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# Raloxifene analogue LY117018 suppresses oxidative stress-induced endothelial cell apoptosis through activation of ERK1/2 signaling pathway

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ABSTRACT

A selective estrogen receptor modulator, raloxifene, has been shown to reduce cardiovascular events in relatively high-risk postmenopausal women with osteoporosis. However, the mechanisms by which raloxifene exerts a pharmacological effect on cardiovascular organs have not been fully elucidated. The present study was designed to examine whether the raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), could inhibit apoptosis and to clarify the signaling pathway in vascular endothelial cells. LY117018 significantly inhibited hydrogen peroxide-induced apoptosis in bovine carotid artery endothelial cells. The anti-apoptotic effect of LY117018 was abolished by an estrogen receptor antagonist, 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780). Mitogen-activated protein kinases (MAPK), including p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase1/2 (ERK1/2), and Akt, have been shown to act as apoptotic or anti-apoptotic signals. Phosphorylation of p38, JNK, ERK1/2 and Akt was examined. LY117018 increased ERK1/2 phosphorylation but did not enhance the phosphorylation of p38, JNK, or Akt. The anti-apoptotic effect of LY117018 was prevented by treatment with 2-[2′-amino-3′-methoxyphenyl]-oxanaphthalen-4-one (PD98059), an upstream inhibitor of ERK1/2. LY117018 stimulated an increase in ERK1/2 phosphorylation, which was diminished by ICI 182,780. The activation of ERK/1/2 by LY117018 was not inhibited by the transcription inhibitor, actinomycin D. These results suggest that estrogen receptors and the ERK1/2 signaling pathway are involved in the anti-apoptotic action of LY117018 in vascular endothelial cells.



## 1. Introduction

The incidence of clinical coronary heart disease in premenopausal women is very low. However, following the menopause, atherogenic risk factors increase and the rate of clinical coronary events accelerates to the level observed in men (Kannel et al., 1976). This difference has been considered to be attributable to the protective effects of estrogen before the menopause (Clarkson, 2007). Recent randomized placebo-controlled trials of hormone replacement therapy, however, have not shown any benefit in either the secondary or the primary prevention of cardiovascular events (Hulley et al., 1998; Grady et al., 2002; Rossouw et al., 2002).

Much current interest is focused on the therapeutic potential of selective estrogen receptor modulators. Interestingly, drugs of this class show estrogen-antagonist effects in the mammary gland and uterus, while they have estrogen-agonist effects in bone and other tissues (Delmas et al., 1997; Grady et al., 2004; Johnell et al., 2004; Cox et al., 2004; Sporn et al., 2004). Thus, they are expected to overcome the adverse effects found with conventional hormone replacement therapy.

Recently, the MORE (*M*ultiple *O*utcomes of *R*aloxifene *E*valuation) study showed that a representative selective estrogen receptor modulator, raloxifene, significantly reduced cardiovascular events in relatively high-risk postmenopausal women with osteoporosis (Barrett-Connor et al., 2002). The death of endothelial and vascular smooth muscle cells is implicated in several pathological vascular conditions, such as atherosclerosis and aneurysm formation. Endothelial damage/dysfunction plays a central role in the clinical manifestation of coronary atherosclerosis (Ross, 1990; Ross, 1999). It has been reported that selective estrogen receptor modulators show a variety of direct actions on vascular cells via estrogen receptors (Simoncini et al., 1999; Simoncini et al., 2002). However, the effect of selective estrogen receptor modulators on endothelial apoptosis has not been clarified.

The aim of this study was to examine the effect of a raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), on endothelial apoptosis and to clarify the mechanisms of action.

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## 2. Materials and methods

### 2.1. Chemicals and reagents

The raloxifene analogue LY117018 was provided by Eli-Lilly (Indianapolis, IN, USA). 1,3,5(10)-estariene-3,17β-diol (17β-estradiol), wortmannin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Phenol red-free Medium199 (M199) was from Gibco (NY, USA). 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from AstraZeneca (Macclesfield, Cheshire, UK). Hydrogen peroxide ($H_2O_2$ 30% solution) and actinomycin D were obtained from Wako (Osaka, Japan). The mitogen-activated protein/extracellular signal-regulated protein kinase (MEK)1 inhibitor, 2-[2′-amino-3′-methoxyphenyl]-oxanaphthalen-4-one (PD98059), and antibodies against Akt, phospho-Akt (Ser-473), c-Jun N-terminal kinase (JNK), phospho-JNK (Thr183/Tyr185), extracellular signal-regulated protein kinase1/2 (ERK1/2) and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p38 (A-12) and phospho-p38 (D-8) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The JNK inhibitor anthrax [1, 9-cd] pyrazol-6(2H)-one (SP600125) and the p-38 inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Gemany). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd., Tokyo, Japan). Charcoal-stripped fetal bovine serum was from MultiSer (ThermoTrace Ltd., Melbourne, Australia). Nitrocellulose membranes were from Amersham (Buckinghamshire, UK). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). Cell Death Detection ELISA $^{plus}$ was purchased from Roche (Mannheim, Germany).

