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Endothelium-dependent hyperpolarization and relaxation in mesenteric arteries of middle-aged rats: influence of oestrogen

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1 We determined whether gender and/or oestrogen deficiency affect endothelium-dependent hyperpolarization and relaxation in mesenteric arteries isolated from middle-aged (44–45 week old) rats.

2 The hyperpolarizing response to acetylcholine (ACh) was significantly greater in females than in males. Ovariectomy caused a marked reduction in ACh-induced hyperpolarization in female arteries, and this was improved by 17β-oestradiol replacement therapy.

3 ACh-induced relaxations in female arteries were not significantly different from those observed in male rats, and were unaffected by ovariectomy, regardless of whether indomethacin was present. However, when endothelial nitric oxide synthase (eNOS) was blocked with N^G-nitro-L-arginine, the sensitivity and maximum relaxant response to ACh was significantly higher in intact females compared with males and ovariectomized females. Treatment with 17β-oestradiol prevented the reduced vasorelaxant response in ovariectomized females.

4 Immunohistochemical examination for eNOS showed no apparent difference in eNOS protein expression in the endothelium of arteries between intact and ovariectomized females.

5 Since circulating concentrations of oestrogen were essentially low in middle-aged female rats, the present results suggest that subtle changes from a critical concentration of oestrogen at this age may strongly affect the vascular actions of endothelium-derived hyperpolarizing factor without effect on eNOS expression and activity.

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Abbreviations: ACh, acetylcholine; 1-EBIO, 1-ethyl-2-benzimidazolinone; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; LDL, low-density lipoprotein; L-NOARG, N^G-nitro-L-arginine; NO, nitric oxide; PBS, phosphate-buffered saline; PSS, physiological salt solution

Introduction

Premenopausal women have a significantly lower incidence of coronary heart disease than do men of a similar age (Isles *et al*., 1992). This gender difference suggests that female sex hormones can prevent the development of coronary heart disease (Eaker *et al*., 1993). A role for oestrogen in the gender-related cardioprotective effect has been shown by the increased prevalence of coronary heart disease in women after menopause (Barret-Connor, 1997). This is reversible by oestrogen replacement therapy (Stampfer *et al*., 1991). Although the direct mechanisms for this beneficial cardiovascular action of oestrogen are less well understood, accumulating evidence suggests that vascular endothelium is an important target of oestrogen. Oestrogen has been shown to have a profound influence on function and expression of endothelial nitric oxide (NO) synthase (eNOS) (Hayashi *et al*., 1995; Vagnoni *et al*., 1998; Geary *et al*., 2000), but this sex hormone may also play a crucial role in regulation of endothelium-derived hyperpolarizing factor (EDHF). Oestrogen-related changes in the EDHF-mediated responses have been indicated by the report showing that EDHF plays a large role in increased endothelium-dependent relaxation in small mesenteric arteries from pregnant rats (Gerber *et al*., 1998). In addition, our recent work has demonstrated that oestrogen-deficient states of both a long-term (ovariectomy) and a short-term (dioestrous cycle) specifically attenuate endothelium-dependent hyperpolarization and relaxation transduced by EDHF in mesenteric arteries from young (12 week old) female rats (Liu *et al*., 2001).

An understanding of the potential importance of oestrogen for regulating endothelium-derived factors is particularly critical in middle-aged females because this age class is when the number of oestrogen users shows a sharp rise for relief of menopausal symptoms. This study was therefore designed to determine whether endogenous oestrogen plays an important role in the vascular effects of EDHF in middle-aged (44–45 week old) rats. The influence of the female sex hormone on EDHF-mediated hyperpolarization and relaxation was investigated in isolated mesenteric arteries. Arteries were taken from intact male and female rats, as well as from ovariectomized rats with and without 17β-oestradiol replacement.

