British Journal of Pharmacology (2005) 144, 178–189
www.nature.com/bjp

Reciprocal changes in endothelium-derived hyperpolarizing factor- and nitric oxide-system in the mesenteric artery of adult female rats following ovariectomy

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1 To explore the effects of estrogen on arterial functions, we examined endothelium-derived hyperpolarizing factor (EDHF)- and NO-mediated responses in isolated mesenteric arteries of female rats, 4 weeks after sham-operation (CON), ovariectomy (OVX) and OVX plus chronic estrogen treatment (OVX + E_2). Tissue levels of connexins-40, 43 (major components of gap junction), inducible NOS (iNOS), endothelial NOS (eNOS) and eNOS regulator proteins such as calmodulin, heat shock protein 90 (hsp90) and caveolin-1 were also examined using Western blot.

2 In OVX, acetylcholine (ACh)-induced EDHF-mediated relaxation and membrane hyperpolarization of arterial smooth muscles were reduced, whereas ACh-induced NO-mediated relaxation was enhanced, leading to no change in ACh-induced relaxation.

3 In OVX, connexin-40 and 43 were decreased. Tissue levels of eNOS and its positive regulators (calmodulin and hsp90) were unchanged, but that of its negative regulator, caveolin-1, was decreased. The levels of iNOS in mesenteric artery and aorta and plasma levels of NO metabolites and cholesterol were elevated.

4 In OVX, contraction of the artery by phenylephrine was reduced, but augmented by nonspecific inhibitor of NOS to the comparable level as that in CON group. The contraction in OVX group unlike that in CON group was augmented by specific iNOS inhibitor, and the difference between contractions in the presence of nonspecific and specific inhibitor as an index of eNOS activity was increased.

5 In OVX + E_2, all these changes were recovered.

6 In all groups, EDHF-mediated relaxation was suppressed by 18β-glycyrrhetinic acid, an inhibitor of gap junction.

7 These results indicate that estrogen deficiency does not change the diameter of mesenteric artery: it reduces EDHF-mediated relaxation by decreasing gap junction, whereas it augments NO-mediated relaxation *via* an increase in NO release. Increased NO result from increased activity of eNOS subsequent to a decrease in caveolin-1 and from induction of iNOS. However, excessive NO generation with elevated plasma cholesterol would raise a risk for atherosclerosis.
British Journal of Pharmacology (2005) **144,** 178–189. doi:10.1038/sj.bjp.0706091
Published online 10 January 2005

Keywords: Estrogen; ovariectomy; endothelium-derived hyperpolarizing factor; connexin-40; connexin-43; gap junction; NO; eNOS; iNOS; caveolin-1

Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NOS; EREs, estrogen responsive elements; 18β-GA, 18β-glycyrrhetinic acid; hsp90, heat shock protein 90; iNOS, inducible NOS; K_{ATP} channel, ATP-sensitive K^+ channel; LDL, low-density lipoprotein; L-NOARG, N^G-nitro-L-arginine; PSS, physiological salt solution; TBS, Tris-buffered saline

Introduction

The incidence of cardiovascular diseases in premenopausal women is significantly lower than that of men of a similar age (Barrett-Connor, 1997), but the incidence in postmenopausal women becomes comparable to that of men (Lerner & Kannel, 1986). Hormone replacement therapy with estrogen in postmenopausal women reduces the risk of vascular events (Grodstein *et al*., 1997). Based on these epidemiological data, it is generally accepted that estrogen plays an essential role in preventing the development of cardiovascular diseases (Bush *et al*., 1987; Stampfer *et al*., 1991), although its mechanism is not fully understood.

In search for the mechanism of the vasoprotective action of estrogen, researchers have usually used administration of estrogen to ovariectomized female animals to mimic estrogen replacement therapy in the postmenopausal women. They analyzed mainly responses mediated by endothelium-derived vasorelaxing factors such as nitric oxide (NO), prostacyclin

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Published online 10 January 2005

and endothelium-derived hyperpolarizing factor (EDHF). There are three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). These isozymes play an important role in physiology and pathophysiology of vascular walls. eNOS and nNOS are constitutively expressed in the vascular endothelial cells and surrounding nerve, respectively, whereas iNOS expression is induced in endothelial and smooth muscle cells after exposure to inflammatory stimuli. Under normal conditions, NO is produced by activation of eNOS and plays critical roles in physiology of vessels such as regulation of vascular tone, inhibition of platelet aggregation (antithrombotic action) and inhibition of proliferation of vascular smooth muscle cells (antiatherosclerotic action) (Schwarzacher *et al*., 1997; Le Tourneau *et al*., 1999). On the other hand, excessive NO generated by iNOS could be toxic through reaction with superoxide anion to yield peroxynitrite, a strong oxidant (Patel *et al*., 1999). Genetic deficiency of iNOS is reported to lower plasma lipid peroxides and reduce atherosclerosis in apolipoprotein E-knockout mice (Detmers *et al*., 2000; Kuhlencordt *et al*., 2001). Thus, the iNOS-mediated increase in NO formation may be critical in the genesis and development of atherosclerosis.

As for NO-mediated relaxation responses, experiments were performed mainly in isolated rat aorta, but the results are divergent. That is, some researchers reported a slight increase in NO-mediated relaxation and the level of eNOS protein following chronic estrogen treatment in ovariectomized female animals (Andersen *et al*., 1999; Gonzales *et al*., 2001). However, others reported no change in these parameters (Lerner & Kannel, 1986; Tamura *et al*., 2000), and still others a decrease (Bolego *et al*., 1997). As for EDHF system, a few researchers reported that EDHF-mediated relaxation responses in mesenteric arteries were reduced following ovariectomy, and the reduction was corrected by estrogen replacement (Liu *et al*., 2001). Gap junctions are considered a molecular entity mediating EDHF responses in rat mesenteric arteries (Sandow & Hill, 2000; Goto *et al*., 2002), and one group implicated a decrease in the level of connexin-43 protein, the main component of gap junctions in the artery, as a possible mechanism for the decreased EDHF-mediated response (Liu *et al*., 2002). However, this report focuses on only connexin-43 of the connexin family; furthermore, it is based on histological findings and lacks quantitative analysis.

Characteristically, most of these studies were limited to one species of vasorelaxing factors in one typical tissue. That is, NO-mediated relaxation was examined in a large conducting artery such as aorta, while EDHF-mediated relaxation was examined in a small resistance artery such as mesenteric artery. It is generally known that small resistance arteries rather than large conducting arteries are physiologically important for regulation of vascular tone in the whole human body. In addition, endothelial cells in some of small resistance arteries are known to release both NO and EDHF, unlike those of large conducting arteries releasing mainly NO (Shimokawa *et al*., 1996; Tomioka *et al*., 1999). Therefore, we asked what types of changes occur for the NO system in such small arteries if the EDHF system is changed and *vice versa*. In this context, it is important to analyze the changes in both NO- and EDHF-mediated responses at the same time and also their molecular mechanisms in a small artery, that is, mesenteric artery, to get insights into antiatherosclerotic actions of estrogen.

For these purposes, we examined the effect of ovariectomy alone and in combination with chronic estrogen treatment on NO- and EDHF-mediated responses in mesenteric arteries of middle-aged female rats. In addition, to clarify the mechanisms for the changes in these responses, we quantitatively analyzed the changes in protein levels of connexin-37, 40, 43 (the main components of gap junctions mediating EDHF responses), iNOS and eNOS together with its regulator proteins such as calmodulin, heat shock protein 90 (hsp90) and caveolin-1.

