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Estrogen augments cyclopiazonic acid-mediated, endothelium-dependent vasodilation

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Abstract

The modulatory effects of chronic estrogen treatment on the responses to cyclopiazonic acid, an endoplasmic reticulum Ca^{2+}-ATPase inhibitor, were studied in rings of aorta and the isolated perfused kidney of the rat. Rings of aorta were obtained from the following groups of age-matched rats (i) male, (ii) female, and two groups of rats implanted with a subcutaneous pellet (iii) ovariectomized, placebo-treated, (iv) ovariectomized, 17β-estradiol-treated (0.5 mg/pellet/21 days). In phenylephrine (2 μM) pre-contracted rings with intact endothelium, cyclopiazonic acid (10^{-7} to 3×10^{-5} M) produced endothelium-dependent relaxations in a concentration-dependent manner. The cyclopiazonic acid dilation as a percentage loss of phenylephrine tone was greater in aortic rings from female ($72.9 \pm 2.4\%$) and estrogen-treated rats ($65.5 \pm 4.8\%$) compared to those from male ($51.5 \pm 3.4\%$) or ovariectomized rats ($40.8 \pm 3.9\%$) ($P < 0.05$, one-way analysis of variance (ANOVA)). These relaxation responses of cyclopiazonic acid were converted to contractions by pre-treatment with an inhibitor of nitric oxide (NO) synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 200 μM; 30 min). There were no differences in cyclopiazonic acid-induced contractions of aortas excised from either estrogen-treated or untreated-ovariectomized rats. In perfused kidneys, cyclopiazonic acid (10^{-5} M) caused a larger decrease in perfusion pressure in kidneys from female rats (110 ± 0.4 mmHg) than it did in kidneys from male rats (80 ± 0.6 mmHg). These results demonstrate that cyclopiazonic acid causes a greater endothelium-dependent dilation in estrogen-treated ovariectomized and control female rats, possibly due to unmasking of estrogen-enhanced Ca^{2+} entry into the endothelial cells.

Keywords: Cyclopiazonic acid; Nitric oxide (NO); Endothelium; Vasodilation; N^{ω}-Nitro-L-arginine methyl ester (L-NAME)

1. Introduction

During their reproductive years, women have a lower incidence of coronary heart disease compared to men of similar age (Zhang et al., 1995). It is thought that estrogen is largely responsible for this decrease in cardiovascular mortality in women (Stampfer and Colditz, 1991). This cardioprotective effect is in part related to the action of estrogen in decreasing circulating serum levels of low-density lipoprotein (LDL) and increasing high-density lipoprotein levels (HDL) cholesterol concentrations (Walsh et al., 1991). It is increasingly clear that estrogen also has a direct effect on vessel wall physiology (Lobo, 1990). A number of reports indicate that nitric oxide (NO) production plays an important role in mediating the effects of estrogen on the vasculature (Gisclard et al., 1988; Williams et al., 1988). A positive correlation has been found between plasma 17β-estradiol concentrations and levels of stable metabolites of NO (nitrite/nitrate) during follicular development in women (Rosseli et al., 1994).

Endothelial cells produce NO, a potent vasodilator (Furchgott and Zawadzki, 1980), by the activity of NO synthase (Palmer et al., 1988). Intracellular Ca^{2+}, a cofactor in the activity of constitutive NO synthase, plays a key role in the synthesis of endothelial NO (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 sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), such

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as cyclopiazonic acid, discharge Ca^{2+} from the endoplasmic reticulum by inhibiting Ca^{2+} uptake (Seidler et al., 1989). Depletion of endoplasmic reticulum Ca^{2+} subsequently activates Ca^{2+} influx (Zhang et al., 1994).

It has been reported that cyclopiazonic acid induces an endothelium-dependent relaxation and guanosine 3,5-cyclic monophosphate (cyclic GMP) production in the rat aorta. These effects were inhibited by inhibitors of NO synthase 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 guanylate cyclase to produce cyclic GMP leading to relaxation of vascular smooth muscle (Moritoki et al., 1994).

There are several studies reporting that chronic estrogen treatment increases the release/production of NO (Hayashi et al., 1992; Rahimian et al., 1996). The present study was carried out to determine whether estrogen modulates cyclopiazonic acid-induced relaxation. We report a gender-based difference in endothelium-dependent cyclopiazonic acid vasodilation in the rat aorta and perfused kidney.