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Methods

Animal preparation

Age-matched male and female Wistar rats (44–45 week old) were used. Female rats at 40 weeks of age were anaesthetized by an intraperitoneal injection of ketamine (100 mg kg^{-1}) under aseptic condition. Some female rats were ovariectomized by making a small incision in the lower abdomen and removing both ovaries as previously described (Liu *et al*., 2001). Sham-operated (intact) female rats received only laparotomy. Some ovariectomized rats were given 17β-oestradiol (10 μg day^{-1}) subcutaneously through an osmotic pump which was implanted in the back (Liu *et al*., 2001). Each group of rats was caged separately (2$\sim$3 animals per cage), housed under a 12 : 12-h light–dark cycle, and fed *ad libitum*. Female rats were euthanized 4–5 weeks after surgery. Blood samples were collected from the inferior cava and analysed for plasma 17β-oestradiol and lipid profiles. Plasma 17β-oestradiol concentrations were determined by radioimmunoassay, and the analysis of lipid profiles in blood was carried out by use of an automated system. The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee.

Electrophysiological experiments

Rats were killed by exsanguination under anaesthesia with gaseous diethyl ether. The main branch of mesenteric arteries was quickly dissected out and placed in oxygenated physiological salt solution (PSS) at room temperature consisting of (in mM): NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. Arterial segments were opened longitudinally. Care was taken to ensure that the endothelial layer was not damaged during processing of the tissue preparation. The preparation was pinned, intimal side upward, to the bottom of an organ chamber (capacity 3 ml) and superfused at a constant flow rate of 7 ml min^{-1} with warmed (37°C) PSS aerated with 95% O_2 and 5% CO_2. After the preparation had been equilibrated for at least 60 min, glass microelectrodes filled with 3 M KCl (tip resistance 40–80 MΩ) were inserted into the smooth muscle cells from the intimal side. Successful impalements were signalled by sudden negative drop in potential from the baseline (zero potential reference). Following a first drop in voltage, the microelectrode was further advanced into the arterial wall. A sudden return to the original baseline potential signified that a smooth muscle cell had been impaled. Electrical signals were monitored continuously on an oscilloscope (Nihon Kohden, VC-10, Tokyo, Japan) and recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan). After voltages were stable for at least 2 min, the membrane potential response to acetylcholine (ACh) or pinacidil was determined by continuous recording of the membrane potential of a single cell. Further details of the experimental procedure have been described elsewhere (Fukao *et al*., 1999).

Tension measurement experiments

Rats mesenteric arterial rings (3 mm length) were prepared as described above. Each ring was suspended by a pair of stainless steel pins in a water-jacketed bath filled with 6 ml of PSS which was gassed with 95% O_2–5% CO_2 and maintained at 37°C. The rings were stretched to a resting tension of 1 g (point where maximal contraction to 80 mM KCl occurred) and then allowed to equilibrate for at least 60 min. Force generation was monitored by an isometric transducer (Unique Medical, UMTB-1, Tokyo, Japan) and a carrier amplifier (Nihon Kohden, AP-621G). The output of the force transducer was registered on a pen recorder (Rikadenki, R-64, Tokyo, Japan). After the equilibration period, the rings were repeatedly challenged with 80 mM K^+ until contractions reached a constant maximal value. High-K^+ PSS was prepared by substitution of KCl for NaCl on an equimolar basis.

To test the relaxant responses to ACh, ring preparations were contracted with 1–10 μM phenylephrine to increase a tension to 500–750 mg in all experimental groups. When the contraction reached a plateau, the cumulative doses of ACh were applied. Three concentration-response curves for ACh were successively separated by washout of at least 30 min. Preliminary time-control experiments demonstrated no difference among responses without intervention, therefore the first curve of each set was performed as a control. The second curve was performed in the presence of 10 μM indomethacin, and the last curve in the presence of 10 μM indomethacin and 100 μM N^G-nitro-L-arginine (L-NOARG). The incubation period with indomethacin and L-NOARG was at least 15 min. Because incubation with L-NOARG markedly enhanced phenylephrine-induced contractions, the concentration of phenylephrine was decreased to equalize the precontraction level to that in the absence of L-NOARG. We defined the ACh responses obtained in the presence of indomethacin and L-NOARG as EDHF-mediated relaxations, since the remaining relaxations to ACh, i.e., the indomethacin- and L-NOARG-resistant relaxations, were completely abolished by 30 mM K^+ PSS as demonstrated in our previous reports (Sugihara *et al*., 1999; Liu *et al*., 2001). Relaxations were expressed as a percentage of the contraction level induced by phenylephrine.