Methods

Experimental animals

Female Wistar rats (40 weeks old) were used in these experiments to mimic the human arteries in menopause. All animals were housed in a temperature-controlled room with 12-h light/dark cycles and given standard laboratory rat chow and water *ad libitum*. All experiments were carried out according to the Guidelines for Hokkaido University School of Medicine Animal Care and Use Committee. Rats were randomly divided into three groups; sham-operated (CON), ovariectomized (OVX) and ovariectomized plus chronic estrogen treated ($OVX + E_2$) group. For ovariectomy, the ovaries on both sides were removed under pentobarbital anesthesia ($50\,mg\,kg^{-1}$, i.p.). In the CON group, sham operation was performed. For $OVX + E_2$ group, osmotic pump (Alza Corporation, 2ML4, Palo Alto, CA, U.S.A.) was implanted immediately after ovariectomy, and 17β-estradiol was administrated *via* the pump at a physiological dose of $3\,\mu g\,day^{-1}$. After completion of surgery, the animals were fed for 4 weeks.

Measurement of plasma 17β-estradiol, cholesterol, nitrate plus nitrite and blood pressure

Under pentobarbital anesthesia, 4 ml blood was collected from inferior vena cava with 5-ml syringe containing 0.1 ml heparin ($1000\,U\,ml^{-1}$). The blood was centrifuged at $3000 \times g$ for 5 min, and the plasma was collected to be stored at $-80^{\circ}C$. Plasma 17β-estradiol concentration was measured by radioimmunoassay, and plasma total cholesterol and low-density lipoprotein (LDL)-cholesterol were measured with an automated system. To evaluate plasma level of NO, the sum of plasma concentration of nitrate and nitrite was measured by high-performance liquid chromatography. Blood pressure was measured with tail cuff method (Ueda, UR-500, Tokyo, Japan).

Membrane potential recording

The rats were anesthetized with diethyl ether, and the main branches of the superior mesenteric arteries were removed carefully and cleaned of surrounding fat and connective tissue in oxygenated physiological salt solution (PSS; constituents 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) at room temperature. The vessels were cut into 3-mm rings and opened longitudinally. Where indicated, the endothelial cells were removed by gently rubbing the intimal surface of the blood vessel with moistened cotton balls. The tissue specimens were carefully pinned to the

rubber bed fixed at the bottom of the chamber and superfused with PSS at 37°C aerated with 95% O_2 and 5% CO_2 (pH 7.4) at a rate of 7 ml min^{-1}; under this condition, they were allowed to equilibrate 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. After a stable recording of membrane potential for at least 2 min was obtained, changes in membrane potential were recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan).

Measurement of relaxation and contraction responses

The rat mesenteric arterial rings with endothelium were prepared as described above. The rings were mounted by a pair of stainless steel pins in a water-jacketed chamber filled with 6 ml of PSS. The solution in the bath was gassed with 95% O_2 and 5% CO_2 (pH 7.4) and its temperature was maintained at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was obtained and then allowed to equilibrate for at least 60 min. Force generation was monitored using an isometric transducer (Unique Medical, UMTB-1, Tokyo, Japan) and a carrier amplifier (Nihon Kohden, AP-621G, Tokyo, Japan). The output of the force transducer was registered on a pen recorder (Rikadenki, R-64, Tokyo, Japan). After the equilibration period, the rings were exposed several times to high-K^+ solution (80 mM $[K^+]_o$) until reproducible contractile responses were obtained. High-K^+ solution was prepared by substituting NaCl with equimolar KCl. The ring specimens were precontracted with 1–10 μM phenylephrine to produce 500–750 mg tension and acetylcholine (ACh)-induced relaxation was determined. In the experiment using 100 μM N^G-nitro-L-arginine (L-NOARG), lower concentrations of phenylephrine (0.1–1 μM) were used to match the precontraction. After the contraction had reached a plateau, ACh was applied in a cumulative manner. Indomethacin (10 μM), L-NOARG (100 μM), apamin (500 nM), charybdotoxin (100 nM) or 18β-glycyrrhetinic acid (18β-GA, 30 μM) was added to the bath solution 15 min before addition of phenylephrine. We regarded the ACh responses in the presence of indomethacin and L-NOARG as EDHF-mediated relaxation and those in the presence of indomethacin, apamin and charybdotoxin as NO-mediated relaxation. Relaxations were expressed as a percentage of the contraction induced by phenylephrine. Phenylephrine-induced contraction was measured in the absence or presence of L-NOARG (100 μM) or aminoguanidine (100 μM) in mesenteric arteries from CON, OVX and OVX + E_2 groups.

RT–PCR for connexins

Total RNA was isolated from rat mesenteric arteries using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). The total RNA was reverse-transcribed to cDNA with random hexamers, using SuperScript™ First-Strand Synthesis System for RT–PCR kit (Invitrogen, Carlsbad, CA, U.S.A.). PCR amplifications using the cDNAs as template were run in 50-μl reaction volumes containing 2.5 mM $MgCl_2$, 200 μM dNTP, 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.) and using 0.4 μM specific primers as follows: connexin-37 (forward, 5′-AAC GGT GCT CTT CAT CTT CC; reverse, 5′-ATG GGG AGG TAG AAG AAA AC; product length, 729 bp), connexin-40 (forward, 5′-TGC CAA AAT GTC TGC TAC GA; reverse, 5′-TTC TCA TCT GGG CTG TTC TT; product length, 647 bp), connexin-43 (forward, 5′-GAG GGA AGG TGT GGC TGT CA; reverse, 5′-CGA GAG ACA CCA AGG ACA CC; product length, 603 bp). PCR was initiated at 94°C for 5 min followed by 35 cycles consisting of 30 s at 94°C, 30 s at 57.3°C (connexin-43), 58.2°C (connexin-40), 58.4°C (connexin-37), and 60 s at 72°C, with the final cycle extended to 5 min at 72°C. Amplified products were electrophoresed on a 1.0% agarose gel and stained with ethidium bromide.

Western blot analysis

The arteries were excised, cleaned and rapidly frozen with liquid nitrogen. The tissues were homogenized with a Kinematic Polytron blender in 50 mM Tris–HCl buffer (pH 7.4) supplemented with Complete™ tablet (protease inhibitor cocktail tablets, Boehringer Mannheim GmbH, Mannheim, Germany). Homogenates were centrifuged at $600 \times g$ for 10 min. The supernatants were collected and again centrifuged at $8000 \times g$ for 10 min. The supernatants were collected and their protein concentrations were determined with DC Protein Assay Reagent kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and the final protein concentration was adjusted to 2 $\mu g\,\mu l^{-1}$. The protein samples (20 μg each lane) were mixed with 10 μl of SDS–PAGE sample buffer and loaded onto 7.5% (for eNOS, iNOS and hsp90), 12.5% (for connexin-43) and 15% (caveolin-1 and calmodulin) polyacrylamide gels. After electrophoresis, the proteins were transferred onto PVDF membranes (Bio-Rad Laboratories) for 4 h at a constant current of 250 mA. The membranes were blocked by Tris-buffered saline (TBS; Tris 50 mM, NaCl 100 mM, adjusted to pH 7.5 by HCl) containing 0.1% Tween 20 plus 5% nonfat dry milk for 60 min at room temperature and subsequently incubated with corresponding antibodies for 2 h (4 h with iNOS antibody) at room temperature. After the incubation, the membranes were washed three times for 10 min each with TBS containing 0.1% Tween 20 and then incubated with secondary anti-rabbit or anti-mouse IgG conjugated to peroxidase labeled-dextran polymer, EnVision + ™ (Dako corporation, Carpinteria, CA, U.S.A.) at a 1 : 100 dilution for 60 min at room temperature. After washing the membranes, each specific protein was detected with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), the membranes were exposed to X-ray film, and the result was quantitatively analyzed using the Micro-Computer Image Device (MCID, Imaging Research Inc., Canada). Each sample was corrected from the mesenteric artery of CON, OVX and OVX + E_2 group, and six data (from 6 rats) were used for statistical analysis.

