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Inhibitory effect of low-dose estrogen on neointimal formation after balloon injury of rat carotid artery

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Abstract

The current regimens of hormone replacement therapy for postmenopausal women, estrogen combined with progestogen, have failed to show beneficial effects for the prevention of atherosclerotic disease. Although the relatively higher dose of estrogen contained in those regimens exerted adverse effects, there are few data examining a lower dose of estrogen in an atherosclerosis model. Therefore, we investigated experimentally whether lower doses of estrogen could inhibit neointimal formation after balloon injury of the rat carotid artery. Ten-week-old Wistar rats were subjected to ovariectomy or sham-operation (n=7). Four days after ovariectomy, rats were implanted with an osmotic mini-pump containing 17-β estradiol (0.2, 1, 2, 10 and 20 μg/kg/day; n=6, 4, 8, 6 and 5, respectively) or placebo (n=10). After 3 days of hormone therapy, balloon injury was performed in the left common carotid artery. Neointimal formation was histologically evaluated 2 weeks after injury. Cross-sectional intimal area and the ratio of intimal area to medial area were dose-dependently reduced by estrogen replacement compared with those in ovariectomized rats without estrogen replacement. The effects of estrogen replacement were identical to those of an angiotensin II type 1 receptor blocker, candesartan. Interestingly, the effect was significant even in rats receiving lower doses of estrogen, in which plasma estradiol concentrations were not increased and the hyperplastic response of the uterus was minimal. These results suggest the efficacy of low-dose estrogen therapy for the protection of atherosclerosis.

Keywords: Estrogen; Low-dose; Neointimal formation

1. Introduction

Previous studies have shown that estrogen administration in ovariectomized animals inhibits the process of atherosclerosis. Different doses of estrogens in combination with or without progestins have decreased the lesion formation in injured vessels or cholesterol-fed animals using rodents, rabbits and swine (Chen et al., 1996; Oparil et al., 1997; Bakir et al., 2000; Chandrasekar and Tanguay, 2000; Finking et al., 2001; Tolbert et al., 2001). Most of the studies, however, have used the estradiol doses of 20 μg/kg/day or higher, which were accompanied by the raised plasma estradiol concentration compared to intact female animals (Chen et al., 1996; Bakir et al., 2000; Tolbert et al., 2001). More importantly, these doses of estrogen (≥20 μg/kg/day of estradiol subcutaneously) elicited adverse effects such as uterine hyperplasia (Bakir et al., 2000; Tolbert et al., 2001; Xu et al., 2003) and dyslipidemia (Joles et al., 1998; Gades et al., 1998; Tomiyoshi et al., 2002). On the other hand, it has been reported that the effect of estradiol on uterine weight was dose-dependent (Kerdelhue and Jolette, 2002) and that low dose estrogen (approximately 3 μg/kg/day of estradiol) could exert its favorable effect on bone metabolism (Chen et al., 2001). Since limited information is

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available on the vascular effect of low dose estrogen therapy, it is intriguing to study whether the lower dose of estrogen could inhibit vascular lesion formation.

In the present study, we hypothesized that lower doses of estrogen could have protective effects on the process of atherosclerosis with minimal adverse effects. To test this hypothesis, we examined neointimal formation of the carotid artery after balloon angioplasty in ovariectomized female rats receiving 10 μg/kg/day or lower doses of estradiol.

2. Materials and methods

2.1. Animals

Ten-week-old female Wistar rats (Oriental Yeast, Tokyo) were used in this study. They were housed in individual cages in a room in which lighting was controlled (12 h on, 12 h off) and room temperature was kept at ≈22 °C. They were given a standard diet and water ad libitum. All the surgical procedures were performed under sterile conditions. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

2.2. Experimental protocols

Rats were randomly divided into 10 groups. Nine groups of rats were subjected to ovariectomy and the other group underwent sham operation (Akishita et al., 1997). After a 4-day recovery period, six groups of ovariectomized rats were subcutaneously implanted with osmotic minipumps (Alzet 2002, 0.5 μl/h; Alza) prefilled with water-soluble 17β-estradiol (0.2, 1, 2, 10 or 20 μg/kg/day; Sigma) or its vehicle (2-hydroxypropyl-β-cyclodextrin; Sigma) under ether anesthesia. To compare the effect of estrogen with that of an angiotensin II type 1 (AT1) receptor blocker, candesartan, the remaining four groups of rats were subcutaneously implanted with an osmotic minipump containing the active metabolite of candesartan, candesartan cilexetil (2, 20 or 200 μg/kg/day; kindly donated by Takeda Chemical Industries, Tokyo) or its vehicle (0.9% saline).