Immunohistochemical analysis

Immunohistochemical analysis for endothelial NO synthase (eNOS) was performed with the use of a mouse monoclonal antibody against eNOS (Transduction Laboratories, Lexington, KY, U.S.A.). The arteries were immersed in 10% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for at least 24 h. Cross sections of the arteries were embedded in paraffin and cut in slices 5-μm thick. The sections were deparaffinized with xylene and rehydrated with graded alcohol. The specimens were preincubated with methanol containing 0.3% hydrogen peroxidase for 30 min, washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X 100 in PBS for 20 min, washed with PBS, incubated with anti-eNOS antibody diluted in PBS (1 : 250) with horse serum for 60 min, and then washed again with PBS. A biotin-labelled secondary antibody was applied for 30 min, followed by avidin-biotin peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.). The standard peroxidase enzyme substitute was 3,3-diaminobenzine. Negative controls included substitution of the primary antibody with either PBS or irrelevant antibodies.

Statistical analysis

All data are expressed as means ± s.e.mean. Statistical assessment of the data was made by Student's *t*-test or ANOVA supported by Scheffé's multiple comparison test when appropriate. $P<0.05$ was taken as significant.

Drugs

ACh chloride was obtained from Wako Pure Chemical (Osaka, Japan), L-phenylephrine hydrochloride, indomethacin, L-NOARG, apamin, charybdotoxin and 17β-oestradiol from Sigma Chemical (St. Louis, MO, U.S.A.), pinacidil from Shionogi (Osaka, Japan), and 1-ethyl-2-benzimidazolinone (1-EBIO) from Tocris Neuramin (Ballwin, MO, U.S.A.). Indomethacin was prepared in 50 mM Tris, L-NOARG and pinacidil were in 0.2 N HCl, and 1-EBIO was in dimethyl sulphoxide. Other compounds were dissolved in distilled water. Further dilutions to the desired concentrations were made with suitable buffer solution.

Results

Blood chemistry

The concentrations of 17β-oestradiol in the plasma of middle-aged intact female rats were scattered from 3.8 to 33.3 pg/ml. Plasma 17β-oestradiol concentrations in ovariectomized rats were significantly less than the level in intact females. As given in Table 1, oestrogen replacement therapy markedly increased plasma 17β-oestradiol to the levels that were not significantly different from those observed in intact females. Males had significantly higher plasma concentrations of 17β-oestradiol than ovariectomized females ($P<0.001$).

Total serum cholesterol was significantly increased when females were subjected to ovariectomy (Table 1). Also, males and ovariectomized females had significantly higher concentrations of low-density lipoprotein (LDL) choresterol than intact females (Table 1). The increase in LDL cholesterol concentrations in ovariectomized females was significantly improved by oestrogen replacement therapy, but total cholesterol concentrations remained elevated (Table 1). There was no significant difference in serum triglycerides among groups (Table 1).

Electrophysiology

The resting membrane potentials of smooth muscle cells of mesenteric arteries were not different between male and female rats at 44–45 weeks of age (Table 2). Ovariectomy significantly depolarized the resting membrane potential in female arteries. This change in the resting membrane potential was restored by treatment with 17β-oestradiol. The resting membrane potential in either of the arteries from intact and ovariectomized female rats was marginally affected by a combination of 500 nM apamin and 100 nM charybdotoxin: the addition of the two toxins produced a small change in the membrane potential (3 mV depolarization). In young rats (12 weeks of age), the resting membrane potential was not changed by ovariectomy (Table 2).