Antibodies and chemicals

Antibodies were purchased from the following sources: rabbit anti-rat connexin-37 polyclonal antibody (1 : 1000 dilution; Alpha Diagnostic, San Antonio, TX, U.S.A.), rabbit anti-mouse connexin-40 polyclonal antibody (1 : 1000 dilution; Chemicon, Temecula, CA, U.S.A.), rabbit anti-rat connexin-43 polyclonal antibody (1 : 1000 dilution; Zymed, San Francisco, CA, U.S.A.), mouse anti-eNOS monoclonal IgG_1 (1 : 1000 dilution; Transduction Lab., Lexington, KY, U.S.A.), rabbit anti-rat iNOS polyclonal antibody (1 : 500

dilution; Transduction Lab., Lexington, KY, U.S.A.), mouse anti-calmodulin monoclonal IgG_1 (1 : 1000 dilution; Upstate Biotechnology, Lake Antonio, NY, U.S.A.), mouse anti-hsp90 monoclonal IgG_1 (1 : 1000 dilution; Transduction Lab., Lexington, KY, U.S.A.) and mouse anti-caveolin-1 monoclonal IgM (1 : 500 dilution; Transduction Lab., Lexington, KY, U.S.A.). Other drugs were from Sigma Chemical (St Louis, MO, U.S.A.).

Statistical analysis

Values were expressed as means ± s.e. Concentration–response curves were analyzed by two-way ANOVA followed by Scheffé's *post hoc* test for multiple comparisons. Other variables were analyzed by a paired Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

Results

Characteristics of experimental animals

Table 1 gives the values for body weight, uterus weight, the ratio of uterus weight to body weight, mean blood pressure, heart rate, plasma levels of 17β-estradiol and nitrate plus nitrite, and plasma cholesterol profile in CON, OVX and OVX + E_2. Ovariectomy increased the body weight, whereas it decreased the uterine weight, resulting in the reduced ratio of uterus weight to body weight. Mean blood pressure and heart rate were not different among the groups. Plasma levels of 17β-estradiol were markedly lower in the OVX than those in the CON, but those of total cholesterol, LDL-cholesterol and NO metabolites such as nitrate plus nitrite were higher. Chronic estrogen treatment reversed all of these changes to the control levels.

Relaxing responses to ACh

Relaxation by ACh of the mesenteric arterial ring specimens with endothelium precontracted with phenylephrine was unaffected by OVX or OVX + E_2 (Figure 1a). Relaxation by ACh in CON group was not affected by indomethacin (10 μM), but the response was partly suppressed by indomethacin in combination with either apamin (500 nM) plus charybdotoxin (100 nM) or L-NOARG (100 μM) (compare the values of CON between a–d in Figure 1): it was completely suppressed by a combination of indomethacin (10 μM), apamin (500 nM) plus charybdotoxin (100 nM) and L-NOARG (100 μM) (data not shown). These data strongly indicate that EDHF and NO play a major role in the ACh-induced endothelium-dependent relaxation in rat mesenteric arteries, whereas prostacyclin has a minimal role.

In the presence of indomethacin, the responses to ACh were unaffected by either OVX or OVX + E_2 (Figure 1b). In the presence of apamin and charybdotoxin in addition to indomethacin, relaxation by ACh was enhanced by OVX, but it was recovered by chronic estrogen treatment (Figure 1c).

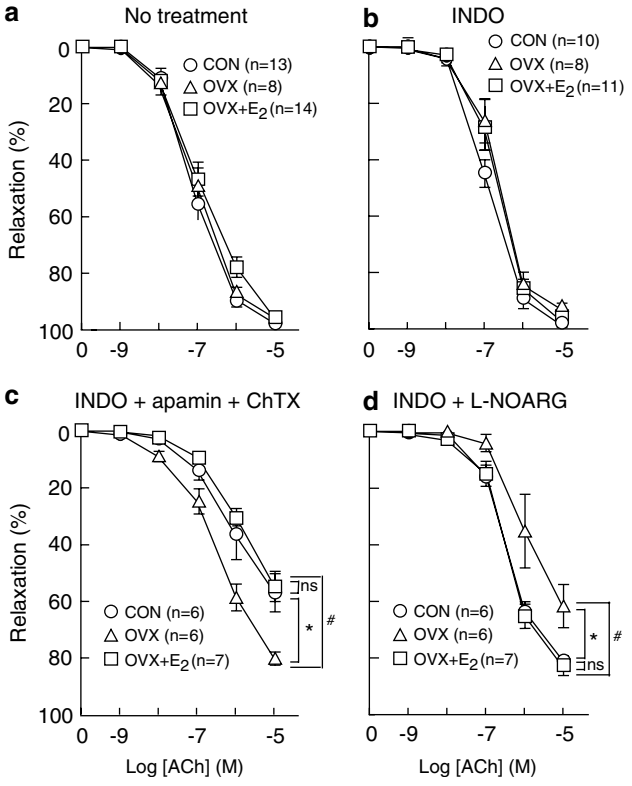

Figure 1 Concentration–response curves for acetylcholine (ACh)-induced relaxation in the mesenteric arteries from control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated (OVX + E_2) group in the absence (a) or presence of drugs : (b) 10 μM indomethacin (INDO), (c) 10 μM INDO in combination with 500 nM apamin plus 100 nM charybdotoxin (ChTX), (d) 10 μM INDO in combination with 100 μM N^G-nitro-L-arginine (L-NOARG). Values are means ± s.e. $^*P<0.05$ CON vs OVX; $^{\#}P<0.05$ OVX vs OVX + E_2. Numbers of animals are shown in the figure.

Table 1 Body weight, uterus weight, blood pressure, heart rate, plasma 17β-estradiol, lipid profile and nitrate plus nitrite levels in control, ovariectomized and ovariectomized plus estrogen treated rats

	CON	*OVX*	*OVX + E_2*
Body weight (g)	220.1 ± 4.2 ($n=13$)	250.0 ± 6.8** ($n=7$)	218.0 ± 4.0 ($n=15$)
Uterus weight (mg)	459.5 ± 23 ($n=13$)	120.7 ± 10*** ($n=7$)	416.9 ± 11 ($n=15$)
Uterus weight/body weight	2.09 ± 0.1 ($n=13$)	0.48 ± 0.1*** ($n=7$)	1.92 ± 0.1 ($n=15$)
Mean BP (mmHg)	100.3 ± 4.6 ($n=4$)	99.8 ± 3.8 ($n=4$)	94.8 ± 4.5 ($n=4$)
HR (bpm)	356.8 ± 7.1 ($n=4$)	357.0 ± 14.2 ($n=4$)	335.3 ± 8.7 ($n=4$)
17β-estradiol (pg ml^{-1})	19.0 ± 2.9 ($n=17$)	5.2 ± 0.5*** ($n=16$)	20.2 ± 2.1 ($n=17$)
Total cholesterol (mg dl^{-1})	89.5 ± 5.0 ($n=11$)	116.1 ± 9.6* ($n=10$)	99.9 ± 7.3 ($n=11$)
LDL-cholesterol (mg dl^{-1})	8.0 ± 0.6 ($n=11$)	17.5 ± 1.1*** ($n=10$)	8.9 ± 0.7 ($n=11$)
Nitrate plus nitrite (μM)	12.9 ± 1.3 ($n=10$)	20.1 ± 3.7* ($n=7$)	13.8 ± 1.0 ($n=10$)

Values are means ± s.e.
$^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs respective control values.