Three days after minipump implantation, balloon injury was performed as previously described (Chen et al., 1996; Nakaoka et al., 1997). General anesthesia was induced by the administration of 90 mg/kg of ketamine intraperitoneally and 15 mg/kg of xylazine intramuscularly. The left carotid artery was exposed and its branches were ligated using 7–0 nylon. After intravenous injection of 75 U/kg of heparin, a portion of the external carotid artery and a portion of the internal carotid artery were cross-clipped using a microclip (2v-clip: S&T, Neuhausen, Switzerland). A 2F Fogarty embolectomy catheter (Baxter, Irvine, CA) was introduced into the artery via the external carotid artery. The common carotid artery was injured by six passes of an embolectomy catheter inflated with 0.2 ml of air. The portion proximal to the incision was ligated with 7–0 nylon, the cross-clip was released and the common carotid artery was reperfused.

2.3. Measurement of hormones and lipids

Blood sampling was performed at sacrifice, after a 16-h overnight fast, to measure serum concentrations of estradiol and progesterone, serum lipids and other biochemical parameters. Serum estradiol, estrone and progesterone concentrations were measured by sensitive radioimmunoassay (Hashimoto et al., 2002). Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein cholesterol concentration was measured by heparin-Ca^{2+} Ni^{2+} precipitation method (Hashimoto et al., 2002).

2.4. Morphometrical analysis of the balloon-injured carotid artery

A portion of the left common carotid artery was harvested at 14 days after balloon injury. The artery was perfusion- and pressure-fixed at 100 mm Hg using 10% neutral formalin buffer and then paraffin-embedded. Five round cross-sections per 1.5-cm length of artery specimens were stained with *Elastica van Gieson staining*, and photographed. Cross-sectional areas of the intima and the media were measured using an image analyzing software package (Scion Image, shared NIH software). The average of five sections was used for analysis as the value of each animal.

2.5. Data analysis

Values are expressed as mean±S.E.M. in the text, table and figures. Data were analyzed by one-factor analysis of variance (ANOVA) followed by Newman–Keuls' multiple comparison test. Differences with a value of $P<0.05$ were considered statistically significant.

3. Results

Sixty-five rats were set up and allocated to each group. Four rats were excluded because of failure of intervention. Estrogen replacement in ovariectomized rats increased serum concentration of estradiol dose-dependently, and replacement of 2 μg/kg/day estradiol achieved a concentration comparable to that in sham-operated rats (Table 1). In all groups, the serum concentration of estrone was below the detection limit (data not shown) and that of progesterone was unchanged. With respect to the lipid profile, the concentration of total cholesterol, triglyceride and high-density lipoprotain (HDL) cholesterol were increased in rats

Table 1
Blood pressure, serum lipids, plasma hormone concentrations and body and uterus weight after balloon injury of left carotid arteries of female Wistar rats