2. Materials and methods

2.1. Animals and procedures

Fifteen (ten ovariectomized and five control) female and five 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 were female rats; group 2 were male rats. Using a 10-gauge trochar, a pellet was implanted subcutaneously at the back of the neck of rats (group 3 and 4) where it remained until killing 21 days later. Group 3 were ovariectomized, placebo-treated; group 4 were ovariectomized, 17β-estradiol-treated (0.5 mg/pellet) rats.

2.2. Measurement of arterial tension

The rats were killed on day 21 with pentobarbital (65 mg/kg, i.p). On the day of killing, blood samples were collected from the vena cava and the plasma fraction was frozen (−70°C) for later analysis of 17β-estradiol levels. Rats were exanguinated by cutting both carotid arteries. 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 2–4 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 min at 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 min 2–3 times until responses were stable.

2.3. Concentration–response curves to cyclopiazonic acid

Rings of aorta were precontracted with phenylephrine (2 μM), which represented a concentration that produced 80% of maximal effect (EC_{80}). Dilator concentration–response curves were obtained by adding increasing concentrations of cyclopiazonic acid (10^{-7} to 3×10^{-5} M). The rings were then washed with Krebs solution for 30 min and N^{ω}-nitro-L-arginine methyl ester (L-NAME; 200 μM), an inhibitor of endothelium-derived NO synthase (Rees et al., 1989), was added for 30 min. This concentration of L-NAME was based on studies by others (Hayashi et al., 1992; Zheng et al., 1994). The same concentration range of cyclopiazonic acid (10^{-7} to 3×10^{-5} M) was then added to phenylephrine (2 μM)-precontracted rings. Tissues were washed with fresh Krebs solution for 30 min to allow relaxation to basal tone. Relaxation is expressed as the percent decrease in tension below that elicited by phenylephrine pretreatment.

2.4. Relaxing effect of sodium nitroprusside

Concentration–response curves to sodium nitroprusside, an endothelium-independent vasodilator agent (10^{-9} to 10^{-5} M), were made before and after pretreatment with L-NAME (200 μM for 30 min).

2.5. Isolated perfused kidney

Kidneys were removed from rats and immediately attached to a cannula connected to a reservoir containing Krebs buffer solution (95% O_2/5% CO_2, 37°C). Perfusion at constant flow (3.0 ml/min) was achieved with a peristaltic pump (Masterflex, Cole Palmer Instrument, IL, USA) and changes in perfusion pressure were monitored by a transducer placed proximal to the cannula (Bhardwaj and Moore, 1989; Ravemacher et al., 1992). Cumulative concentration–response curves to cyclopiazonic acid (10^{-7} to 10^{-4} M) were made by addition to the inflow reservoir that contained phenylephrine (2 μM). Under these conditions, there were no spontaneous decreases in the phenylephrine-induced increase in basal tone. Vasodilation due to cyclopiazonic acid is given in absolute decreases in perfusion pressure.

2.6. Radioimmunoassay for estradiol measurement

Plasma concentrations of 17β-estradiol were measured by using a ^{125}I radioimmunoassay (RIA) kit (ICN Biomedical, Carson, CA, USA). 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 min and the contents of tubes was aspirated or decanted. The empty tubes were counted for ^{125}I in a gamma counter. A standard curve was used to estimate the estradiol concentration of each sample.

2.7. Chemical reagents and drugs

Cyclopiazonic acid was purchased from Research Biochemicals International (Natick, MA, USA). L-Phenylephrine hydrochloride, sodium nitroprusside, N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO, USA). 17β-Estradiol (0.5 mg/pellet) and placebo pellets were purchased from Innovative Research of America (Toledo, OH, USA), and were designed to release 17β-estradiol over a 21-day period. All drugs were prepared as aqueous solutions except cyclopiazonic acid which was dissolved in dimethyl sulfoxide (DMSO) as stock solution and diluted before use. DMSO at the applied concentrations had no effect.

2.8. Data analysis

Values are expressed as means ± standard error of the mean (S.E.M.). Comparisons of means were made by using the Student's *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% was considered significant. Sensitivity is expressed as the $-\log M$ concentration required for 50% of maximal relaxation or contractor (EC_{50}) determined.