Representative effects of 1 μM ACh on the membrane potential in mesenteric arteries from intact male and female rats, as well as from ovariectomized rats with and without 17β-oestradiol replacement are illustrated in Figure 1. In tissues with endothelium, ACh hyperpolarized the membrane potential in all groups. Consistent with our previous reports (Fukao *et al*., 1997; Liu *et al*., 2001), neither indomethacin (10 μM) nor L-NOARG (100 μM) affected the hyperpolarizing response to ACh of rat mesenteric arteries, but this was eliminated by high K^+ medium (data not shown). As shown in Figure 2, ACh-induced hyperpolarization was significantly greater in intact females than in males. Ovariectomy caused a marked reduction in ACh-induced hyperpolarization (Figures 1 and 2). Thus, the maximal hyperpolarizing response to ACh in ovariectomized females was much smaller not only than that observed in intact females but also than that in males (Table 2). The striking attenuation of ACh-induced hyperpolarization was significantly prevented by treatment with 17β-oestradiol (Figure 2). The effect of ovariectomy on ACh-induced hyperpolarization was the same as that seen in arteries from young rats, although the ACh responses in arteries from intact middle-aged rats were less than those from intact young rats (Table 2).

In contrast, pinacidil-induced hyperpolarization was unaffected by the gender and deficiency of female sex hormones. The peak amplitude of hyperpolarization induced by 10 μM pinacidil was 22.4 ± 0.5 ($n=5$) in male, 22.6 ± 1.4 ($n=7$) in intact female, 26.3 ± 0.3 ($n=3$) in ovariectomized female and 24.3 ± 1.3 mV ($n=4$) in ovariectomized and 17β-oestradiol-treated female rats.

Hyperpolarization evoked by 1-EBIO, an opener of intermediate-conductance K^+ channels (Devor *et al*., 1996; Jensen *et al*., 1998), was small in arteries from middle-aged female rats. No difference in the hyperpolarizing response to 500 μM 1-EBIO was found between intact and ovariectomized arteries (4.0 ± 0.0 vs 3.7 ± 0.3 mV, $n=3$ for each).

Table 1 Plasma 17β-oestradiol levels and serum lipid profiles in intact male and female rats, as well as ovariectomized female rats with and without 17β-oestradiol

	17β-oestradiol (pg ml^{-1})	*Total cholesterol* (mg dl^{-1})	*LDL cholesterol* (mg dl^{-1})	*Triglyceride* (mg dl^{-1})
Male	4.4 ± 0.5*	88 ± 8	14.3 ± 0.9***	166 ± 19
Female (intact)	12.7 ± 3.3	76 ± 4	6.0 ± 0.2	196 ± 41
Ovariectomy	1.7 ± 0.3*	106 ± 6***	10.8 ± 1.1**	175 ± 31
Ovariectomy + 17β-oestradiol	27.3 ± 9.3#	106 ± 3***	7.9 ± 0.7*#	218 ± 40

Values are means ± s.e.mean ($n=9$). *$P<0.05$, **$P<0.01$, ***$P<0.001$, vs the corresponding values obtained in intact females. #$P<0.05$ vs the values in ovariectomized rats.

Table 2 Resting membrane potentials and maximal hyperpolarizations to ACh in intact male and female rats, as well as ovariectomized female rats with and without 17β-oestradiol

	Middle-aged rats		*Young rats*[a]	
	Resting membrane potential (mV)	*Maximal ACh hyperpolarization* (mV)	*Resting membrane potential* (mV)	*Maximal ACh hyperpolarization* (mV)
Male	-51.6 ± 0.5	7.8 ± 0.4*	-52.4 ± 1.0	15.9 ± 0.5
Female (intact)	-50.9 ± 1.2	11.2 ± 1.4	-52.5 ± 0.6	15.4 ± 0.5
Ovariectomy	-40.3 ± 2.2**	2.5 ± 0.4**	-50.6 ± 0.9	3.5 ± 1.0***
Ovariectomy + 17β-oestradiol	-49.3 ± 2.1#	8.8 ± 0.6###	-53.6 ± 0.7	12.8 ± 1.5###

