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Effects of Raloxifene on Caveolin-1 mRNA and Protein Expressions in Vascular Smooth Muscle Cells

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Abstract Caveolin-1 is regulated by estrogen in vascular smooth muscle cells. Raloxifene, a selective estrogen receptor modulator that possibly has cardioprotective properties without an increased risk of cancer or other side effects of estrogen, may be used in women with risk of coronary artery disease. However, the relationship between raloxifene and caveolin-1 is still unknown. Therefore, this study was designed to see whether raloxifene regulates caveolin-1 expression and if so, whether such regulation is mediated by estrogen receptor. Rat aortic smooth muscle cells were cultured in the absence or presence of raloxifene (10^{-8} to 10^{-6} M) for 12 or 24 h. Both mRNA and protein levels of caveolin-1 were increased significantly after 24 h treatment with raloxifene. These increases were inhibited by estrogen receptor antagonist ICI 182780 (10^{-5} M). Results of this study suggest that raloxifene stimulates caveolin-1 transcription and translation through estrogen receptor mediated mechanisms.

Key words raloxifene; caveolin-1; estrogen receptor; vascular smooth muscle cell

Caveolae, the flask-shaped vesicular invaginations of the plasma membrane, are present in many cell types including vascular smooth muscle cells. Caveolae have been implicated to be important for cellular functions such as signaling, transport and proliferation [1–3]. The principal coat proteins of caveolae are the caveolins. Thus far, three distinct mammalian caveolin genes have been identified, and caveolin-1, a protein of 21–22 kDa, is so far the best biochemical marker for caveolae [4–6].

Women experience initial manifestations of coronary artery disease 10 years later than men, suggesting that estrogen might play a cardioprotective role. In vascular smooth muscle cells, estrogen stimulated the binding of ERα with caveolin-1 and augmented the production of caveolin-1 through a transcriptional mechanism [7]. Mice lacking the caveolin-1 gene show impaired endothelium-dependent relaxation, contractility and maintenance of myogenic tone of the aorta [3]. Thus, estrogen-mediated upregulation of caveolin-1 might be related to the improvement of vascular function [8].

Raloxifene belongs to a class of drugs recently described as selective estrogen receptor modulators. It binds to the estrogen receptor and shows tissue-specific effects such as estrogen agonist effects on bone and lipids, and estrogen antagonist effects on the breast and uterus. Because the side effects, especially the high risk of breast cancer, prohibit the use of estrogen as a chronic prophylactic therapy for many postmenopausal women who are at risk of cardiovascular disease, more tissue-specific agents, such as raloxifene, may provide a good alternative. Recent findings suggested that raloxifene also possesses beneficial effects on the cardiovascular system [9–12].

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However, there are no reports demonstrating the relationship between raloxifene and caveolin-1 in vascular smooth muscle cells. Hence, the present study was designed to determine the effects of raloxifene treatment on caveolin-1 expression in cultured rat aortic smooth muscle cells (RASMCs).

Materials and Methods

Chemicals

Raloxifene and 17β-estradiol were purchased from Sigma. ICI 182780 was bought from Tocris. Anti-caveolin-1 antibody and anti-β-actin antibody were purchased from Santa Cruz (Santa Cruz, USA). All other reagents and solvents used in this study were of analytical grade.

Cell culture

RASMCs were isolated from the aorta of 8-week-old female Wistar rats by enzymatic digestion, according to the method described previously [13]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol red-free DMEM were used to avoid contaminations of steroids and estrogen receptor agonist. Subcultured RASMCs (4–8 passages) were used in the following experiments.