3. Results

3.1. Plasma estradiol level

Estrogen treatment significantly ($P < 0.01$) increased the concentrations of serum estradiol (Table 1). 17β-Estradiol concentrations were significantly ($P < 0.01$) lower in male and ovariectomized rats compared with those in female and estrogen-treated-ovariectomized rats. In agreement with Ferrer et al. (1996), the body weights of estrogen-treated rats were significantly ($P < 0.01$) lower than those of non-treated rats at the time of killing (339 ± 3.5 g and 389 ± 6.6 g, respectively).

3.2. Effect of cyclopiazonic acid on the contraction induced by phenylephrine

Fig. 1 shows a typical trace of a concentration–response curve to cyclopiazonic acid (10^{-7} to 3×10^{-5}) in phenylephrine-precontracted aortic rings from a control group of rats. In agreement with the finding of Zheng et al. (1994), low cyclopiazonic acid concentrations (10^{-7} to 10^{-5} M) caused persistent vasodilation, whereas higher concentrations of cyclopiazonic acid ($> 10^{-5}$) produced a biphasic response, a relaxation followed by gradual reversal to contraction (data not shown). In the case of biphasic

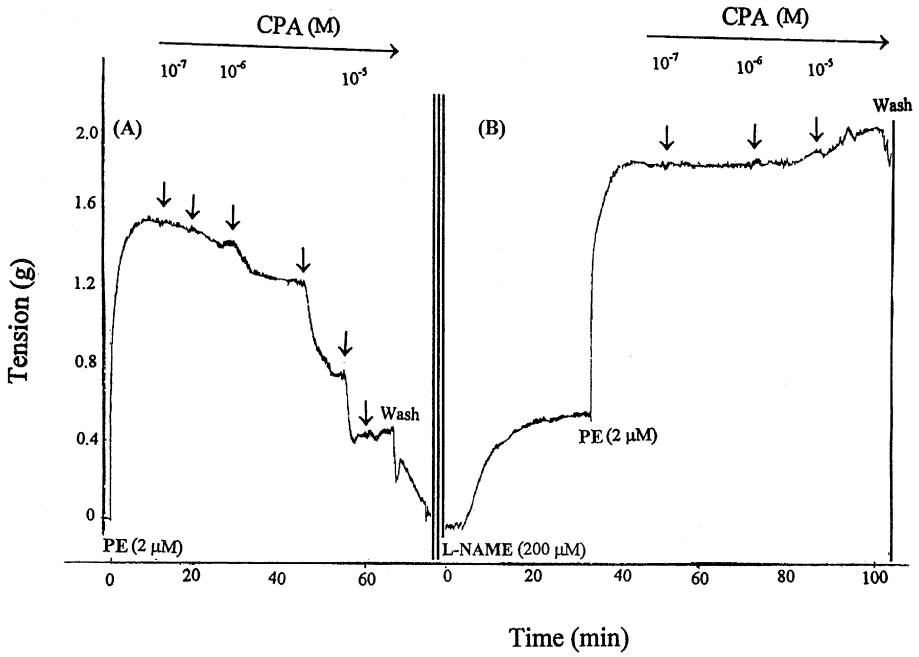

Fig. 1. Representative traces of rat aortic rings showing: (A) Relaxation-response curve induced by cyclopiazonic acid (CPA, 10^{-7} to 3×10^{-5} M) in rings precontracted with phenylephrine (PE, 2 μM). (B) After pretreatment of the same ring with L-NAME (200 μM) for 30 min, cyclopiazonic acid (10^{-7} to 3×10^{-5} M) induced contraction in PE (2 μM) precontracted rings.

Table 1
Mean plasma concentrations of 17β-estradiol (pg/ml) in the various groups of rats

Rat	17β-Estradiol (pg/ml)
Male	35.86 ± 3.96 [a]
Ovariectomized	11.76 ± 0.05 [a]
Ovariectomized + 17β-estradiol	54.96 ± 1.71
Female	67.01 ± 5.50

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

responses, the maximal relaxation was taken for statistical purposes. Fig. 2 summarizes the results of the concentration-dependent effects of cyclopiazonic acid on aortic rings precontracted with phenylephrine (2 μM). Aortic rings from female (72.9 ± 2.4%) and ovariectomized rats receiving 17β-estradiol (65.5 ± 4.8%) relaxed more ($P < 0.05$) to cyclopiazonic acid (3×10^{-5} M) compared to those from ovariectomized (40.8 ± 3.9%) and male (51.5 ± 3.4%) rats. Responses to cyclopiazonic acid 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 cyclopiazonic acid (Fig. 3). The EC_{50} values of cyclopiazonic acid in aortae from male and female rats were 2.99 ± 0.33 μM and 1.66 ± 0.18 μM, respectively ($P < 0.01$).

