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Vasoprotective effects of an endothelin receptor antagonist in ovariectomized female rats

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ABSTRACT

Aims: The effects of hormone replacement therapy with estrogen on cardiovascular disease in postmenopausal women are still controversial. In the present study, we examined the effects of an endothelin (ET) receptor antagonist (ERA) and/or angiotensin receptor blocker (ARB) on neointimal formation following vascular injury in ovariectomized (OVX) female rats.

Main methods: Female rats were divided into intact female and OVX groups. The right carotid artery was subjected to balloon injury, and harvested 2 weeks later.

Key findings: In the intact female groups, treatment with ARB (L-158809; 1 mg/kg/day) for two weeks after the injury significantly decreased neointimal formation, whereas treatment with the ERA (J-104132; 10 mg/kg/day) did not affect neointimal formation. On the other hand, the ERA markedly decreased neointimal formation after the injury in the OVX groups; however, neointimal formation was not significantly improved by the ARB treatment. In addition, the combined treatment with 17β-estradiol (20 μg/kg/day) or the ERA and ARB markedly suppressed neointimal formation after the balloon injury in the OVX groups, whereas no combinational effects were observed due to the combined treatment with 17β-estradiol and the ERA.

Significance: These results suggest that ERAs have estrogen-like vasoprotective effects on neointimal formation following balloon injury in OVX rats. ERAs may be useful as an alternative therapy to prevent vascular disease in postmenopausal women.

Introduction

The incidence of cardiovascular disease has been shown to be lower in women prior to menopause than in men and postmenopausal women (Mahendru and Morris, 2013; Maranon and Reckelhoff, 2013). The mechanisms underlying these lower risks in premenopausal women have not been elucidated completely, but can at least be partly explained by the vasoprotective effects of estrogen (Bernelot Moens et al., 2012; Farhat et al., 1996; Pare et al., 2002). Previous studies showed that estrogen increased endothelial nitric oxide production and suppressed smooth muscle proliferation/migration, oxidative stress, and inflammation in the vessels (Florian et al., 2004; Miller et al., 2003; Tolbert et al., 2001). Several clinical studies reported the beneficial effects of estrogen replacement therapy (ERT) on the risk of cardiovascular diseases in postmenopausal women (Grady et al., 1992; Walsh et al., 1991). In contrast, other clinical trials, including the Heart and Estrogen–Progestin Replacement Study (HERS) and Women's Health Initiative (WHI) Clinical Trial and observational study, found no beneficial effects of ERT (Hulley et al., 1998; Rossouw et al., 2002). Thus, the efficacy of ERT on cardiovascular disease is still controversial in clinical settings. Elucidating the mechanisms of estrogen-induced vasoprotective effects and alternative estrogen therapies in postmenopausal women in more detail remains a critical issue.

Endothelin (ET)-1 exhibits potent vasoconstriction and mitogenic effects on vascular smooth muscle cells (VSMCs) (Kirchengast and Munter, 1998; Kitada et al., in press; Ohkita et al., 2012). The ET-1/ET receptor system-induced proliferation of VSMCs and neointimal formation are known to be involved in the development of vascular diseases such as atherosclerosis, restenosis, and hypertension or diabetes-induced arterial hypertrophy; therefore, endothelin receptor antagonists (ERAs) may be used as vasoprotective drugs to treat vascular diseases (Kirchengast and Munter, 1998; Kitada et al., in press).

Previous studies showed that the ET-1/ET receptor system is closely related to the sex differences associated with cardiovascular diseases (Kawanishi et al., 2007; Kitada et al., 2011; Lekontseva et al., 2010; Tostes et al., 2008). For example, plasma ET-1 concentrations were shown to be lower in women than in men and old women had higher plasma ET-1 levels than young women (Best et al., 1998; Maeda et al., 2003; Miyauchi et al., 1992). In addition, the administration of

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17β-estradiol has been suggested to decrease plasma ET-1 levels in postmenopausal women (Best et al., 1998). Moreover, previous studies reported that estrogen could regulate the production and function of ET-1 as well as ET receptor expression in the cardiovascular system (Dubey et al., 2001; Kitada et al., in press; Lekontseva et al., 2010; Pedersen et al., 2008; Tostes et al., 2008). We previously demonstrated that ET receptor-mediated ET-1 actions were stronger in the vascular lesion sites of males than in those of intact females (Kitada et al., 2011). These results indicate that the inhibitory effects of estrogen on the ET system contribute to the mechanisms underlying the sex differences associated with cardiovascular diseases. Thus, the vascular ET-1/ET receptor system may be augmented and contribute to the increase in the incidence of cardiovascular events in postmenopausal women because of estrogen deficiencies. Taken together, ERAs may be a new target to replace estrogen and become an effective therapy for reducing the risk of cardiovascular diseases after menopause.

In the present study, we hypothesized that an ERA may effectively prevent the development of vascular lesions in OVX female rats as a replacement for estrogen. To confirm this hypothesis, we examined the vasoprotective effects of an ERA on balloon injury-induced neointimal formation in intact and ovariectomized (OVX) female rats and compared these effects with those of angiotensin receptor blockade (ARB), which is one of the key vasoprotective drugs used in clinical cases (Hernandez Schulman et al., 2007; Nakashima et al., 2006).