[a]Source of data: male data from Tomioka *et al*. (1999); female data from Liu *et al*. (2001). Young intact females were in the proestrous to oestrous stage. Values are means$\pm$s.e.mean ($n=4-13$). *$P<0.05$, **$P<0.01$, ***$P<0.001$, vs the corresponding values obtained in intact females at each age. #$P<0.05$, ###$P<0.001$ vs the values in ovariectomized rats at each age.

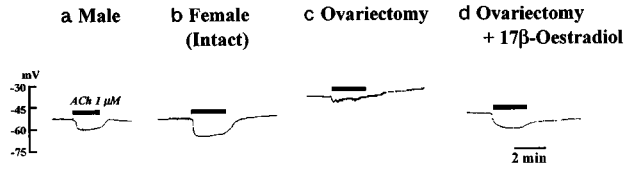

Figure 1 Actual recordings of hyperpolarizations induced by ACh in mesenteric arteries from male (a), intact female (b), ovariectomized (c) and ovariectomized and 17β-oestradiol-treated (d) rats. ACh (1 μM) was applied during the period indicated by horizontal bars.

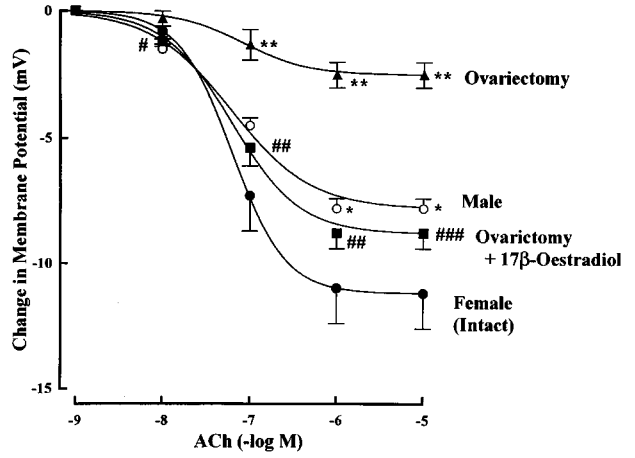

Figure 2 Concentration-response curves for ACh-induced hyperpolarization in mesenteric arteries from male and intact female rats, as well as ovariectomized rats with and without 17β-oestradiol. Values are means$\pm$s.e.mean ($n=4-6$). *$P<0.05$ and **$P<0.01$ vs the corresponding values obtained in intact females. #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ vs the values obtained in ovariectomized rats.