In contrast, in the presence of L-NOARG in addition to indomethacin, relaxation by ACh was reduced by ovariectomy, but it was again recovered by chronic estrogen treatment (Figure 1d). These results indicate that NO-mediated relaxation is enhanced by ovariectomy, whereas EDHF-mediated relaxation is reduced, and that chronic treatment with estrogen recovers all of these changes.

To examine whether the changes in relaxation response to ACh of the arteries result from changes in relaxation mechanisms in vascular smooth muscles by themselves, we compared the effects of direct smooth muscle relaxants such as sodium nitroprusside (SNP) or pinacidil, an ATP-sensitive K^+ (K_{ATP}) channel opener (Brayden, 2002) among three groups. Relaxation to SNP or pinacidil of the arteries precontracted with phenylephrine was unaffected by OVX or OVX + E_2 (open circles in Figure 3b and c). This result indicates that the relaxation mechanisms in vascular smooth muscles are unchanged and that changes in ACh-induced relaxation result from changes in biosynthesis and/or release and/or transmission of EDHF and NO.

Membrane hyperpolarization by ACh

The average resting membrane potentials of smooth muscle cells in rat mesenteric artery measured by microelectrodes were not different from each other among the CON (-51.5 ± 0.4 mV, $n=24$), OVX (-48.7 ± 0.6 mV, $n=30$) and OVX + E_2 (-51.6 ± 0.6 mV, $n=25$). ACh elicited membrane hyperpolarization in only arteries with intact endothelium; the response was not affected by treatment with either L-NOARG or indomethacin, but it was abolished by combined application of apamin and charybdotoxin as reported previously (Fukao *et al*, 1997b; Liu *et al*., 2001).

ACh induced membrane hyperpolarization in CON group in a concentration-dependent manner and its effect reached the maximum at $\geqslant 1\,\mu$M (Figure 2a and b). The hyperpolarizing response to ACh in OVX group was decreased in comparison with that in the CON group, but it was recovered by chronic estrogen treatment (Figure 2a and b).

We also examined the effect of OVX or OVX + E_2 on endothelium-independent hyperpolarization induced by pinacidil, which hyperpolarizes smooth muscle cell membrane by directly activating K_{ATP} channels in these cells. Notably, pinacidil-induced endothelium-independent hyperpolarization was not different among the groups. The peak amplitude of hyperpolarization induced by 1 μM pinacidil was 12.8 ± 0.5 ($n=5$) in CON group, 14.4 ± 0.8 ($n=4$) in OVX group and 13.0 ± 0.6 mV ($n=5$) in OVX + E_2 group; hyperpolarization by 10 μM pinacidil was 21.2 ± 0.7 ($n=6$) in CON group, 22.5 ± 1.2 ($n=4$) in OVX group and 21.3 ± 0.7 in OVX + E_2 group ($n=5$).

Involvement of gap junctions in EDHF-mediated relaxation

EDHF may not be a molecule but transmission of membrane hyperpolarization from endothelial cells to vascular smooth muscle cells through myoendothelial gap junctions (Chaytor *et al*., 1998; Edwards *et al*., 1999; Goto *et al*., 2002). 18β-GA is reported to inhibit the gap junctional communication (Yamamoto *et al*., 1998). To clarify the involvement of gap junctions in the EDHF-mediated relaxation in rat mesenteric artery, we

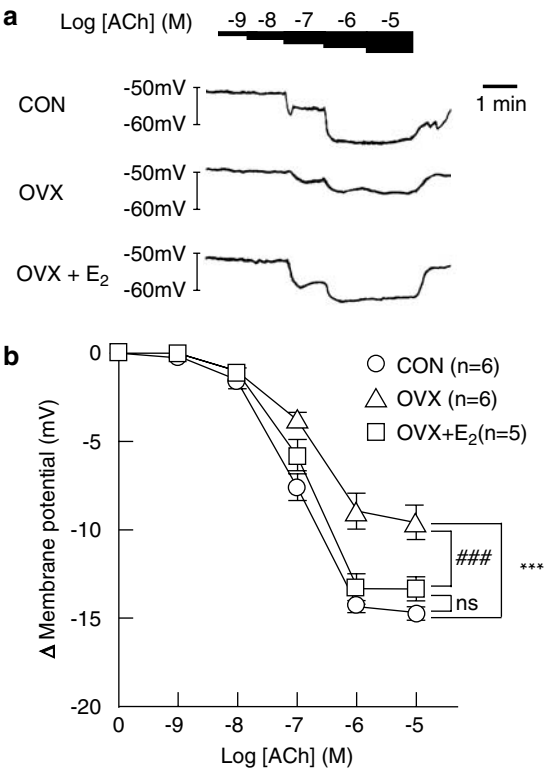

Figure 2 Acetylcholine (ACh)-induced endothelium-dependent hyperpolarization of membrane potentials of mesenteric arterial smooth muscle cells in CON, OVX and OVX + E_2 group. (a) Actual recordings of membrane potentials. Increasing concentrations of ACh (1 nM–10 μM) were applied during the period indicated by horizontal bars. (b) Concentration–response curves for ACh-induced hyperpolarization in CON, OVX and OVX + E_2 group. Values are means ± s.e. $^{***}P<0.001$ CON vs OVX; $^{\#\#\#}P<0.001$ OVX vs OVX + E_2. Numbers of animals are shown in the figure.

attempted to use this compound. However, some researchers reported that high concentrations (100 μM) of 18β-GA are reported to exert nonspecific effects (Santicioli & Maggi, 2000). We also regarded that 18β-GA (100 μM) reduce the arterial contraction by phenylephrine (data not shown). Therefore, we decided to use lower concentrations ($\leqslant 30\,\mu$M) of 18β-GA and examined whether such concentrations of the drug had nonspecific effects. Firstly, 18β-GA at 30 μM was found to exert no effect on the resting tension and phenylephrine-induced contraction in rat mesenteric artery (Figure 3a). Secondly, 18β-GA at 30 μM had no significant effect on relaxation responses of rat mesenteric artery induced by SNP and pinacidil in CON, OVX and OVX + E_2 groups (Figure 3b and c). Based on these data, we thought that 18β-GA (30 μM) had almost no nonspecific actions, in terms of relaxation and contraction of vascular smooth muscles.

Notably, this concentration of 18β-GA almost completely inhibited the ACh-induced relaxation in the presence of indomethacin and L-NOARG in CON, OVX and OVX + E_2 group (Figure 4), suggesting that the relaxation responses are mediated by gap junctions.

Downregulation of gap junctions following ovariectomy

Gap junction is a membrane pore-forming protein complex connecting two adjacent cells, and it is composed mainly of