RT-PCR analysis of caveolin-1 mRNA in RASMCs

RASMCs were seeded in 10 cm-culture dishes and grown to 80%–90% confluence. The medium was then replaced with phenol red-free DMEM containing vehicle, 17β-estrodial (10^{-7} M), raloxifene (10^{-8} to 10^{-6} M), or in combination with ICI 182780 (10^{-5} M). Experiments were terminated after 12 h or 24 h of incubation with the above agents. Cells were washed twice with phosphate-buffered saline (PBS) and homogenized immediately in Trizol reagent. Total RNA was extracted according to the manufacturer's instructions. RT-PCR was performed using the primers based on the rat caveolin-1 cDNA sequence. The sequences of primers for caveolin-1 were: 5′-GCATCCTCTTTCCTGCA-3′ (sense), 5′-TGGAATTAGCACGGCTGATG-3′ (antisense). β-actin was used as a control for the quantity of the RNA. The product of β-actin is a 275 bp fragment and the sequences of the primers were: 5′-CTACAATGAGCTGCGTGTGGC-3′ (sense) and 5′-CAGGTCCAGACGCAGGATGGC-3′ (antisense). The amplification was performed with denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 60 s for 31 cycles with an initial denaturation at 95 ºC for 5 min and a final extension of 5 min at 72 ºC. The PCR product was fractionated by size on 2% agarose gels and visualized with ethidium bromide.

Western blot analysis of caveolin-1 protein in RASMCs

The confluent RASMCs were exposed to phenol red-free DMEM-containing vehicle, 17β-estrodial (10^{-7} M), raloxifene (10^{-8} to 10^{-6} M) or in combination with ICI 182780 (10^{-5} M) for 12 h or 24 h. Cells were then rinsed twice with PBS and lysed in an ice-cold lyses buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4 and 0.02 mM leupeptin. The resulting lysates were cleared by centrifugation. The protein in the supernatant was quantified using Bradford assay. Total protein (40 μg) from each sample was subjected to 10% SDS-PAGE for 1.5 h and electroblotted onto nitrocellulose membranes for 3 h. The membrane was blocked for 6 h at room temperature in TBS containing 5% skimmed milk powder, followed by 2 h with the appropriate primary antibody (polyclonal rabbit anti-caveolin-1 polyclonal antibodies, 1:500; rabbit anti-β-actin polyclonal antibodies, 1:500). Afterwards, membranes were washed 4 times (15 min each time) in TBS and incubated with horseradish peroxidase labeled anti-rabbit IgG secondary antibody for another 2 h. Finally, the immunoblots were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB) solution. The relative expression levels of caveolin-1 (caveolin-1/β-actin) were measured using an imager in terms of the absorbance.

Statistical analysis

RT-PCR and Western blot experiments were carried out independently in samples prepared from a minimum of four separate cultures. Data were presented as mean±SEM. Analysis was performed with SPSS in Windows. Statistical comparison was determined by analysis of variance. $P<0.05$ was considered as being significantly different.

Results

Effects of raloxifene and 17β-estrodial on caveolin-1 expression

Compared with controls, the mRNA and protein

expressions of caveolin-1 were upregulated significantly by raloxifene (10^{-8} to 10^{-6} M), as well as 17β-estrodial (10^{-7} M) after 24 h treatment. No significant difference could be found between 17β-estrodial and raloxifene, and no dose-dependent response effect of raloxifene could be found (**Figs. 1** and **2**).

Time- and dose-dependent responses of raloxifene

In order to assess the effects of raloxifene on caveolin-1 fully, further experiments were performed. As shown in **Figs. 3** and **4**, the expression of caveolin-1 mRNA was increased after 24 h treatment of cells with raloxifene. The increase in mRNA coincided with a statistically significant increase in protein expression. Both caveolin-1 mRNA and protein were observed to increase with the low dose of raloxifene (10^{-8} M) and not to increase further with high concentration of raloxifene (10^{-6} M). Treatment with raloxifene for 12 h did not significantly alter caveolin-1 mRNA and protein expressions (**Figs. 3** and **4**).

