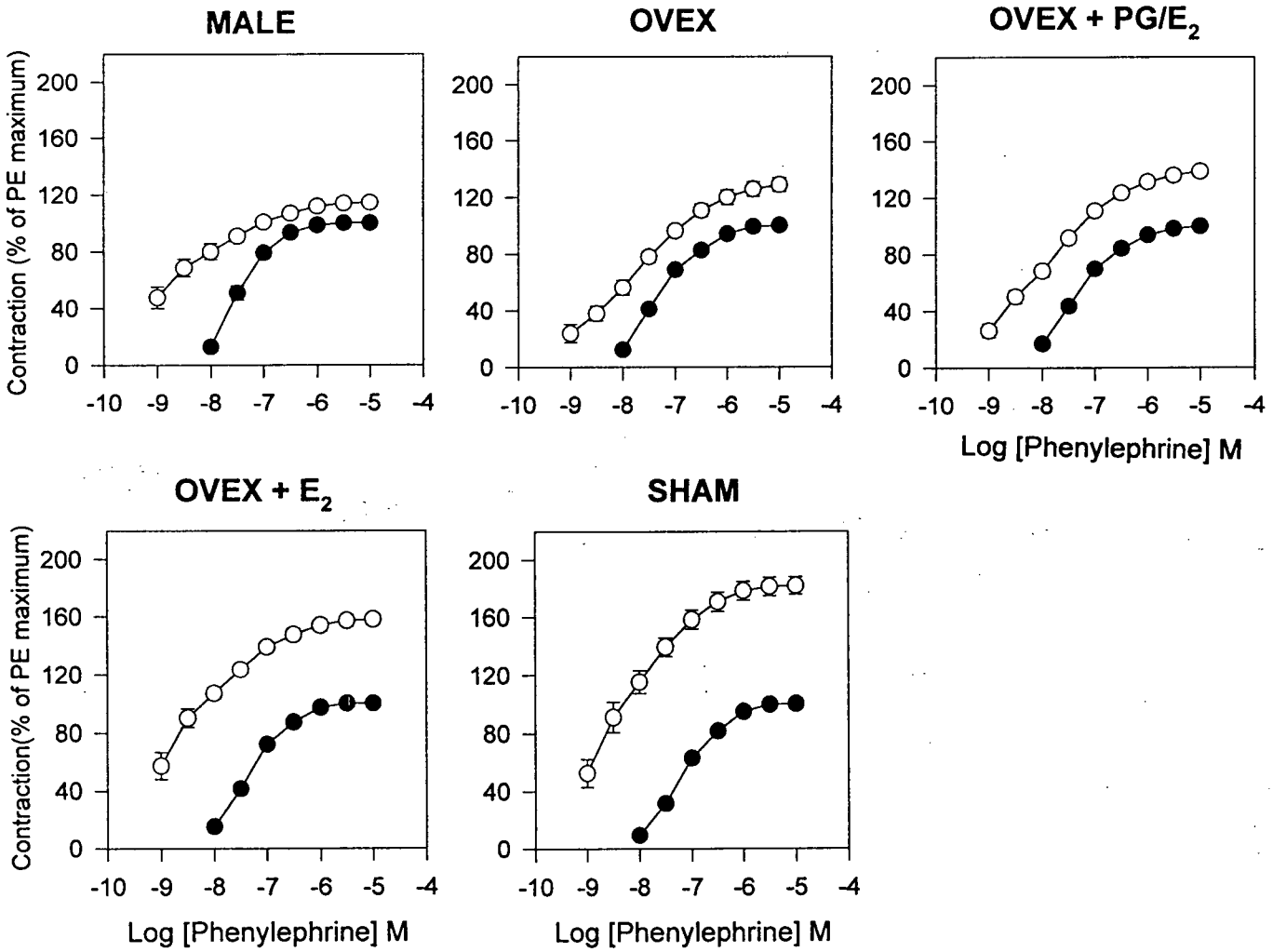

Fig. 3.4. Concentration-response curves for phenylephrine (PE) in thoracic aorta of male, sham-operated, treated and untreated ovariectomized rats (implanted category), in the absence (•) or presence (O) of L-NAME ($2x10^{-4}$ M).
Results are expressed as a percent of the control maximal response to PE (10^{-5} M) obtained in the absence of L-NAME. The upward shift in the curves induced by L-NAME is significant ($P < 0.05$, ANOVA) in all groups. The results are shown as the mean ± S.E.M. of 4-5 rats per group.

category (Fig. 3.4) through the entire concentration-response range of PE (10^{-9} to 10^{-5} M). Aortic rings from sham, estrogen and LY117018-treated rats had a greater maximal ($P < 0.05$) potentiation of the PE responses after inhibition of NOS when compared to those in male, ovariectomized, and progesterone plus estrogen-treated ovariectomized rats (Fig. 3.5). The sensitivity of α-adrenoceptors is not significantly affected by estrogen status either before or after inhibition of NOS as indicated by the absence of significant differences (at the level of 95%) in PE EC_{50} values between estrogen-treated and untreated ovariectomized groups before and after L-NAME treatment (EC_{50}: 0.08 ± 0.006 μM, 0.05 ± 0.0003 μM, before L-NAME treatment, and 0.007 ± 0.001 μM, 0.009 ± 0.003 μM, after L-NAME treatment, respectively; $P > 0.05$, ANOVA) (Fig. 3.6). However, the slopes of the dose-response relationships were less steep in L-NAME-pretreated versus control preparations.

3.1.4. Effect of L-NAME on dilation induced by SNP

Relaxation to SNP was used to examine the effect of estrogen treatment on the response to NO donors. SNP is a NO donor, leading to a rise of cGMP mediated endothelium-independent relaxation in smooth muscle cells (Ignarro *et al.*, 1981). The sensitivity of smooth muscle to SNP was not significantly different in aortic rings from sham-operated, LY117018-treated and untreated ovariectomized rats (EC_{50}: 0.04 ± 0.01 μM, 0.04 ± 0.01 μM and 0.02 ± 0.003 μM, respectively; $P > 0.05$, ANOVA) (Fig. 3.7). Furthermore, addition of L-NAME ($2x10^{-4}$ M; 30 min) did not inhibit SNP (10^{-9} to 10^{-6} M) induced relaxation (data not shown).

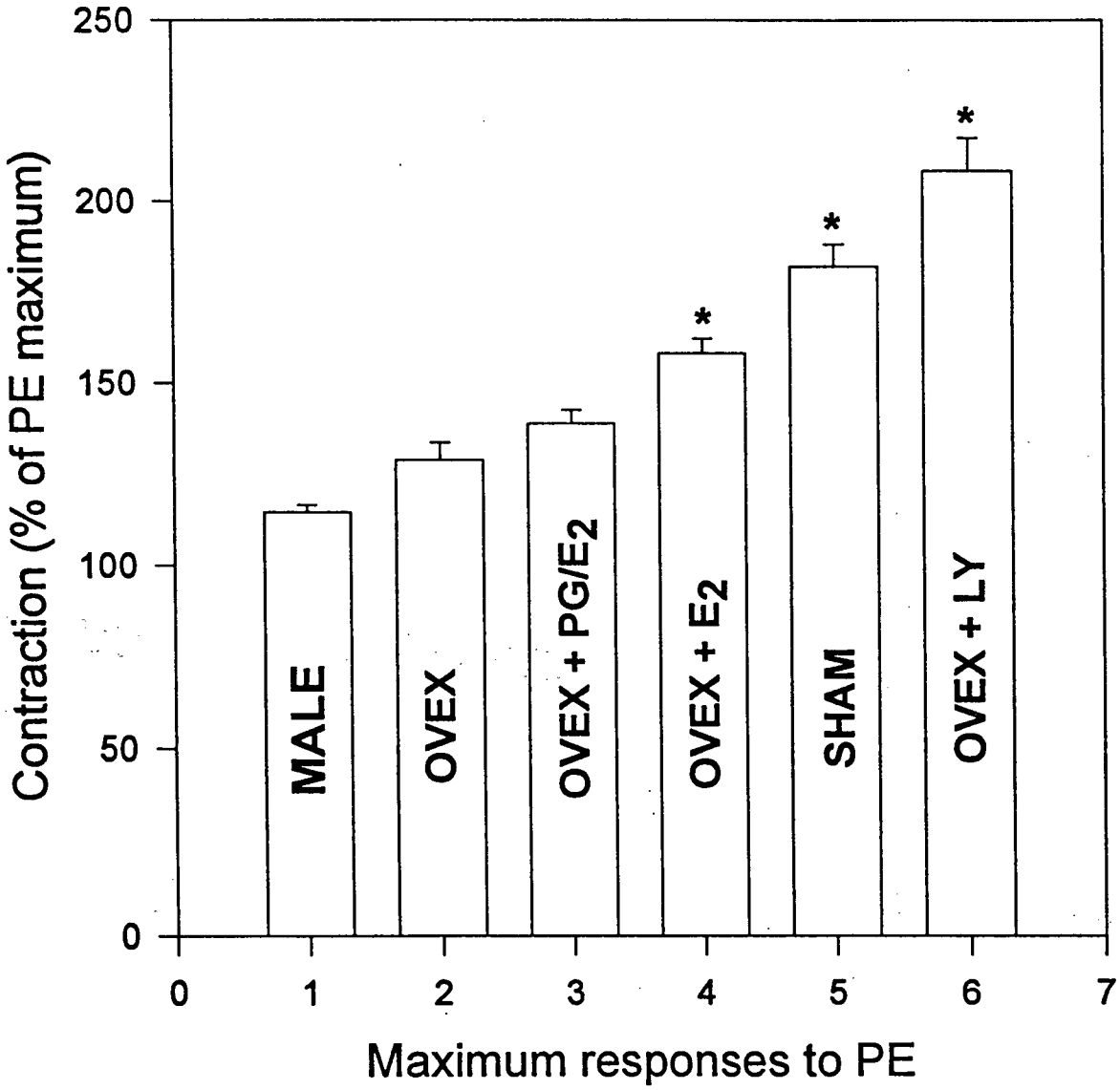

Fig. 3.5. Comparison of maximum responses to phenylephrine (PE, 10^{-5} M) in the presence of L-NAME ($2x10^{-4}$ M; 30 min) to control responses obtained in the absence of L-NAME. Data are means ± S.E.M. of 4-5 rats per group. Only the LY117018-treated ovariectomized rats are from the orally treated category.

* Significantly different ($P < 0.05$) from ovariectomized (OVEX) group by ANOVA and multiple comparison.

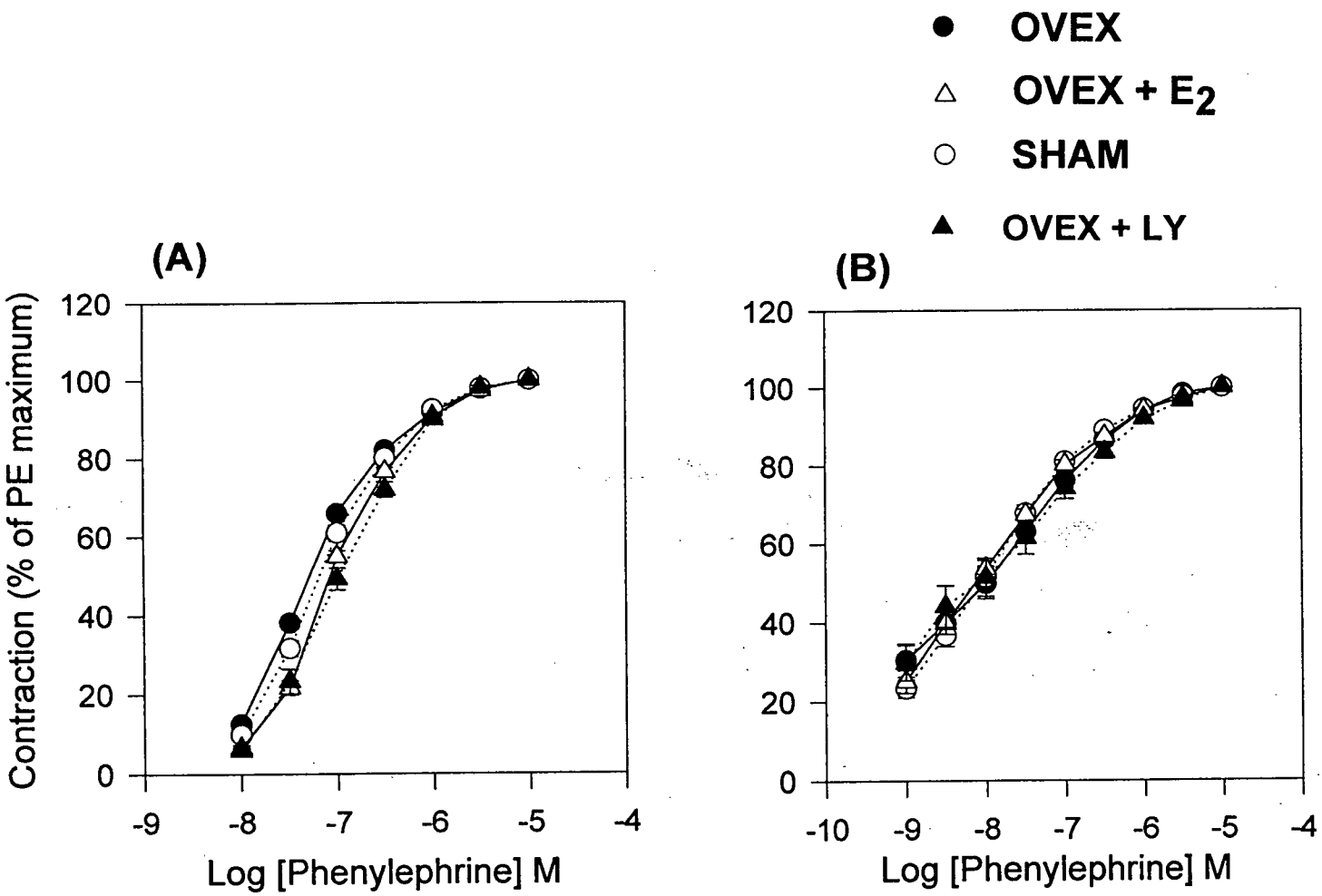

Fig. 3.6. Concentration response curves to phenylephrine (PE) in thoracic aorta of sham-operated and ovariectomized rats (with and without estrogen or LY117018 replacement). The responses are normalized to the respective maximal contractile responses to PE (10^{-5} M) before (A) and after (B) pretreatment with L-NAME ($2x10^{-4}$ M; 30 min). The results are shown as the mean ± S.E.M. of 4-5 rats per group. The sensitivity to PE was not significantly different between aortic rings from estrogen-treated and untreated ovariectomized rats either before or after L-NAME ($P > 0.05$, ANOVA).

