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Chronic treatment with 17β-estradiol increases susceptibility of smooth muscle cells to nitric oxide

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Abstract

The purpose of this study was to evaluate the role of estrogen as a vasodilator or relaxing modulator during vascular tonus through chronic estrogen treatment. Experiments were conducted using isolated basilar arteries from ovariectomized female rabbits divided into two groups (the with and without estrogen replacement groups, respectively). Both acetylcholine and carbachol relaxed the basilar arteries of rabbits in the with estrogen replacement group (pre-contracted by 30 mM K^+) more strongly than in the without estrogen replacement group. Vasodilatation effects of (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3 -hexenamide (NOR1) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) were greater in rabbits in the with estrogen replacement group than the without estrogen replacement both with endothelium-intact and denuded preparations. On the other hand, vasodilatation effects of nicardipine, 17β-estradiol and membrane-permeable cyclic-GMP or cyclic-AMP were the same in both groups. These results suggest that chronic administration of estradiol potentiates reactivity to nitric oxide (NO) in smooth muscle cells, which could be a therapeutic target for cardiovascular diseases in postmenopausal women.

Keywords: 17β-Estradiol; Estrogen; Vascular smooth muscle

1. Introduction

It is widely accepted that the low incidence of cardiovascular mortality and morbidity in women can be ascribed to differences in their hormonal status compared to men. Moreover, cessation of ovarian hormones after menopause is known to increase susceptibility to vascular dysfunction such as atherosclerosis (Glasser et al., 1995) and possibly hypertension (Staessen et al., 1998). An early report on hormone replacement therapy in menopausal women showed the effectiveness of this therapy in vascular protection (Stampfer et al., 1991) with the beneficial action of estrogen mediated indirectly by its effect on lipid metabolism (Miller et al., 1994) and directly by its effect on the vessel wall itself (Hayward et al., 2000). On the other hand, a randomized prospective controlled clinical trial (the Heart and Estrogen-progestin Replacement Study) showed no benefits of hormone replacement therapy (Hulley et al., 1998). The current recommendation of the Women's Health Initiative Steering Committee is that both combined equine estrogens plus continuous administration of progestin and equine estrogen do not offer benefits in preventing coronary heart disease, and therefore, this therapy should not be used even for primary prevention of heart disease (Writing Group for the Women's Health Initiative Investigators, 2002; The Women's Health Initiative Steering Committee, 2004). However, in response to these negative results, Gray et al. (2001) commented on the need to reconsider the study design of such trials, especially with regard to the recruitment of patients, and emphasized the importance of elucidating the effects of hormone replacement therapy at the cellular level. Indeed, recent estrogen experiments at cellular

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and molecular levels are continuing to accumulate evidence of a positive relationship between ovarian hormones and vascular protection via nitric oxide synthesis (Venema et al., 1994; Hishikawa et al., 1995; Mendelsohn and Karas, 1999).

Upregulation of estrogen-mediated endothelial nitric oxide synthase (eNOS) in endothelial cells accounts for the acute and chronic actions of estrogen (Venema et al., 1994; Hayashi et al., 1995; Hishikawa et al., 1995). McCrohn et al. (1997) demonstrated that nitroglycerin, an NO donor, significantly increased vasodilatation in estrogen-treated men, suggesting possible direct action of estrogen on human smooth muscle cells via an NO-mediated pathway. Thus, elucidation of the contribution of smooth muscle itself in the estrogen effect would be important in understanding NO production, since membrane and cytosolic estrogen receptors were recently shown to be expressed in both endothelial and smooth muscle cells (Enmark and Gustafsson, 1999; Colburn and Buonassisi, 1978; Bayard, 1996; Bei et al., 1996; Losordo et al., 1994; Karas et al., 1994). In this study, we conducted experiments focusing on NO-related responses to elucidate the cellular responses of endothelial and arterial muscle cells to chronic estradiol treatment.