Influence of ICI 182780 on the effect of raloxifene

The pure estrogen receptor antagonist ICI 182780 (10^{-5} M) inhibited the increase of caveolin-1 mRNA and protein expressions in response to raloxifene treatment for 24 h

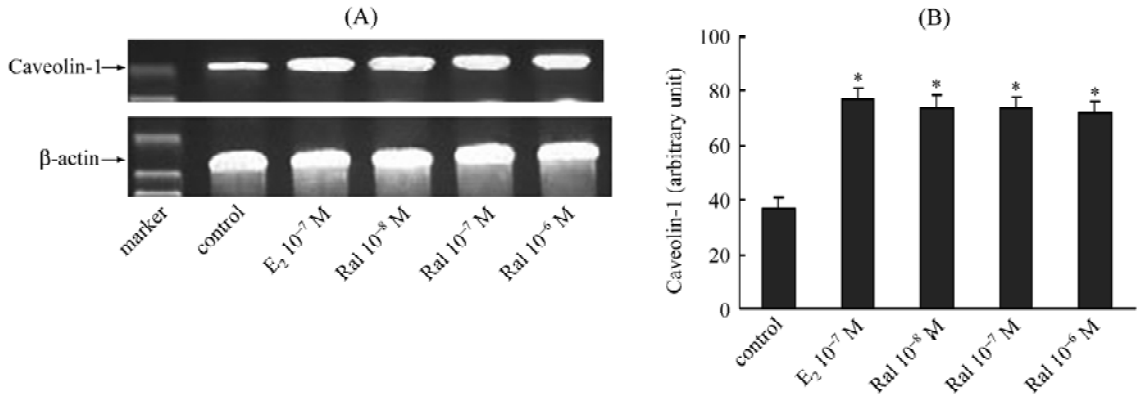

Fig. 1 RT-PCR analysis of effects of raloxifene and 17β-estrodial on caveolin-1 mRNA expression in rat aortic smooth muscle cells (RASMCs)

(A) Compared with control, caveolin-1 mRNA expression was increased significantly after 24 h treatment of 17β-estrodial (10^{-7} M), raloxifene (10^{-8} to 10^{-6} M), and no dose-dependent response could be found. (B) Densitometric value of caveolin-1 mRNA expression in RASMCs. **P*<0.05 compared with control, *n*=4. Ral, raloxifene; E_2, 17β-estrodial.

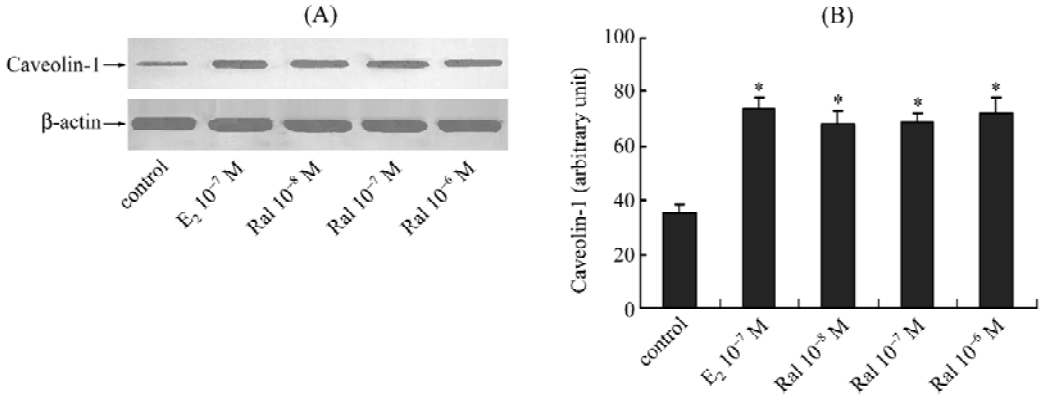

Fig. 2 Western blot analysis of caveolin-1 in rat aortic smooth muscle cells (RASMCs) in response to 17β-estrodial and raloxifene treatments

(A) Caveolin-1 protein expression increased significantly after 24 h treatment of 17β-estrodial (10^{-7} M) and raloxifene (10^{-8} to 10^{-6} M), and no significant difference could be found among different concentrations of raloxifene. (B) Densitometric analysis of caveolin-1 protein in RASMCs. **P*<0.05 compared with control, *n*=4. Ral, raloxifene; E_2, 17β-estrodial.