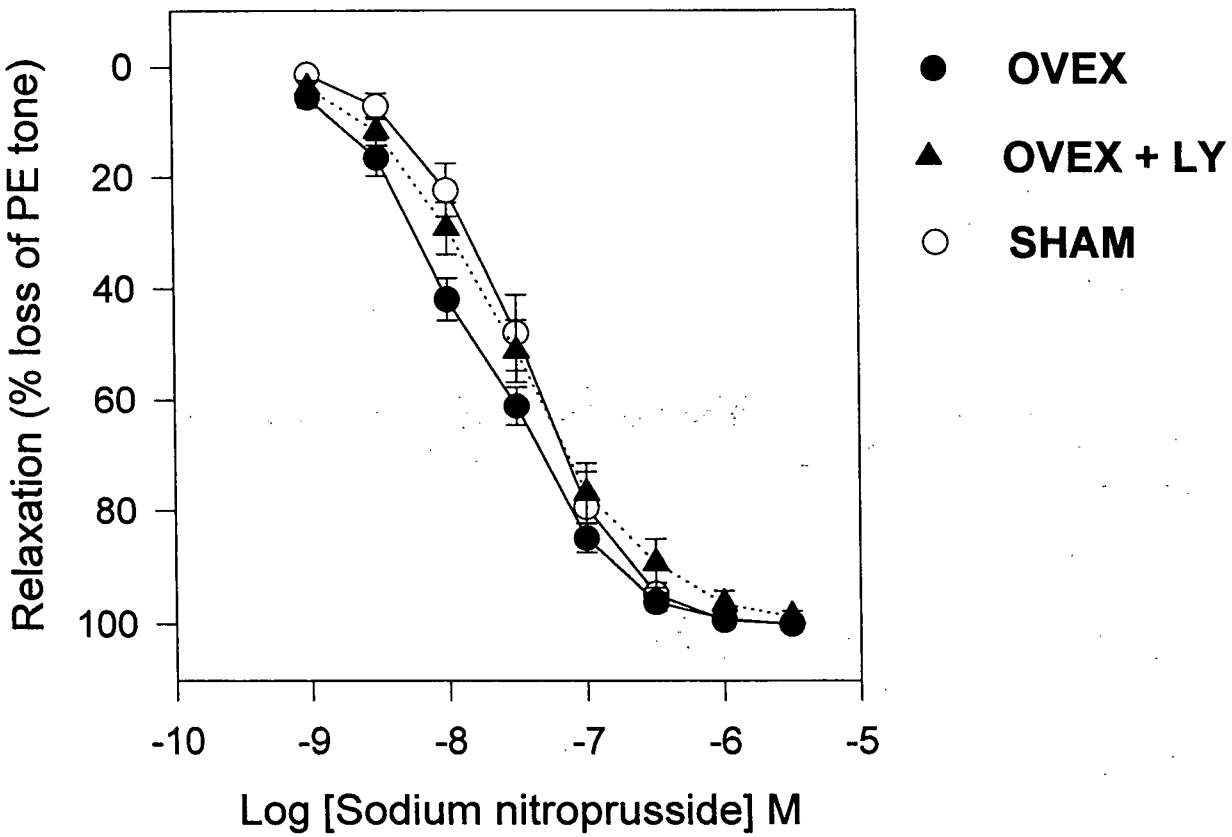

Fig. 3.7. Concentration response curves to sodium nitroprusside (SNP) in intact aortic rings of sham-operated and ovariectomized rats (with and without LY117018 replacement) precontracted with phenylephrine (PE, $2x10^{-6}$ M). Relaxation to SNP is expressed as a percentage of PE ($2x10^{-6}$ M) maximum contraction. The results are shown as the mean ± S.E.M. of 3-4 rats per group. The SNP EC_{50} values were not significantly different in aortic rings from sham-operated, LY117018-treated and untreated ovariectomized rats (0.04 ± 0.01 μM, 0.04 ± 0.01 μM and 0.02 ± 0.003 μM, respectively; $P > 0.05$, ANOVA).

3.2. STUDY OF THE EFFECTS OF ESTROGEN AND RALOXIFENE ON BASAL NO RELEASE IN RAT AORTA

3.2.1. Effect of L-NAME on contraction induced by PE

To compare the effects of estrogen and raloxifene on modulation of arterial function, concentration response curves to PE were generated, before and after pretreatment with L-NAME, in rings of aorta taken from estrogen/ raloxifene-treated, sham operated and vehicle-treated ovariectomized rats. In figure 3.8, we show that incubation of the aortic ring segments with L-NAME ($2x10^{-4}$ M) resulted in a significant potentiation of the contractile responses to PE in all four groups of aortae through the entire concentration-response range of PE (10^{-9} to 10^{-5} M). Aortic rings from sham, estrogen and raloxifene-treated rats had a greater maximal ($P < 0.05$) potentiation of the PE responses after inhibition of NOS when compared to those in vehicle-treated ovariectomized rats. Figure 3.9 shows the comparison of maximum responses to PE (10^{-5} M) in the presence of L-NAME ($2x10^{-4}$ M; 30 min) among different groups.

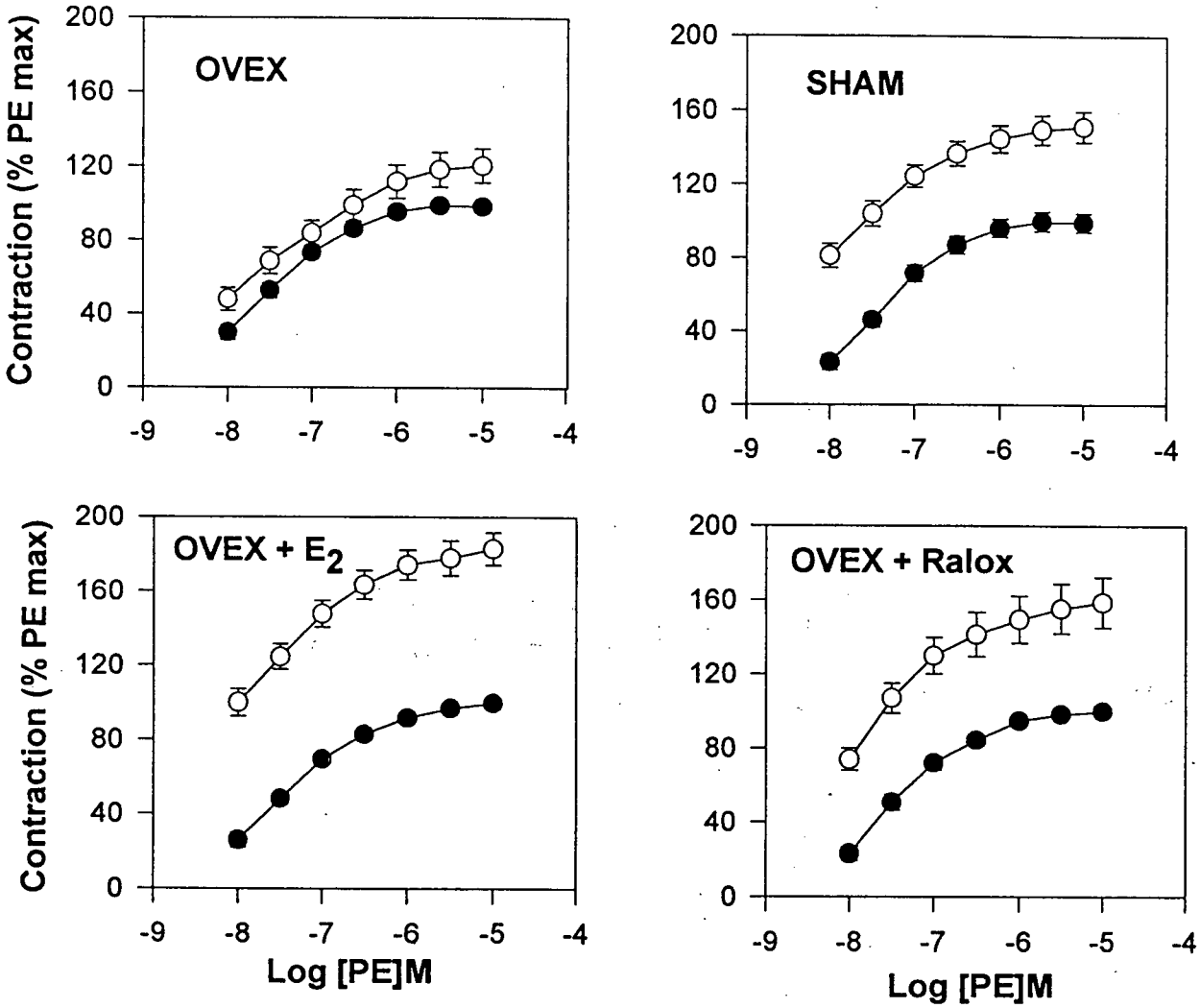

Fig. 3.8. Concentration-response curves for phenylephrine (PE) in thoracic aorta of sham-operated, treated and untreated ovariectomized rats, in the absence (•) or presence (O) of L-NAME ($2x10^{-4}$ M).
Results are expressed as a percent of the control maximal response to PE (10^{-5} M) obtained in the absence of L-NAME. The upward shift in the curves induced by L-NAME is significant ($P < 0.05$, ANOVA) in all groups. The results are shown as the mean ± S.E.M. of 6-7 rats per group.

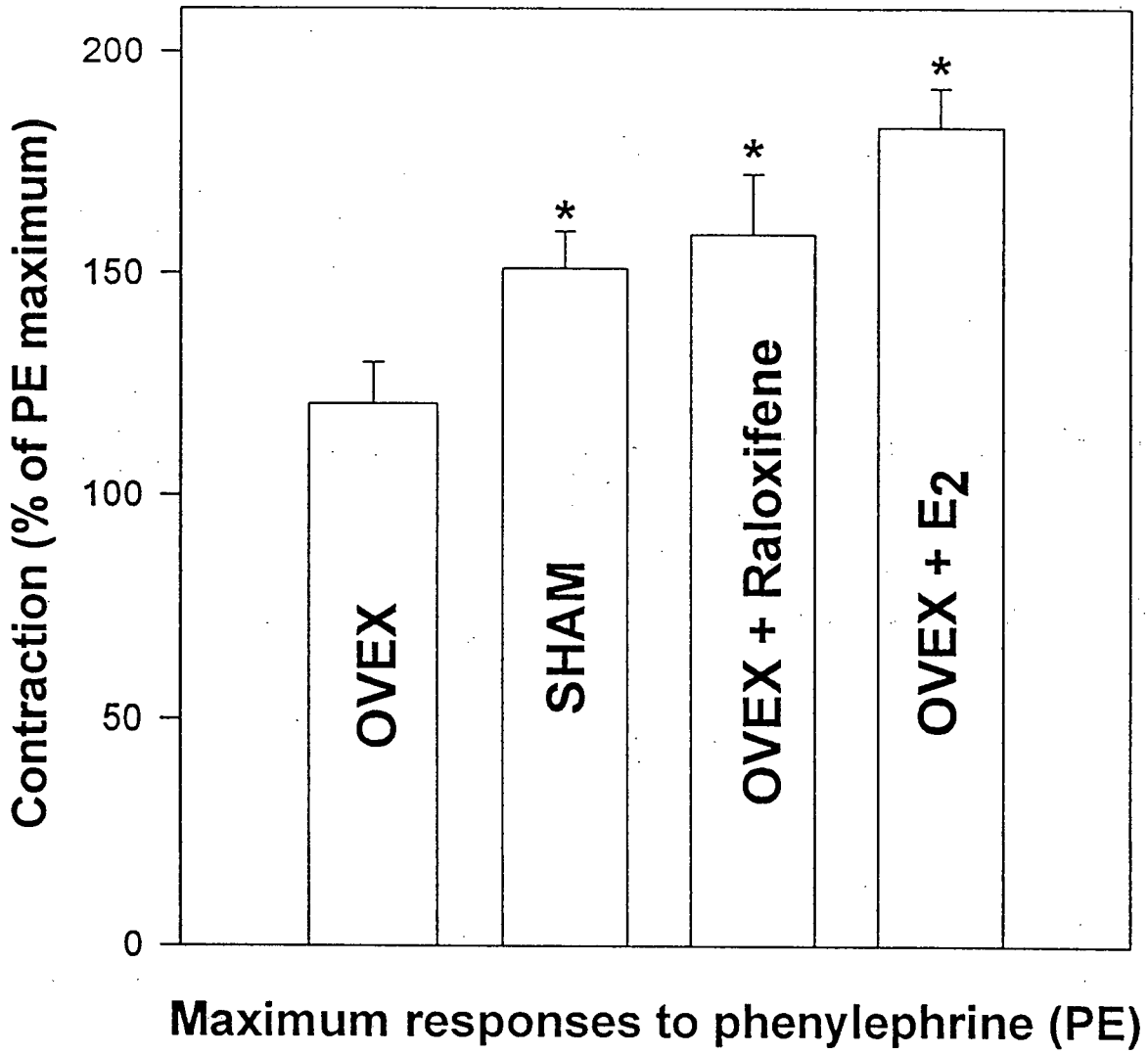

Fig. 3.9. Comparison of maximum responses to phenylephrine (PE, 10^{-5} M) in the presence of L-NAME ($2x10^{-4}$ M; 30 min) to control responses obtained in the absence of L-NAME. Data are means ± S.E.M. of 6-7 rats per group.
* Significantly different ($P < 0.05$) from ovariectomized (OVEX) group by ANOVA and multiple comparison.

3.3. STUDY OF THE EFFECT OF ESTROGEN ON CPA-MEDIATED ENDOTHELIUM-DEPENDENT VASODILATATION IN RAT AORTA

3.3.1. Effect of CPA on the contraction induced by PE

To evaluate the modulatory effects of chronic estrogen treatment on responses to CPA, concentration response curves to the CPA were generated in PE precontracted rings of aorta from estrogen-treated and non-treated ovariectomized rats. Figure 3.10 shows a typical trace of a concentration-response curves to CPA (10^{-7} to $3x10^{-5}$ M) in PE precontracted aortic rings from a control group of rats. In agreement with the finding of Zheng *et al* (1994), low CPA concentrations (10^{-7} to 10^{-5} M) caused persistent vasodilation, whereas higher concentrations of CPA ($>10^{-5}$) produced a biphasic response, a relaxation followed by gradual reversal to contraction (data not shown). In the case of biphasic responses, the maximal relaxation was taken for statistical analysis. Figure 3.11 summarizes the results of the concentration-dependent effects of CPA on aortic rings precontracted with PE ($2x10^{-6}$ M). Aortic rings from female (72.9 ± 2.4%) and ovariectomized rats receiving 17 β-estradiol (65.5 ± 4.8%) relaxed more ($P < 0.05$) to CPA ($3x10^{-5}$ M) compared to those from ovariectomized (40.8 ± 3.9%) and male (51.5 ± 3.4%) rats. Responses to CPA were similar in rats with low levels of estrogen (male and ovariectomized rats), whereas tissues from rats with higher estrogen levels (female and estrogen treated-ovariectomized rats) were significantly more sensitive to CPA (Fig. 3.12). The EC_{50} values of CPA in aortae from male and female rats were 2.99 ± 0.33 μM and 1.66 ± 0.18 μM, respectively ($P < 0.01$).

