Raloxifene Prevents Cardiac Hypertrophy and Dysfunction in Pressure-Overloaded Mice

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Abstract—17β-Estradiol reduces myocardial hypertrophy and left ventricular mass, suggesting that the selective estrogen receptor modulator raloxifene may have similar effects. However, it is not clear whether raloxifene inhibits both cardiac hypertrophy and dysfunction. We used transverse aortic-banded mice to produce pressure-overload cardiac hypertrophy and used neonatal rat ventricular cardiomyocytes to investigate the cellular mechanisms of raloxifene on cardiac hypertrophy. Left ventricular mass and fractional shortening of mice hearts were measured by transthoracic echocardiography. Protein synthesis of cardiomyocytes was evaluated by incorporation of [3H]leucine into cardiomyocytes exposed to angiotensin II. Phosphorylation of mitogen-activated protein (MAP) kinase was also observed in cardiomyocytes. Raloxifene prevented increases in left ventricular mass and decreases of fractional shortening at 4 weeks after aortic banding. Pretreatment with raloxifene before angiotensin II stimulation inhibited the increase in [3H]leucine incorporation into neonatal rat cardiomyocytes in a concentration-dependent manner. This inhibition was partially but not significantly attenuated by Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, and completely abolished by IC182780, an estrogen receptor antagonist. Although the phosphorylation of p38 MAP kinase, c-Jun N-terminal kinase (JNK), or extracellular signal-regulated protein kinase (ERK) in cardiomyocytes was significantly increased by angiotensin II stimulation as compared with the control, pretreatment with raloxifene attenuated p38 MAP kinase phosphorylation, but neither JNK nor ERK phosphorylation. We conclude that raloxifene inhibits cardiac hypertrophy and dysfunction and that the inhibition of p38 MAP kinase phosphorylation after the stimulation of estrogen receptors may be involved in the cellular mechanisms of this agent. (Hypertension. 2004; 43:237-242.)

Key Words: echocardiography ▪ heart failure ▪ hormones ▪ hypertrophy ▪ signal transduction

The incidence of left ventricular hypertrophy caused by essential hypertension is one of the major causes of impaired cardiac function followed by heart failure.1,2 Although the increase in left ventricular mass may represent adaptation of the heart in response to pressure overload, left ventricular hypertrophy is associated with an increased risk of cardiovascular complications and mortality/morbidity.2-5 Reduction or prevention of left ventricular hypertrophy by treatment with appropriate antihypertensive drugs reduces the risk of cardiovascular diseases or death.6,7 In postmenopausal women, left ventricular hypertrophy is common and is likely to be a strong cardiovascular risk factor compared with men, suggesting that estrogen has potential protective effects in the cardiovascular system.2 In fact, hormone replacement therapy in hypertensive postmenopausal women contributes to reducing left ventricular mass, improving cardiac function, and decreasing future cardiovascular events.8-10 However, the application of long-term hormone replacement therapy is limited by its side effects, which include an increased risk of breast and endometrial cancer, venous thromboembolism, vaginal bleeding, mastodynia, and weight gain.11

Selective estrogen receptor modulators (SERMs) have recently been approved as a new type of hormone replacement therapy to prevent osteoporosis and improve lipid profiles in postmenopausal women without producing uterine proliferation.12 The beneficial effects of raloxifene, one of the SERMs, on cardiovascular risks and events have been investigated,13 and a double-blind, placebo-controlled, randomized, clinical trial called the Raloxifene Use for The Heart (RUTH) trial is currently being conducted to determine whether raloxifene decreases the occurrence of coronary events in postmenopausal women with cardiovascular diseases or risk factors.14 Although 17β-estradiol has been reported to prevent the development of cardiac hypertrophy...
in the aortic-banded mouse model, it has not been demonstrated whether raloxifene reduces the increase in left ventricular mass or prevents cardiac contractile dysfunction caused by pressure-overload hypertrophy and what mechanisms are involved in those effects. To investigate the effects of raloxifene on cardiac hypertrophy, we administered raloxifene to transverse aortic-constricted (TAC) mice and examined the effects and cellular mechanisms of this agent.