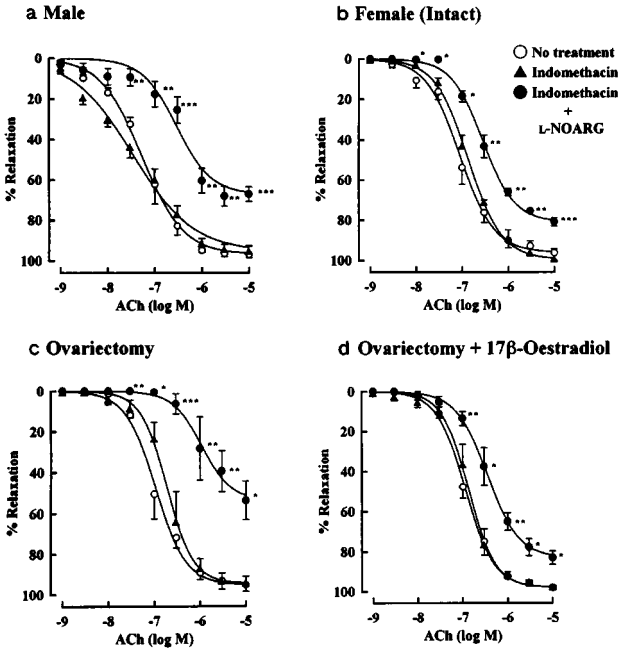

Figure 3 Concentration-response curves for ACh-induced relaxation in the absence, presence of 10 μM indomethacin, and presence of 10 μM indomethacin and 100 μM L-NOARG in mesenteric arteries from male (a), intact female (b), ovariectomized (c) and ovariectomized and 17β-oestradiol-treated (d) rats. Values are means$\pm$s.e.mean ($n=4-6$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs the corresponding values without any treatment.

Endothelium-dependent relaxations

In mesenteric arterial rings precontracted with phenylephrine, ACh caused concentration-dependent relaxations only in rings with endothelium. In arteries from male rats ($n=6$), the concentration-response curve for ACh-induced relaxation was described by an EC_{50} of 76 ± 17 nM and a maximum relaxation of 97 ± 2% (Figure 3a). These values were not statistically different from those obtained in arteries from intact female rats ($EC_{50}=97\pm23$ nM, maximum $=96\pm2$%, $n=5$) (Figure 3b). The endothelium-dependent relaxant response to ACh of female arteries were unaffected by ovariectomy. Thus the concentration-response curves for ACh constructed in ovariectomized (Figure 3c) and in ovariectomized and 17β-oestradiol-treated rat arteries (Figure 3d) were essentially the same as the curve determined in intact female rat arteries (ovariectomy, $EC_{50}=116\pm31$ nM, maximum $=95\pm4$%, $n=4$; ovariectomy and 17β-oestradiol treatment, $EC_{50}=127\pm31$ nM, maximum $=98\pm1$%, $n=5$).

Indomethacin alone did not affect the relaxant responses to ACh of mesenteric arteries from male and female rats. The concentration-response curves for ACh were not significantly altered by the presence of 10 μM indomethacin in any of the four groups (Figure 3).

When both 10 μM indomethacin and 100 μM L-NOARG were given, the concentration-response curve for ACh was significantly shifted to the right and the maximum relaxation

was significantly reduced in all groups (Figure 3). In the presence of indomethacin and L-NOARG, arteries from female rats exhibited a higher sensitivity to ACh compared to arteries from males ($EC_{50}=288\pm63$ nM vs 465 ± 62 nM, $P<0.05$) with significantly greater maximum relaxation ($81\pm2\%$ vs $71\pm4\%$, $P<0.05$). Furthermore, the maximum relaxant response to ACh of arteries was far less after ovariectomy ($59\pm9\%$, $P<0.05$) than the value in intact females, and the EC_{50} value for ACh was much greater in the ovariectomized group (2.1 ± 0.7 μM, $P<0.05$). Treatment with 17β-oestradiol of ovariectomized rats restored the L-NOARG-resistant relaxant response to ACh ($EC_{50}=363\pm77$ nM, $P<0.05$; maximum $=83\pm3\%$, $P<0.05$).

Immunohistochemistry

The immunohistochemical localization of eNOS in mesenteric arteries from intact and ovariectomized female rats is shown in Figure 4. Positive staining for eNOS was observed in the endothelium of the arteries. There was no qualitative difference in eNOS protein expression between the two groups of arteries.