3.3. Effect of L-NAME on relaxation induced by cyclopiazonic acid

In Fig. 4, we show the effect of L-NAME on cyclopiazonic acid relaxation responses. We used L-NAME, a NO

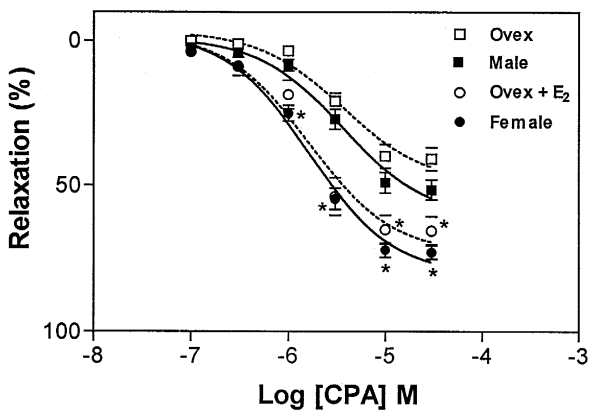

Fig. 2. Effect of chronic estrogen treatment of ovariectomized rats on the relaxation response to cumulative concentrations of cyclopiazonic acid (CPA) in intact aortic rings precontracted with phenylephrine (PE, 2 μM). Relaxation to CPA is expressed as a percentage of PE (2 μM) maximum contraction.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

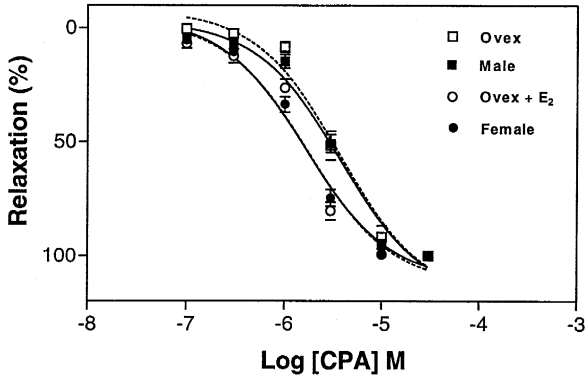

Fig. 3. Concentration–response curves to cyclopiazonic acid (CPA) in thoracic aorta of rats. Responses are normalized to the respective maximal dilator responses to cyclopiazonic acid (3×10^{-5} M). Results are shown as the mean ± S.E.M. of 5 rats per group. The EC_{50} values of cyclopiazonic acid in aortae from male and female rats were 2.99 ± 0.33 μM and 1.66 ± 0.18 μM, respectively ($P < 0.01$, Student's *t*-test).

synthase inhibitor, to uncover responses occurring in the absence of released NO. Incubation of the same aortic rings with L-NAME (200 μM) for 30 min not only abolished the endothelium-dependent relaxation induced by cyclopiazonic acid in all groups, but converted these to contractile responses. There were no differences in the maximum cyclopiazonic acid responses as a percent of phenylephrine tone in aortic rings from female (111 ± 1.0), ovariectomized rats receiving 17β-estradiol (116 ± 1.1), ovariectomized (116 ± 1.0) and male rats (111 ± 1.4).

3.4. Effect of L-NAME on dilation induced by sodium nitroprusside

Sodium nitroprusside releases NO leading to a rise of cyclic GMP-mediated endothelium-independent relaxation in smooth muscle cells (Ignarro et al., 1981). In aortic rings precontracted with phenylephrine (2 μM), L-NAME (200 μM; for 30 min) did not significantly inhibit the relaxation induced by sodium nitroprusside over the entire