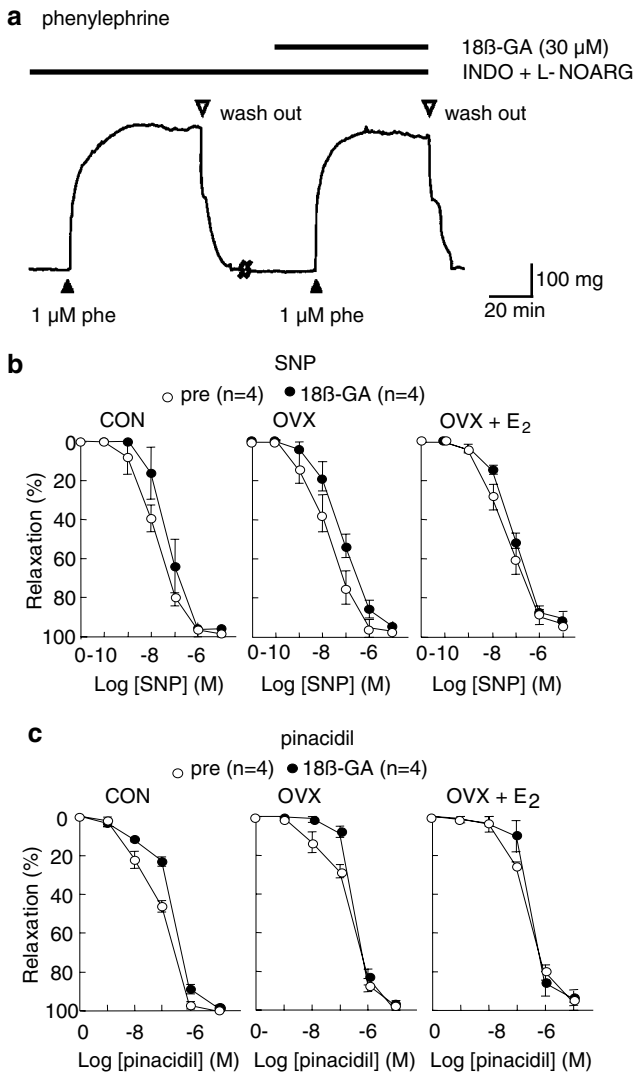

Figure 3 Effects of 18β-glycyrrhetinic acid (18β-GA) on basal and phenylephrine-induced tension (a) and on sodium nitroprusside (SNP) (b) and pinacidil (c)-induced relaxation in rat mesenteric arteries. (a) Actual recordings showing the effects of 18β-GA. Experiments were performed in the continuous presence of 10 μM INDO plus100 μM L-NOARG, and 1 μM phenylephrine and 30 μM 18β-GA were added at the indicated time. (b, c) Concentration–response curves of SNP (b) and pinacidil (c) in the control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated (OVX + E_2) group in the absence (pre) or presence (18β-GA) of 30 μM 18β-GA. Values are means ± s.e. Numbers of animals are shown in the figure.

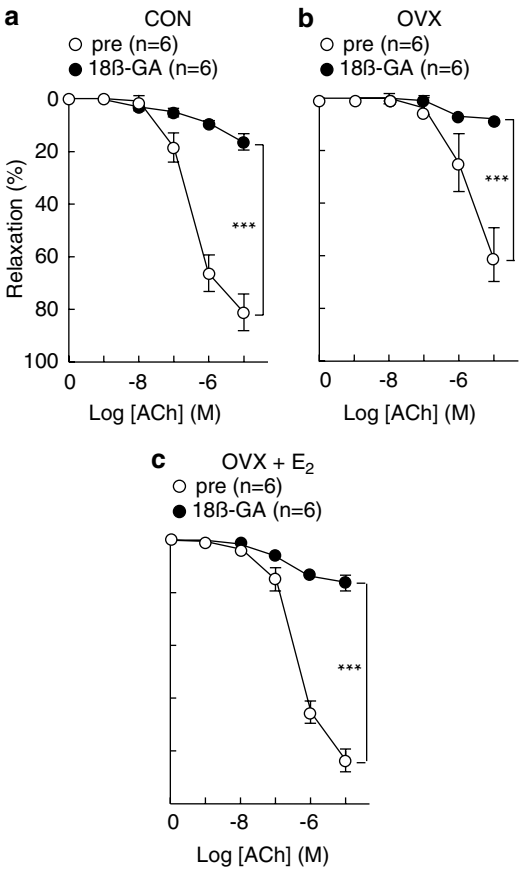

Figure 4 Effects of 18β-glycyrrhetinic acid (18β-GA) on EDHF-mediated relaxation in rat mesenteric arteries. Concentration–response curves of ACh-induced relaxation in the presence of 10 μM indomethacin (INDO) and 100 μM N^G-nitro-L-arginine (L-NOARG) in the control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated (OVX + E_2) group were evaluated in the absence (pre) or presence (18β-GA) of 30 μM 18β-GA. Values are means ± s.e. ***$P<0.001$ vs pre. Numbers of animals are shown in the figure.

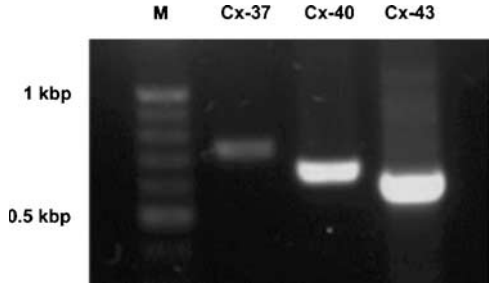

Figure 5 RT–PCR analysis for expression of mRNA of connexin-37, 40 and 43 in rat mesenteric arteries. Amplified products by PCR were separated on 1.0% agarose gel and were identified by ethidium bromide. M, 100-base pair ladder; Cx-37, connexin-37; Cx-40, connexin-40; Cx-43, connexin-43.

connexins (Kumar & Gilula, 1996; Yeager *et al*., 1998). Connexin-37, 40 and 43 were reported to exist in most of arteries (Hill *et al*., 2001), and relative abundance of each connexin is different depending on the arteries. In rat mesenteric artery, the existence of connexin-37, 40 and 43 have been reported using immunohistochemistry (Gustafsson *et al*., 2003). To confirm these connexins are expressed in rat mesenteric artery, we first performed RT–PCR using mRNA prepared from the artery. As shown in Figure 5, bands corresponding to the expected sizes of connexin-37, 40 and 43 were detected in the artery. These PCR products were sequenced and identified as connexin-37, 40 and 43, respectively. We also attempted to confirm the presence of these three types of connexin proteins using Western blot. We could detect connexin-40 and 43 in the artery, but not connexin-37, probably because of a low level of connexin-37 protein.

Changes in protein level of connexin-43 and 40 in the mesenteric artery following OVX and OVX + E_2 were determined using Western blot (Figure 6). The protein level of connexin-43 in OVX group was reduced to about 50% of

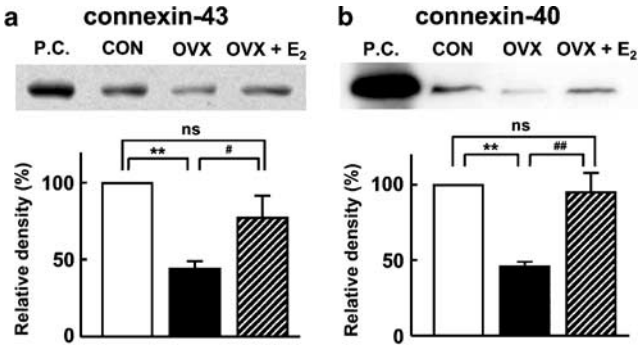

Figure 6 Western blot analysis of the expression of connexin-43 (a) and connexin-40 (b) protein in mesenteric arteries of the control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated ($OVX + E_2$) group. An upper panel represents typical electrophoretic data, and a lower bar graph represents the relative density of the immunostained band for connexins. The relative density was expressed as percentages of the value in CON group. P.C. indicates the positive control. Values are means ± s.e. of six rats. $^{**}P<0.01$ CON vs OVX; $^{\#}P<0.05$ OVX vs $OVX + E_2$; $^{\#\#}P<0.01$ OVX vs $OVX + E_2$.