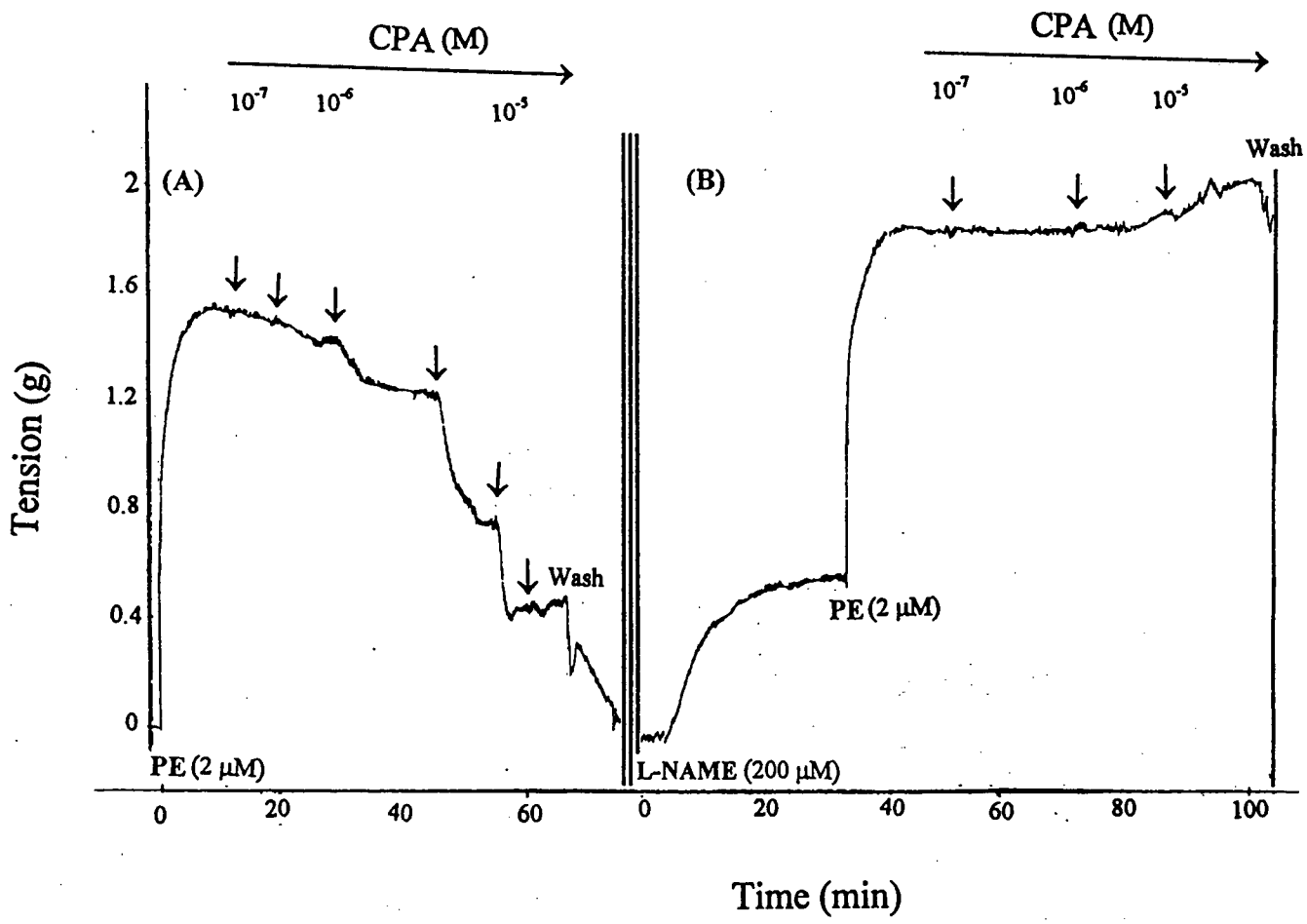

Fig. 3.10. Representative traces of rat aortic rings showing: **A)** Relaxation-response curve induced by cyclopiazonic acid (CPA, 10^{-7} to $3x10^{-5}$ M) in rings precontracted with phenylephrine (PE, $2x10^{-6}$ M). **B)** After pretreatment of the same ring with L-NAME ($2x10^{-4}$ M; 30 min), CPA (10^{-7} to $3x10^{-5}$ M)-induced contraction in PE ($2x10^{-6}$ M)-precontracted rings.

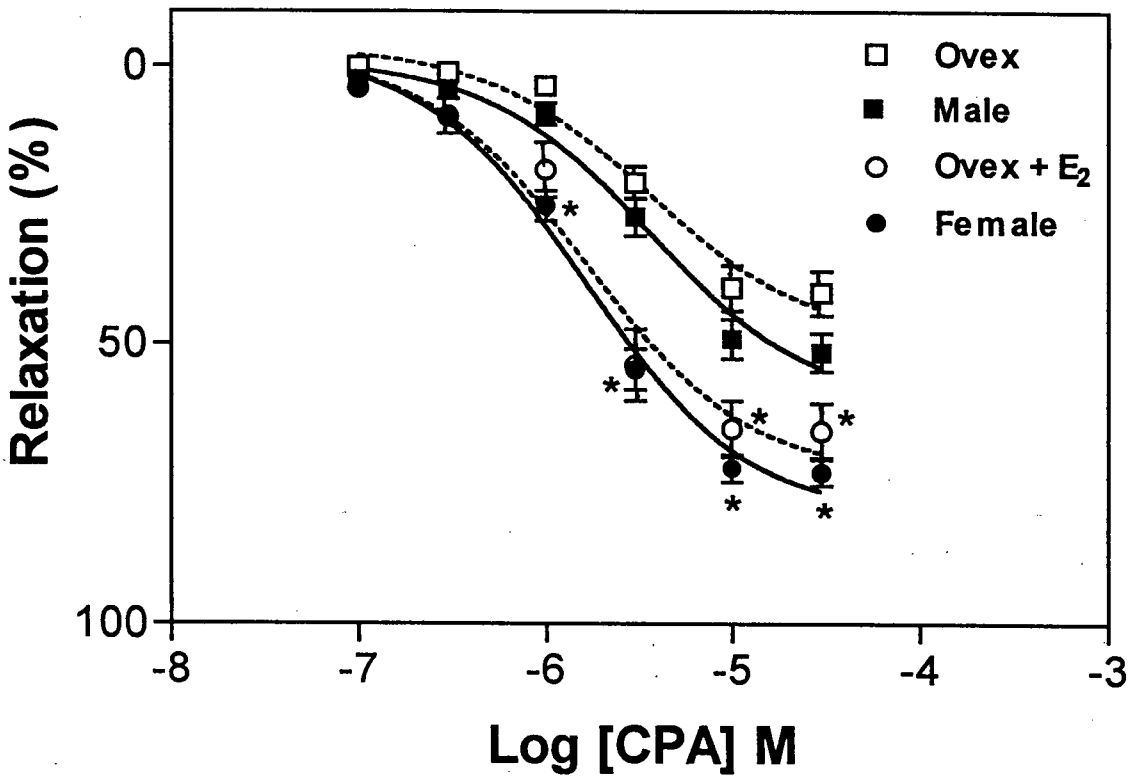

Fig. 3.11. Effect of chronic estrogen treatment of ovariectomized rats on the relaxation-response to cumulative concentrations of cyclopiazonic acid (CPA) in intact aortic rings precontracted with phenylephrine (PE, $2x10^{-6}$ M). Relaxation to CPA is expressed as a percentage of PE ($2x10^{-6}$ M) maximum contraction. Points are shown as means ± S.E.M. of 5 rats per group.
Asterisk denotes that relaxations of female and estrogen-treated rats are significantly different ($P < 0.05$) from those of the ovariectomized and male rats by ANOVA and multiple comparison.

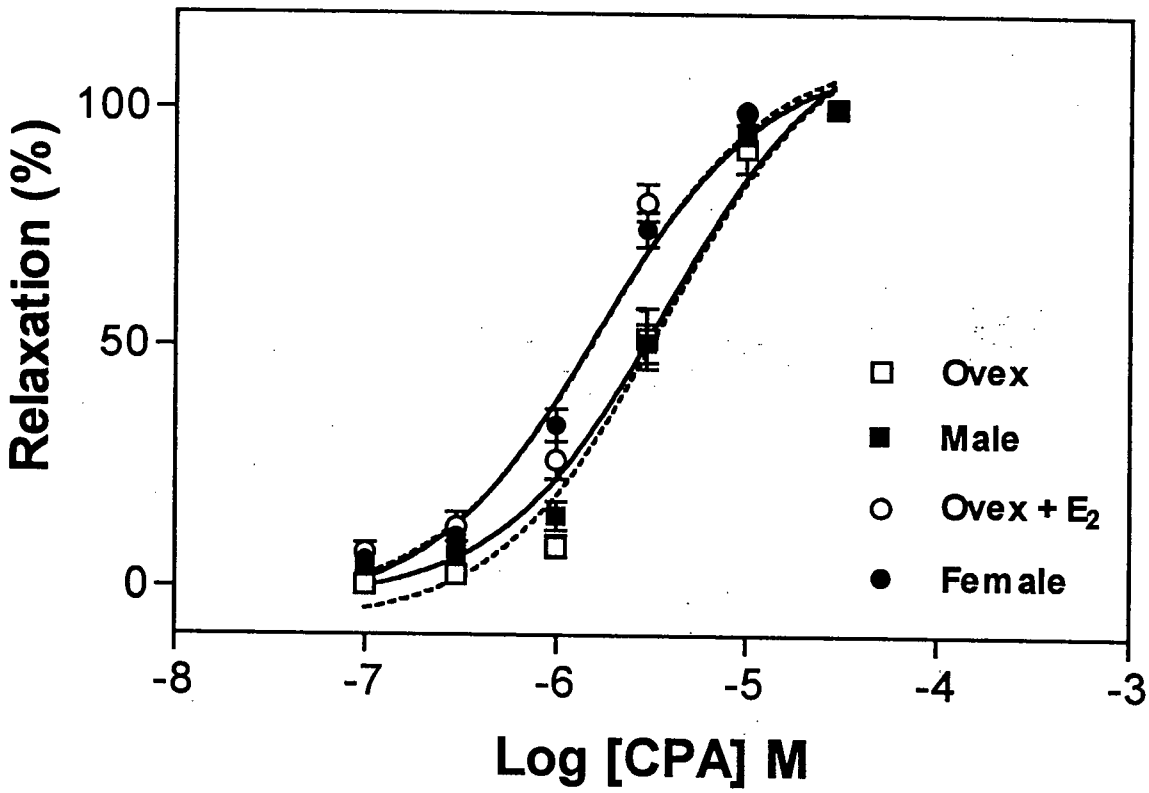

Fig. 3.12. Concentration response curves to cyclopiazonic acid (CPA) in thoracic aorta of rats. Responses are normalized to the respective maximal dilator responses to CPA ($3x10^{-5}$ M). Results are shown as the mean ± S.E.M. of 5 rats per group. The EC_{50} values of CPA in aortae from male and female rats were 2.99 ± 0.33 μM and 1.66 ± 0.18 μM , respectively ($P < 0.01$, Student' t- test).

3.3.2. Effect of L-NAME on relaxation induced by CPA

In figure 3.13, we show the effect of L-NAME on the CPA relaxation responses. We used L-NAME, a NOS inhibitor, to uncover responses occurring in the absence of release of NO. Incubation of the same aortic rings with L-NAME ($2x10^{-4}$ M) for 30 minutes not only abolished the endothelium-dependent relaxation induced by CPA in all groups, but converted these to contractile responses. There were no differences in the maximum cyclopiazonic acid responses as a percentage of PE tone, in aortic rings from female (111 ± 1.0%), ovariectomized rats receiving 17 β-estradiol (116 ± 1.1%), ovariectomized (116 ± 1.0%) and male rats (111 ± 1.4%).

3.4. STUDY OF REGULATION OF ACH- AND CPA-INDUCED CA^{2+} ENTRY BY MEMBRANE POTENTIAL IN RABBIT AORTIC ENDOTHELIAL CELLS

3.4.1. Identification of rabbit aortic endothelial cells

To identify endothelial cells, they were incubated with 10 μg/ ml DiI-Ac-LDL for 4 h at 37°C and subsequently examined by fluorescence microscopy. As shown in figure 3.14, rabbit aortic endothelial cells were brightly stained and the fluorescence was predominantly punctate within the cytoplasm. A negative control is provided by rabbit aortic smooth muscle cells (data not shown). Low background fluorescence was observed in smooth muscle cells.

3.4.2. Effect of PE and Ach on $[Ca^{2+}]_i$

To examine whether endothelial cells have functional α_1 adrenergic receptors, the cells were stimulated by PE, a selective α_1 adrenergic agonist, followed by Ach, a non - selective cholinergic agonist. Figure 3.15 is a representative trace of fluorescence ratio signals of rabbit aortic endothelial cells in response to PE followed by application of Ach.

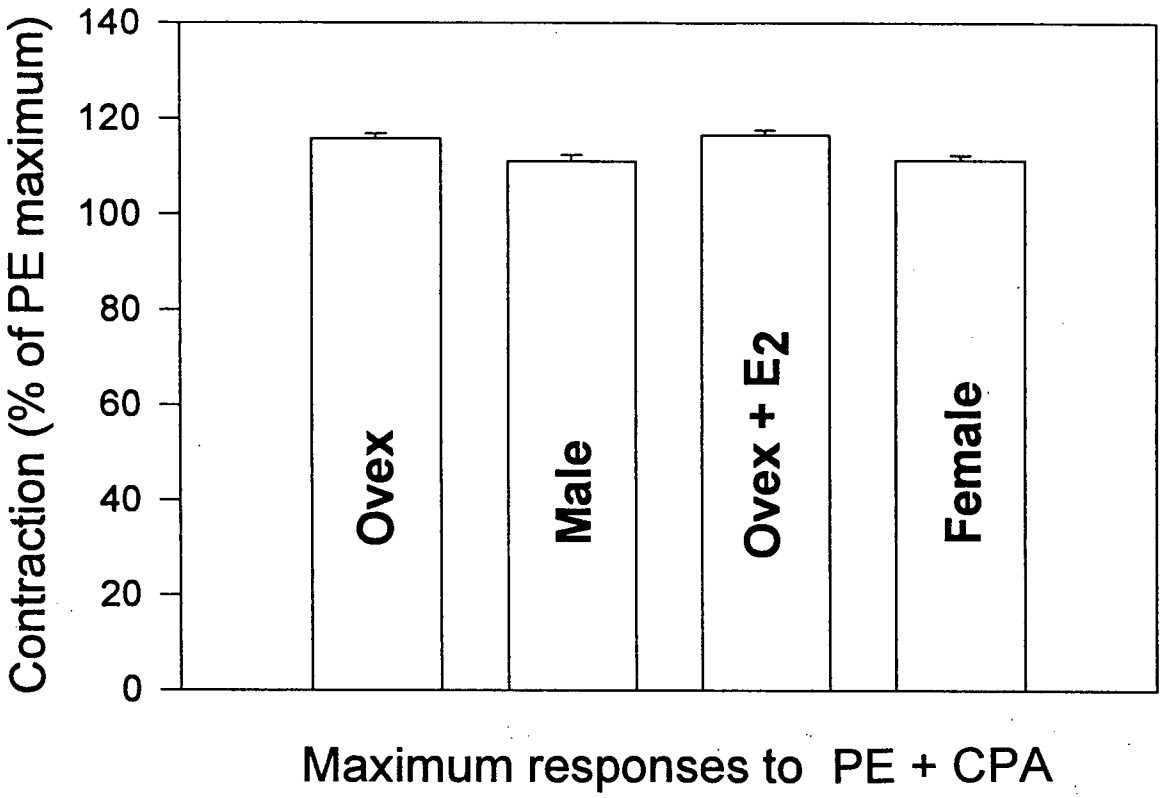

Fig. 3.13. Responses to phenylephrine (PE, $2x10^{-6}$ M) plus cyclopiazonic acid (CPA, $3x10^{-5}$ M) in the presence of L-NAME ($2x10^{-4}$ M; 30 min) relative to control PE response obtained in the absence of L-NAME. Data are means ± S.E.M. of 5 rats per group. There were no differences in CPA-induced contractions of aortae in the various groups.