	Sham	Ovariectomy+17β-estradiol (μg/kg/day)						Ovariectomy+TCV-116 (μg/kg/day)		
		0	0.2	1	2	10	20	0	2	20
No. of rats	7	10	6	4	8	6	5	4	4	4
SBP (mm Hg)	121±4	113±7	123±2	120±5	127±2	121±4	121±4	121±7	122±7	116±8
T.chol (mg/dl)	76±9	75±5	86±4	78±10	84±6	96±5[a]	113±3[b]	79±2	89±4	81±8
HDL-C (mg/dl)	20±2	21±3	20±2	16±3	23±2	27±1	30±1[a]	17±2	21±2	22±2
Triglyceride (mg/dl)	41±6	53±8	46±9	64±16	91±13[a]	87±10[a]	153±31[b]	64±11	25±6	35±10
Estradiol (pg/ml)	19±4[b]	8±1	9±1	12±2	20±2[b]	54±5[b]	96±3[b]	11±3	11±1	14±2
Progesterone (ng/ml)	20±5	13±2	6±3	21±5	9±2	11±3	5±2	16±4	21±6	15±6
Body weight (g)	269±6	282±8	281±8	260±6	264±6	257±5[a]	263±7	285±10	290±5	290±3
Uterus (mg)	661±102[b]	174±29	321±23	577±46[b]	511±76[b]	–	–	148±22	149±5	156±7

Values are expressed as mean±S.E.M. SBP, systolic blood pressure; T.chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; –, not examined.
[a] $P<0.05$ vs. OVX+0 μg/kg/day of 17β-estradiol.
[b] $P<0.01$ vs. OVX+0 μg/kg/day of 17β-estradiol.

receiving higher doses of estrogen, as previously reported (Gades et al., 1998; Joles et al., 1998; Tomiyoshi et al., 2002), whereas those were unchanged in rats receiving 2 μg/kg/day or a lower dose of estrogen. The body weight of rats treated with higher doses was significantly lower than that in rats without estrogen replacement. In contrast, uterine weight in rats receiving lower doses of estrogen was greater than that in rats without estrogen.

Morphometric analysis showed that the neointimal area of the carotid artery was dose-dependently decreased by estrogen replacement (Figs. 1 and 2). As shown in Fig. 2, neointimal formation was sufficiently attenuated even in rats treated with 0.2 μg/kg/day of estradiol compared to that in ovariectomized rats without estrogen replacement. The inhibitory effect of estrogen on neointimal formation was compared with that of candesartan because the effects of AT1 receptor blockers including candesartan have been established (Kim et al., 2002; Liu et al., 2002; Nozawa et al., 1999; Tazawa et al., 1999). The effect of 20 μg/kg/day estradiol was more potent than that of subdepressor dose of candesartan (20 μg/kg/day) and was as potent as that of 200 μg/kg/day candesartan; a dose that lowered blood pressure and body weight as well as neointimal formation (intima/media ratio was 0.66±0.07, data not shown). Importantly, the effect of 2 μg/kg/day or a lower dose of estradiol on neointima formation was comparable to that of 20 μg/kg/day candesartan (Fig. 2). Medial area was not different among all groups of rats. Small non-significant differences in several measurements between the control for estrogen and that for candesartan were likely to be due

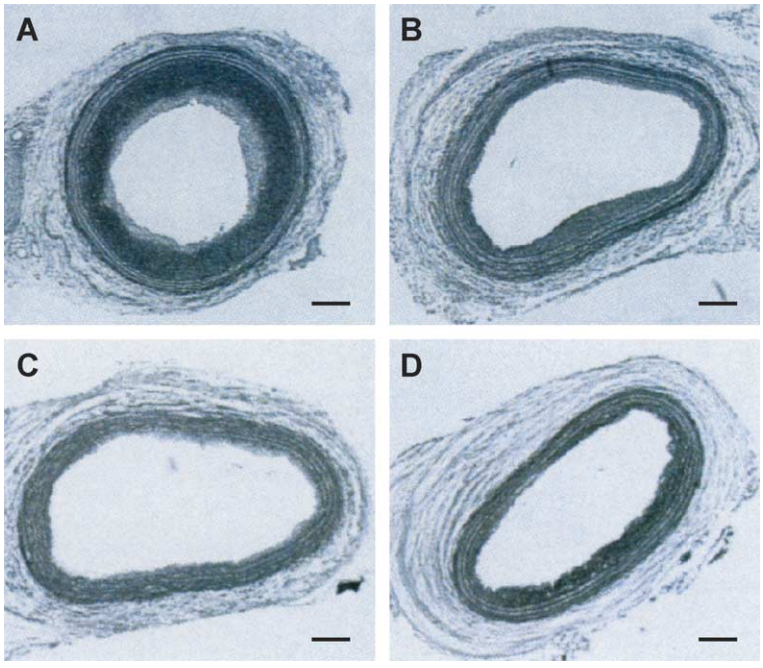

Fig. 1. Representative cross-sections of the rat carotid artery 2 weeks after balloon injury (elastica van gieson staining, magnification ×100). Rats were treated with 20% cyclodextrin vehicle (A), 0.2 μg/kg/day of 17-β estradiol (B), 20 μg/kg/day of 17-β estradiol (C) and 20 μg/kg/day of candesartan (D). Bars: 100 μm.