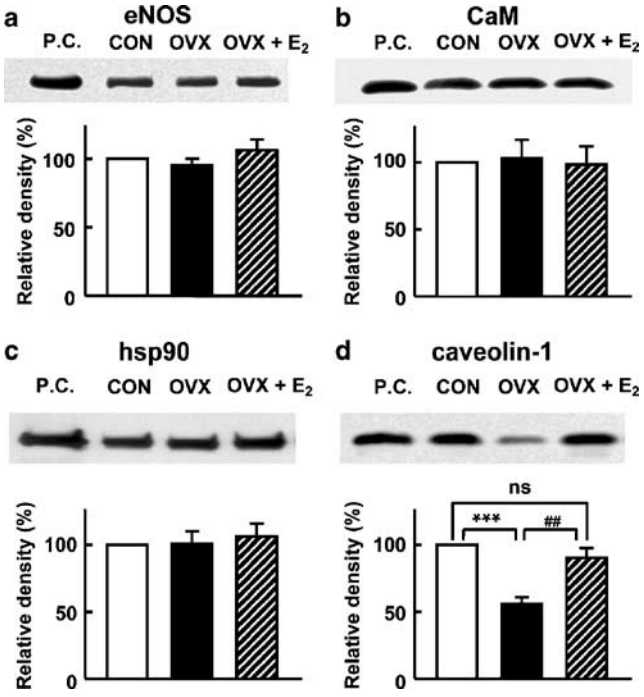

Figure 7 Western blot analysis of eNOS (a) and its regulators, calmodulin (CaM) (b), hsp90 (c), and caveolin-1 (d) proteins in the mesenteric arteries of the control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated ($OVX + E_2$) group. Upper panels represent typical electrophoretic data, and lower bar graphs represent the relative density of the immunostained band. The relative density was expressed as percentages of the value in CON group. P.C. indicates the positive control. Values are means ± s.e. of six rats. $^{***}P<0.001$ CON vs OVX; $^{\#\#}P<0.01$ OVX vs $OVX + E_2$.

CON group, but it was recovered following chronic estrogen treatment (Figure 6a). The protein level of connexin-40 showed essentially similar changes as that of connexin-43 following OVX and $OVX + E_2$ (Figure 6b).

Protein levels of eNOS and related proteins such as calmodulin, hsp90 and caveolin-1

To explore the mechanism for the enhancement of ACh-induced NO-mediated vasorelaxation following OVX, we examined the protein levels of eNOS using Western blot. The protein level of eNOS was unchanged following either OVX or $OVX + E_2$ (Figure 7a).

It has recently become known that the activity of eNOS is regulated by several associated proteins such as calmodulin, hsp90 and caveolin-1 (Fulton *et al*., 2001). Calmodulin and hsp90 stimulate the catalytic activity of eNOS, whereas caveolin-1 inhibits it (Fulton *et al*., 2001). Therefore, we wondered whether levels of these regulatory proteins were altered following OVX, leading to a change in eNOS activity. As shown in Figure 7b and c, the protein levels of calmodulin and hsp90 were unchanged following OVX and $OVX + E_2$. In contrast, the protein level of caveolin-1 was decreased following OVX, but it was recovered by chronic estrogen treatment (Figure 7d).

Protein levels of iNOS

In the present study, plasma levels of NO metabolites such as nitrate and nitrite were elevated following OVX (Table 1). As it is generally accepted that plasma NO metabolites are mainly derived from NO produced by iNOS, we determined to examine protein levels of iNOS in mesenteric artery and aorta.

The protein signal for iNOS in rat mesenteric artery was undetectable using the same Western blot protocol as that for eNOS. To increase the sensitivity of Western blot for iNOS protein, we varied the concentration of the first specific antibody against iNOS and the incubation time with the iNOS antibody (Figure 8a). With the incubation time fixed at 1 h, an elevation of the concentration of iNOS antibody from 1 : 1000 to 1 : 500 dilution did not visualize the iNOS. However, when the incubation time was prolonged from 1 to 2 h with the concentration of iNOS antibody fixed at 1 : 500, the iNOS became faintly visible: it was clearly visualized with the incubation time of 4 h. Therefore, in the following experiment, we used the incubation time of 4 h and the concentration of iNOS antibody of 1 : 500 dilution.

Using this protocol, we could detect the protein of iNOS even in rat mesenteric artery as well as rat aorta of the CON group (Figure 8b). The protein level of iNOS in mesenteric artery and aorta was elevated to about 150% following OVX, and it was recovered to the control level by chronic estrogen treatment.

Effects of L-NOARG and aminoguanidine on phenylephrine-induced contraction

To further analyze the physiological consequences of OVX-induced changes in regulator proteins of eNOS (caveolin-1) and iNOS protein, we examined the effects of L-NOARG (nonspecific NOS inhibitor) and aminoguanidine (iNOS-specific inhibitor) on phenylephrine-induced contraction of mesenteric arteries from CON, OVX and $OVX + E_2$ group.

In CON group, phenylephrine-induced contraction was enhanced by L-NOARG, in comparison with that in the absence of the drug (Figure 9a). The contraction tended to be increased by aminoguanidine, but the change did not reach a statistical significance (Figure 9a).

Following OVX, the phenylephrine-induced contraction by itself was reduced to about half of that in the CON group

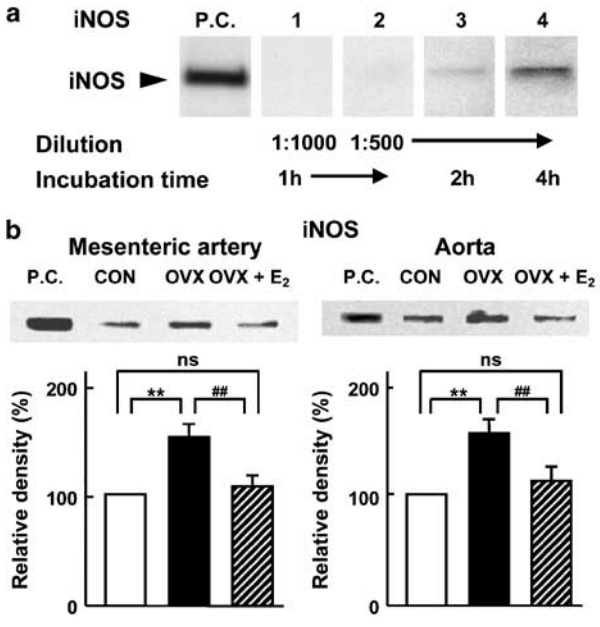

Figure 8 Immunoblot analysis of iNOS protein in rat mesenteric arteries. (a) Effects of concentrations of specific iNOS antibody and incubation time with the antibody on the density of the band corresponding to iNOS protein. As a positive control (P.C.) for iNOS, mouse macrophage lysate (RAW 264.7) stimulated with interferon γ ($10\,ng\,ml^{-1}$) and lipopolysaccharide ($1\,\mu g\,ml^{-1}$) was used. (b) Immunoblot analysis of iNOS proteins in mesenteric arteries of the control (CON), ovariectomized (OVX) and ovariectomized with estrogen treated ($OVX + E_2$) group. Upper panels represent typical electrophoretic data, and lower bar graphs represent the relative density of the immunostained band. The relative density was expressed as percentages of the value in CON group. Values are means ± s.e. of six rats. $^{**}P<0.01$ CON vs OVX; $^{\#\#}P<0.01$ OVX vs $OVX + E_2$.