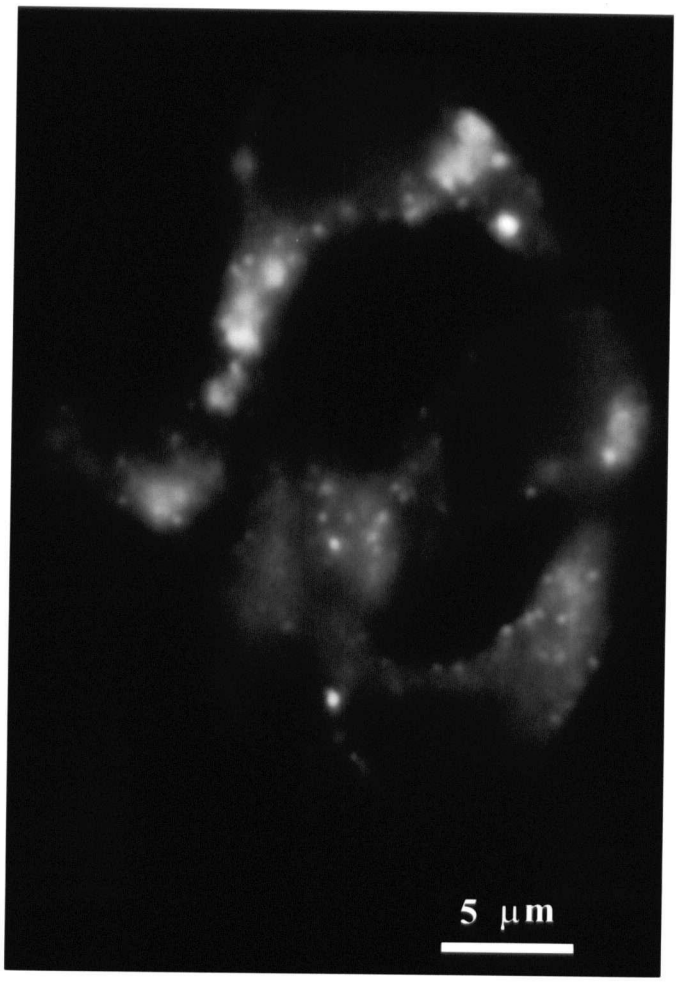

Fig. 3.14. Uptake of DiI-Ac-LDL by rabbit aortic endothelial cells. The cells have been incubated with 10 μg ml^{-1} DiI-Ac-LDL for 4 h at 37°C. The cells were visualized using a standard rhodamine excitation: emission filter set.

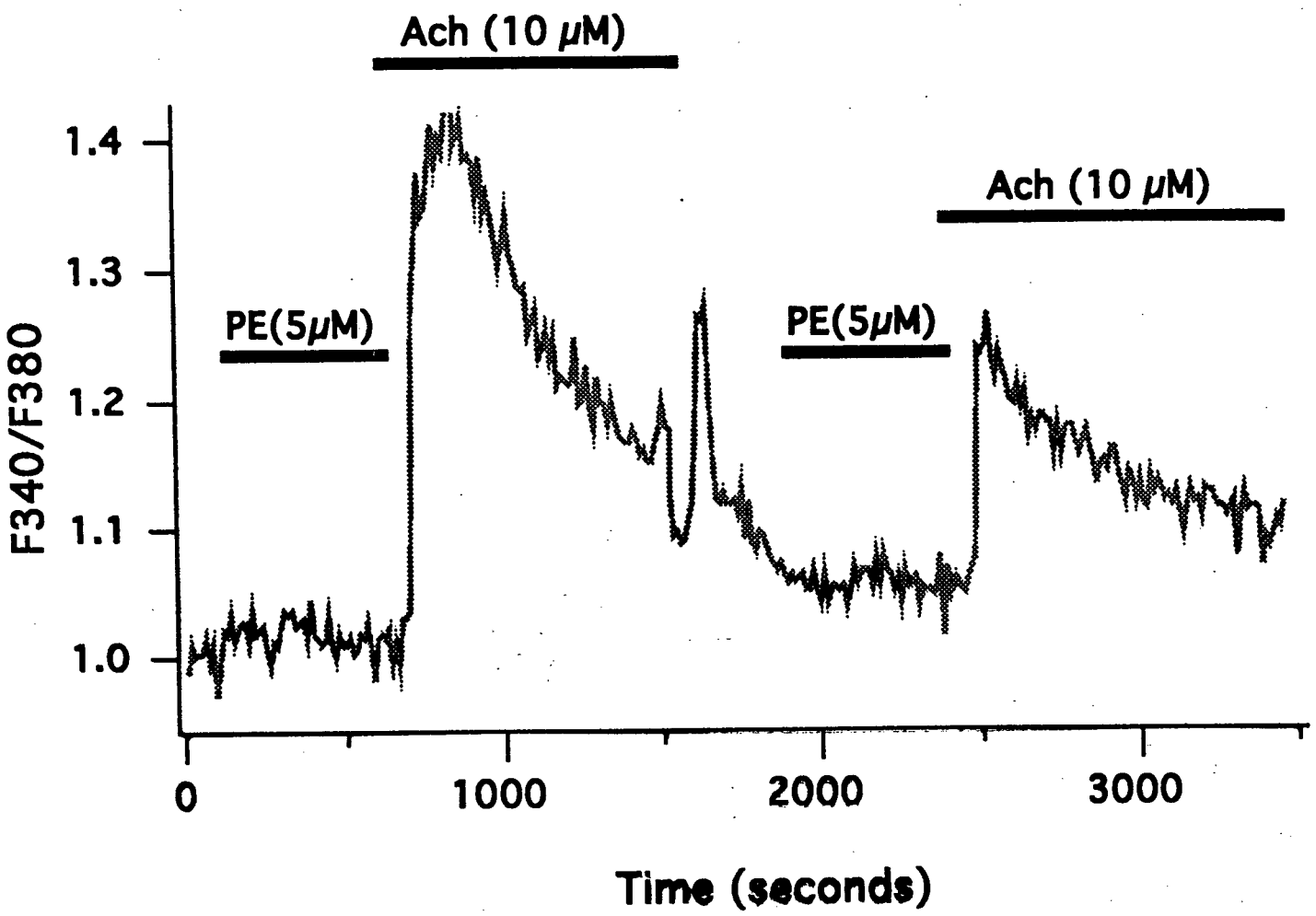

Fig. 3.15. A representative trace of the fura-2 fluorescence ratio (F_{340}/F_{380}) signals of rabbit aortic endothelial cells in response to phenylephrine (PE, 5 μM) followed by subsequent application of acetylcholine (ACh, 10 μM).

These cells were then washed with n-PSS and the same protocol repeated. We observed no changes in $[Ca^{2+}]_i$ in response to PE (1-10 μM) in fura-2 loaded rabbit aortic endothelial cells. The activation of endothelial cell surface receptors by Ach evoked a biphasic increase in $[Ca^{2+}]_i$. The initial transient component reflected the release of Ca^{2+} from intracellular stores by inositol 1,4,5-triphosphate, whereas the subsequent elevation in $[Ca^{2+}]_i$ resulted from the influx of Ca^{2+} from the extracellular space (Wang *et al.*, 1995a, 1997).

3.4.3. Effect of K channel blockade on Ach- and CPA-induced $[Ca^{2+}]_i$ changes

The agonist Ach (10 μM)- and the inhibitor of SERCA-, CPA (10 μM), induced increases in $[Ca^{2+}]_i$. In order to investigate the contribution of K^+ channels in Ach-/CPA-induced Ca^{2+} entry, a non-selective K^+ channel inhibitor TEA (3 mM) was used. The maintained $[Ca^{2+}]_i$ increase upon agonist or Ca^{2+} pump blocker application were significantly ($P < 0.05$, n=36) blocked by TEA (Figs. 3.16 & 3.17A/B). TEA inhibited the Ach- and CPA-stimulated $[Ca^{2+}]_i$ increase by an average of 73 ± 3% and 56 ± 4%, respectively. The inhibitory effect of TEA on CPA-induced $[Ca^{2+}]_i$ increase was only slightly less than that produced by removal of extracellular Ca^{2+} (77 ± 4%; $P < 0.05$, n=18) (Fig. 3.17B).

Points to be consider: 1) There was a persistent increase in $[Ca^{2+}]_i$ with CPA in the absence of extracellular Ca^{2+} (0Ca), 2) The Ca^{2+} oscillations were frequently observed upon application of TEA on the top of Ach or CPA, and 3) TEA (5 mM) itself had no effect on the resting $[Ca^{2+}]_i$ of the rabbit aortic endothelial cells (data not shown).

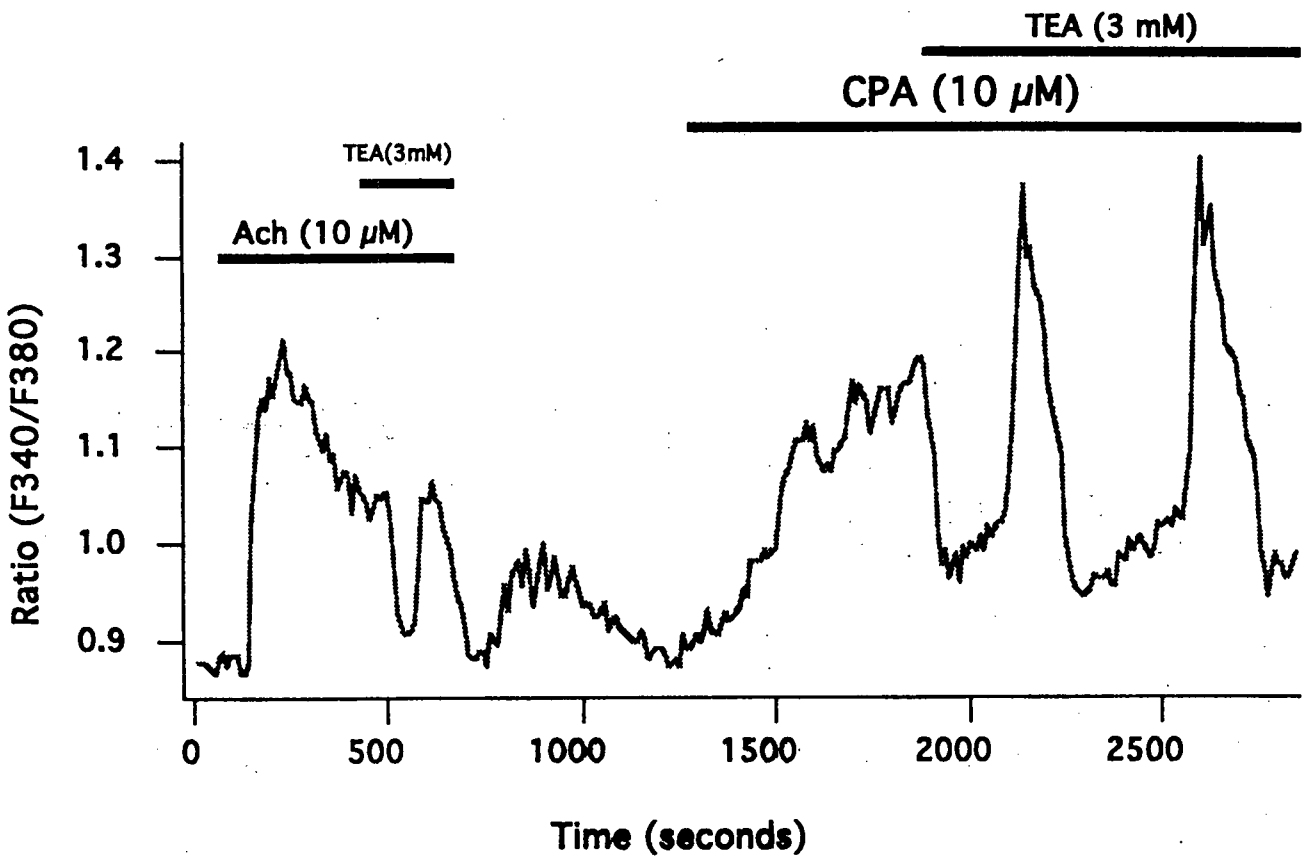

Fig. 3.16. A representative trace of the fura-2 fluorescence ratio (F_{340}/F_{380}) signals of rabbit aortic endothelial cells in response to acetylcholine (Ach, 10 μM) followed by subsequent application of tetraethylamonium (TEA, 3 mM). The cells were then washed with n-PSS and stimulated by cyclopiazonic acid (CPA, 10 μM) followed by subsequent application of TEA (3 mM).

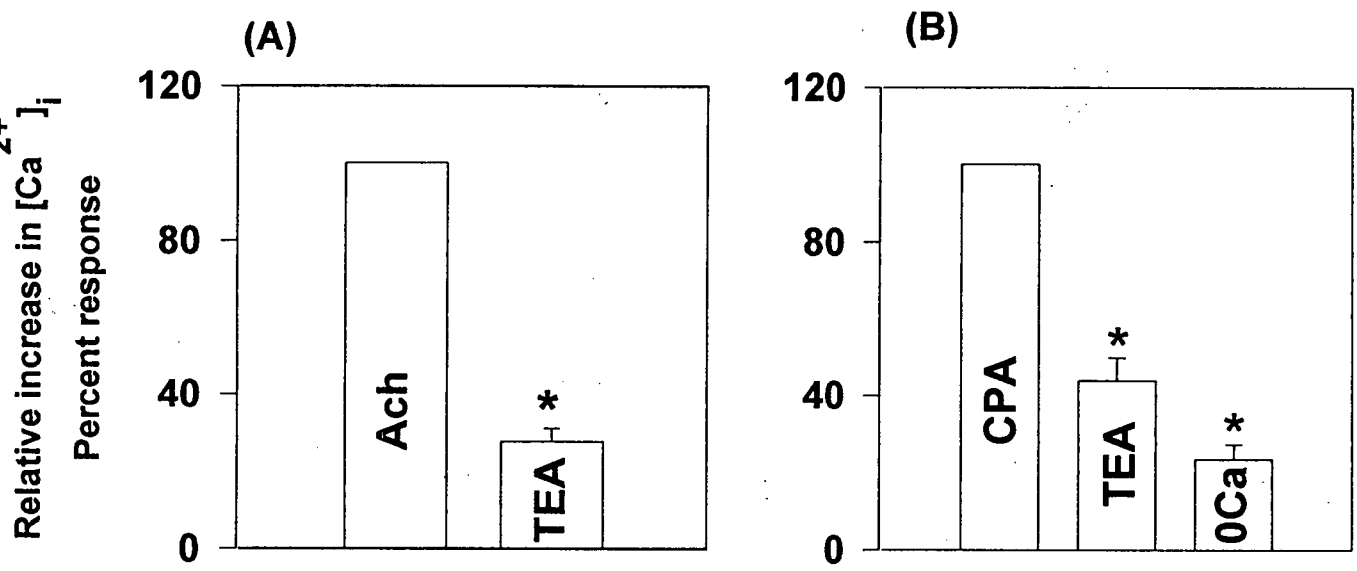

Fig. 3.17. Inhibitory effect of TEA (3 mM) on **(A)** Ach (10 μM), and **(B)** CPA (10 μM)-induced $[Ca^{2+}]_i$ elevation in freshly isolated rabbit aortic endothelial cells (n=36). The Ach and CPA responses before TEA were set as 100%.
*, Significantly different ($P < 0.05$) from Ach or CPA responses by Student't- test.

3.4.4. Effect of ROC and Cl^- channel blockers on Ach/ CPA-induced $[Ca^{2+}]_i$ changes

The application of CPA on top of Ach markedly enhanced $[Ca^{2+}]_i$ to a level higher than that application of Ach alone (by an average of 23 ± 5%; $P < 0.05$, n= 26). In order to investigate that the ROCs are responsible for Ca^{2+}-influx triggered by agonist/ or ER Ca^{2+}-ATPase inhibitors, SK&F96365, a ROC blocker was used. SK&F96365 (50 μM) greatly reduced the maintained $[Ca^{2+}]_i$ increase caused by Ach and CPA (by an average of 88 ± 3%; $P < 0.05$, n= 26) (Figs. 3.18 & 3.20). Furthermore, when Ach and CPA were added together the induced Ca^{2+} plateau was less sensitive to TEA but could be abolished by a combination of TEA (5 mM) and the Cl^- channel inhibitor NPPB (50 μM) (by an average of 50 ± 7% vs 88 ± 3%, respectively; $P < 0.05$, n= 26) (Figs. 3.19 & 3.20).