2. Materials and methods

2.1. Animals

Three-month-old female albino rabbits (Nippon White, 1.9–2.3 kg) from Kyudou Animal Institute (Fukuoka, Japan) were ovariectomized under anesthesia with sodium pentobarbital (Mochida Pharmaceutical, Tokyo, Japan) by continuous intravenous infusion. Overiectomized rabbits were then divided into two groups. One (the with estrogen replacement group) was administrated estradiol valerate by intramural injection (5 mg/0.5 ml: Mochida Pharma., Tokyo, Japan) every 2 weeks (the first estrogen administration was conducted at overiectomy), while the other (the without estrogen replacement group) was injected with an equivalent amount of saline at simultaneous intervals. Rabbits were housed individually in stainless-steel cages at a room temperature of 23±2 °C with 50±10% humidity in the Animal Center of Kyushu University. Food and water were available ad libitum. Venous blood samples were taken to measure blood concentrations of estradiol and cholesterols. For tissue dissection, animals were anesthetized with sodium pentobarbital by intravenous injection (40 mg/kg) then exsanguinated 12 weeks later for tension recording. All procedures were approved by the Animal Research Committee of Kyushu University.

2.2. Solution and drugs

Modified Krebs solution with the following ionic composition (mM) was used: NaCl (121.9), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (15.5), KH_2PO_4 (1.2) and glucose 11.5. The pH of the solution was adjusted to 7.3–7.4 with 5% CO_2:95% O_2. Drugs used in the present experiments were acetylcholine chloride, carbachol, 17β-estradiol, nicardipine, indomethacin (Sigma Chem., St. Louis, MO, U.S.A.), (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1), *S*-nitroso-*N*-acetyl-penicillamine (SNAP), (Wako, Tokyo, Japan), 8-bromoguanosine-3′, 5′-cyclic monophosphate sodium salt (8-bromo-cGMP), 2′-O-dibutyryladenosine-3′, 5′-cyclic monophosphate sodium salt (dibutyryl-cGMP), 8-bromoguanosine-3′, 5′-cyclic monophosphate sodium salt (8-bromo-cAMP) and 2′-O-dibutyryladenosine-3′, 5′-cyclic monophosphate sodium salt (dibutyryl-cAMP: Biomol Res. Lab., Plymouth Meetings, PA, U.S.A.). Water-soluble 17β-estradiol rather than a solvent form (ethanol) was used to avoid the effects of the solvent in the vascular response of 17β-estradiol. The hydrophobicity of water-soluble 17β-estradiol is reduced in the presence of 2-hydroxypropyl-β-cyclodextrin (=1 mM), but this agent had no effect on the rabbit basilar arteries.

2.3. Preparation for mechanical recording

Basilar arteries were dissected and isolated by removing the surrounding connective tissue. A circular preparation of each artery (1 mm in length) was then held in a small organ bath using a pair of hooks, one of which was fixed to the organ bath and the other to a force displacement transducer (Ufer UC-5TD; Iwashiya, Kyoto, Japan). Each preparation was equilibrated in warmed Krebs solution (35 °C) for 30 min. In some experiments, endothelium-denuded preparations were prepared by scrubbing the inner vessel wall with fine threads. Removal of endothelial cells was confirmed by the lack of relaxation with acetylcholine (1 μM) administration.

2.4. Recording of signals

Before obtaining dose response curves, we confirmed that 30 mM K^+ had produced a sustained contraction of 20–30 min in the vessels of both groups of rabbits. Both vessels were regarded as having contracted to the same level (maximally) before assessing dilation. No passive tension was applied to the vessels in this procedure. After steady state tension was reached, acetylcholine, carbachol, NOR1 and SNAP were added cumulatively to both vessels at 1, 3, 10, 30, 100 and 300 nM followed by 1, 3 and 10 μM then the relative tension was calculated. IC_{20} and IC_{50} values of these drugs were calculated by a single interpolation to compare their relaxing potencies during K^+-induced contraction of rabbit basilar arteries in both groups.