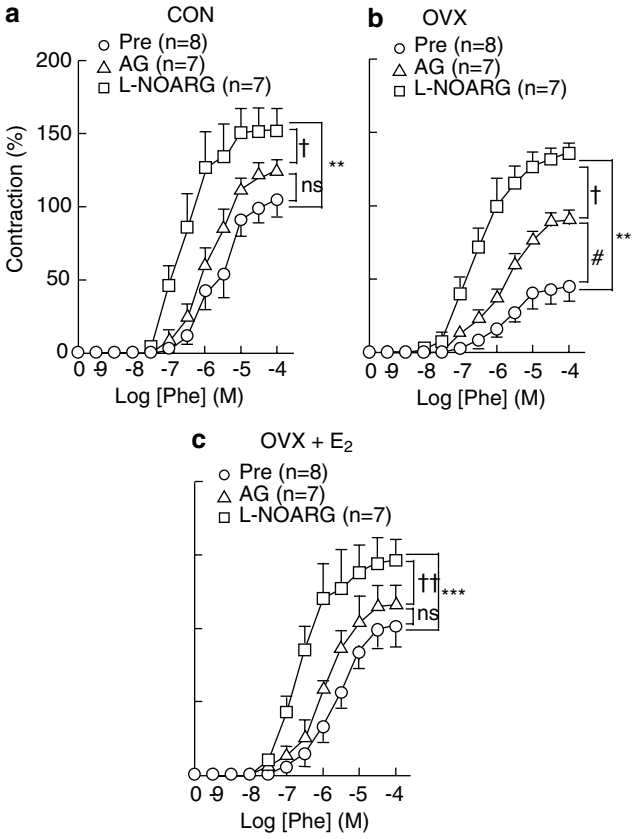

Figure 9 Concentration–response curves for phenylephrine (Phe)-induced contraction in the mesenteric arteries from control (CON) (a), ovariectomized (OVX) (b) and ovariectomized with estrogen treated ($OVX + E_2$) group (c) in the absence or presence of 100 μM N^G-nitro-L-arginine (L-NOARG) or 100 μM aminoguanidine. Values are means ± s.e. $^{**}P<0.01$ pre vs L-NOARG; $^{***}P<0.001$ pre vs L-NOARG; $^{\#}P<0.05$ pre vs aminoguanidine; $^{\dagger}P<0.05$ L-NOARG vs aminoguanidine; $^{\dagger\dagger}P<0.01$ L-NOARG vs aminoguanidine. Numbers of animals are shown in the figure.

(Figure 9b). Characteristically, the contraction was markedly augmented by L-NOARG to the level comparable to that in the CON group treated by the same drug (Figure 9b), indicating that the artery of the OVX group releases more NO than that of the CON group. Notably, the phenylephrine-induced contraction was also significantly enhanced by aminoguanidine (Figure 9b), indicating that the artery of the OVX group releases more iNOS-derived NO than that of the CON group. The difference between the contraction in the presence of L-NOARG and that in the presence of aminoguanidine was expanded, indicating that the artery of the OVX group releases more eNOS-derived NO than that of the CON group.

After chronic estrogen treatment of OVX rats, the phenylephrine-induced contractions in the absence or presence of L-NOARG and aminoguanidine were recovered to levels comparable to those in the CON group (Figure 9c).

Discussion

Effects of ovariectomy on ACh-induced endothelium-dependent relaxation

In the present study, ACh-induced endothelium-dependent relaxation in rat mesenteric artery was resistant to indomethacin, an inhibitor of cyclooxygenase. In contrast, the relaxation was partially sensitive to either apamin plus charybdotoxin (blockers of K^+ channel responsible for the EDHF action) or to L-NOARG (an inhibitor of NOS), and it was abolished by their combination (data not shown). These results are consistent with our previous reports (Fukao *et al*., 1997b; Liu *et al*., 2001) and indicate that both EDHF and NO play an important role in the ACh-induced endothelium-dependent relaxation in rat mesenteric artery, whereas prostanoids are not involved.

Total endothelium-dependent relaxation by ACh in rat mesenteric artery appear to be independent of estrogen level, because they were unaffected by OVX alone or $OVX + E_2$. However, when the EDHF- and NO-mediated responses were separately examined, it became apparent that following OVX, the EDHF-mediated responses were reduced, whereas the NO-mediated responses were enhanced.

Effects of ovariectomy on EDHF-mediated responses

Both EDHF-mediated relaxation of rat mesenteric artery and membrane hyperpolarization of vascular smooth muscles were reduced following OVX, and these responses were reversed by chronic estrogen treatment. These results confirm previous reports that EDHF-mediated responses are positively regu-

lated by estrogen levels (Liu *et al*., 2001; Sakuma *et al*., 2002). However, the present findings are discrepant with the recent report in which ovariectomy is not associated with a reduction in relaxation and hyperpolarization (Chataigneau *et al*., 2004). The reasons for such a discrepancy remain unclear but might be due to different ages of experimental animals (12 vs 40 weeks old in our experiment) and/or different experimental protocols.

Regarding the molecular entity of EDHF, several factors have been proposed: a P450 epoxygenase product such as epoxyeicosatrienoic acid (Fulton *et al*., 1992; Bauersachs *et al*., 1994), K^+ (Edwards *et al*., 1998) and hydrogen peroxide (Matoba *et al*., 2000). Apart from these candidate molecules, several studies have shown that at least in mesenteric arteries of rabbit, guinea-pig and rat, EDHF is not a molecule but transmission of membrane hyperpolarization through gap junctions from endothelial cells to vascular smooth muscle cells (Fukao *et al*., 1997a; Chaytor *et al*., 1998; Edwards *et al*., 1999; Goto *et al*., 2002). This theory is based on the following findings. Firstly, the EDHF-mediated responses are suppressed by derivatives of glycyrrhetinic acid, a chemical inhibitor of gap junctions (Yamamoto *et al*., 1998; Goto *et al*., 2002). Secondly, EDHF-mediated responses are suppressed by a peptide possessing sequence homology with the second extracellular loop of connexins, major components of gap junctions: this peptide is a specific inhibitor of both hetero- and homocellular gap junctional communication (Chaytor *et al*., 1998).

In the present study, EDHF-mediated relaxation of rat mesenteric artery was virtually abolished by 18β-GA at a concentration of 30 μM. The action of 18β-GA at this concentration was considered to be specific for gap junctional communication but not to be the result of nonspecific action on vascular smooth muscles, based on the following findings. (1) 18β-GA did not affect the resting tension and the phenylephrine-induced contraction of rat mesenteric artery. (2) 18β-GA (30 μM) had little effect on relaxation of rat mesenteric artery in CON, OVX and OVX + E_2 groups induced by SNP or pinacidil, which directly relaxes vascular smooth muscles by generating NO or opening K_{ATP} channel, respectively. This result is in good agreement with the previous report that glycyrrhetinic acid derivatives such as 18α-GA and carbenoxolone inhibit EDHF-mediated responses of rat mesenteric artery but not responses induced by levcromakalim, a K_{ATP} channel opener (Goto *et al*., 2002). Taken together, these data strongly indicate that gap junctions play an important role in the EDHF-mediated responses in rat mesenteric artery, although we could not exclude the possibility that 18β-GA specifically inhibited the yet unidentified K^+ channel responsible for EDHF action.

Gap junctions are composed mainly of connexins (Kumar & Gilula, 1996; Yeager *et al*., 1998). In the present study, RT–PCR analysis showed that mRNAs for connexin-37, 40 and 43 were expressed in rat mesenteric artery. Accordingly, connexin-40 and 43 proteins were detected in rat mesenteric artery using Western blot, although connexin-37 was not detected possibly because of its low level.

Protein levels of connexin-40 and 43 as detected by Western blot were reduced following OVX, and they were recovered with chronic estrogen treatment, indicating that levels of these proteins are regulated by estrogen levels. Notably, it is reported that half-palindromic estrogen responsive elements (EREs) but not complete EREs exist in the promoter region of the connexin-40 and 43 genes of the rat, and that the half EREs are sufficient to activate transcription of the gene controlled by these elements (Yu *et al*., 1994). Taken together, these data suggest that estrogen deficiency following OVX downregulates connexin-40 and 43 proteins, which are main components of gap junctions in rat mesenteric artery; this may lead to reduction of EDHF-mediated responses.