**Methods**

**Transverse Aortic-Banded Mice Model**

After C57BL/6 male mice (aged 9 to 10 weeks, 18 to 23 g, n = 18) were anesthetized with a mixture of pentobarbital sodium (50 mg/kg IP) and ketamine (25 mg/kg IP), the surgical procedure of TAC was performed as previously described. Sham-operated mice underwent a similar surgical procedure without constriction of the aorta. All animal procedures conformed to the guidelines of Osaka University Graduate School of Medicine with regard to animal care and the position of the American Heart Association on research animal use.

**Hemodynamic and Echocardiographic Measurement**

Both noninvasive blood pressure and heart rate were measured before and at 2 and 4 weeks after surgery in the unanesthetized condition by the tail-cuff plethysmography method (model BP-98A, Softron). After the mice were lightly anesthetized with pentobarbital sodium (30 mg/kg IP), we performed 2-dimensional guided M-mode echocardiography as previously described. The percentage of left ventricular fractional shortening was calculated as

\[
\text{LVFS} = \frac{\text{LVDD} - \text{LVSD}}{\text{LVDD}} \times 100\%.
\]

LVDD and LVSD indicate left ventricular end-diastolic and end-systolic chamber dimensions, respectively. Left ventricular mass was calculated as

\[
1.055(\text{PWTD}^3 - \text{LVDD}^3 - \text{VSTD}^3)(\text{mg}),
\]

where PWTD indicates diastolic posterior wall thickness, and VSTD indicates diastolic ventricular septal thickness.

**Experimental Protocol**

After the initial assessment using tail-cuff plethysmography and echocardiography, the mice were randomly divided into transverse aortic-banding or sham-operated groups. At the end of the transverse aortic-banding operation, TAC mice were also assigned to two groups; in one group, the mice were administered by vehicle (0.1% DMSO IP), and in the other group, the mice were treated with raloxifene (10 mg/kg per day IP). We followed up with these three groups (sham, n = 6; TAC, n = 6; TAC + raloxifene, n = 6) for 4 weeks and used them for data analysis.

**Histopathological Examination**

Hearts were excised after the mice were euthanized with an overdose injection of pentobarbital sodium. Transverse sections of the heart, which were prepared as previously described, were stained with hematoxylin and eosin or anti-desmin antibody and observed by light microscopy.

**[3H]Leucine Uptake Assay**

Neonatal rat cardiomyocytes cultured in DMEM containing 10% fetal bovine serum were used to assess protein syntheses by incorporation of [3H]leucine (Amersham Biosciences Japan) into the cardiomyocytes. Twenty-four hours after the onset of serum starvation, the cardiomyocytes were treated with the drugs indicated in Figure 3 for 1 hour before angiotensin II (1 μmol/L) stimulation and 1 μCi of [3H]leucine addition. Then the cells were further incubated for 24 hours to measure radioactivity.

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<th>Systemic Hemodynamic Changes Among Each Group During the Experimental Period</th>
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<td>Heart rate (bpm)</td>
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TAC indicates transverse aortic contraction.

**Analysis of Mitogen-Activated Protein Kinases**

Neonatal cardiomyocytes were cultured and starved as described and treated with or without raloxifene (10 μmol/L) or ICI182780 (20 μmol/L) for 1 hour before angiotensin II (1 μmol/L) stimulation. After 24 hours of post-stimulation, equal amounts of lysates of the cardiomyocytes were immunoblotted. The remaining equal amounts of lysates were subjected to immunoprecipitation with anti-p38 mitogen-activated protein (MAP) kinase antibody, c-Jun fusion protein beads, or anti-extracellular signal-regulated kinase (ERK) 1/2 antibody. The immunoprecipitates were collected and then immunoblotted. The antibodies used for immunoprecipitation or immunoblotting were all purchased from Cell Signaling Technology (Beverly, MA).