To clarify differences between the groups in reactivity to NOR1 and SNAP mainly as a result of their actions on smooth muscle cells, arteries were superfused with nicardipine and 17β-estradiol at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M in an organ bath. Similarly, arteries

Table 1
Changes in body weights and serum lipid profiles of rabbits in the with ($n=8$) and without ($n=8$) estrogen replacement groups before and 12 weeks after ovariectomy

	Without estrogen replacement ($n=8$)		With estrogen replacement ($n=8$)	
	Before	12 weeks after	Before	12 weeks after
Body weight	2143.5±34.8	2637.8±35.5	2147.5±44.6	2836.3±51.5*
Total cholesterol	49.5±3.0	58.8±6.0	49.8±4.2	45.4±5.3
HDL cholesterol	17.2±2.1	19.0±1.6	19.6±1.4	20.2±0.6
Triglyceride	82.3±11.8	56.8±9.5	67.8±6.9	40.0±10.2
LDL cholesterol	15.9±1.3	28.5±4.7	16.6±2.1	17.2±3.0

*$P<0.01$ versus rabbits in the without estrogen replacement group.

were also superfused with each membrane permeable cyclic nucleotide (8-bromo-cGMP, dibutyryl cGMP, 8-bromo-cAMP, dibutyryl-cAMP) at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M.

2.5. Serum concentrations of estradiol and cholesterol

To measure serum cholesterol levels, two blood samples were taken from each individual (once before and at 12 weeks after ovariectomy) after fasting for 24 h. Serum levels of total cholesterols, triglycerides and high- and low-density lipoprotein cholesterol (HDL and LDL cholesterol, respectively) were determined using an enzymatic method with HITACHI 7450 (Hitachi, Tokyo, Japan) and the following kits: T-CHOL-HR (Wako), TG-II, (Daiichi), and HDL-C auto (Daiichi). To measure serum concentrations of estradiol, blood samples were also taken 1, 4, 7, 10 and 14 days after estrogen injection. Serum estradiol levels were measured using a radioimmunoassay method with a 17β-estradiol Correlate-EIA kit (Cosmo Bio, Tokyo, Japan).

2.6. Data analysis and statistics

In the tension recording experiments, the results were corrected to remove the baseline tension. Results are expressed as the mean±standard error of the mean (SEM). Statistical significance was assessed using the paired Student's t test and repeated measurements ANOVA with a P value <0.05 considered significant.

IC_{50} values of carbachol, NOR1 and SNAP were estimated by fitting to the Hill equation with the least square method. For acetylcholine, IC_{20} values estimated by interpolation were used due to the weak relaxing effects.

Table 2
Changes in serum estradiol concentrations in ovariectomized rabbits before (day 0) and after single administration of estrogen ($n=4$)

	Day 0	Day 1	Day 4	Day 7	Day 10	Day 14
Mean	218.3	10322.5	1840.0	260.3	237.5	215.0
SEM	36.4	1652.9	397.5	26.3	18.5	15.8

(pg/ml).

3. Results

3.1. Body weight

As shown in Table 1, rabbits in the without estrogen replacement group gained 494.3±21.8 g of body weight by 12 weeks after ovariectomy. On the other hand, the mean body weight gain of the with estrogen replacement group was 688.8±22.3 g. Body weights measured 12 weeks after ovariectomy and gains in body weight were statistically significant between groups ($P<0.01$ and $P<0.05$, respectively).

