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Divergent effects of estrogen and nicotine on Rho-kinase expression in human coronary vascular smooth muscle cells

Junko Hiroki, Hiroaki Shimokawa*, Yasushi Mukai, Toshihiro Ichiki, Akira Takeshita

Department of Cardiovascular Medicine, The 21st Century COE Program on Lifestyle-Related Diseases, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

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Abstract

Recent studies have demonstrated that up-regulated Rho-kinase plays an important role in the pathogenesis of coronary arteriosclerosis and vasospasm. We have shown that inflammatory stimuli, such as angiotensin II and interleukin-1β, up-regulate Rho-kinase expression and activity in human coronary vascular smooth muscle cells, for which intracellular signal transduction mediated by protein kinase C and NF-κB is involved. Here, we show that estrogen down-regulates while nicotine up-regulates Rho-kinase and that nicotine counteracts the inhibitory effect of estrogen on angiotensin II-induced Rho-kinase expression. Furthermore, we demonstrated that the intracellular signal transduction of the inhibitory effect of estrogen is mediated by an estrogen receptor. These results demonstrate that inflammatory stimuli up-regulate Rho-kinase, for which estrogen (mediated by an estrogen receptor) and nicotine exert divergent inhibitory and stimulatory effects on the Rho-kinase expression, respectively, and may explain in part why the incidence of arteriosclerotic and vasospastic disorders is increased in postmenopausal women and smokers.

Keywords: Rho-kinase; Nicotine; Estrogen; Estrogen receptor; Vascular smooth muscle; Coronary arteriosclerosis

Recent studies in vitro have demonstrated that the small GTP-binding protein Rho and its effector Rho-kinase/ROK/ROCK [1–3] play an important role in various cellular functions, including smooth muscle contraction [4,5], actin cytoskeleton organization [6,7], cell adhesion and motility [8], cytokinesis [9], and gene expressions [10], all of which may be involved in the pathogenesis of arteriosclerosis. There are two isoforms of Rho-kinase, Rho-kinase/ROKα/ROCKII and ROKβ/ROCKI [2,3,11]. Hereafter, we will refer to them as Rho-kinase α and Rho-kinase β, respectively. Recently, we have demonstrated that Rho-kinase is upregulated at inflammatory arteriosclerotic lesions and causes coronary vasospastic responses through inhibition of myosin light chain phosphatase in both a porcine model of coronary artery spasm [12,13] and arteriosclerotic human arteries [14]. We have also demonstrated that Rho-kinase is substantially involved in the pathogenesis of arteriosclerosis in general [15]. Furthermore, the long-term inhibition of Rho-kinase causes a marked regression of coronary arteriosclerosis and disappearance of coronary vasospastic activities in vivo [16,17]. These results suggest that Rho-kinase is a novel therapeutic target for the treatment of arteriosclerotic cardiovascular diseases [15]. Furthermore, we have recently demonstrated that inflammatory stimuli, such as angiotensin II and interleukin-1β, up-regulate Rho-kinase expression and activity in human coronary vascular smooth muscle cells (hcVSMC), for which intracellular signal transduction mediated by protein kinase C (PKC) and NF-κB is involved [18]. Indeed, coronary vascular lesion formation induced by long-term

* Corresponding author. Fax: +81 92 642 5374.
E-mail address: shimo@cardiol.med.kyushu-u.ac.jp (H. Shimokawa).

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treatment with angiotensin II was markedly suppressed in NF-κB-deficient mice with reduced expression and activity of Rho-kinase in vivo [18]. Thus, our series of studies in animal models and humans suggest that the Rho-kinase expression is linked to inflammatory responses [12–18].

It is well known that premenopausal women with normal estrogen levels rarely manifest coronary artery disease. In addition, the administration of exogenous estrogens to healthy postmenopausal women may reduce the incidence of coronary events [19]. However, the mechanisms underlying the beneficial effects of estrogen remain to be examined. The modest cholesterol-lowering effect of estrogens does not explain their substantial protective effects [20]. An alternative hypothesis is that estrogens exert protective effects against coronary artery disease through genomic mechanisms in blood vessels. Estrogens inhibit the growth of vascular smooth muscle cells (VSMC) that characterizes obstructive atherosclerotic lesions [21,22]. Functional estrogen receptors (ERs) are present in VSMC [23]. Furthermore, in addition to menopause, cigarette smoking also is a major risk factor for coronary artery disease [24,25]. Although the underlying mechanisms for the adverse effects of smoking are not completely understood, there is substantial evidence indicating that the primary role of cigarette smoking in coronary artery disease is due in part to vascular injury by its direct cytotoxicity, leading to endothelial and VSMC dysfunction, and thereby initiating the process of coronary atherosclerosis [26–28].