**Statistical Analysis**

The data are expressed as mean±SE and were compared by ANOVA. A value of P<0.05 was considered to be statistically significant.

**Results**

**Hemodynamic and Echocardiographic Examinations**

There was no significant difference in heart rate or mean arterial pressure as measured by tail-cuff plethysmography among the 3 groups before and at 2 and 4 weeks after the operation (Table). The pressure gradient between the right and left carotid arteries, measured invasively after TAC, was ∼50 mm Hg, which was not significantly different between the TAC and TAC + raloxifene groups (data not shown). Echocardiographic study showed that in the TAC group, posterior wall thickness increased at 2 and 4 weeks after the operation (Table). There was no significant difference in heart rate or mean arterial pressure among each group during the experiment.
that raloxifene has cardioprotective effects against cardiac pressure overloading. In regard to left ventricular end-diastolic diameter, there was no difference among the three groups. This indicates that cardiac hypertrophy induced by TAC results in afferent myocardial thickening (Figure 1).

Myocardial Histopathological Analysis

Hematoxylin-eosin stain of myocardium revealed that the size of cardiomyocytes was significantly larger in the TAC group than the control group, and that the increase in the size of the cardiomyocytes was attenuated by administration of raloxifene (Figure 2A). The severity of fibrosis detected by Azan-Malory staining was similar among the three groups (data not shown). To further examine why the cardiac function in the TAC mice was reduced we performed desmin staining of the myocardium in each group. We found that the desmin staining was strongly positive in the TAC group compared with the other groups, suggesting that the increased amount of desmin in the cardiomyocytes may be associated with reduced cardiac function (Figure 2B).

Cellular Effects of Raloxifene on Hypertrophic Stimulation

To investigate the protective effects of raloxifene against hypertrophic stimulation, we performed 2 in vitro experiments. First, the amount of $[^{3}\text{H}]$leucine incorporated into the cardiomyocytes was measured for one of the indices of protein syntheses with or without raloxifene or inhibitors under angiotensin II ($1 \mu\text{mol/L}$) stimulation. This experiment showed that raloxifene inhibits the increase of $[^{3}\text{H}]$leucine uptake caused by angiotensin II stimulation in a dose-dependent manner, and that this inhibitory effect is partially but not significantly attenuated by an inhibitor of nitric oxide synthase, $N^\text{G}$-nitro-$L$-arginine methyl ester ($L$-NAME; $100 \mu\text{mol/L}$), and completely abolished by an estrogen

![Figure 1](image1.png)

**Figure 1.** A, Effects of TAC and raloxifene on posterior wall thickness. B, Left ventricular end-diastolic dimension. C, Left ventricular mass. D, Fractional shortening. TAC increased posterior wall thickness and left ventricular mass and decreased fractional shortening, whereas treatment with raloxifene abolished and prevented the increase in posterior wall thickness and left ventricular mass at 2 and 4 weeks after TAC operation and the decrease in fractional shortening at 4 weeks. Note that left ventricular end-diastolic dimension did not significantly change among three groups during the 4 weeks of observation.

![Figure 2](image2.png)

**Figure 2.** A, Hematoxylin-eosin stained sections of mice hearts. TAC enlarged the size of cardiomyocytes compared with sham operation, and raloxifene treatment inhibited the increase in the size of cardiomyocytes (bars: $20 \mu\text{m}$). B, Immunohistochemical analysis with anti-desmin antibody demonstrated that TAC induced desmin filaments assembly and that raloxifene reduced that assembly (bars: $20 \mu\text{m}$).