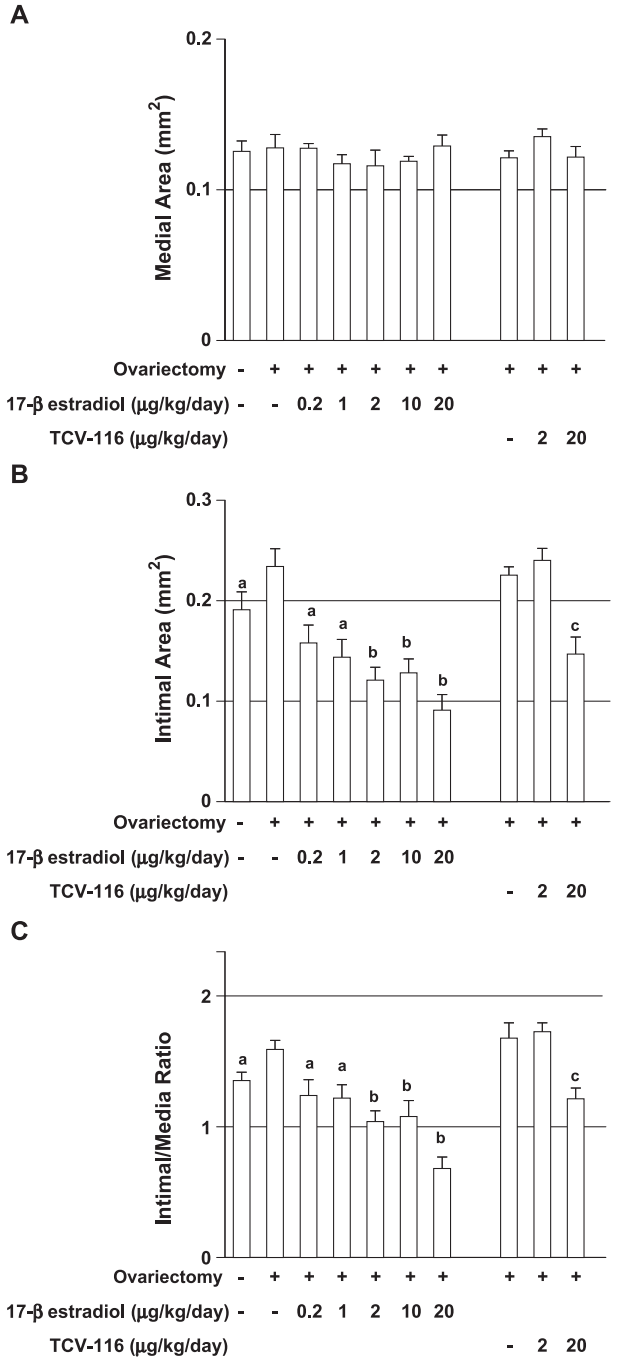

Fig. 2. Morphometric analyses of intimal area (A), medial area (B) and intima/media area ratio (C) in the carotid artery 2 weeks after balloon injury. The results are expressed as mean±S.E.M. [a]$P<0.05$, [b]$P<0.01$ vs. ovariectomized rats without 17-β estradiol, [c]$P<0.01$ vs. ovariectomized rats without candesartan.

to the variation of the measurements rather than the effect of vehicle for each group.

4. Discussion

This study showed that subcutaneous administration of 2 μg/kg/day or lower doses of estradiol inhibited neointimal formation after vascular injury with minimal adverse effects on the uterus and lipid metabolism, suggesting the efficacy of lower doses of hormone replacement therapy for the prevention of atherosclerosis.