Thus, the present study was designed to examine how estrogens and nicotine modulate Rho-kinase expression in human coronary VSMC (hcVSMC).

Materials and methods

This experiment was reviewed by the Committee of Ethics on Animal Experiments of the Kyushu University and was carried out according to the Guidelines for Animal Experiments of the Kyushu University, and the Law (No.105) and the Notification (No.6) of the Japanese Government.

Reagents. The reagents used in this study were as follows: angiotensin II (the Peptide Institute, Osaka, Japan); PD98059 (Research Biochemicals International, Natick, USA), [α-^{32}P]dCTP (DuPont NEN, Boston, USA), ICI182780 (Tocris Cookson, Ballwin, USA), and PD123319 (Takeda Chemical Industries, Osaka, Japan). Other reagents were purchased from WAKO Pure Chemicals (Osaka, Japan).

Cell culture. hcVSMC were obtained from Amersham (Piscataway, USA) and maintained as previously described [18,29]. Passages 4–10 were used for the experiments. Cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), growth-arrested in DMEM with 0.1% bovine serum albumin (BSA) for 1 day, and used for the experiments.

Northern blot analysis. Total RNA was prepared by an acid guanidinium–phenol–chloroform extraction method [30]. Since we confirmed in the previous study that Rho-kinase α is a major isoform in hcVSMC as compared with Rho-kinase β [18], we examined the expression of Rho-kinase α in the present study. The sequence of the primer for RT-PCR analysis of human Rho-kinase α was amplified from a human blood cDNA library. We obtained direct purification products of DNA from these PCR amplifications used by the Wizard PCR Preps DNA purification system (Promega, Madison, USA). A human Rho-kinase α cDNA was used as a probe. Northern blot analysis (with 10 μg total RNA) was performed as previously described [18,29]. The radioactivity of hybridized bands of Rho-kinase α mRNA and α-actin mRNA was quantified by a MacBAS Bioimage Analyzer (Fuji film, Tokyo, Japan). For quantitative analysis, the density of the bands was measured by an NIH image analyzer, and the levels of Northern products for Rho-kinase α were normalized to those for α-actin as an internal control [18,29].

Statistical analysis. Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc test. Results are shown as means ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Estrogen concentration-dependently inhibits Rho-kinase mRNA expression in cultured human coronary VSMC

It is well known that the incidence of coronary arteriosclerosis and vasospasm is increased in postmenopausal women [17]. Furthermore, our series of studies in animal models and humans have suggested that the Rho-kinase expression is linked to inflammatory responses at coronary atherosclerotic lesions [12–18]. Accordingly, we examined how estrogen modulates Rho-kinase expression in hcVSMC. We have previously demonstrated that angiotensin II and IL-1β up-regulate Rho-kinase mRNA expression in a time- and concentration-dependent manner, with a peak response noted at 0.5 and 1 h after stimulation with angiotensin II and IL-1β, respectively [18]. Thus, in the present study, hcVSMC were treated for 24 h with 17β-estradiol before the treatment with either angiotensin II or IL-1β and the Rho-kinase mRNA expression was examined at 0.5 and 1 h after the stimulation with angiotensin II and IL-1β, respectively. The results showed that the Rho-kinase α mRNA expression induced by angiotensin II or IL-1β was concentration-dependently inhibited by 17β-estradiol at its concentration in premenopausal (10^{-7} mol/L) and pregnant (10^{-4} mol/L) women (Figs. 1A and B). Considerable clinical information has supported the notion that estrogens exert protective effects against atherosclerosis in general and coronary artery disease in particular [19,20]. Although the relationship between estrogen deficiency and vascular disease is poorly understood, estrogens may have genomic effects on the vascular wall, which are probably mediated through estrogen receptors. These effects include the inhibition of VSMC growth and migration in vitro, and the inhibition of arterial intimal hyperplasia in vivo [21,22]. It has been reported that the newly discovered estrogen receptor β subtype is the most prevalent receptor in human VSMC, which may explain, at least in part, the differences in the