![Figure 3](image3.png)

**Figure 3.** Treatment with raloxifene ($10 \mu\text{mol/L}$) significantly reduced the increase in $[^{3}\text{H}]$leucine incorporation into cardiomyocytes induced by angiotensin II stimulation. $L$-NAME (100 $\mu\text{mol/L}$) partially but not significantly inhibited and ICI182780 (20 $\mu\text{mol/L}$) completely abolished the effects of raloxifene on $[^{3}\text{H}]$leucine incorporation. Results are mean±SE of at least 4 experiments. $^*P<0.05$. 

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receptor blocker, ICI182780 (20 μmol/L), suggesting that the effects of raloxifene against angiotensin II are completely mediated by estrogen receptors (Figure 3). Furthermore, we examined what signaling pathway was associated with the downstream of stimulated estrogen receptors. Here we observed the phosphorylation of each MAP kinase by stimulation of angiotensin II and treatment with raloxifene. Angiotensin II stimulation increased each MAP kinase phosphorylation, whereas co-treatment with raloxifene attenuated only p38 MAP kinase phosphorylation and affected neither c-Jun N-terminal kinase (JNK) nor ERK phosphorylation. Moreover, the inhibitory effect of raloxifene on p38 MAP kinase phosphorylation against angiotensin II stimulation was abrogated by addition of ICI182780 (upper panels of Figure 4). These results suggest that raloxifene might prevent cardiac hypertrophy and dysfunction by inhibiting p38 MAP kinase phosphorylation and that estrogen receptors are involved in this signaling pathway. The lower panels of Figure 4 indicate that the total amount of p38 MAP kinase, JNK, or ERK was equal within each group.

**Discussion**

To our knowledge, this is the first study to date to demonstrate that raloxifene, one of the SERMs, prevented the progression of cardiac hypertrophy and dysfunction induced by TAC in mice. Because, as shown in Table 1, raloxifene did not alter either mean arterial pressure or heart rate in TAC mice, the effects of raloxifene on both reduction of cardiac hypertrophy and inhibition of left ventricular dysfunction are not likely to depend on systemic hemodynamics. This suggests that raloxifene exerts direct effects on cardiomyocytes to attenuate their size and maintain their mechanical function. Furthermore, we did not detect any significant increase in inflammatory cells, extracellular matrix, or fibrosis in the interstitial space in the hearts of the TAC group compared with either the control or the TAC plus raloxifene group by light microscopic examination. This observation was similar to that of a previous study.\(^\text{29}\) To elucidate why TAC deteriorated cardiac contractile function without proliferating interstitial tissue in the heart, we investigated the extent of desmin filament accumulation in the cardiomyocytes. Immunohistochemical study of heart sections against anti-desmin antibody revealed that desmin filaments in the cardiomyocytes increased in the TAC mice compared with the control mice and that treatment with raloxifene reduced the desmin accumulation. This result suggests that desmin filaments might play a role in cardiac contractility.

Desmin filaments, muscle-specific intermediate filaments that are located around the Z-disk of sarcomeres and connect neighboring sarcomeres, are essential for myocardial contraction–relaxation movement.\(^\text{18}\) It has been reported that desmin null mice showed degeneration of cardiac muscle and that desmin augmentation was found in failing human myocardium as a compensatory mechanism to maintain cardiac function.\(^\text{19,20}\) However, cardiac hypertrophy caused by chronic pressure overload was possible to excessively upregulate desmin filaments in the heart, and significant accumulation of mutated desmin filaments in cardiomyocytes has been reported to cause cardiac functional abnormalities in mutated desmin transgenic mice.\(^\text{21}\) Thus, extraordinary increases in desmin protein in cardiomyocytes may be involved in cardiac dysfunction in the pressure-overloaded heart.