In addition, NO derived from iNOS is reported to decrease the protein level of connexin-43 (Sladek *et al*., 1999; Roh *et al*., 2002). As there was excessive NO production catalyzed by eNOS and iNOS in the mesenteric artery as discussed below, the increase in NO may also contribute to reduction of EDHF-mediated response by decreasing connexin-40 and 43 proteins.

Effects of ovariectomy on eNOS system

ACh-induced NO-mediated relaxation of rat mesenteric artery was enhanced following OVX, and it was recovered by chronic estrogen treatment, indicating that the NO-mediated relaxation is regulated by estrogen level. However, the protein level of eNOS *per se* was unchanged following OVX alone or OVX + E_2. The present data on the NO-mediated relaxation and the protein level of eNOS are discrepant with some of the previous reports: chronic estrogen treatment increases either ACh-induced relaxation of rat pulmonary artery and aorta (Andersen *et al*., 1999; Gonzales *et al*., 2001) or protein level of eNOS in rat pial tissue (Xu *et al*., 2001). Yet, the present data are consistent with the other report on ACh-induced relaxation of female rat aorta (Bolego *et al*., 1997). The mechanisms for the discrepancy remain to be elucidated, but it might be due to the difference of the tissues, ages or experimental designs.

ACh-induced NO production in the endothelium is mediated exclusively by eNOS (Arnal *et al*., 1999). Several lines of evidence indicate that the activity of eNOS is regulated by associated proteins such as calmodulin, hsp90 and caveolin-1, which interact with eNOS: calmodulin and hsp90 stimulate the enzyme activity, while caveolin-1 inhibits it (Michel *et al*., 1997; Garcia-Cardena *et al*., 1998; Fulton *et al*., 2001). Based on these data, we assumed that the expression of these regulatory proteins is altered following ovariectomy. The present study clearly demonstrated that the protein level of caveolin-1 but not of calmodulin and hsp90 was regulated by estrogen levels in rat mesenteric artery. The decrease in the protein level of caveolin-1 following OVX would enhance the catalytic activity of eNOS without a change in its protein level and thereby augment ACh-induced NO-mediated relaxation.

The present findings on changes in the protein level of caveolin-1 following OVX and its recovery by chronic estrogen treatment are an extension of previous reports using culture of bovine aortic endothelial cells and human vascular smooth muscle cells, which show that incubation of the cells with estrogen increases the expression of caveolin-1 (Jayachandran *et al*., 2001; Razandi *et al*., 2002). The estrogen-induced increase in the protein level of caveolin-1 seems quite relevant, because an ERE is present in the promoter region for caveolin-1 gene in rat. In contrast, our data are discrepant with several reports, in which the protein level of caveolin-1 in cerebral arterioles and uterus of rat was elevated following OVX (Turi *et al*., 2001; Xu *et al*., 2001). The mechanism for the discrepancy is at present unknown, but it might be due to the presence of another signaling pathway such as an NFκB

system in different tissues, which is known to be negatively regulated by estrogen (Galien & Garcia, 1997).

Effects of ovariectomy on iNOS system

The protein level of iNOS in mesenteric artery and aorta was increased following OVX, and it was reversed by chronic estrogen treatment, indicating that the protein levels of iNOS in these arteries are negatively regulated by the level of estrogen. These data are in line with recent reports that show an increase in iNOS protein in bone stromal cells and osteoblasts of mice following OVX or suppression of cytokines-induced increase in iNOS protein in the cultured rat aortic smooth muscle cells following chronic estrogen treatment (Zancan *et al*., 1999; Cuzzocrea *et al*., 2003; Maggi *et al*., 2003). Sequence analysis of the rat iNOS promoter does not reveal any EREs, but this promoter region contains binding sites for transcription factors such as NF-κB and AP-1 (Diaz-Guerra *et al*., 1996). Estrogen receptors that have bound estrogen are known to suppress the promoter activities controlled by NF-κB and AP-1 (Diaz-Guerra *et al*., 1996; Paech *et al*., 1997). A decrease in estrogen level would release the inhibitory influence of estrogen receptor on transcription of iNOS mRNA, resulting in the increased level of iNOS protein. It is generally known that iNOS does not require a calcium signal for its activation but that it is continuously active once expressed, leading to generation of high concentration of NO (Coleman, 2001). In fact, excessive NO generation by iNOS is reflected by the elevation in plasma levels of NO metabolites in the OVX group, because an increase in the metabolites is generally derived from iNOS-mediated NO production (Ellis *et al*., 1998). Our data on changes in plasma nitrate/nitrite levels following OVX and OVX + E_2 are consistent with those of Takahashi *et al*. (1997), but discrepant with several previous reports (van Bezooijen *et al*., 1998; Zhai *et al*., 2000). The mechanism for the discrepancy is unknown.

Physiological consequences of increased activities of eNOS and iNOS

Phenylephrine-induced contraction of rat mesenteric artery was reduced in OVX, but it was augmented by a nonspecific NOS inhibitor (L-NOARG) to the level comparable with that in CON group treated by the same drug. This result demonstrates that the artery in OVX group releases more NO than that in CON group.

Phenylephrine-induced contraction of the artery in CON group was not enhanced by a specific iNOS inhibitor (aminoguanidine), but that in OVX group became enhanced by the drug, indicating that production of NO by iNOS is very low in CON group, whereas it is augmented in OVX group. These results are considered to result from an increase in protein levels of iNOS as detected by Western blot.

The difference between phenylephrine-induced contraction in the presence of the nonspecific NOS inhibitor and that in the presence of the specific iNOS inhibitor is considered to represent the part which is contributed by the NO derived from eNOS, and notably, this difference was also increased. This result clearly indicates that the artery in OVX group releases more eNOS-derived NO than that in CON group, and confirms the increase in the catalytic activity of eNOS resulting from reduction of a negative regulator, caveolin-1.

Pathological consideration of the present findings

Several lines of evidence demonstrate that excessive NO derived from iNOS triggers atherosclerosis by producing reactive nitrogen oxide species (Patel *et al*., 1999; Detmers *et al*., 2000; Kuhlencordt *et al*., 2001). In the present study, plasma cholesterol level was elevated following OVX, as in postmenopausal women (Mosca, 2000). Considering that high plasma cholesterol level is a major risk factor for atherosclerosis, it is possible that excessive NO production by iNOS together with high plasma cholesterol levels would promote the progression of atherosclerosis. In contrast, the diameter of arteries seems to be unaffected by a decrease in estrogen, as reflected by no change in blood pressure. That is, in resistance arteries, which can generate both EDHF and NO, reciprocal changes in EDHF-mediated relaxation and NO-mediated relaxation cancel each other. In large arteries such as aorta, which can generate only NO, there might be an increase in production of vasoconstricting substances such as angiotensin and endothelin, leading to minimal change in diameter.

In summary, (1) estrogen deficiency following ovariectomy reduced EDHF-mediated relaxation of rat mesenteric artery, whereas it augmented NO-mediated relaxation, causing no change in ACh-induced endothelium-dependent relaxation. (2) Reduction of EDHF-mediated relaxation may result from a decrease in protein levels of connexin-40 and 43, main components of gap junction. (3) Enhancement of NO-mediated relaxation is due to an increase in synthesis and release of NO, which results from an increase in the level of iNOS protein and in the catalytic activity of eNOS subsequent to a decrease in the level of a negative regulator of eNOS, caveolin-1. (4) Plasma cholesterol level was elevated, whereas blood pressure was unchanged. (5) Excessive NO release and high plasma cholesterol level would promote the progression of atherosclerosis in postmenopausal women.

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Smoking Research Foundation, Japan.

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(Received September 9, 2004
Revised October 5, 2004
Accepted October 5, 2004)