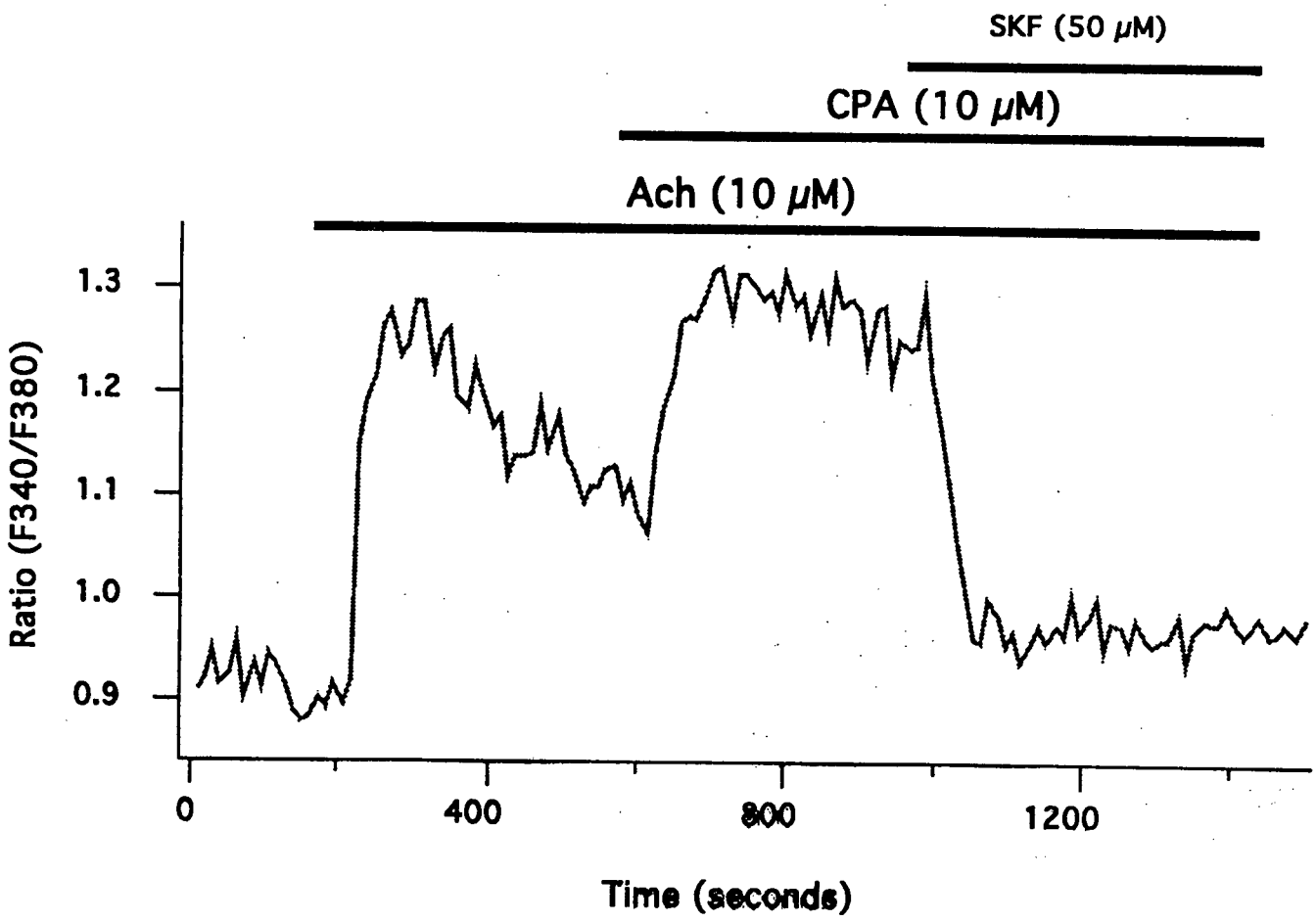

Fig. 3.18. A representative trace of the fura-2 fluorescence ratio (F_{340}/F_{380}) signals of rabbit aortic endothelial cells in response to Ach (10 μM), followed by subsequent application of CPA (10 μM) and SK&F96365 (50 μM).

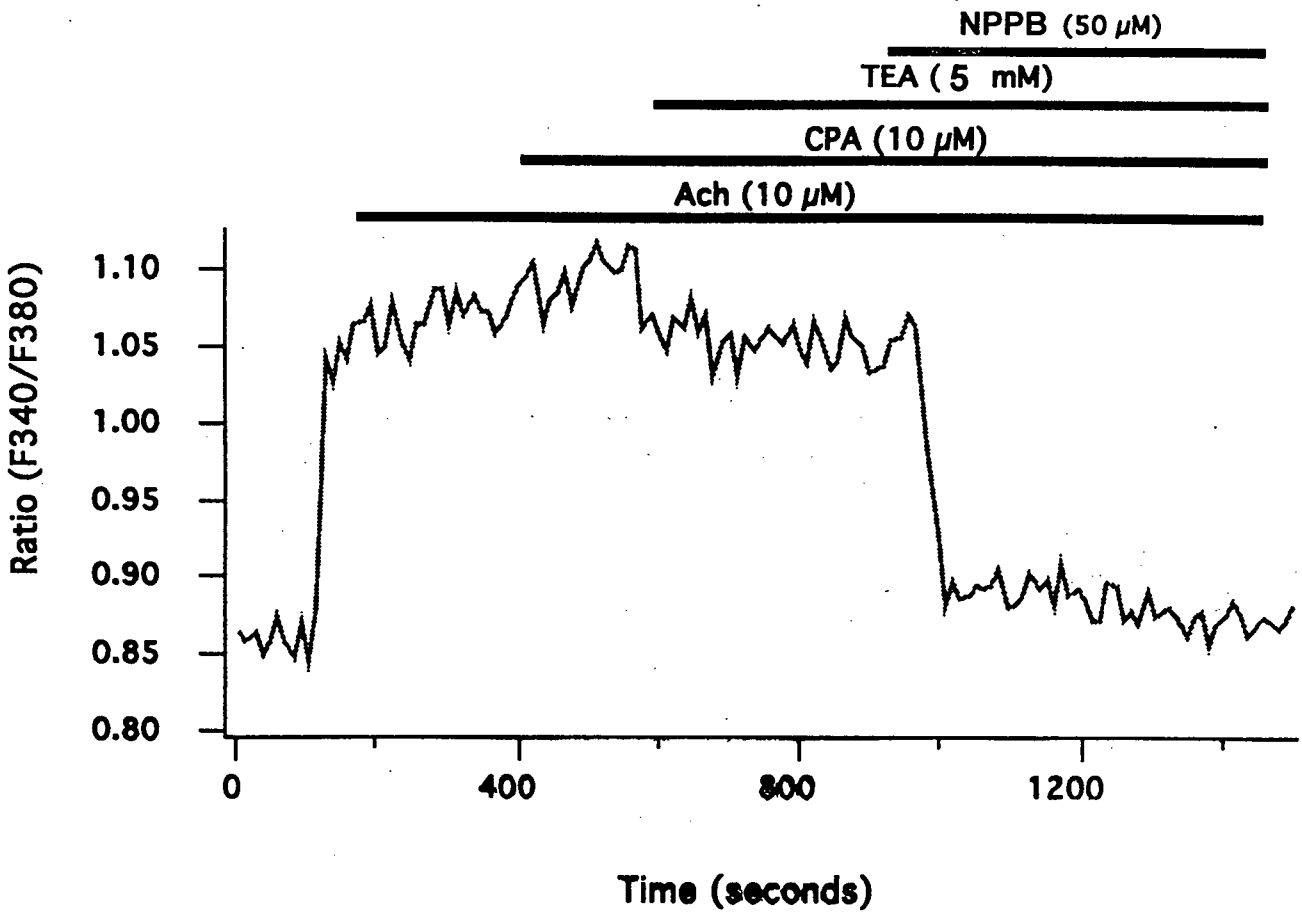

Fig. 3.19. A representative trace of the fura-2 fluorescence ratio (F_{340}/F_{380}) signals of rabbit aortic endothelial cells in response to Ach (10 μM), followed by subsequent application of CPA (10 μM), TEA (5 mM) and NPPB (50 μM).

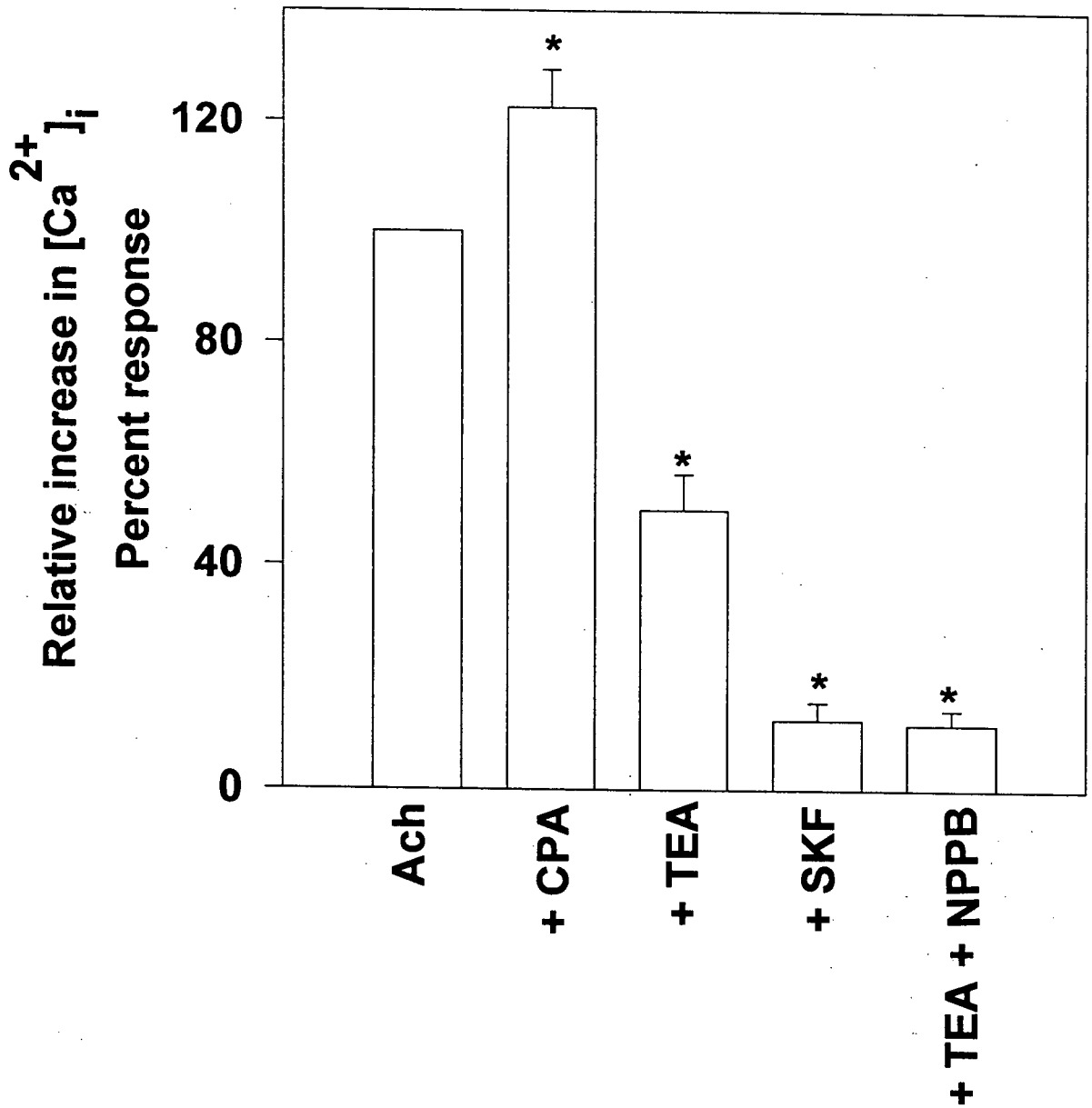

Fig. 3.20. Effects of CPA (10 μM), TEA (5 mM), SK&F96365 (50 μM), and TEA (5 mM) plus NPPB (50 μM) on Ach (10 μM)-induced $[Ca^{2+}]_i$ elevation in freshly isolated rabbit aortic endothelial cells (n=26). The Ach response was set as 100%.
*, Significantly different ($P < 0.05$) from Ach response by Student't- test.

3.5. STUDY OF THE EFFECT OF ESTROGEN ON THE BASAL $[CA^{2+}]_I$ IN RAT VALVULAR ENDOTHELIAL CELLS

In order to investigate that the enhanced Ca^{2+} stimulation of cNOS contributes to estrogenic effects, we measured $[Ca^{2+}]_i$ in valvular endothelial cells taken from female and male rats.

3.5.1. Effect of estrogen on the basal $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in isolated rat valvular endothelial cells superfused with n-PSS (Fig. 3.21). The basal $[Ca^{2+}]_i$ was significantly elevated in female valvular endothelial cells when compared to males (239 ± 19 nM *vs* 156 ± 21 nM, respectively; $P < 0.05$) (Fig. 3.22). CPA, a SERCA inhibitor, was used to investigate the role of the ER in regulating Ca^{2+} entry (Fig. 3.21). CPA (10 μM), in the presence of extracellular Ca^{2+} (1 mM), induced a consistent increase in $[Ca^{2+}]_i$ in both female and male endothelial cells. The extent to which CPA induced an increase in $[Ca^{2+}]_i$ was significantly greater in female endothelial cells than in males (to 228 ± 18 nM and 150 ± 19 nM, respectively; $P < 0.05$). Removal of extracellular Ca^{2+} returned the $[Ca^{2+}]_i$ signal to the basal level, but did not abolish the difference in the basal level of $[Ca^{2+}]_i$ between male and female endothelium (Fig. 3.22). This difference was not observed after washing out of CPA in Ca^{2+}-free PSS (data not shown). The rate of $[Ca^{2+}]_i$ decline upon removal of extracellular Ca^{2+} in the presence of CPA was significantly slower in female endothelial cells compared to males ($1.10 \times 10^{-2} \pm 7.25 \times 10^{-4}$ s^{-1} *vs* $1.90 \times 10^{-2} \pm 1.60 \times 10^{-3}$ s^{-1}, respectively; $P < 0.05$) (Fig. 3.23).