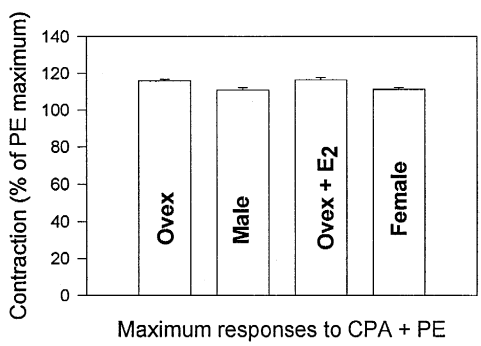

Fig. 4. Responses to phenylephrine plus cyclopiazonic acid (CPA, 3×10^{-5} M) in the presence of L-NAME (200 μM; 30 min) relative to control phenylephrine response obtained in the absence of L-NAME. Data are means ± S.E.M. of five rats per group. There were no differences in cyclopiazonic acid-induced contractions of aortae in the various groups.

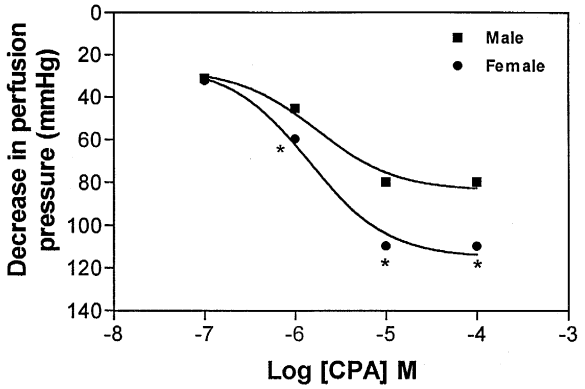

Fig. 5. Loss of perfusion pressure (mmHg) induced by cyclopiazonic acid (CPA) in isolated kidneys from female and male rats. Kidneys were perfused at constant flow, and dilator responses are expressed as the mean ± S.E.M. of four rats per group. Asterisks indicate significant differences in responses ($P < 0.05$).

concentration–response range (10^{-9} to 10^{-5} M) (data not shown).

3.5. Effect of cyclopiazonic acid on the isolated perfused kidneys

When perfused at constant flow, the basal perfusion in kidneys from female and male rats was similar. Phenylephrine (2 μM) caused a sustained increase in basal perfusion pressure that was of similar magnitude in kidneys isolated from female (120 ± 6 mmHg) and male (131 ± 8 mmHg) rats. Cumulative additions of cyclopiazonic acid decreased perfusion pressure (due to vasodilation), reaching a maximum effect at 10^{-5} M. At this concentration, cyclopiazonic acid caused a greater loss of perfusion pressure in kidneys from female (109.7 ± 0.4 mmHg) than from male (79.7 ± 0.6 mmHg) rats ($P < 0.05$) (Fig. 5). In kidneys pretreated with L-NAME (200 μM, 30 min), cyclopiazonic acid induced increases in perfusion pressure, i.e. vasoconstriction, that were similar in magnitude in kidneys from female and male rats ($n = 3$, data not shown).

4. Discussion

In the current study, chronic treatment with 17β-estradiol increased the plasma estradiol levels in rats. The ranges of plasma estradiol values reported in the literature for estradiol-treated and untreated-ovariectomized rats are 26.6 ± 3.3 to 180 ± 17.5 pg/ml and 12.2 ± 4.7 to 21 ± 2.4 pg/ml, respectively (Cheng et al., 1994; Hayashi et al., 1992). Chronic treatment with 17β-estradiol also decreased body weight as reported by others (Conrad et al., 1994; Ferrer et al., 1996).

The main finding of our study is that chronic treatment of ovariectomized rats with 17β-estradiol enhanced ($P < 0.05$) endothelium-dependent relaxations to cyclopiazonic acid in phenylephrine-precontracted aortic rings when compared to the vasodilation in aortic rings from ovariectomized rats or male rats. The presence of estrogen (female, estrogen treated-ovariectomized rats) thus enhanced the sensitivity of the endothelium to the action of cyclopiazonic acid that results in vasodilation. Cyclopiazonic acid also induced a greater vasodilator response in isolated perfused kidneys from female rats, a vascular bed that contains a large number of resistance arteries (Navar, 1978). In agreement with Zheng et al. (1994), NO appears to be the vasodilator mediator released by cyclopiazonic acid in our study since dilations due to cyclopiazonic acid were abolished and converted to contraction by L-NAME, an inhibitor of NO synthase. An agent which raises intracellular Ca^{2+} in vascular smooth muscle (e.g., cyclopiazonic acid) without concomitant endothelial effects, would be expected to initiate contraction (Deng and Kwan, 1991).