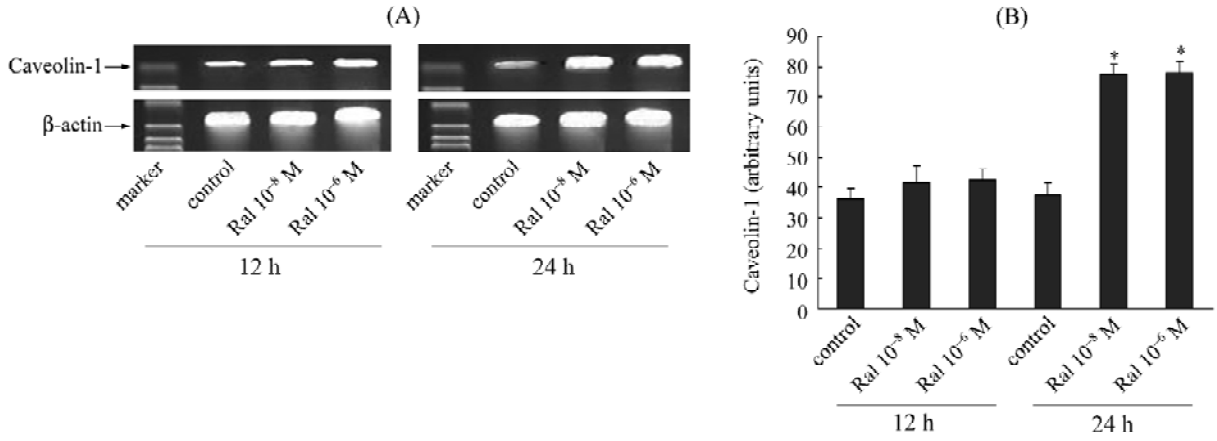

Fig. 3 RT-PCR analysis of effects of raloxifene on caveolin-1 mRNA expression in rat aortic smooth muscle cells (RASMCs)
(A) Raloxifene (10^{-8} or 10^{-6} M) treatment increases caveolin-1 mRNA expression after 24 h treatment but not after 12 h treatment. (B) Densitometric value of caveolin-1 mRNA expression in RASMCs. **P*<0.05 compared with control, *n*=4. Ral, raloxifene.

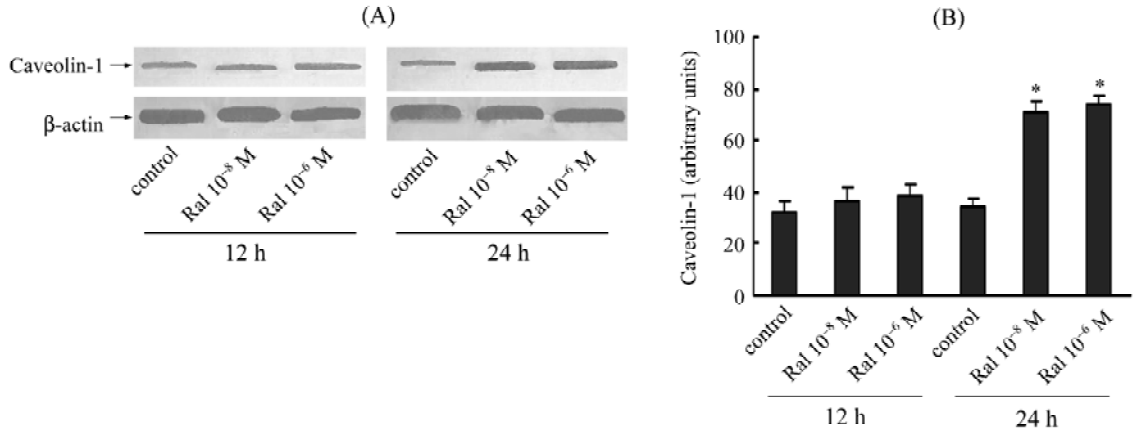

Fig. 4 Western blot analysis of caveolin-1 in rat aortic smooth muscle cells (RASMCs) in response to raloxifene treatment
(A) Caveolin-1 protein expression increased significantly after 24 h treatment of raloxifene (10^{-8} or 10^{-6} M), but not after 12 h. (B) Densitometric analysis of caveolin-1 protein in RASMCs. **P*<0.05 compared with control, *n*=4. Ral, raloxifene.