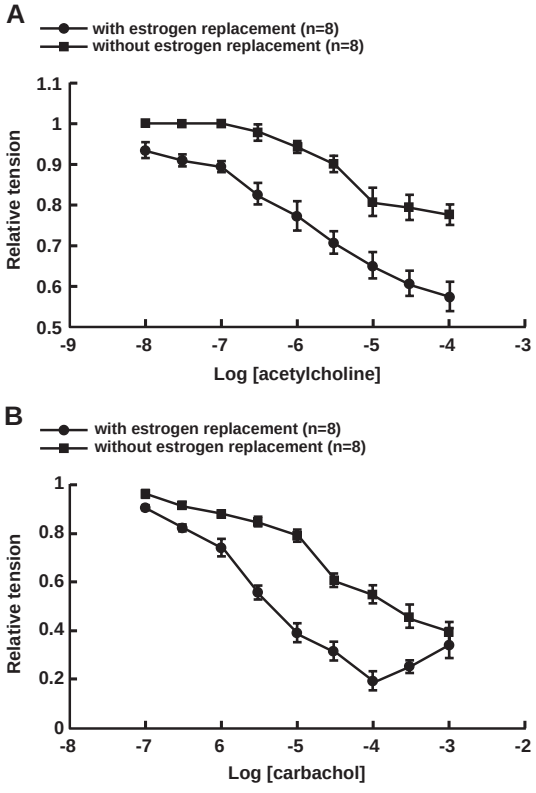

Fig. 1. Effects of acetylcholine (A) and carbachol (B) on rabbit basilar arteries pre-contracted by 30 mM K^+. Circles and squares indicate the relative tension observed in arteries from ovariectomized rabbits in the with and without estrogen replacement groups, respectively ($n=6$–8). The amplitude of the 30 mM K^+-induced contraction was set at 1.0, and each contraction in the presence of either drug was expressed in a relative manner.

3.2. Serum concentrations of cholesterol

As shown in Table 1, serum concentrations of cholesterol were the same between the without and with estrogen replacement groups before and 12 weeks after ovariectomy.

3.3. Serum concentrations of estradiol

Serum estradiol levels before and after intramural injection of estradiol valerate were measured in preliminary sampled rabbits ($n=4$; Table 2). Serum estradiol concentrations immediately increased 47-fold compared to control rabbits after a single injection of estradiol, decreasing gradually to the control level by 14 days after injection. We therefore injected rabbits in the with estrogen replacement group with estradiol every 2 weeks.

Serum concentrations of estradiol in rabbits in the without estrogen replacement group ($n=8$) were 225.0±13.0 before and less than 35 at 12 weeks after ovariectomy. Concentrations were 222.4±8.1 and 245.0±28.4, respectively, in the with estrogen replacement group ($n=8$). There were no significant differences in serum concentrations of estradiol

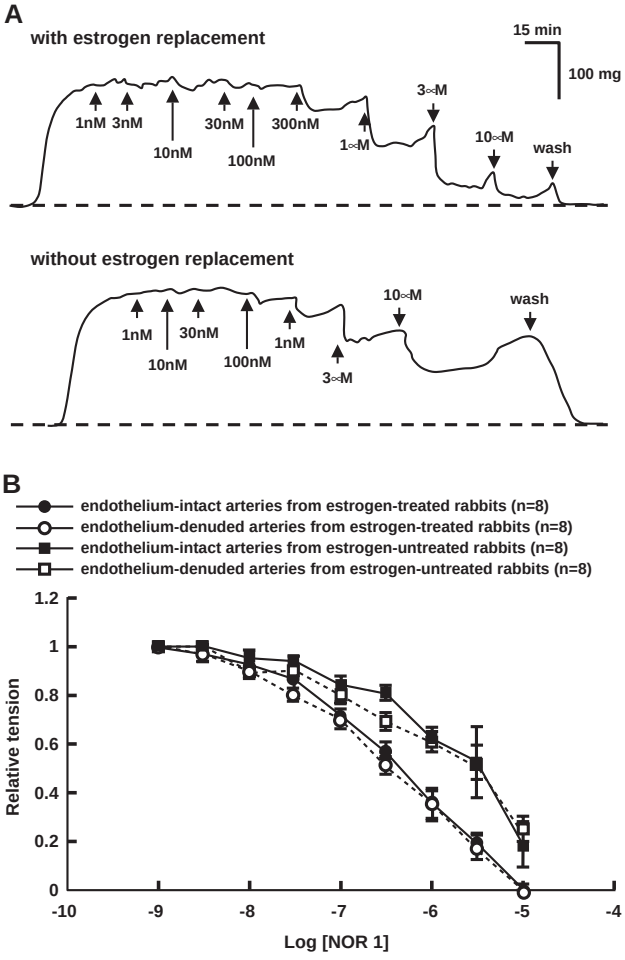

Fig. 2. Effects of NOR1 on rabbit basilar arteries pre-contracted by 30 mM K^+. (A) Inhibitory actions of NOR1 in pre-contracted arteries from ovariectomized rabbits in the with (upper trace) and without (lower trace) estrogen replacement groups. Various concentrations of NOR1 (1 nM–10 μM) were applied cumulatively. Arteries were superfused with excess K^+ in an organ bath to obtain maximum contraction before assessing dilation. (B) Relationship between relative tension and NOR1 concentration. Closed circles: endothelium-intact arteries from estrogen-treated rabbit ($n=8$). Open circles: endothelium-denuded arteries from estrogen-treated rabbit ($n=8$). Closed squares: endothelium-intact arteries from estrogen-untreated rabbit ($n=8$). Open squares: endothelium-denuded arteries from estrogen-untreated rabbit ($n=8$). Values indicate the mean±SD. The amplitude of the 30 mM K^+-induced contraction was set at 1.0, and each contraction in the presence of either drug was expressed in a relative manner.