Estrogen has been reported to inhibit neointimal formation after vascular injury in rodents using balloon angioplasty of the rat carotid artery (Bakir et al., 2000; Chen et al., 1996; Oparil et al., 1997, 1999), cuff placement around the rat femoral artery (Akishita et al., 1997) and ligation of the mouse carotid artery (Tolbert et al., 2001). Oparil and her colleagues have shown using the rat carotid balloon-injury model that subcutaneous administration of 20 μg/kg/day estradiol reduced neointimal formation by more than 50% compared to that without estradiol treatment (Chen et al., 1996; Oparil et al., 1997, 1999; Bakir et al., 2000). In their studies, plasma estradiol levels in estrogen-replaced rats (135.0±5.7 pg/ml, Chen et al., 1996, or 32.0±4.8 pg/ml, Bakir et al., 2000) were higher than those in intact female rats (51.9±5.8 pg/ml, Chen et al., 1996, or 25±6.9 pg/ml, Bakir et al., 2000). In the present study, administration of 10 or 20 μg/kg/day estradiol in ovariectomized rats inhibited neointimal formation with the increased plasma estradiol concentration beyond that in sham-operated rats as well. These results suggest that the estradiol doses used in the previous studies (>10 μg/kg/day) may be relatively high although plasma estradiol concentration fluctuates in rats with the estrous cycle (ranged from 16±2 to 39±7 pg/ml, Anisimov and Okulov, 1980, or from 1±1 to 44±15 pg/ml, Hawkins et al., 1975), and changes with development and age (Meijs-Roelofs et al., 1975). In contrast, replacement of 2 μg/kg/day estradiol achieved serum estradiol concentrations comparable to those in sham-operated rats in the present study. Replacement of 1 μg/kg/day or a lower dose of estradiol did not increase the serum estradiol concentration. However, the inhibition of neointimal formation was significant at the lower doses and was comparable to the effect of 20 μg/kg/day of candesartan (Fig. 2). Moreover, 1 μg/kg/day or a lower dose of estradiol did not increase the serum triglyceride concentration, and 0.2 μg/kg/day of estradiol caused the minimal and non-significant increase of uterus weight. This could be a new finding with respect to the adverse effects on lipid profiles and uterus. Taken these findings together, a local effect of estrogen replacement on organs or cells was observed even if circulating estrogen was not elevated, providing some hints on determining the dose of hormone replacement therapy.

In the present study, we did not demonstrate the mechanisms by which estrogen inhibited neointimal formation. Previous reports have shown that re-endothelialization (White et al., 1997), preservation of endothelial survival (Sudoh et al., 2001) and function (White et al., 1997), inhibition of smooth muscle cell proliferation (Akishita et al., 1997) and inhibition of fibroblast proliferation and differentiation in the adventitia (Oparil et al., 1999) contribute to the effect of estrogen on the response to

vascular injury. Stimulation of nitric oxide synthesis as well as modulation of other vasoactive substances has been implicated in these effects, although activation of endothelial nitric oxide synthase may play a major role (Chambliss and Shaul, 2002). Further investigation is needed to elucidate the contribution and interaction of these factors in the effects of lower doses of estrogen on neointimal formation.

Recent randomized trials (Hulley et al., 1998; Rossouw et al., 2002) have suggested that hormone replacement therapy with the standard regimen should not be recommended for postmenopausal women. Improvement of the regimen, such as the dose, route (oral or subcutaneous) or schedule (continuous or cyclic), could resolve the adverse effects of hormone replacement therapy, although few data are currently available (Grodstein et al., 2000; Jick et al., 1996; Hashimoto et al., 2002; Wakatsuki et al., 2003, 2004). Direct comparisons of animal studies to clinical studies are inadequate because several major differences can be pointed including route of administration, duration of the treatment, cardiovascular risk profile of subjects and body fat distribution. However, our experimental result that lower doses of estrogen inhibited the response to vascular injury with relatively small adverse effects may imply the potential efficacy of low dose hormone replacement therapy in postmenopausal women.

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