### 2.2. Cell culture

Bovine carotid endothelial cells (BCEC) were provided by Dr. Sudoh and prepared as described previously (Sudoh et al., 2001; Akishita et al., 1998). Cells were cultured in a 37 °C humidified atmosphere of 95% air/5% $CO_2$ in DMEM containing 10% FBS and 100 units/ml penicillin/100 μg/ml streptomycin. For all experiments, BCEC were used at passages 5 to 7, and plated at a concentration of $10^4$ cells/ml. Raloxifene experiments were performed with phenol red-free M199. DMSO was used as a solvent for LY117018, 17β-estradiol, ICI 182,780 and PD98059. DMSO was present at equal concentrations (0.05%) in all groups, including the vehicle group.

### 2.3. Apoptosis induction

Apoptosis was induced by addition of hydrogen peroxide ($H_2O_2$). At 70–80% confluence, cells were washed with phosphate-buffered saline (PBS), and then replenished with phenol red-free M199 without serum, and proliferation was stopped. Cells were exposed to 100 μM $H_2O_2$ for 1 h after 6 h starvation, washed twice again with PBS (−), then replenished with phenol red-free M199 containing 5% DCC–FBS. In the same experiments, LY117018 or 17β-estradiol was added for 30 min before $H_2O_2$ stimulation in the apoptosis assay. In experiments on inhibitors, the inhibitors were added for 60 min before LY117018 addition. After 24 h of stimulation by $H_2O_2$, cell apoptosis was evaluated.

### 2.4. Assay of endothelial cell apoptosis (DNA fragmentation assay)

Cell apoptosis was quantified by means of DNA fragmentation, using a photometric enzyme-linked immunosorbent assay (Cell Death Detection ELISA $^{plus}$) kit. Cells with each treatment were lysed in 300 μl lysis buffer, and a fraction of the supernatant was subjected to reaction for 2 h with the immunocomplex of anti-DNA conjugated with peroxidase, which binds to nucleosomal DNA, and antihistone–biotin, which interacts with streptavidin-coated wells in a microtiter plate. At the end of the incubation, substrate was added, and development was quantified at 405 nm wavelength.

### 2.5. Western blot analysis

After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1 mM PMSF. For Western blot analysis, total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to a polyvinilidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38, and anti-p38. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody. Immunoreactive bands were visualized using a LumiGLO Reserve Chemiluminescent Substrate Kit and quantified by densitometry in the linear range of NIH image 1.60.

### 2.6. Statistics

Values are expressed as means ± S.E.M. Statistical comparisons were performed by ANOVA followed by Fisher's protected least significance difference (PLSD) test. A probability value $<0.05$ was considered significant.

## 3. Results

### 3.1. Effect of LY117018 on endothelial cell apoptosis

On the basis of concentration- and time-response experiments (data not shown), $H_2O_2$ (100 μM) was added to BCEC for 1 h to induce apoptosis. BCEC apoptosis induced by $H_2O_2$ was significantly attenuated by treatment with LY117018 in a concentration-dependent manner (Fig. 1), while LY117018 per se did not show any effect on apoptosis (data not shown).

### 3.2. Involvement of MEK/ERK pathway in anti-apoptotic action of LY 110718

Phosphorylation levels of p38, JNK, ERK1/2, and Akt were examined because these kinases have been shown to regulate apoptosis (Xia et al.,

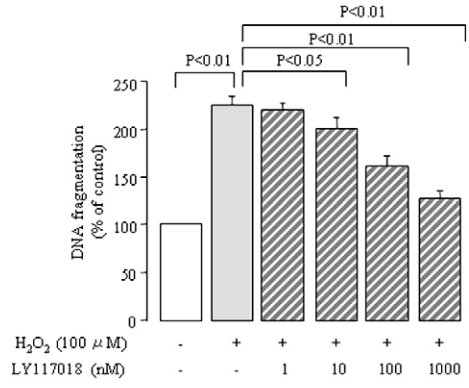


**Fig. 1.** Effect of LY117018 on $H_2O_2$-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μM $H_2O_2$ for 1 h as described in Materials and methods. Various concentrations of LY117018 (1 nM–1 μM) were added to the culture medium 30 min before $H_2O_2$ stimulation in the apoptosis assay. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation (with a Cell Death Detection ELISA $^{plus}$ kit) as described in Materials and methods. Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).