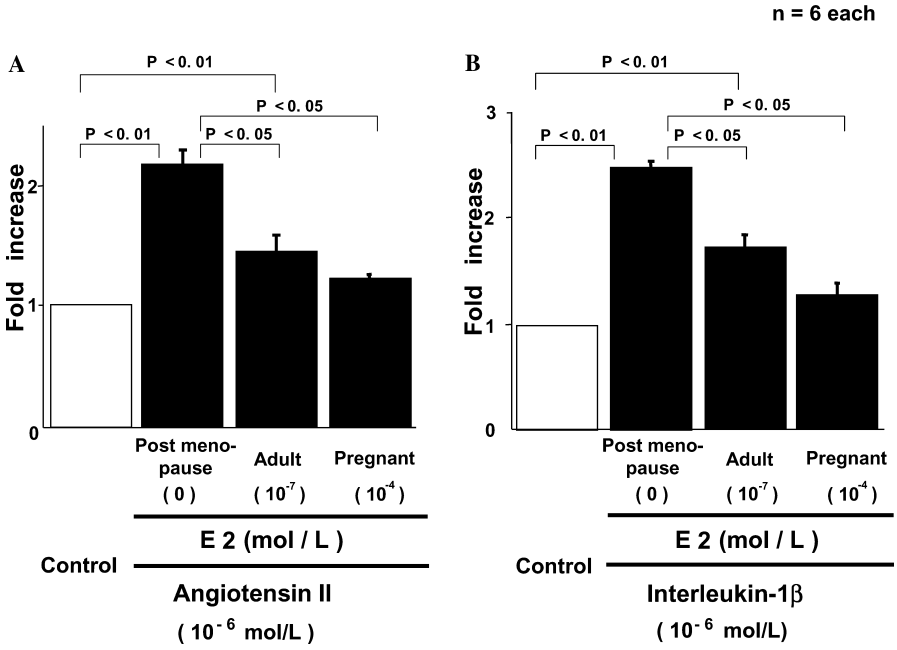

Fig. 1. Estrogen concentration-dependently inhibits Rho-kinase mRNA expression in cultured human coronary VSMC. (A) The Rho-kinase α mRNA expression induced by angiotensin II (10^{-6} mol/L, for 0.5 h) was inhibited by 17β-estradiol (E2) at its physiological concentration in premenopausal (10^{-7} mol/L, adult) and pregnant (10^{-4} mol/L, pregnant) women. (B) The Rho-kinase α mRNA expression induced by IL-1β (10^{-6} mol/L, for 1 h) was inhibited by 17β-estradiol (E2) at its physiological concentration in premenopausal (10^{-7} mol/L, adult) and pregnant (10^{-4} mol/L, pregnant) women, $n = 6$ each.

effects of estrogens between the vascular bed and other organs [31].

Nicotine up-regulates Rho-kinase mRNA expression in cultured human coronary VSMC

Cigarette smoking is a major risk for coronary artery disease [24,25]. Accordingly, we examined how nicotine modulates Rho-kinase expression in hcVSMC. hcVSMC were pretreated for 24 h with and without nicotine (10^{-7} mol/L, an average concentration in smokers) for 24 h and thereafter were stimulated with angiotensin II (10^{-10}–10^{-8} mol/L). The Rho-kinase mRNA expression was examined 30 min after the stimulation with angiotensin II. The results showed that nicotine significantly enhanced the angiotensin II-induced Rho-kinase mRNA expression (Fig. 2). It is noteworthy that the Rho-kinase mRNA expression induced by a low concentration of angiotensin II (10^{-8} mol/L, Fig. 2) in the presence of nicotine was greater than that induced by a higher concentration of angiotensin II alone (10^{-6} mol/L) [18], indicating that nicotine enhances the angiotensin II-induced Rho-kinase mRNA expression by more than 100 times.

Cigarette smoking has been implicated as a major risk factor for atherosclerosis and related cardiovascular diseases. The mechanism for the nicotine-induced increase in the risk of cardiovascular diseases is not well understood. It has been postulated that tobacco smoke

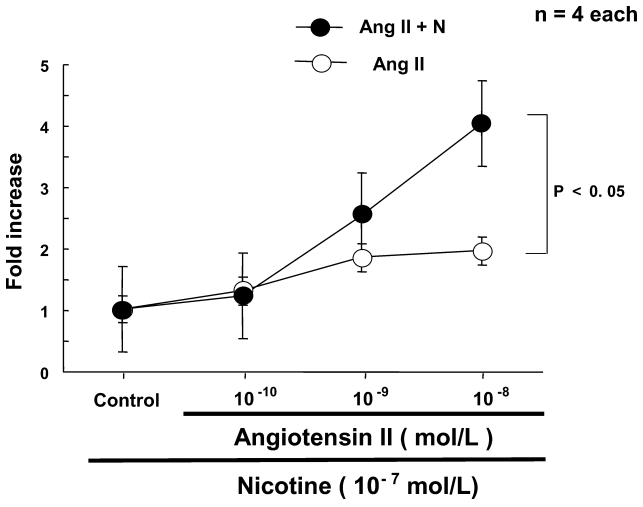

Fig. 2. Nicotine up-regulates Rho-kinase mRNA expression in cultured human coronary VSMC. Nicotine, at its average concentration in smokers (10^{-7} mol/L, for 24 h), markedly enhanced the Rho-kinase α mRNA expression induced by low doses of angiotensin II (for 0.5 h), $n = 4$ each.

constituents may adversely affect cell function but the true mediator(s) remains to be elucidated. Endothelin-1 is a potent vasoconstrictor and mitogenic agent [32]. In humans, cigarette smoking results in a significant increase in plasma endothelin-1 levels [33], and regular cigarette smoking is associated with functional and morphological changes [34–37]. PKC has been impli-

cated in the regulation of endothelin-1 production by endothelial cells, and recent studies have shown that cigarette smoke condensate activates PKC in endothelial cells [38] and that the extract stimulates endothelin-1 gene expression via PKC-mediated pathway in endothelial cells [39].