Materials and methods

Animals

Animals were housed in a light-controlled room with a 12-hour light/dark cycle and were allowed *ad libitum* access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences, and all studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocols

Female Sprague–Dawley (SD) rats (9 weeks old, Japan SLC, Shizuoka, Japan) were divided into intact female and OVX groups. Ovariectomy or sham surgery was performed under anesthesia using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Rats underwent balloon injury to the right carotid artery one week after surgery. Some intact female and OVX rats were gavaged with J-104132 (an ET_A/ET_B dual receptor antagonist: 10 mg/kg/day), (+)-(5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl)cyclopenteno[1,2-b]pyridine-6-carboxylic acid, and/or L-158809 (an AT_1 receptor antagonist: 1 mg/kg/day), 5,7-dimethyl-(2-ethyl-3-[[2′-(1H-tetrazol-5-yl)[1,1′]-biphenyl-4-yl]methyl]-3H-imidazo[4,5-b] pyridine, for 2 weeks starting 12 h after the balloon injury. Furthermore, some OVX rats were treated daily with the subcutaneous administration of 17β-estradiol (20 μg/kg/day) and/or ICI 182,780 (an estrogen receptor inhibitor: 5 mg/kg/day) starting 24 h before the balloon injury, with or without J-104132 or L-158809. The administration of J-104132, L-158809, 17β-estradiol, ICI 182,780, or vehicle was continued until 2 weeks after the balloon injury. The doses of these drugs were determined based on previous studies (Bakir et al., 2000; Huckle et al., 1996; Kitada et al., 2009, 2011). We also determined the effects of these doses of J-104132 and L-158809 in male rats in separate experiments. Relative to the vehicle treatment, J-104132 or L-158809 significantly suppressed the neointima/media ratio to the same extent (0.71 ± 0.09 vs 0.42 ± 0.06 or 0.46 ± 0.06, respectively, $P < 0.05$, $n = 4$ per group). We used the following 12 groups in this study.

1) intact + vehicle
2) intact + ERA
3) intact + ARB
4) OVX + vehicle
5) OVX + ERA
6) OVX + ERA + ICI 182,780
7) OVX + ARB
8) OVX + 17β-estradiol
9) OVX + 17β-estradiol + ICI 182,780
10) OVX + 17β-estradiol + ERA
11) OVX + 17β-estradiol + ARB
12) OVX + ERA + ARB

Balloon injury model

Rats were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), and the right carotid artery was injured with a 2 F Fogarty balloon catheter (Baxter International, Deerfield, IL, USA), as described previously (Kurumazuka et al., 2006). The left carotid artery was not damaged. Rats were sacrificed 2 days and 2 weeks after the balloon injury with a sodium pentobarbital overdose (75 mg/kg), and both the left and right carotid arteries were harvested.

Morphometric analysis

The bilateral carotid arteries 2 weeks after the balloon injury were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick sections. Tissue sections were stained using the Elastica Van Gieson method. Morphometric analysis of each arterial segment was performed with a computer-based Motic Image Plus 2.0 Morphometric system (Shimadzu, Kyoto, Japan). The border of the lumen, internal elastic lamina, and external elastic lamina were traced and the neointimal and medial areas were measured. The ratio of the neointimal to medial area (neointima/media ratio) was calculated by dividing the neointimal area by the medial area.

NADPH-dependent superoxide production

NADPH oxidase activity was measured based on the degree of NADPH-dependent superoxide production in the isolated carotid arteries, as assessed by a lucigenin-enhanced assay. We examined NADPH-dependent superoxide production 2 days after the balloon injury based on our previous study (Kurumazuka et al., 2006). Briefly, both injured (right) and uninjured (left) common carotid arteries were cleared of adherent adipose and loose connective tissue *in situ* and were harvested in ice-cold modified Krebs–HEPES buffer containing (in mmol/L): NaCl 99.01, HEPES 20, KCl 4.69, $MgSO_4$ 0.59, KH_2HPO_4 1.03, $NaHPO_4$ 25, $CaCl_2$ 1.41, and glucose 11.1 (pH 7.4). The tissues were then gently flushed with cold buffer to remove blood from the lumen and were cut into 3 segments. The segments were incubated with NADPH (100 μmol/L) in buffer at 37 °C for 15 min. Lucigenin-enhanced chemiluminescence was measured in 2 mL Krebs–HEPES buffer containing lucigenin (5 μmol/L) using a Berthold FB12 single-tube luminometer, modified to maintain a sample temperature of 37 °C. Chemiluminescence was measured continuously for 15 min after allowing for dark adaptation and was expressed as relative light units per minute per milligram vessel dry weight (RLU/min/mg).

Drugs

17β-Estradiol and ICI 182,780 were obtained from Nacalai Tesque (Kyoto, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively and dissolved in cottonseed oil. J-104132 and L-158809 were provided by Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan) and Merck & Co. Inc (Rahway, USA), respectively, and dissolved in distilled water. Other chemicals were purchased from Sigma Chemical Co., Nacalai Tesque (Kyoto, Japan), and Wako (Osaka, Japan).

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