Discussion

The results of the present study clearly indicate that EDHF-mediated hyperpolarization and relaxation of mesenteric arteries are significantly diminished in middle-aged (44–45 week old) female rats when oestrogen levels are severely reduced by ovariectomy. The diminished arterial responses to EDHF were sufficiently improved by 17β-oestradiol replacement therapy during the ovariectomy-induced oestrogen-deficient period. These findings are in accordance with our recent work using young (12 week old) female rats (Liu *et al*., 2001). However, the average value of plasma 17β-oestradiol concentrations in middle-aged females (13 pg ml^{-1}) was much lower than in young females which showed plasma 17β-oestradiol levels of 126 and 38 pg ml^{-1} at the oestrous and dioestrous stage, respectively (Liu *et al*., 2001). Arteries from young female rats at the dioestrous stage have also been found to exhibit less reactivity to EDHF than those at the oestrous stage (Liu *et al*., 2001). The plasma 17β-oestradiol levels estimated in middle-aged female rats used in this study somewhat varied possibly related to physiological fluctuations in the sex steroid hormone during the oestrous cycle, but middle-aged female rats did not exhibit an obvious oestrous cycle, i.e., proestrous, oestrous, and dioestrous, which was confirmed by vaginal smears. Despite the fact that circulating oestrogen is low, further reduction in plasma 17β-oestradiol concentrations occurred following ovariectomy caused a striking decrease in the EDHF-mediated responses in middle-aged female rats. This would suggest that subtle changes from a critical concentration of circulating oestrogen can influence the vascular actions of EDHF.

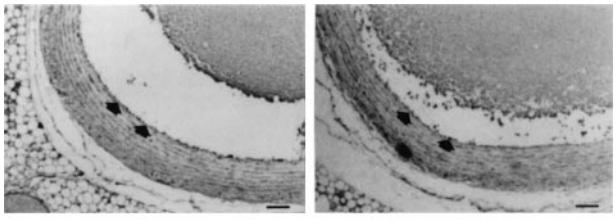

Figure 4 Immunohistochemical staining of mesenteric arteries from intact (a) and ovariectomized (b) female rats. Positive staining (brown) for eNOS was associated with endothelium as shown by arrows. Note that staining appeared to be the same between the two groups of arteries. Scale bar is 50 μm. The same results were obtained with two other vessels in each group.

We have previously found that prostacyclin plays a negligible role in ACh-induced endothelium-dependent relaxations in mesenteric arteries from male rats (Tomioka *et al*., 1999) and from female young rats independent of circulating oestrogen concentrations (Liu *et al*., 2001). In accordance with this, pharmacological analysis using indomethacin, which inhibits the production of prostanoids, revealed that the contribution of prostacyclin to the endothelium-dependent relaxant response to ACh of mesenteric arteries from middle-aged rats appeared to be minimal.

There was no difference in the endothelium-dependent relaxant effect of ACh between mesenteric arteries from male and female rats. Furthermore, ACh-induced endothelium-dependent relaxation was not being altered in arteries from ovariectomized rats. Only when eNOS was blocked by maximally effective concentration of L-NOARG, relaxations to ACh were significantly greater and shifted leftward in mesenteric arteries from female compared to male rats, and were impaired after ovariectomy. These results suggest that the NO-mediated endothelium-dependent relaxations in mesenteric arteries from middle-aged rats are substantially unaffected by sex or oestrogen deficiency. This is consistent with our recent observations using young female rats that the endothelium-dependent relaxation to ACh of mesenteric arteries treated with the combination of apamin and charybdotoxin, which can selectively block the EDHF response (Doughty *et al*., 1999), were unchanged after ovariectomy (Liu *et al*., 2001). Several studies suggest a potential role of oestrogen in regulation of eNOS (Hayashi *et al*., 1995; Vagnoni *et al*., 1998; Pelligrino *et al*., 2000; Geary *et al*., 2000). In contrast, our immunohistochemical analysis showed no apparent difference in the eNOS expression in mesenteric arteries between intact and ovariectomized middle-aged female rats. A possible explanation for this difference is that circulating concentrations of oestrogen are actually low in intact females employed in this study. Furthermore, oestrogen-related changes in eNOS-dependent vasorelaxations may differ by species, ages, and/or anatomic origin of the artery. However, further study is needed to certify no influence of ovariectomy on vascular eNOS expression in middle-aged rats, because this study did not allow us to analyse quantitatively its protein level.