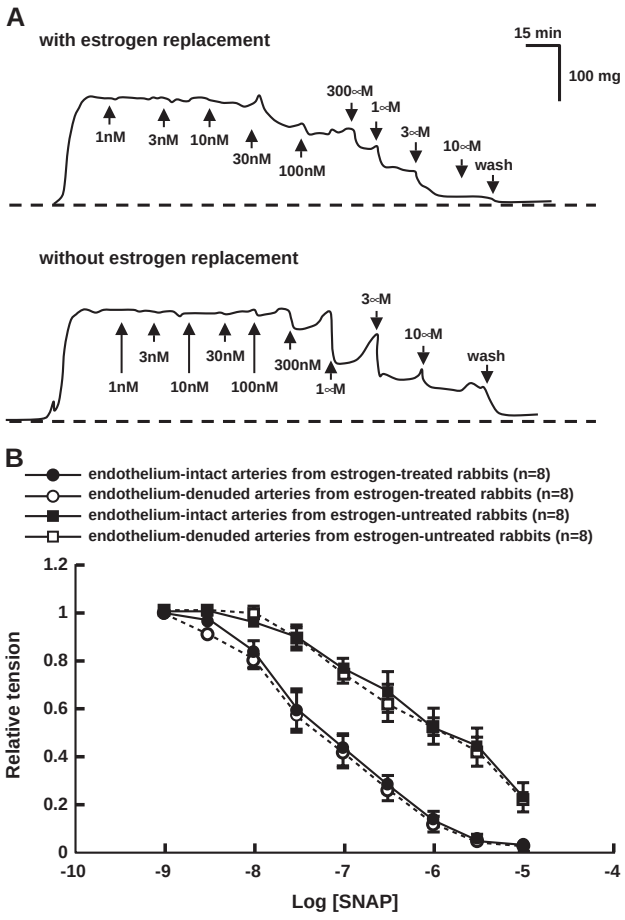

Fig. 3. Effects of SNAP on rabbit basilar arteries pre-contracted by 30 mM K^+. (A) Inhibitory actions of SNAP on pre-contracted arteries from ovariectomized rabbits in the with (upper trace) and without (lower trace) estrogen replacement groups. Various concentrations of SNAP (1 nM–10 μM) were applied cumulatively. Arteries were superfused with excess K^+ solution in an organ bath to obtain maximum contraction before assessing dilation. (B) Relationship between relative tension and SNAP concentration. Closed circles: endothelium-intact arteries from estrogen-treated rabbit ($n=8$). Open circles: endothelium-denuded arteries from estrogen-treated rabbit ($n=8$). Closed squares: endothelium-intact arteries from estrogen-untreated rabbit ($n=8$). Open squares: endothelium-denuded arteries from estrogen-untreated rabbit ($n=8$). Values indicate the mean±SEM. The amplitude of the 30 mM K^+-induced contraction was set at 1.0, and each contraction in the presence of either drug was expressed in a relative manner. Values were measured from blood samples taken before and at 12 weeks after ovariectomy. Values indicate the mean±SEM ($n=8$).