(**Figs. 5** and **6**).

Discussion

Results of the present study demonstrate for the first time the relationship between raloxifene and caveolin-1 in vascular smooth muscle cells. Caveolin-1 mRNA and protein expressions increased in response to raloxifene treatment in RASMCs. The pure estrogen receptor antagonist ICI 182780 inhibited the increase, which suggests that raloxifene stimulates caveolin-1 transcription and translation through estrogen receptor-mediated mechanisms. The lack of a dose-dependent response effect of raloxifene treatment on caveolin-1 expression might reflect saturation of the estrogen receptors with the lowest dose of raloxifene. Caveolin-1 expression directly correlates with the caveolae number [14,15]. Therefore, the results also suggest that raloxifene might influence the number of caveolae present on the cell surface through regulation of caveolin-1. Consistent with previous reports, the present study also showed that estrogen upregulated caveolin-1 in vascular smooth muscle cells.

Though recent findings suggest that raloxifene might possess cardioprotective properties such as reducing total and low-density lipoprotein cholesterol, decreasing homocysteine levels and inhibiting vascular smooth muscle cell proliferation [9–12], the exact role of raloxifene in the

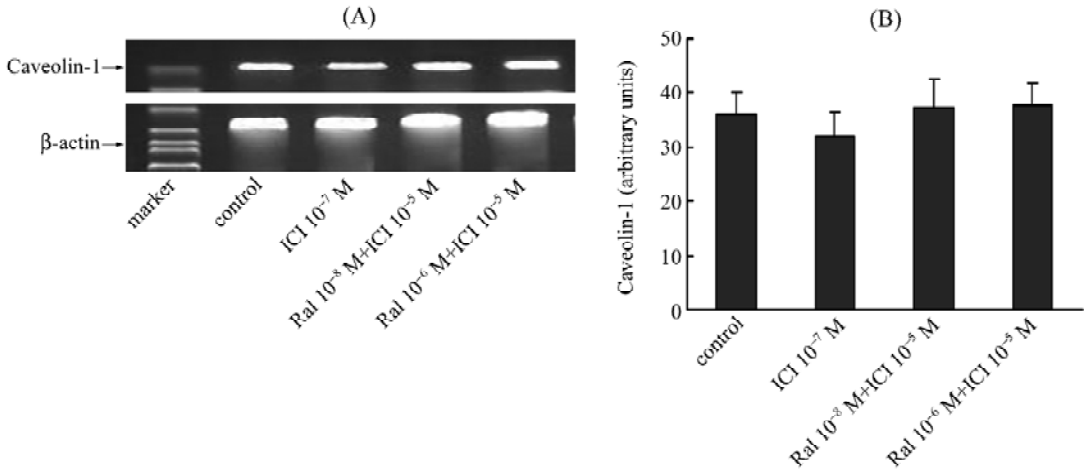

Fig. 5 RT-PCR analysis of effects of raloxifene and ICI 182780 on caveolin-1 mRNA expression in rat aortic smooth muscle cells (RASMCs)

(A) Caveolin-1 mRNA expression after 24 h treatment with raloxifene (10^{-8} or 10^{-6} M) plus ICI 182780 (10^{-5} M) in RASMC. (B) Densitometric analysis of caveolin-1 mRNA. There was no change in caveolin-1 mRNA expression after 24 h treatment with raloxifene plus estrogen receptor antagonist ICI 182780 in RASMCs. Ral, raloxifene; ICI, ICI 182780.