The present study showed that EDHF-mediated hyperpolarization and relaxation were significantly greater in females than males. Interestingly, the plasma concentrations of 17β-oestradiol were higher in male rats than in females after ovariectomy, probably as a result of metabolism of testosterone by aromatase in the adipose tissue. ACh produced greater membrane hyperpolarization of mesenteric arteries in male rats than in ovariectomized female rats. A possible explanation for the gender difference in the EDHF-mediated responses could relate to oestrogen concentrations.

However, it is possible that interactions among plural sex hormones may be important in this gender difference. Hyperpolarization induced by ACh in mesenteric arteries from middle-aged rats was less than those from young rats regardless of gender (see Table 2). Thus, EDHF-mediated hyperpolarizations appear to be reduced with age.

In middle-aged female rats, the resting membrane potentials were markedly decreased after ovariectomy and reversed by 17β-oestradiol. This suggests that the notable reduction in oestrogen concentrations after ovariectomy alters K^+ channels responsible for the resting membrane potential in middle-aged rats. The identity of the target K^+ channels for EDHF has not been unequivocally established, but a combination of apamin, a blocker of the small-conductance Ca^{2+}-activated K^+ channel, and charybdotoxin, a blocker of the large- and intermediate-conductance Ca^{2+}-activated K^+ channel, can completely prevent the hyperpolarizing and relaxant action of EDHF in various vascular tissues (Waldron & Garland, 1994; Corriu *et al*., 1996; Chen & Cheung, 1997), implying that Ca^{2+}-activated K^+ channels on which the two toxins act are involved in the EDHF responses. We cannot rule out the possibility that such K^+ channels for the EDHF responses might be altered in middle-aged females when oestrogen concentrations are severely reduced by ovariectomy. Chataigneau *et al*. (1998) have shown that the amplitude of ACh-induced hyperpolarization is correlated negatively with the absolute values of the resting membrane potential in guinea-pig carotid artery. However, it seems unlikely that the marked depolarized resting membrane potential might have prevented ACh-induced hyperpolarization in ovariectomized arteries, because ovariectomy diminished ACh-induced hyperpolarization without affecting the resting membrane potential in young rats (see Table 2). The present study revealed that inhibition of the Ca^{2+}-activated K^+ channel for EDHF by combined application of apamin and charybdotoxin produced minor changes in the resting membrane potential in both of the arteries from intact and ovariectomized rats. We also observed that hyperpolarization induced by pinacidil, an ATP-sensitive K^+ channel opener, was not substantially affected by ovariectomy. 1-EBIO, an opener of intermediate-conductance Ca^{2+}-activated K^+ channel (Devor *et al*., 1996; Jensen *et al*., 1998), has been reported to elicit endothelium-dependent hyperpolarization of rat hepatic artery, an indication that 1-EBIO might selectively open endothelial K^+ channels (Edwards *et al*., 1999). In the present study, 1-EBIO evoked only small hyperpolarization in mesenteric arteries from middle-aged female rats, and the hyperpolarizing response was unaffected by ovariectomy. Thus, it seems that ovariectomy-induced oestrogen deficiency does not alter the K^+ channel activated by 1-EBIO in mesenteric arteries from middle-aged rats.

In conclusion, the present study demonstrates that endothelium-dependent relaxations of mesenteric arteries from middle-aged rats, which are mediated entirely by NO, are not affected by gender or oestrogen deficiency. However, when the EDHF-mediated component of vasorelaxation is unmasked in the absence of NO, an evident decrease in this component can be detected in males and ovariectomized females compared with intact females. In the light of the fact that circulating concentrations of oestrogen are essentially low in middle-aged female rats, it may be inferred that subtle changes from critical concentrations of the sex hormone at this age can modify EDHF-mediated vascular responses. EDHF acts as a backup mechanism under circumstances where endothelium-derived NO is impaired, and assumes greater importance than NO in resistance beds, possibly including coronary microcirculation. We thus suggest that the importance of oestrogen for regulating EDHF may be relevant to the apparent protection in females against coronary heart disease.

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