Table 3
Effects of estradiol and nicardipine on basilar arteries from ovariectomized rabbits in the with and without estrogen replacement groups

		Endothelium intact			Endothelium denuded		
		With estrogen (n=8)	Without estrogen (n=8)		With estrogen (n=8)	Without estrogen (n=8)	
Estradiol	10^{-8}M	1.000±0.000	1.000±0.000	n.s.	1.000±0.000	1.000±0.000	n.s.
	10^{-7}M	0.965±0.005	0.965±0.005	n.s.	0.980±0.008	0.983±0.006	n.s.
	10^{-6}M	0.765±0.025	0.785±0.018	n.s.	0.780±0.010	0.765±0.011	n.s.
	10^{-5}M	0.300±0.025††	0.290±0.017†	n.s.	0.515±0.025††	0.505±0.032†	n.s.
	10^{-4}M	0.158±0.021††	0.173±0.008††	n.s.	0.428±0.008††	0.443±0.010††	n.s.
Nicardipine	10^{-9}M	1.000±0.000	1.000±0.000	n.s.	1.000±0.000	1.000±0.000	n.s.
	10^{-8}M	0.898±0.004	0.900±0.006	n.s.	0.926±0.009	0.913±0.014	n.s.
	10^{-7}M	0.720±0.008	0.730±0.006	n.s.	0.765±0.011	0.765±0.011	n.s.
	10^{-6}M	0.400±0.017	0.390±0.025	n.s.	0.418±0.012	0.418±0.016	n.s.
	10^{-5}M	0.203±0.008	0.185±0.009	n.s.	0.248±0.013	0.223±0.011	n.s.

††indicates $P<0.01$, † indicates $P<0.05$.

between groups before ovariectomy, but the with estrogen replacement group showed significantly higher estradiol levels than the without estrogen replacement group at 12 weeks after ovariectomy.

3.4. Tension recording

Acetylcholine relaxed endothelium-intact rabbit basilar arteries prepared from rabbits in both the with and without estrogen replacement groups in a dose-dependent manner (Fig. 1). However, the relative amplitudes of acetylcholine-induced relaxation were always larger in arteries isolated from rabbits in the with estrogen replacement group (Fig. 1). Acetylcholine (=0.1 mM) only inhibited 30 mM K^+-induced contraction by 40% and 20% in the with and without estrogen replacement groups, respectively. Estimated IC_{20} values for rabbits in the with and without estrogen replacement groups were 0.41 and 32.4 μM, respectively with statistical significance ($P<0.05$; Fig. 1A). Carbachol (=1 mM) induced more relaxation than the same concentration of acetylcholine. Estimated IC_{50} values of rabbits in the with and without estrogen replacement groups differed significantly (3.5 vs. 200 μM, respectively; $P<0.05$; Fig. 1B). These results indicate that chronic estradiol treatment enhanced the NO-dependent pathway in endothelial cells.

Table 4
Effects of 8br-cGMP, Db-cGMP, 8br-cAMP and db-cAMP on basilar arteries from ovariectomized rabbits in the with and without estrogen replacement groups

		With estrogen replacement (n=8)	Without estrogen replacement (n=8)	
8-bromo-cGMP	10^{-8} M	0.987±0.006	0.970±0.002	n.s.
	10^{-7} M	0.905±0.011	0.885±0.009	n.s.
	10^{-6} M	0.792±0.021	0.799±0.010	n.s.
	10^{-5} M	0.579±0.013	0.593±0.007	n.s.
Dibutyryl-cGMP	10^{-8} M	0.986±0.005	0.990±0.004	n.s.
	10^{-7} M	0.908±0.007	0.927±0.008	n.s.
	10^{-6} M	0.817±0.007	0.829±0.003	n.s.
	10^{-5} M	0.710±0.007	0.744±0.004	n.s.
8-bromo-cGMP	10^{-8} M	0.989±0.004	0.970±0.002	n.s.
	10^{-7} M	0.924±0.008	0.899±0.007	n.s.
	10^{-6} M	0.824±0.004	0.807±0.002	n.s.
	10^{-5} M	0.724±0.004	0.709±0.015	n.s.
Dibutyryl-cAMP	10^{-8} M	0.980±0.004	0.997±0.002	n.s.
	10^{-7} M	0.906±0.012	0.923±0.012	n.s.
	10^{-6} M	0.805±0.009	0.764±0.027	n.s.
	10^{-5} M	0.642±0.013	0.543±0.020	n.s.

n.s.: not significant.