Inhibition of NO synthase enhanced phenylephrine-induced tone of the aorta suggesting that there was 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 cyclopiazonic acid 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 cyclopiazonic acid-induced relaxation were independent of the initial tension developed in response to different concentrations of phenylephrine.

L-NAME did not inhibit relaxation by sodium nitroprusside which is thought to act by releasing NO in smooth muscle cells (Ignarro et al., 1981). It is also important to note that estrogen treatment did not directly affect smooth muscle function, as judged by the unchanged sensitivity of rat aorta to cyclopiazonic acid contractions or to sodium nitroprusside relaxation.

The results obtained in the present study could be explained by assuming that chronic estrogen treatment of ovariectomized rats either enhances the cyclopiazonic acid-induced $[Ca^{2+}]_i$ increase in endothelial cells or increases expression of NO synthase. Some studies have demonstrated that the A23187-stimulated, endothelium-dependent relaxation of blood vessels was unaltered by estrogen (Gisclard et al., 1988; Miller and Vanhoutte, 1991). This calcium ionophore stimulates NO synthesis by raising $[Ca^{2+}]_i$ to maximal levels. Therefore, the absence of an estrogen effect on endothelium-dependent relaxation induced by A23178 suggests that enhancement of receptor-mediated relaxation by estrogen is not due to increased expression of endothelial NO synthase.

The enhanced cyclopiazonic acid dilation in estrogen-treated ovariectomized and control female rats may, therefore, more likely result from elevation of endothelial $[Ca^{2+}]_i$. By blocking sarco-endoplasmic reticulum Ca^{2+}-ATPase, cyclopiazonic acid has two immediate effects on supply of Ca^{2+} to the cytoplasm: (1) the unopposed Ca^{2+} leak from the endoplasmic reticulum and (2) the removal of buffering of Ca^{2+} influx. The former mechanism leads

to a transient rise in endothelial $[Ca^{2+}]_i$ while the latter leads to maintained $[Ca^{2+}]_i$ elevation (Li and Van Breemen, 1996). These components are difficult to dissect because receptor-operated channel blockers also inhibit phenylephrine-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 cyclopiazonic acid relaxation in rat thoracic aortic rings. In contrast, the voltage-dependent Ca^{2+} channel blocker, nifedipine, did not affect the relaxation caused by cyclopiazonic acid. Therefore, the maintained relaxation induced by low and intermediate doses of cyclopiazonic acid could be best explained by lack of buffering of leak-mediated Ca^{2+} entry or opening of store-operated channels. Thus if estrogen enhances either the leak- or store-operated channel activity it would augment cyclopiazonic acid-mediated relaxation.

Although the Ca^{2+} store-operated channels that allow Ca^{2+} influx into the endothelial cells are not voltage regulated, the membrane potential plays an important role in regulating Ca^{2+} entry by virtue of its effect on the electrical driving force (Adams et al., 1989). Estrogen directly hyperpolarizes arteries by activating an outward K^+ current (Harder and Coulson, 1979), a finding further supported by our recent report that acute administration of 17β-estradiol (1–30 μM) markedly enhanced the activity of the large Ca^{2+}-dependent K^+ channels in rabbit aortic endothelial cells and caused an increase in $[Ca^{2+}]_i$ (Rusko et al., 1995). Taken together, our results may be explained by assuming that estrogen hyperpolarizes the endothelial cell and increases the electrochemical gradient for Ca^{2+} entry. The resultant increase in endothelial $[Ca^{2+}]_i$ would be expected to augment NO release, consistent with the enhanced cyclopiazonic acid-induced, L-NAME-sensitive, aortic relaxations observed in the present study. Although the above interpretation is consistent with the data presented, it is not possible to rule out estrogen modulation of endothelial NO synthase expression or changes in its Ca^{2+} sensitivity.

In summary, our study indicates that inhibition of endoplasmic reticulum Ca^{2+} uptake in endothelial cells leads to vasodilation that is greater in blood vessels from estrogen-treated animals. This vasodilation is mediated by NO release, and may in part be responsible for the cardioprotective effects of estrogen.

Acknowledgements

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