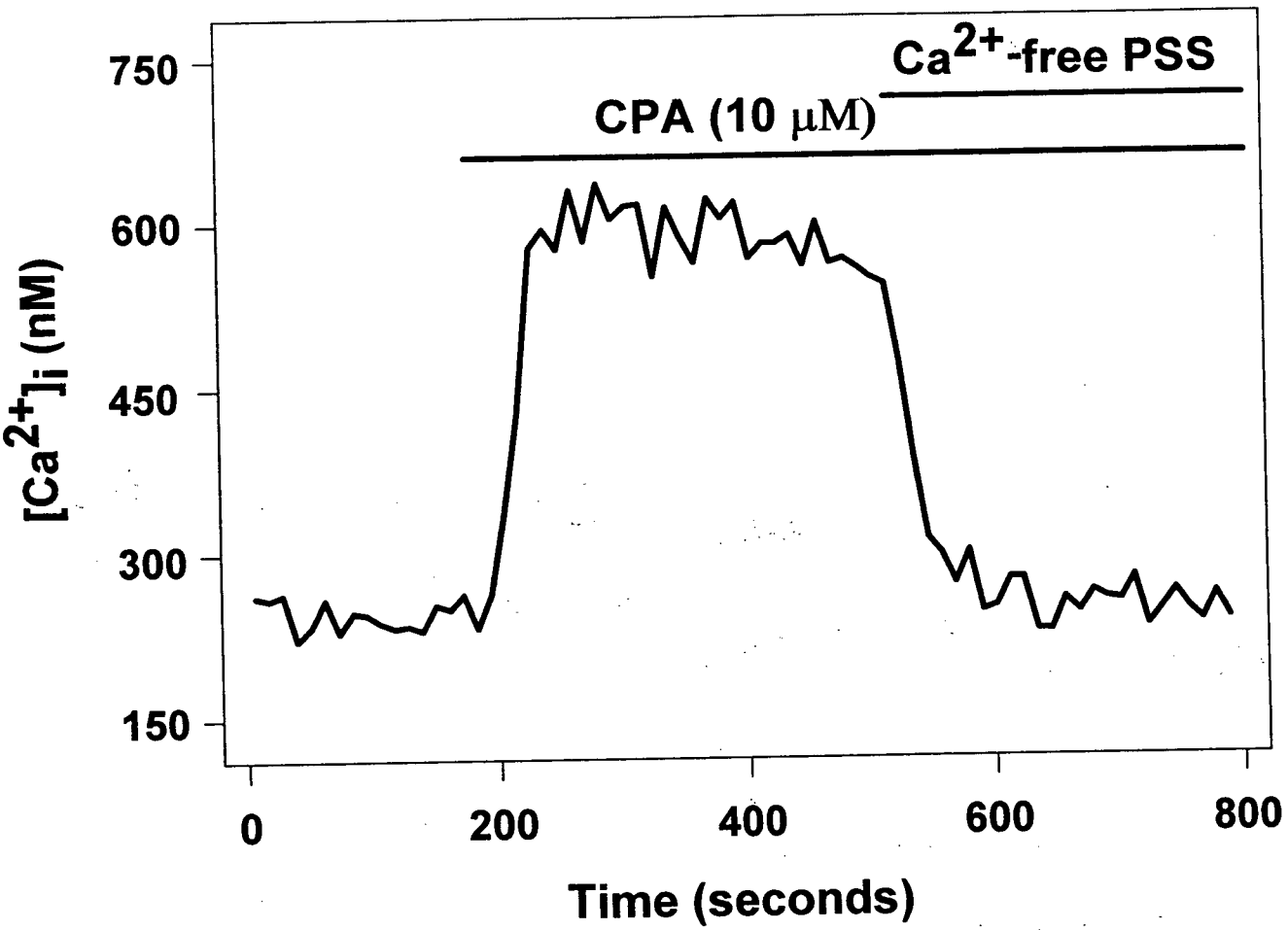

Fig. 3.21. A representative trace of the response of $[Ca^{2+}]_i$ of female rat valvular endothelial cells to CPA (10 μM) followed by removal of extracellular Ca^{2+}.

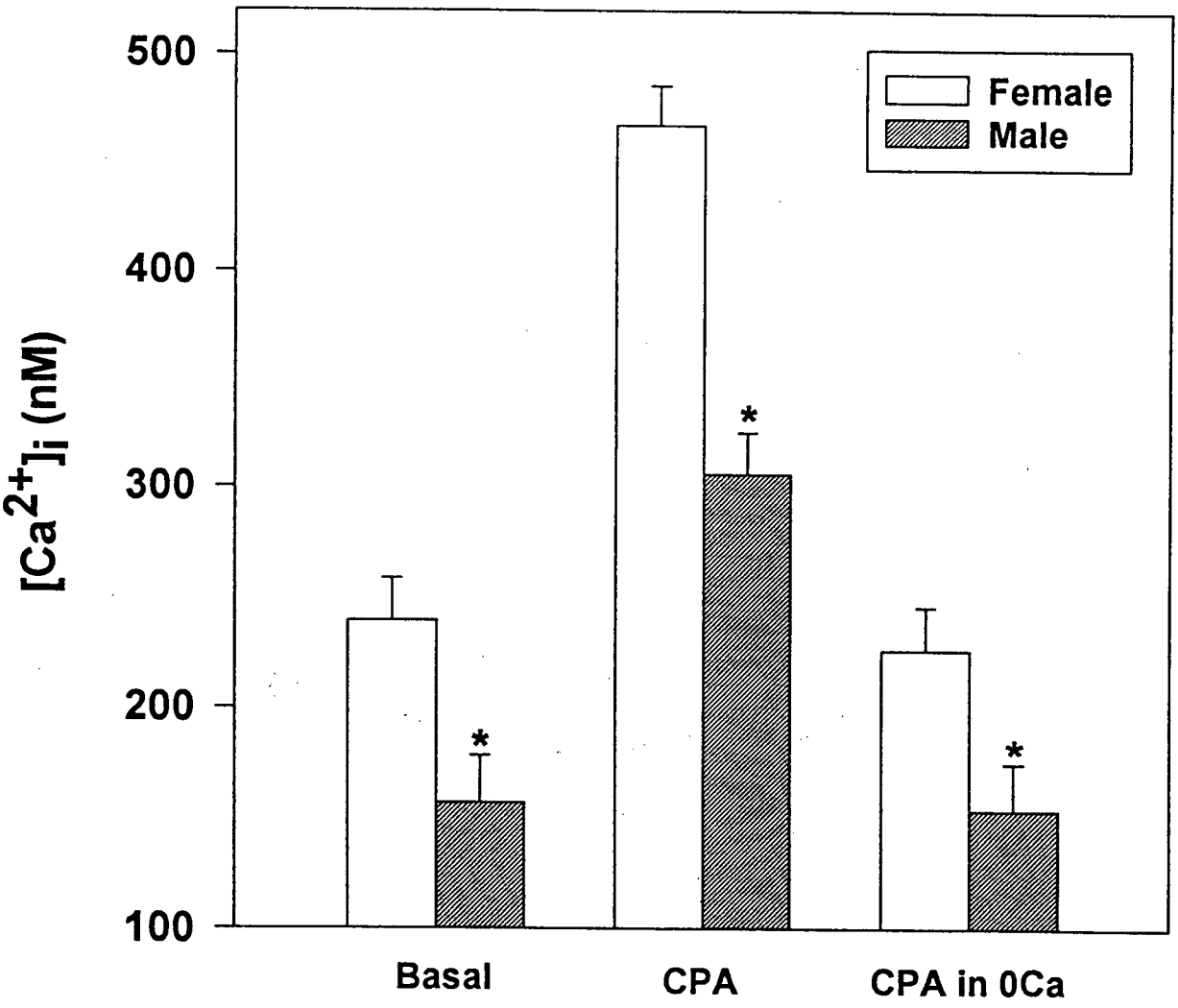

Fig. 3.22. The mean ± S.E.M $[Ca^{2+}]_i$ of 43 to 47 valvular endothelial cells of 10 female and 8 male rats under control condition and after CPA (10 μM) in the presence and absence of extracellular Ca^{2+}.
*, Significantly different ($P < 0.05$) from female group by Student't- test.

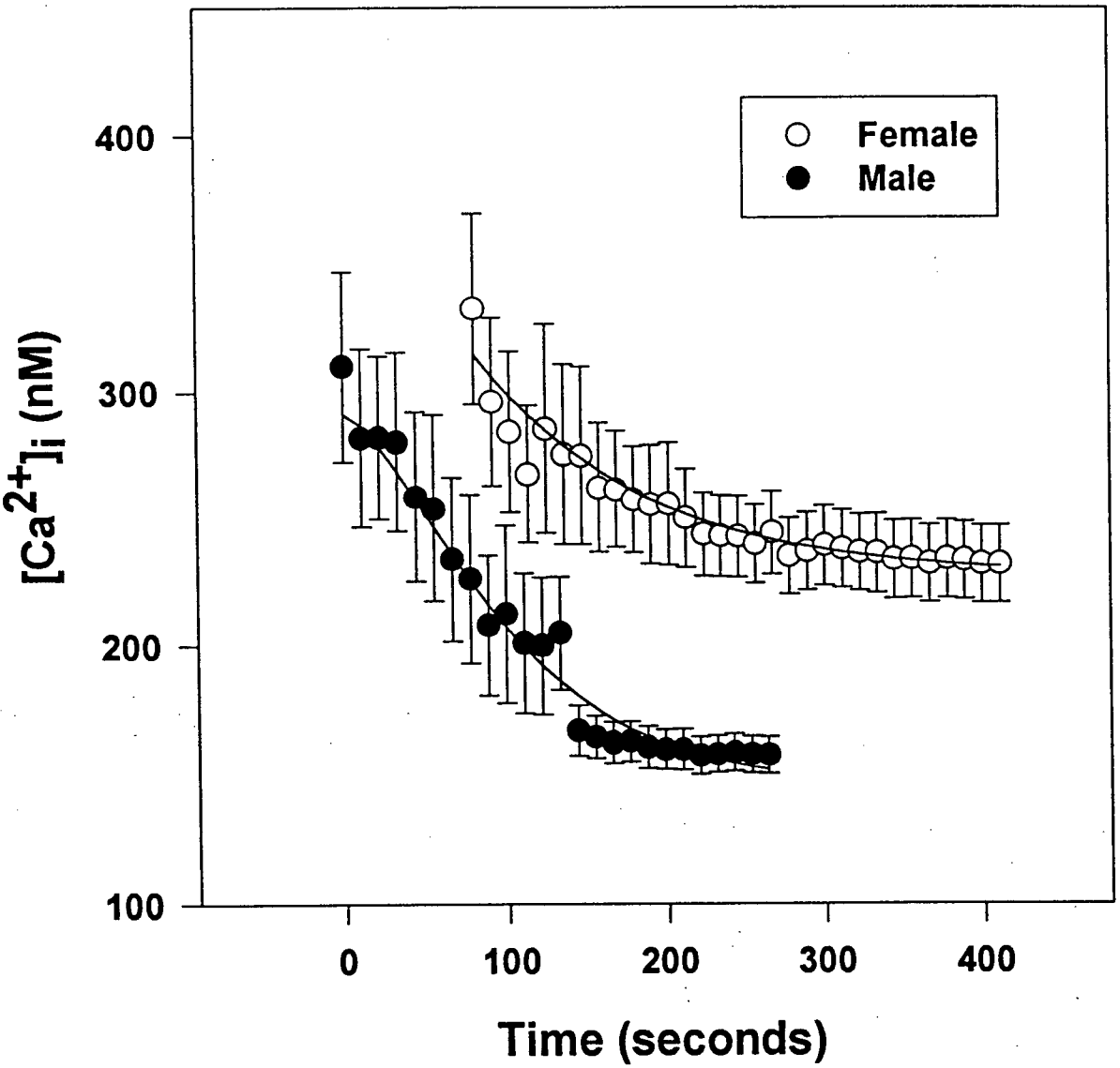

Fig. 3. 23. The mean ± S.E.M $[Ca^{2+}]_i$ decline upon removal of extracellular Ca^{2+}, of 43 to 47 valvular endothelial cells of 10 female and 8 male rats. Below the concentration of 350 nM Ca^{2+}, a biexponential curve fitting equation was used to estimate the rate of $[Ca^{2+}]_i$ decline in Ca^{2+}-free solution. The rate of $[Ca^{2+}]_i$ decline was significantly slower in female endothelial cells compared to males.

3.5.2. Effect of estrogen on the rate of Mn^{2+} quenching

Measurements of $[Ca^{2+}]_i$ provide indirect evidence for Ca^{2+} entry mechanisms. To investigate the possible contribution of Ca^{2+} entry to the elevated basal $[Ca^{2+}]_i$ in female endothelial cells, we measured Mn^{2+} entry as an indicator of divalent cation influx by recording its quenching of cytoplasmic fura-2 fluorescence. Figure 3.24 shows that Mn^{2+} entered the cell and progressively quenched the fura-2 fluorescence at 360-nm in a nearly linear manner, at least until ~30% of the fluorescence was quenched. The slope of the nearly linear part of the quenching trace can be regarded as a measurement of the rate of Mn^{2+} entry into the endothelium. In the current study, there were no differences in the non-stimulated rate of Mn^{2+} quenching between two groups (female: $4.01 \times 10^{-2} \pm 3.00 \times 10^{-3}$ s^{-1}, male: $4.00 \times 10^{-2} \pm 4.00 \times 10^{-3}$ s^{-1}; $P > 0.05$).

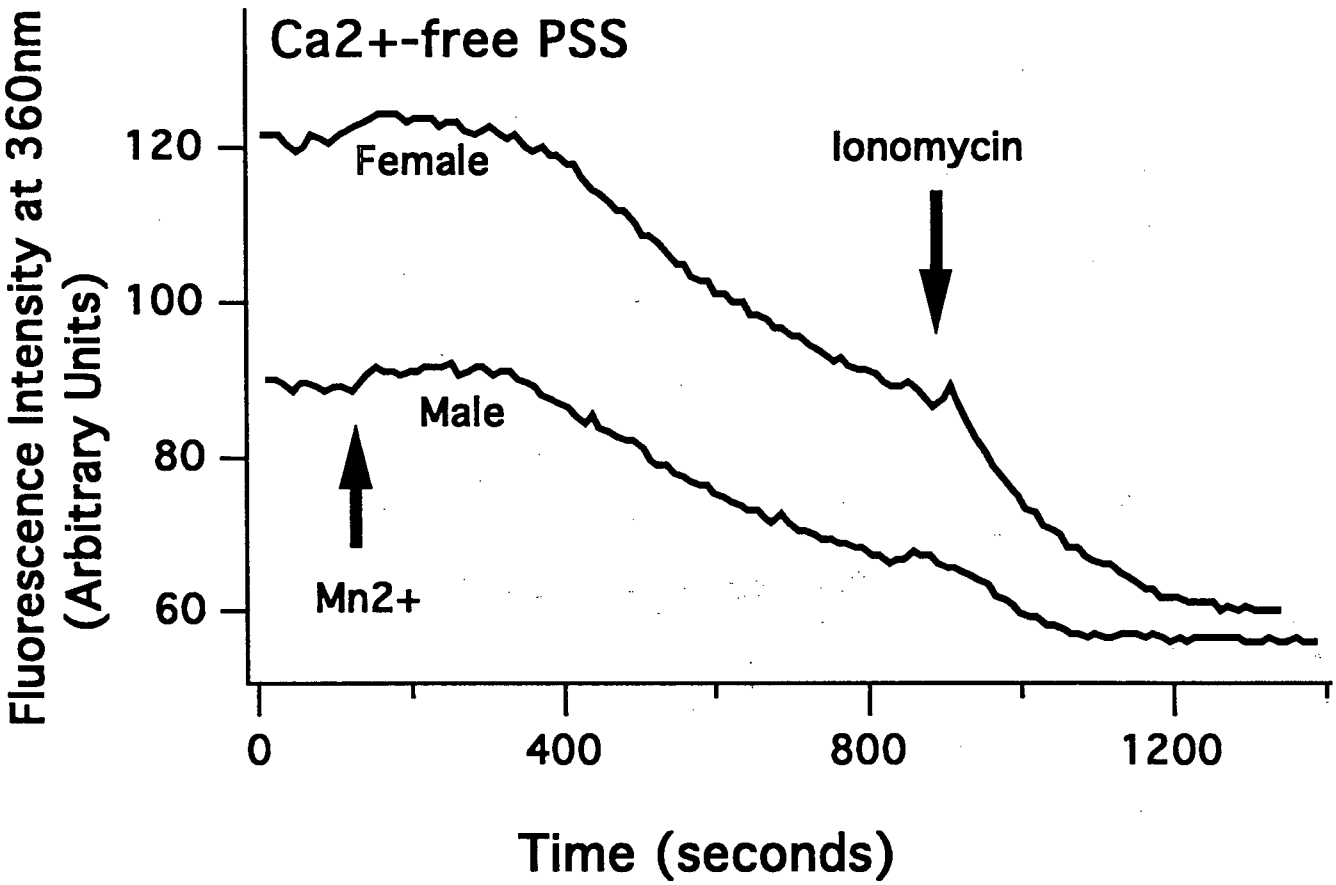

Fig. 3.24. Mn^{2+} quenching of fura-2 fluorescence in female and male rat valvular endothelial cells recorded at the excitation wavelength of 360-nm. The incubating solution (n-PSS) was first replaced with Ca^{2+}-free PSS whereupon the fluorescence intensity decay upon addition of 150 μM Mn^{2+} and 10 μM ionomycin was recorded. The slope of the nearly straight portion of fura-2 quenching trace was assumed to be proportional to rate of Mn^{2+} entry. The ordinate scale indicates fura-2 fluorescence intensity excited at 360-nm (arbitrary units).