To determine the effect of chronic estradiol treatment on the NO reactivity of smooth muscle cells, arteries were superfused with NOR1, an NO donor that releases NO molecules at a 1 : 1 ratio with NOR1 for 10–15 min (half-life: 1.8 min), in an organ bath. As shown in Fig. 2A, NOR1 relaxed both endothelium-intact and denuded rabbit basilar arteries in a concentration-dependent manner. Larger relaxation occurred in rabbits in the with estrogen replacement group than the without estrogen replacement group (IC_{50}=0.33 vs. 2.2 μM, respectively, for endothelium-intact preparations; $P<0.05$; IC_{50}=0.32 vs. 1.78 μM, respectively, for endothelium-denuded preparations; $P<0.05$; Fig. 2B). There was no significant difference between IC_{50} values of endothelium-intact and denuded preparations within groups.

SNAP, a nitrosothiol derivative that spontaneously releases NO, similarly relaxed both endothelium-intact and denuded rabbit basilar arteries in a concentration-dependent manner (Fig. 3A). Larger relaxation occurred with SNAP in rabbits in the with estrogen replacement group than the without estrogen replacement group (IC_{50}=0.056 vs. 0.52 μM, respectively, for endothelium-intact preparations; $P<0.05$; IC_{50}=0.050 vs. 0.46 μM, respectively, for endothelium-denuded preparations; $P<0.05$; Fig. 3B). There was no significant difference between IC_{50} values of intact and endothelium-denuded preparations within groups.

To clarify the differences between groups in reactivity to NOR1 and SNAP mainly as a result of their actions on smooth muscle cells, arteries were superfused with nicardipine and 17β-estradiol in an organ bath. As shown in Table 3, both nicardipine and estradiol inhibited 30 mM K^+-induced contraction. There was no statistical significance between the inhibitory actions of these agents in rabbits in

the with and without estrogen replacement groups, and there was no significant difference in the effect of nicardipine on intact and endothelium-denuded preparations within groups. On the other hand, 17β-estradiol produced significantly stronger relaxation in the endothelium-intact preparation than the endothelium-denuded preparation. These results suggest that 17β-estradiol, but not nicardipine, additionally stimulates endothelial cells to release relaxing substance(s) as well as having a direct effect on smooth muscle cells.

The effects of the membrane permeable cyclic nucleotides on the endothelium-denuded preparations are summarized in Table 4. There was no statistical significance between rabbits in the with and without estrogen replacement groups with regard to the relaxing actions of 8-bromo-cGMP, dibutyryl-cGMP, 8-bromo-cAMP and dibutyryl-cAMP.

4. Discussion

The vascular endothelium maintains vascular tone and structure by regulating the balance between vasodilation and vasoconstriction, and is involved in the release of three major vasodilators (nitric oxide (NO), prostanoids, and endothelium-derived hyperpolarizing factors) as well as vasoconstrictors. Firstly, NO diffuses from the endothelial cell into adjacent smooth muscle fiber activating guanylate cyclase, which promotes the formation of cGMP, inducing relaxation of the arterial smooth muscle (Regoli, 2004). Secondly, prostacyclin, the major prostanoid produced by endothelial cells, causes relaxation predominantly via the adenylate cyclase-cAMP transduction system (Kukovetz et al., 1979; Ignarro et al., 1985). cAMP then activates protein kinase A, which phosphorylates selected target proteins and gives rise to vascular relaxation. Thirdly, endothelium-derived hyperpolarizing factor is widely hypothesized to hyperpolarize and relax vascular smooth muscle following stimulation of the endothelium by agonists. Candidates such as K^+ ions, eicosanoids, hydrogen peroxide and C-type natriuretic peptide have been implicated as the putative mediator. An alternative explanation for the endothelium-derived hyperpolarizing factor-type vasodilation is direct intercellular communication via gap junctions, which allows passive spread of agonist-induced endothelial hyperpolarization through the vessel wall (Tudor, 2004). None of the vasodilators described above functions independently, and a dynamic reciprocal relationship exists among these mechanisms.