Estrogen inhibits Rho-kinase mRNA expression via the estrogen receptor in cultured human coronary VSMC

We have shown that estrogen inhibits Rho-kinase mRNA expression induced by angiotensin II. To examine whether the effect of estrogen is genomic or non-genomic, we used the estrogen receptor antagonist, ICI 182,780. hcVSMC were treated for 24 h with 10^{-7} mol/L ICI 182,780 before the treatment with estrogen followed by that with angiotensin II. ICI 182,780 reversed the inhibitory effect of estrogen, indicating that the effect of estrogen was mediated by estrogen receptors (Fig. 3). Importantly, nicotine also completely reversed the inhibitory effect of estrogen (Fig. 3). These results may explain, at least in part, why the incidence of coronary arteriosclerosis and vasospasm is increased in postmenopausal women and smokers.

The molecular mechanism for the inhibitory effect of estrogen on the angiotensin II-induced up-regulation of Rho-kinase remains to be fully elucidated. ICI 182,780 can reverse the inhibitory effect of estrogens on VSMC growth in vitro [22]. This observation supports the notion that the effects of estrogens on VSMC are primarily mediated through classic estrogen receptor-dependent transcriptional mechanisms. It has been previously demonstrated that a selective PKC inhibitor can suppress the actions of estrogens [40], suggesting an involvement of the PKC-mediated pathway in the effect of estrogens. Furthermore, it has been reported that contraction and PKC activity of rat VSMC were greater in males compared with females, suggesting an involvement of the PKC-mediated pathway in the gender differences in VSMC contraction [41]. There is a gender difference in the sensitivity of the PKC-mediated pathway to endogenous sex hormones in VSMC [42]. A gender-specific reduction in VSMC reactivity in female rats with intact gonadal function compared with males is associated with a reduction in the expression and activity of vascular PKC [43]. On the other hand, it also has been reported that activation of estrogen receptor α inhibits NF-κB activation and VSMC proliferation in aged female rats, demonstrating the importance of classical estrogen receptor α in regulating inflammatory and growth-promoting genes implicated in atherogenesis [44].

In summary, we were able to demonstrate that estrogen and nicotine exert divergent effect on Rho-kinase expression in hcVSMC, which may explain, at least in part, why the incidence of arteriosclerotic and vasospastic disorders is increased in postmenopausal women and smokers.

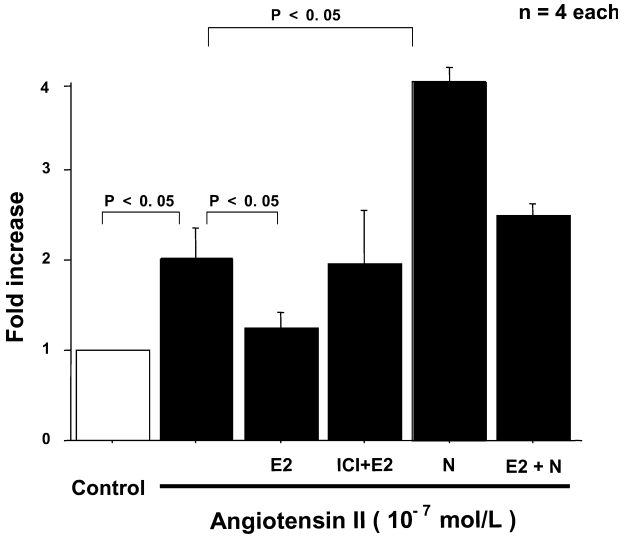

Fig. 3. Estrogen inhibits Rho-kinase mRNA expression via the estrogen receptor in cultured human coronary VSMC. The cells were treated with ICI 182,780 (10^{-6} mol/L, for 24 h, ICI) before the treatment with estrogen (E2, 10^{-7} mol/L, for 24 h). ICI 182,780 reversed the inhibitory effect of estrogen, indicating that the effect of estrogen was mediated by estrogen receptors. Furthermore, nicotine (N, 10^{-7} mol/L, for 24 h) completely reversed the inhibitory effect of estrogen, $n = 4$ each.

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