CHAPTER IV. DISCUSSION

4.1. Study of the effects of estrogen and SERMs on NO release in rat aorta

The main findings of this study are that treatment of ovariectomized rats with estrogen and SERMs enhance endothelial-dependent vasodilation by increasing basal and stimulated release of NO. These effects occur without changes in the sensitivity of smooth muscle cells to either NO donors or to an adrenergic receptor agonist.

Epidemiological data indicate that pre-menopausal women are at lower risk of CHD than are men of similar age (Castelli, 1988; Barret-Connor, 1994). There is increasing evidence that treatment with replacement estrogen after menopause will reduce cardiovascular mortality events (Stampfer *et al.,* 1991; Nabulsi *et al.,* 1993).

Animal studies have provided further evidence of estrogen's effect on the vascular system. These studies lend experimental support to the hypothesis, brought forward by epidemiological studies, that female sex hormones are protective against CHD. What these studies do not address, however, is the exact mechanism(s) whereby the presence of estrogen is translated into an effect on the biology of the arterial wall.

Endogenous and exogenous estrogens have been observed to alter the levels of serum lipids and lipid metabolism in humans (Hong *et al.,* 1992). The changes in serum lipids noted in human patients, however, fail to fully account for the discrepancy in the incidence of coronary disease between men and premenopausal (Bush *et al.,* 1987).

A direct or non-genomic effect of estrogen on the arterial wall is suggested by animal and human experimental data. Ach vasodilation in atherosclerotic coronary arteries of ovariectomized monkeys was improved 20 min following estrogen infusion (Williams *et al.,* 1992). Similarly, intravenous administration of estrogen also attenuated

the abnormal coronary vasomotor responses to Ach in postmenopausal women 15 min after administration of the hormone (Reis *et al.,* 1994). Data from isolated organ perfusion and arterial segment preparations further support a non-genomic effect of estrogen on vasomotor tone. Raddino *et al* (1986) showed, estradiol (10^{-7}M) elicited immediate vasodilation in isolated rabbit heart during vasopressin-induced coronary vasospasm. This effect was independent of gender and may be mediated by an effect on smooth muscle cell calcium transport. In all these experiments, the acute nature of vascular responses to estrogen indicates the involvement of membrane-mediated mechanisms of action. However, the fact that micromolar concentrations of estrogen are required to produce these responses would suggest a non-specific effect of the hormone.

Establishing a direct, genomically mediated mechanism of estrogen on the vessel wall, however, requires expression of functional estrogen receptor in the target tissue. Estrogen receptor has been demonstrated in canine peripheral arteries (Horwitz & Horwitx, 1982), rat aortic endothelial cells (Lin *et al.,* 1986), VSMCs (Nakao *et al,* 1981), and human vascular endothelial cells (Colburn & Buonassisi, 1978). Binding of estrogen to its receptor can increase transcription of a variety of genes that have ERE in their promoters. Antiestrogens bind to the estrogen receptor in a manner that is competitive with estrogen. Among antiestrogens, SERMS are of particular interest and utility because of their effectiveness in suppressing the estrogen-stimulated proliferation and metastatic activity of estrogen receptor-containing breast and uterine cancers.

The estrogen receptor also interacts with the transcription factors, Fos and Jun (Paech *et al.,* 1997). c-fos and c-Jun expression has been shown to be associated with increased production of both endothelial (Eizirik *et al.,* 1993), and neuronal (Herdegen *et*

al., 1993, 1994) isozymes of constitutive NOS (eNOS and nNOS). A number of reports indicate that NO production may play an important role in mediating the effects of estrogen on the vasculature. Gisclard *et al* (1988) reported that femoral arteries from estrogen-treated rabbits show an enhanced endothelium-dependent relaxation to Ach. Williams *et al* (1990) reported that "*in situ*" atherosclerotic coronary arteries of ovariectomized cynomoglus monkeys responded to intracoronary infused Ach with "paradoxical" constriction and that chronic estrogenic treatment reverted the constriction to a moderate dilation. Keaney *et al* (1994) observed that chronic estrogenic treatment of ovariectomized hypercholesterolemic miniature swine preserve the endothelium-dependent relaxation of coronary artery rings to bradykinin and substance P, whereas vessels from untreated animals exhibited impaired relaxations to these agonists. However, there are also studies reporting that chronic estrogenic treatment did not effect receptor-mediated release of NO by Ach. For example, Miller and Vanhoutte (1990 & 1991) observed no differences in receptor-mediated (Ach, adenosine diphosphate, bradykinin) relaxations of arteries from estrogen-treated and untreated ovariectomized rabbits. Similar negative findings were reported by Hayashi *et al* (1992).

In view of these conflicting reports, we compared NO-dependent responses in intact aortic rings from sham-operated, estrogen-/ SERM-treated, and untreated ovariectomized and male rats both in the basal state and after stimulation by Ach.

Ach stimulates the production of NO from L-arginine within the endothelium, which then relaxes the underlying smooth muscle by stimulating production of cGMP within the vascular smooth muscle (Furchgott & Zawadzki, 1980). Nitrovasodilators (endothelium-independent vasodilators) such as nitroglycerin and SNP are metabolized to

NO in smooth muscle which then activates GC (Ignarro *et al.*, 1981). The effect of basal release of NO was monitored indirectly by observing the effects of L-NAME on the concentration response curve to PE. L-arginine is converted to L-citrulline in endothelial cells (Palmer *et al.*, 1988) by the enzyme NOS (Furchgott & Zawadzki, 1980; Forstermann *et al.*, 1991). NO synthesis is competitively inhibited by certain analogs of L-arginine such as L-NAME (Rees *et al.*, 1989). Since the endothelial cells appear to be devoid of α adrenergic receptors, differences in basal release of NO would be reflected as differences in the degree of PE-induced contraction in the presence and absence of L-NAME.

In our study, aortic rings from sham and ovariectomized rats receiving estrogen and LY117018 showed a significantly greater potentiation of the PE responses by addition of L-NAME when compared to L-NAME-mediated potentiation of PE contractions in ovariectomized rats receiving placebo or progesterone plus estrogen and male rats. In the light of the absence of functional α adrenoreceptors in endothelial cells (see below), these observations are consistent with chronic estrogen- and raloxifene-dependent maintenance of basal NO release from rat aortic endothelium following ovariectomy. In agreement with this result, Hayashi *et al* (1992) reported that basal release of NO is greater from the endothelium of aortic rings from female rabbits than from either ovariectomized or male rabbits.

In the present study L-NAME did not inhibit relaxation by SNP which is thought to act by releasing NO in smooth muscle cells (Ignarro *et al.*, 1981). It is also important to note that estrogen and LY117018 treatment did not affect the sensitivity of rat aorta either to PE contraction or to SNP relaxation.

In addition to the gender difference in the basal release of NO, we also observed that chronic treatment of ovariectomized rats with estrogen or LY117018 enhances endothelium-dependent relaxation to high concentrations (10^{-6} to 10^{-5} μM) of Ach in PE precontracted aortic rings. Activation of endothelial muscarinic receptors induces synthesis and release of NO (Furchgott & Zawadzki, 1980). Our results show that estrogen treatment can increase receptor-mediated NO release. In agreement with these results, Weiner *et al* (1989 & 1991) observed that Ach-induced NO-mediated relaxation of guinea pig uterine and carotid arteries was increased during pregnancy.

Although the focus of our study was to examine the effects of estrogen and SERMs on vascular function, estrogen is usually administered in combination with progesterone when used therapeutically. We therefore included progesterone plus estrogen-treated group of rats in our study. Interestingly, the vasomotor effects of chronic estrogen were reduced when progesterone was combined with estrogen. The mechanism(s) of the interaction we report in the current study is unclear, but it is known that estrogen and progesterone can act in ways that are antagonistic to each other. Related to this, it has been demonstrated that progesterone attenuates estrogen-induced stimulation of the endothelium-dependent responses in isolated dog coronary artery rings (Miller & Vanhoutte, 1991). These antagonistic effects may, in turn, be receptor-mediated. In the productive tissues such as the uterus and breast, estrogen has been shown to up-regulate progesterone receptors while progesterone down-regulates its own receptor (Kreitmann *et al.,* 1979; Read *et al.,* 1988; May *et al,* 1989). It has also been demonstrated that in the chick oviduct and rodent uterus, progesterone prevents the continuation of an estrogen effect (Means & O` Malley, 1971; Bronson & Hamilton,

1972), possibly by interfering with replenishment of cytosolic estrogen receptors (Hsueh *et al,* 1976).

In conclusion, this study indicates that a) chronic estrogen and SERM treatment enhances release of NO in aortic rings of rats, and b) the vasomotor effects of chronic estrogen is reduced when progesterone is combined with estrogen.

The enhanced NO production observed in the present study may result from either greater expression of NOS or elevated basal Ca^{2+} concentrations in endothelial cells. Weiner *et al* (1994) showed that estrogen treatment and pregnancy in the guinea pig increased the activity of Ca^{2+}-dependent NOS in the uterine artery, heart, kidney, skeletal muscle and cerebellum as well as the levels of mRNA expression for both the endothelial and neuronal isoforms of the constitutive NOS (eNOS and nNOS) in skeletal muscle. In agreement with this finding, Hishikawa *et al* (1995) demonstrated that treatment of cultured human aortic endothelial cells with estrogen enhances both Ca^{2+}-dependent NO production and NOS protein. These findings suggest that estrogen increases release of NO, at least in part, by enzyme induction.

Ca^{2+} plays an essential role in NO synthesis/release in endothelial cells (Laskey *et al.,* 1991; Mayer *et al.,* 1989), and it has been shown that cNOS of endothelial cells is Ca^{2+} dependent (Forstermann *et al.,* 1991; Mayer *et al.,* 1989). Regulation of endothelial $[Ca^{2+}]_i$ is composed of activating mechanisms which supply Ca^{2+} to the cytoplasm and homeostatic mechanisms which remove cytoplasmic Ca^{2+} after stimulation.

In endothelial cells, agonist induced increases in $[Ca^{2+}]_i$ are due to a combination of Ca^{2+} influx from the extracellular pool and the release of intracellular stored Ca^{2+} (Schilling *et al.,* 1992; Dolor *et al.,* 1992). Inhibitors of SERCA, CPA, discharge Ca^{2+}

from the ER by inhibiting Ca^{2+} uptake (Seidler *et al.,* 1989). Depletion of ER Ca^{2+} subsequently activates Ca^{2+} influx (Zhang *et al.,* 1994).

4.2. Study of the effect of estrogen on CPA-mediated endothelium-dependent vasodilation in rat aorta

It has been reported that CPA induces an endothelium-dependent relaxation and cGMP production in the rat aorta (Moritoki *et al.,* 1994; Zheng *et al.,* 1994). In order to determine whether estrogen modulates CPA-induced relaxation in rat aorta, we compared CPA endothelium-dependent responses in intact aortic rings from female, estrogen-treated and untreated ovariectomized and male rats.

Chronic treatment of ovariectomized rats with 17 β-estradiol in the present study enhanced endothelium-dependent relaxations to CPA in PE-precontrated aortic rings when compared to the vasodilation in aortic rings from ovariectomized rats treated with placebo or male rats. The presence of estrogen (female, estrogen treated-ovariectomized rats) thus enhanced the sensitivity of the endothelium to the action of CPA that result in vasodilation. In agreement with Zheng *et al* (1994), NO appears to be the vasodilator mediator released by CPA in our study since dilations due to CPA were abolished and converted to contraction by L-NAME, an inhibitor of NOS. An agent which raises intracellular Ca^{2+} in vascular smooth muscle (e.g. CPA) without concomitant endothelial effects, would be expected to initiate contraction (Deng & Kwan, 1991).

It is known that EDHF, distinct from NO, in part contributes to endothelium-dependent vasorelaxation induced by A23187 or bradykinin (Nagao & Vanhoutte, 1991; 1992), and that EDHF mediated component of the relaxations is dependent on Ca^{2+}/calmodulin and resistant to L-NAME (Illiano *et al.,* 1992). From these

considerations, it is conceivable that CPA-induced relaxation may be in part mediated by EDHF. Since we found that the inhibitor of the NO pathway, L-NAME, abolished the relaxant effect of CPA, it is likely that the contribution of EDHF in CPA-induced relaxation in the rat aorta is negligible, and that NO plays the major role in the CPA-induced relaxation.

Inhibition of NOS enhanced PE-induced tone of the aorta suggesting that there is a continuous basal production of NO in our preparation. It could be argued that the enhancement of tone by L-NAME could possibly obscure the relaxation by CPA if it were not related to endothelial NO production. However, Moritoki *et al* (1994) have shown that the inhibitory effects of L-NAME on the CPA-induced relaxation were independent of the initial tension developed in response to different concentrations of PE. In our study estrogen treatment did not directly affect smooth muscle function, as judged by the unchanged sensitivity of rat aorta to CPA contractions or to SNP relaxation.

The results obtained in the present study could be explained by assuming that chronic estrogen treatment of ovariectomized rats either increases expression of NOS in part or enhances the CPA-induced $[Ca^{2+}]_i$ increase in endothelial cells.

By blocking SERCA, CPA has three immediate effects on the supply of Ca^{2+} to the cytoplasm: 1) the unopposed Ca^{2+} leak from ER, 2) the removal of buffering of Ca^{2+} influx through ROCs, and 3) the activation of putative channels gated by store depletion called store-operated channels (SOCs) (Inazu *et al.,* 1994; Pasyk *et al.,* 1995). The former mechanism leads to a transient rise in $[Ca^{2+}]_i$ while the latter lead to maintained $[Ca^{2+}]_i$ elevation (Li & van Breemen, 1996). These components are difficult to dissect because ROC blockers also blocked PE induced contractions. In spite of these obstacles,

Moritoki *et al* (1996) reported that SK&F96365, a putative inhibitor of receptor-mediated Ca^{2+} entry, inhibited CPA relaxation in rat thoracic aortic rings. In contrast, the voltage-dependent Ca^{2+} channel blocker, nifedipine, did not affect the relaxation caused by CPA. Therefore, the maintained relaxation induced by low and intermediate doses of CPA are best explained by lack of buffering of leak mediated Ca^{2+} entry or opening of ROCs/ or SOCs.

Although the passive Ca^{2+} leak, ROCs/ or SOCs that allow Ca^{2+} influx into the endothelial cells are voltage independent, membrane potential (E_m) plays an important role in regulating Ca^{2+} entry (Adams *et al.,* 1989; Laskey *et al.,* 1991; Rusko *et al.,* 1992; Schilling, 1989). The E_m determines the electrochemical gradient (E_m - E_{Ca}) that provides the driving force for Ca^{2+} influx. It is important therefore to consider the ion channels that modulate the E_m of the vascular endothelial cells, as they can influence NO release by regulating Ca^{2+} influx (Adams *et al.,* 1989;Luckhoff & Busse, 1990;Olesen *et al.,* 1988; Rusko *et al.,* 1992; Schilling, 1989). K^+ and Cl^- channels play the most important role in the regulation of the endothelial cell membrane potential.