At present, the effect of estrogen on vascular function at a cellular and/or molecular level is incompletely understood, but it appears to be multifactorial. It seems clear that estrogen lowers the concentration of calcium in vascular smooth muscle either directly by affecting calcium mobilization or indirectly through opening of potassium channels (Harder and Coulson, 1979); the latter causes repolarization of membrane potential, thereby closing voltage-dependent calcium channels. Also, a preponderance of data has indicated a role for estrogen in NO production (Hayashi et al., 1992; Conrad et al., 1993; Kharitonov et al., 1994). It has been suggested that estrogen stimulates the release of NO from vascular cells by a mechanism dependent upon (Weiner et al., 1994; Binko and Majewski, 1998) or independent of (Lantin-Hermoso et al., 1997; Caulin-Glaser et al., 1997) gene expression, but how estrogen enhances NO production has yet to be completely defined. Thus, we designed the present experiments to elucidate any chronic action of estrogen on smooth muscle cells rather than endothelial cells, as there is abundant evidence for the latter.

As shown in Fig. 1, acetylcholine is a multifactorial vasodilating agent. The vasodilating activity of acetylcholine is thought to be mostly mediated by muscarinic acetylcholine receptors that trigger the release of the actual vasodilating agent, NO (Furchgott and Zawadzki, 1980; Rosenblum, 1986; Huang et al., 1995; Faraci and Sigmund, 1999); however, some investigators have documented large endothelium-derived hyperpolarizing factor-type responses to acetylcholine (Savage et al., 2003), which are capable of promoting endothelial synthesis of cAMP (Kamata et al., 1996; Taylor et al., 2001.), H_2O_2 (Matoba et al., 2000, 2002; Rabelo et al., 2003) and C-type natriuretic peptide (Wennberg et al., 1999) and enhancing cell to cell coupling via gap junctional communication (Burghardt et al., 1995; Chanson et al., 1996; Abudara et al., 2000; Paulson et al., 2000; Van Rijen et al., 2000; Gladwell and Jefferys, 2001; Grazul-Bilska et al., 2001). Therefore, chronic treatment with estrogen enhances acetylcholine-induced endothelium-derived hyperpolarizing factor-type vasodilation distinct from NO-mediated vasodilation (Fig. 1). To investigate the potential contribution of vascular smooth muscle cells in NO-mediated vasodilation responses, we studied NO donor-mediated vasodilation effects in different vascular preparations (Figs. 2 and 3). Probable mechanisms of estrogen-induced increases in endothelium-derived NO production have been suggested (Tostes et al., 2003; Wagner et al., 2001; Barbacanne et al., 1999); however, we suggest that endothelial derived up-regulation of NO is negligible because of the result obtained after endothelial deprivation. Vascular smooth muscle cell-derived NO up-regulation caused by chronic estrogen treatment, as reported by Binko et al. (1998), might have occurred in this experiment (Figs. 2 and 3); therefore, we suggest that the increment of NO in the smooth muscle cells was nonsignificant compared to the amount produced by NO donors. These results therefore suggest the possibility that chronic estrogen treatment increases the susceptibility of smooth muscle cells to nitric oxide. On the other hand, direct application of membrane-permeable cGMP (8-bromo-cGMP and dibutyryl-cGMP), which is produced as a second messenger via NO-mediated vasodilation, resulted in comparable vasodilation of rabbit basilar arteries both from rabbits in the with and without estrogen replacement groups (Table 4), suggesting that the cGMP-regulated process and its downstream pathway might not be involved in enhancement of NO reactivity.

It was previously reported that acute administration of estrogen inhibits contraction in cultured aortic smooth muscle cells (Nakajima et al., 1995) and rabbit basilar artery (Ogata et al., 1996) by suppression of voltage-dependent L-type Ca^{2+} channels. However, these acute inhibitory effects did not contribute to the vasodilation observed in the present study, as serum estrogen concentrations were restored to the control level by the time of sacrifice (Table 2). Furthermore, channel activity of L-type Ca^{2+} channels might not be altered by chronic estrogen treatment, because the inhibitory action of nicardipine, a selective channel blocker of L-type Ca^{2+} channels, was unchanged between the two rabbit groups (Table 3). Additionally, chronic changes in lipid profiles and body weights between groups might have altered the vascular responsiveness to NO, and therefore, this also needs further investigation.

In conclusion, chronic estrogen treatment increased the susceptibility of smooth muscle cells to NO reactivity. This finding indicates that vascular smooth muscle could be a possible therapeutic target for cardiovascular diseases in postmenopausal women in the near future.

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