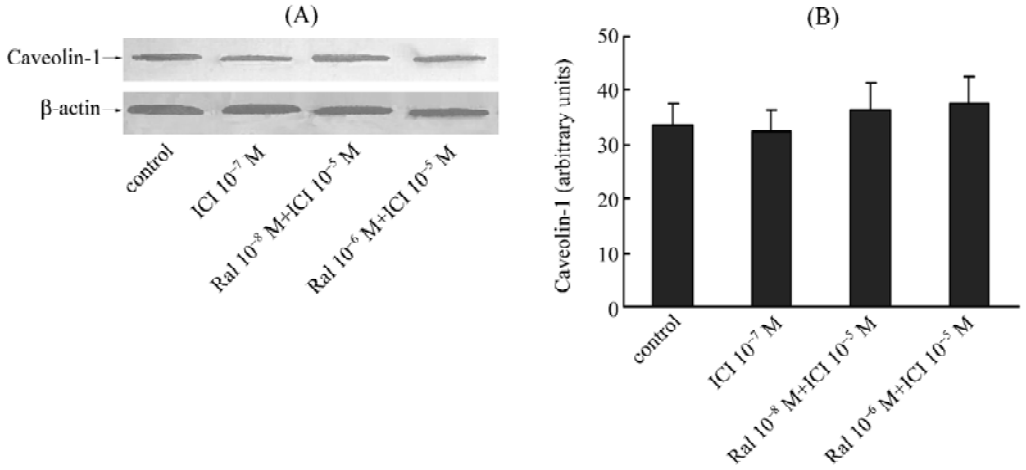

Fig. 6 Western blot analysis of caveolin-1 in rat aortic smooth muscle cells (RASMCs) treated with raloxifene and ICI 182780

(A) Caveolin-1 protein expression in rat aortic smooth muscle cells (RASMCs) after treated for 24 h with raloxifene plus ICI 182780. (B) Densitometric analysis of caveolin-1 protein in RASMC. There was an inhibition in raloxifene-induced caveolin-1protein expression in ICI 182780 (10^{-5} M) treatment with (10^{-8} or 10^{-6} M) raloxifene. Ral, raloxifene; ICI, ICI 182780.

cardiovascular system has remained unresolved until now. Recent studies *in vivo* and *in vitro* dramatically show that caveolae and caveolin-1 play prominent roles in various pathobiological conditions, especially those related to the cardiovascular system. Caveolin-1 contains a cytosolic N-terminal juxtamembrane domain (scaffolding domain), which binds to signaling molecules and inhibits their usual activation after growth factor ligation of receptors [16, 17]. Such examples include receptor tyrosine kinases, serpentine receptors and regulated enzymes. The interactions of caveolin-1 with these signaling molecules have important consequences for cellular functions such as proliferation. In the development of atherosclerosis, the proliferation of smooth muscle cells is a crucial pathophysiological process. Recent findings reported that caveolin-1 could negatively regulate the proliferative activity of vascular smooth muscle cells and inhibit neointimal hyperplasia [18,19]. Caveolin-1 is also thought to play an

important role in the regulation of cellular cholesterol homeostasis, a process that needs to be properly controlled in order to limit and prevent cholesterol accumulation and eventually atherosclerosis. For example, Batetta *et al.* [20] found that compared with adjacent serial sections of the same artery, atherosclerotic segments manifested higher levels of cholesterol esters, ACAT (acyl-coA:cholesterol acyltransferase) and multidrug resistance 1(MDR) mRNA and lower levels of caveolin-1 mRNA. Therefore, upregulation of caveolin-1 by raloxifene in vascular smooth muscle cells might contribute to protect the cardiovascular system against atherogenesis.

The present study also demonstrated that the upregulation effect of raloxifene on caveolin-1 expression was blocked by ICI 182780. Two types of estrogen receptors, estrogen receptors α and β were found in vascular smooth muscle cells, and emerging evidence shows that there is a plasma membrane estrogen receptor that could associate with and regulate the production of caveolin [7]. It is unknown whether this blockade is mediated by estrogen receptor α or β, and it is also unclear whether this blockade is related to the nuclear estrogen receptor or membrane estrogen receptor. A further study is needed to clarify the molecular mechanism by which raloxifene upregulates caveolin-1 mRNA and protein expressions.

In summary, raloxifene could influence the progression of coronary disease and the incidence of cardiovascular events through several mechanisms. Results of the present study demonstrate that raloxifene increases gene transcription and translation of caveolin-1 in vascular smooth muscle cells, providing a new pathway through which raloxifene affects vascular functions.

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