4.3. Regulation of Ach and CPA-induced Ca^{2+} entry by membrane potential in rabbit aortic endothelial cells

The main finding reached from this study is that endothelial K channels and Cl^- channels are important for agonist and SERCA inhibition-induced $[Ca^{2+}]_i$ elevation and the subsequent release of NO.

In the present study, we observed no changes in $[Ca^{2+}]_i$ in response to PE (1-10 μM) in freshly isolated rabbit aortic endothelial cells. This observation is important for the following reasons: 1) it confirms that the isolated cells are endothelial, not smooth

muscle and 2) since the endothelial cells do not have functional α_1 adrenergic receptors, PE contraction may be used as a test system for basal NO release.

Depletion of ER Ca^{2+}, by agonist Ach or SERCA inhibitor CPA in our endothelial cell preparation increased $[Ca^{2+}]_i$ in a biphasic manner, with an initial peak due to IP_3-mediated Ca^{2+} release from intracellular stores, followed by a sustained plateau. It has been proposed that discharge of Ca^{2+} from intracellular stores in endothelial cells triggers influx of Ca^{2+} from the extracellular space via ROCs/ or SOCs of the plasma membrane (Hallam *et al.,* 1989; Jacob, 1990; Dolor *et al.,* 1992; Schilling *et al.,* 1992), and subsequently stimulates NOS (Moritoki *et al.,* 1996). This may explain the enhancement of $[Ca^{2+}]_i$, upon application of CPA on top of Ach, to a level higher than that seen upon application of Ach alone. However, Wang *et al* (1996) recently reported that store depletion is not a prerequisite for the Ach-induced Ca^{2+} entry in rabbit aortic endothelial cells.

We showed that TEA inhibits the Ach/or CPA-induced maintained $[Ca^{2+}]_i$ elevation in rabbit aortic endothelial cells. However, TEA itself had no effect on the resting $[Ca^{2+}]_i$ of the rabbit aortic endothelial cells. It has been shown that TEA inhibits the different types of K channels (Cook & Quast, 1990). Interestingly, application of TEA on the top of Ach or CPA induced spontaneous Ca^{2+} oscillations. Although, the mechanisms by which TEA induces the Ca^{2+} oscillation has not been addressed here, this effect could be from a variety of factors including increased Ca^{2+} entry through ROCs/ or SOCs.

The secretory process in several exocrine glands is thought to be associated with the stimulation of the K_{Ca} channels (Petersen & Marruyama, 1984). Endothelial cell

hyperpolarization in response to Ach is correlated with increases in $[Ca^{2+}]_i$ (Busse *et al.*, 1988; Chen & Cheung, 1992; Danthuluri *et al.*, 1988), whereby an increase in $[Ca^{2+}]_i$ activates K_{Ca} channels in endothelial cells (Rusko *et al.*, 1992; Sakai, 1990).

We showed that the inhibitory effect of TEA on CPA-induced $[Ca^{2+}]_i$ increase was only slightly less than that produced by removal of Ca^{2+} from the medium. Based on the data from membrane potential measurement (Wang & van Breemen, 1998), this observation may suggest that the E_m plays a role in maintaining the open state of the SOCs. In other words, depolarization does not merely exerts its effect on Ca^{2+} entry by decreasing the electrochemical driving force but largely by inactivation of the open state of the SOCs. Inactivation of ROC/SOC by depolarization is not unique to endothelial cells, and has been recently reported by Tabo *et al* (1996) for smooth muscle cells.

Another point to be consider is that there was a persistent increase in $[Ca^{2+}]_i$ with CPA in the absence of extracellular Ca^{2+} (0Ca). This observation could be explained by contribution of ER Ca^{2+} leak pathways.

We showed that SK&F96365 at the concentration of 50 μM in rabbit aortic endothelial cells greatly reduced the maintained $[Ca^{2+}]_i$ increase, caused by Ach and CPA. These results suggest that the channels mediating Ca^{2+}-influx triggered by the ER Ca^{2+}-ATPase inhibitors as well as Ach are ROCs (Johns *et al.*, 1987) or SK&F96365-sensitive, non selective cation channels as observed in HL-60 cells (Krautwurst *et al.*, 1993).

At high concentrations SK&F96365 has been shown also to block dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels in arterial smooth muscle cells (Merritt *et al.*, 1990). However, it has been reported that vascular endothelium and endothelial cells are devoid of voltage dependent Ca^{2+} channels (Colden-Stanfield *et al.*,

1987; Jayakody *et al.,* 1987) and that Ca^{2+} channel blockers had no significant effect on that endothelium-dependent relaxation (Jayakody *et al.,* 1987; Adeagbo & Triggle, 1991). Therefore, it is unlikely that the inhibitory effect of SK&F96365 observed in our experiments is due to inhibition of Ca^{2+} influx via voltage-dependent channels.

SK&F96365 has been shown to interfere with release of Ca^{2+} from internal stores (Merritt *et al.,* 1990), but this effect is not the major cause of decrease in $[Ca^{2+}]_i$ induced by the Ach/ or SERCA inhibitors for the following reasons: (1) it has been reported that SK&F96365 at 50 μM had little or no effect on agonist-induced mobilization of stored Ca^{2+} (Krautwurst *et al.,* 1993); (2) concentrations of SK&F96365 necessary to inhibit release of Ca^{2+} are 10 fold higher than those affecting ROC Ca^{2+} entry (Merritt *et al.,* 1990).

The possibility cannot be ruled out that SK&F96365 blocks K_{Ca} channels, resulting depolarization of the endothelial as has been observed in human umbilical endothelial cells (Schwarz *et al.,* 1994).

Besides K^+ channels, Cl^- channels have also been described in a variety of endothelial cells (Groschner *et al.,* 1994; Watanabe *et al.,* 1994; Himmel *et al.,* 1993; Nilius *et al.,* 1996; Nilius *et al.,* 1997). Wang & van Breemen (1998) have showed the existence of a Cl- current in rabbit aortic endothelial cells. Cl^- channels may contribute to cell membrane potential regulation (Voets *et al.,* 1996). More recently several reports suggested that the Cl^- channels may modulate agonist or store depletion-induced $[Ca^{2+}]_i$ increase. Kremer *et al.,* 1995 reported that the presence of extracellular Cl^- is necessary for agonists-induced Ca^{2+} entry in mesangial cells. Similar finding were reported by other investigators in human aortic endothelial cells (Yumoto *et al.,* 1995; Hosoki &

Iijima, 1994) and rat sublingual mocus acini (Zhang & Melvin 1993).

In our endothelial cell preparation, when agonist Ach and ER Ca^{2+}-ATPase inhibitor CPA were added together the induced Ca^{2+} plateau was less sensitive to TEA but could be abolished by a combination of TEA and NPPB. It has been shown that NPPB potently inhibits the Cl^- channels in endothelial cells (Nilius *et al.,* 1997). These results indicate that the activation of Cl^- channels as well as K^+ channels in vascular endothelial cells contributes to the agonist-, Ca^{2+}-ATPase inhibitor-induced Ca^{2+} entry through endothelial ROCs and/or SOCs. Although, the possibility that NPPB also blocks ROCs/ or SOCs in endothelial cells should not be ruled out (Nilius *et al.,* 1997).

In intact rat aorta (Marchenko & Sage, 1994) and rabbit aortic endothelial cells (Wang *et al.,* 1995b, 1996) has been shown that Ach-induced a transient membrane hyperpolarization carried by K^+ currents. In these cases Cl^- current may contribute to the membrane potential after K^+ conductance is largely inactivated. If Cl^- and K^+ conductance coexist, Wang & van Breemen (1998) showed that the blocking of the Cl^- current leads to membrane depolarization of the endothelial cells, which in turn inactivates the SOCs.

In conclusion of this phase of study, we propose that the maintenance of a polarized membrane potential by the activity of both K^+ and Cl^- channels is a requisite for Ca^{2+} influx through endothelial ROCs and/or SOCs, and, therefore, for the synthesis/release of NO.

Harder & Coulson (1979) demonstrated that a synthetic estrogen, diethylstilbestrol (DES), directly hyperpolarizes canine coronary smooth muscle cells by activating an outward K^+ current, a finding further supported by our recent laboratory report that acute

administration of 17 β-estradiol (1-30 μM) markedly enhanced the activity of the large K_{Ca} in rabbit aortic endothelial cells and caused an increase in $[Ca^{2+}]_i$ (Rusko *et al.*, 1995). However, these investigators used supraphysiological levels of estrogen in their preparation, and no study was performed to determine whether physiological level of estrogen, chronic "*in vivo*" treatment, contributes to enhanced $[Ca^{2+}]_i$.

In order to elucidate whether estrogen increases NO release at least in part, by raising the $[Ca^{2+}]_i$ levels in endothelial cells, we compared the basal levels of $[Ca^{2+}]_i$ in female and male rat valvular endothelial cells.

4.4. Study of the effect of estrogen on the basal $[Ca^{2+}]_i$ in rat valvular endothelial cells

In this study, we found that the basal levels of $[Ca^{2+}]_i$ were significantly elevated in female valvular endothelial cells when compared to males. The elevation of $[Ca^{2+}]_i$ in female endothelial cells could be explained by assuming that estrogen either enhances Ca^{2+} influx into the cells or decreases Ca^{2+} extrusion. We measured Mn^{2+} entry as an indicator of divalent cation influx by recording its quenching of cytoplasmic fura-2 fluorescence. The observation that Mn^{2+} entered the cells under resting condition confirmed the existence of a leak pathway in these cells. The direct measurement of divalent cation entry revealed that the basal Ca^{2+} entry was not different between the two groups of animals, suggesting that a direct modulatory effect of estrogen on Ca^{2+} influx is unlikely. Inhibition of the ER Ca^{2+}-ATPase pump with CPA caused an increase in the $[Ca^{2+}]_i$ in both female and male endothelial cells. The extent to which CPA induced an increase in $[Ca^{2+}]_i$ was significantly greater in female endothelial cells than in those from males. This could be explained by enhanced ER Ca^{2+} uptake from the elevated $[Ca^{2+}]_i$ in the females and is consistent with the gender difference in CPA induced endothelium-

dependent relaxation of rat aorta, which we recently reported (Rahimian *et al,* 1997b). Although Ca^{2+} release from the ER contributes to the initial increase, the maintained plateau is due to the Ca^{2+} entry through the passive leak and ROCs/ or SOCs as we have also shown in our previous study. Removal of Ca^{2+} from the extracellular space in the presence of CPA allowed measurement of the decay in $[Ca^{2+}]_i$ as a function of Ca^{2+} extrusion (Nazer & van Breemen, 1997). Ca^{2+} extrusion is a physiological process that is essential for keeping the resting $[Ca^{2+}]_i$ low in response to the Ca^{2+} entry that is due to the activity of a variety of Ca^{2+} channels in the presence of a steep electrochemical gradient across the plasma membrane. The observation that this process was slower in the female than male identifies inhibition of Ca^{2+} extrusion as a probable mechanism for the estrogen induced enhancement of basal $[Ca^{2+}]_i$ and consequent enhanced NO secretion (Rahimian *et al.,* 1998). Removal of Ca^{2+} from the cytosol is an active process that in endothelial cells appears to depend primarily on the PMCA, and to a lesser extend on the Na^+-Ca^{2+} exchanger (Li & van Breemen, 1995) and SERCA. Since washing out of the SERCA blocker in Ca^{2+}-free PSS returned the $[Ca^{2+}]_i$ signal to the same basal level for both female and male endothelial cells, the direct modulatory effect of estrogen on SERCA seems to be unlikely. Whether the slower rate of Ca^{2+} extrusion observed in female endothelium is related to inhibition of PMCA or Na^+/Ca^{2+} exchanger remains to be investigated.

In conclusion, our results indicate a novel mechanism for the protective action of estrogen in the blood vessels. It shows that a difference in Ca^{2+} homeostasis leading to greater basal $[Ca^{2+}]_i$ in female than male rats may be responsible for enhanced CPA endothelium-dependent vasodilation and NO secretion in female and estrogen-treated

ovariectomized female rats, when compared to male or ovariectomized rats (Rahimian *et al.,* 1997a, 1997b).

CHAPTER V. CONCLUSION

5.1. The rat as a model

Using the rat as a model to study the influence of plasma estrogen on the function of aortic endothelium has several advantages. First, the rat represents a cost effective animal model where plasma estrogen levels can be altered relatively easily (ovariectomy and estrogen replacement). Secondly, viable, small arteries, aortae and cardiac valves can be consistently isolated from the rat. Although plasma estrogen levels are slightly lower in female rats (10-200 pg/ml) compared to women (50-300 pg/ml), the relative change in estrogen levels through the menstrual cycle is similar.

The other point to consider is that the measurements of tension in isometric aortic rings, and $[Ca^{2+}]_i$ in isolated aortic and valvular endothelial cells were made using a variety of sources, techniques and could not be made simultaneously. However, the data from each of the approaches were internally consistent; e.g. enhancement of basal NO-, and basal endothelial Ca^{2+} concentration, suggesting a common cellular mechanism in each of the tissue configurations.

Ovariectomy may alter the plasma levels of hormones and factors (progesterone and trophic hormones) other than estrogen, and such changes could also influence aortic responses to vasodilators. Our studies do not rule out this possibility. However, estrogen replacement in ovariectomized rats restores the "female" responses. This is arguing against a significant role of other factors that might be altered by ovariectomy. Therefore, we believe our interpretations and conclusion drawn from these studies are reasonable.

5.2. Potential cardiovascular protective effects of estrogen

This study provides basic evidence to support the abundant clinical data which suggest that estrogen is largely responsible for the decreased mortality women experience prior to menopause. Our demonstration that inhibition of NOS causes a greater enhancement of adrenergic vasoconstriction in aortic rings from animals with physiological levels of 17 β-estradiol defines the basal endothelial NO as an important therapeutic site for the cardioprotective actions of estrogen and SERMs. Furthermore, we showed that inhibition of ER Ca^{2+} uptake in endothelial cells leads to vasodilation that is greater in blood vessels from estrogen-treated animals. This vasodilation is also mediated by NO release.

An estrogen-induced increase in NO production within the aortic rings could provide several benefits with respect to protection against CHD. One action of NO is to reduce platelet aggregation and adhesion to the vascular endothelium. This effect of NO combined with the favorable alterations in plasma lipids induced by associated with estrogen would decrease the incidence of thrombosis and development of atherosclerosis. Secondly, basal release of NO plays a major role in regulating coronary vascular tone and blood flow, therefore a basal increase in NO may reduce the likelihood or severity of an ischemic event. Increased NO production in response to estrogen could also decrease or prevent ischemic events associated with pathological conditions such as hypertensive heart disease.

5.3. Therapeutic potential for cardioprotective action of estrogen

An understanding of the mechanisms underlying the actions of estrogen on blood vessels could lead to the development of new therapeutic approaches towards the prevention of cardiovascular disease. These strategies could provide a decrease in cardiovascular mortality in both men and women without the undesirable side effects associated with estrogen use.

In the current research, we indicated a novel mechanism for the protective action of estrogen in the blood vessels. Our results suggest that a difference in Ca^{2+} homeostasis may be responsible for enhanced NO secretion in female and estrogen-treated ovariectomized female rats, when compared to male or ovariectomized rats (Rahimian *et al.,* 1998). The objective of the future research, therefore, would be to mimic the estrogenic action on the Ca^{2+} extrusion pathway, PMCA or Na^{+}-Ca^{2+} exchanger, in order to raise endothelial $[Ca^{2+}]_i$ and subsequently enhance NO secretion.

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