We used neonatal rat cardiomyocytes to understand the intracellular and molecular mechanisms of raloxifene against hypertrophic stimulation. In in vitro experiments, raloxifene inhibited angiotensin II stimulation, mainly via the stimulation of estrogen receptors, as demonstrated by the finding that a specific blocker of estrogen receptors, ICI182780, completely abrogated the effects of raloxifene. The precise pathway for the action of raloxifene and whether raloxifene mediates either estrogen receptor α or estrogen receptor β is not known. Raloxifene has a 4-fold higher affinity for estrogen receptor α than β compared with 17β-estradiol.\(^\text{22}\) However, because it has been reported that receptor-ligand affinity properties are not likely to make a major contribution to the molecular mechanisms of SERMs,\(^\text{23}\) the different activities of SERMs in various tissues expressing estrogen receptor α and β should be further examined in the future. It is well known that estrogen exerts its cardioprotective effects rapidly or chronically via nitric oxide,\(^\text{24,25}\) and that estrogen regulates nitric oxide synthase function and nitric oxide production through caveolin-1, which is the principle coat protein of caveolae.\(^\text{26}\) However, the role of nitric oxide in raloxifene-induced cardioprotection seems to be minimal, because an inhibitor of nitric oxide synthase, L-NAME, did not show significant inhibitory effects of raloxifene. In previous studies, either raloxifene or idoxifene was shown to cause vasorelaxation, mainly in a nitric oxide-dependent manner.\(^\text{27,28}\) These differences between our data and other studies may be because of the differences of SERMs, target tissues, or animals.

To further examine the intracellular mechanisms by which raloxifene exerted its inhibitory effects against angiotensin II stimulation, we studied the phosphorylation of MAP kinases, (ie, p38 MAP kinase, JNK, and ERK) in angiotensin II. Although angiotensin II increased these three MAP kinase phosphorylations, raloxifene abolished the phosphorylation
of p38 MAP kinase but did not affect that of JNK and ERK. It is reported that ERK mediates multiple cellular pathways, which are critical to cell proliferation, differentiation, and hypertrophy, and that 17β-estradiol or its metabolites inhibit ERK activation induced by hypertrophic or mitogenic stimulation. The difference between our data and previous studies is uncertain, but the effects of 17β-estradiol and raloxifene may vary on some signal transduction pathways. However, previous studies have demonstrated that p38 MAP kinase is involved in cardiac hypertrophy and dysfunction in a spontaneously hypertensive rat model,33 activated p38 MAP kinase increases desmin expression in cardiomyocytes via Hsp25,34 and estrogen inhibits p38 MAP kinase phosphorylation induced by TAC in mice.15 Our results and those of other studies suggest that the inhibition of p38 MAP kinase phosphorylation by raloxifene or estrogen may represent one of the cardioprotective mechanisms against cardiac hypertrophy and dysfunction caused by hypertrophic stimulation. Because it is well known that multiple signal pathways apart from the MAP kinase pathway and the crosstalk between MAP kinases are involved in the progression of cardiac hypertrophy, further studies are needed to reveal the complete mechanisms responsible for the progression of cardiac hypertrophy and the cardioprotective effects of raloxifene against cardiac hypertrophy, because the possibility that raloxifene may protect cardiac hypertrophy induced via other mechanisms except the MAP kinase pathway was not investigated in this study.

Perspectives
When we consider the clinical implications of raloxifene for hormone replacement therapy, raloxifene is thought to be more beneficial than estrogen because, although estrogen exerts many cardioprotective effects, it increases the risk of carcinogenesis in the breasts and uterus. Previously, SERMs have been shown to improve lipid profiles and endothelial function, inhibit smooth muscle cell proliferation, and have beneficial effects on ischemic heart diseases in human subjects and experimental models.12,35–37 Moreover, we have for the first time revealed that raloxifene, one of the SERMs, prevents cardiac hypertrophy and dysfunction in TAC-induced pressure-overloaded mice and elucidated the possible intracellular mechanisms of raloxifene against hypertrophic stimulation. Based on these findings, raloxifene may be considered as a therapeutic drug for postmenopausal women who are at high risk for cardiac hypertrophy.

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References


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