Raloxifene Increases Proliferation and Up-regulates Telomerase Activity in Human Umbilical Vein Endothelial Cells*

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Vascular endothelial senescence is involved in human atherosclerosis. Telomerase activity is known to be critical in cellular senescence and its level is modulated by regulation of telomerase catalytic subunit (telomerase reverse transcriptase (TERT)) at both the transcriptional and post-transcriptional levels. Since the cardioprotective effect of estrogen itself has not been ruled out, we examined that of raloxifene, which has been classified as a selective estrogen receptor modulator, on the proliferation and telomerase activity of human umbilical vein endothelial cells (HUVECs). Raloxifene, like estrogen, clearly induced the telomerase activity and human TERT (hTERT) expression via estrogen receptor (ER) α and ERβ. Treatment with raloxifene for 5 days significantly induced cell growth, and either cotreatment with a telomerase inhibitor, 3′-azido-3′-deoxythymidine, or transfection with hTERT-specific interfering RNA significantly attenuated the raloxifene-induced cell growth. Raloxifene also induced the phosphorylation of Akt, and pretreatment with a phosphatidylinositol 3-kinase inhibitor, LY294002, significantly attenuated the raloxifene-induced telomerase activity. In addition, raloxifene induced both the phosphorylation of hTERT and IκB. Moreover, cotreatment with an IκBα phosphorylation inhibitor, BAY-11–7082, or a specific NFκB nuclear translocation inhibitor, SN50, significantly attenuated the raloxifene-induced telomerase activity and the association of NFκB with hTERT. These results show that raloxifene induced the up-regulation of telomerase activity not only by the transcriptional regulation of hTERT but also by post-translational regulation of the phosphorylation of Akt and hTERT and the association of hTERT with NFκB in HUVECs. Thus, the up-regulation of telomerase activity in vascular endothelial cells might be one mechanism contributing to the potential atheroprotective effect of raloxifene.

The risk of cardiovascular disease steeply increases after menopause. Many epidemiological and basic studies have shown that estrogen has the significant function in the vasculature of preventing the primary development of cardiovascular disease in women (1–2). In the Women’s Health Initiative, a large prospective randomized controlled study, although women on the conjugated equine estrogen-medroxyprogesterone acetate arm had an increase in the relative risk of cardiovascular events and breast cancer (3), the more recent reports indicated that on women on the conjugated equine estrogen-only treatment arm experienced a significant increase in the risk of stroke compared with women treated with placebo but did not show an increase in cardiovascular disease (4). Thus, the cardioprotective effect of estrogen itself has not been ruled out by the results of the Women’s Health Initiative study.

Recently, we reported that medroxyprogesterone acetate attenuates the induction of both endothelial nitric-oxide synthase (eNOS) activity and NO production by estrogen in human umbilical vein endothelial cells (HUVECs) (5). Thus, it is possible that progesterin has adverse effects on the cardiovascular system. Therefore, there is a need for an ideal estrogen analog that does not require the addition of progesterin to protect the uterine endometrium. Raloxifene is a nonsteroidal benzo-thiophene that has been classified as a selective estrogen receptor modulator based on the fact that it exerts estrogen-agonistic effects on bone (6) and lipid metabolism (7) and estrogen-antagonistic effects on the uterine endometrium (6) and breast tissue (8). However, its ability to protect against cardiovascular disease has yet to be proven.

We demonstrated previously that the effects (and the mechanisms thereof) of raloxifene on vascular endothelial and smooth muscle cells were similar to those of estrogen. For instance, in vascular endothelial cells, raloxifene induces eNOS phosphorylation, as estrogen does. Its effect is mediated by estrogen receptor (ER) α via a transcription-independent mechanism, a so-called nongenomic mechanism, and is differ-

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‡ The abbreviations used are: eNOS, endothelial nitric-oxide synthase; HUVEC, human umbilical vein endothelial cell; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; TERT, telomerase reverse transcriptase; siRNA, small interfering RNA; TRAP, telomeric repeat amplification protocol; CSS, charcoal-stripped serum; RT, reverse transcription; E2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase; KO, knock-out.
entially mediated by an Akt- and ERK-dependent cascade (9–10). In vascular smooth muscle cells, raloxifene exerts an antiproliferative effect on cells treated with platelet-derived growth factor mediated by Eκα, as estrogen does, in part via a transcription-dependent mechanism, a so-called genomic mechanism (11), and in part via a nongenomic mechanism (12).

The incidence of atherosclerosis increases with age. Aging is associated with endothelial dysfunction. On a cellular level, aging leads to an irreversible state of cell cycle arrest known as senescence (13). It is generally believed that an important factor in regulating cellular life span is the telomere length (14). Telomerase, an RNA-dependent DNA polymerase, catalyzes the addition of telomeric repeat sequences to chromosome ends and delays the development of senescence (14). It was reported that telomerase inactivation precedes the aging of vascular endothelial cells (15) and cells with senescence-associated phenotypes are present in human atherosclerotic lesions (16). In addition, the expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, in endothelial cells was reported to cause resistance to the induction of apoptosis (17). Thus, endothelial cell senescence induced by telomere shortening may contribute to atherogenesis.

We reported that raloxifene induces eNOS phosphorylation via the Akt cascade (9). NO was reported to activate telomerase and delay endothelial cell senescence (15). In addition, it was reported that the region surrounding Ser-824 in hTERT conforms to a consensus sequence for phosphorylation by Akt and that Akt kinase enhances human telomerase activity through phosphorylation of hTERT (18).

These considerations led us to examine whether raloxifene up-regulates telomerase activity and to attempt to clarify the molecular mechanism involved. In the present report, we show that raloxifene up-regulates telomerase activity not only by transcriptional regulation of hTERT but also by post-translational regulation of the phosphorylation of hTERT and the association of hTERT with NFκB.

**EXPERIMENTAL PROCEDURES**

**Materials**—3′-Azido-3′-deoxothymidine (AZT) and LY294002 were obtained from Sigma. BAY-11–7082 and SN50 were obtained from Calbiochem. Anti-phospho-Akt substrate, anti-Akt, anti-phospho-Akt, anti-phospho–ERK, and anti-phospho-IκB antibody were obtained from Cell Signaling Technology (Beverly, MA). Anti-hTERT, anti-NFκB antibody, scramble (control) siRNA, and ERβ siRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ICI 182780 was obtained from Tocris (Ballwin, MO). The cell titer 96-well proliferation assay kit was obtained from Promega (Madison, WI). The telomeric repeat amplification protocol (TRAP) assay kit (TRAPeze) was obtained from Chemicon (Billerica, MA).

**Isolation of Endothelial Cells**—Umbilical cords were obtained from term deliveries. The umbilical cord vein was filled with trypsin solution and incubated for 30 min to cause the release of endothelial cells. The detached endothelial cells were washed in phosphate-buffered saline and re-suspended in endothelial cell growth medium-2 (HUMEDIA EG-2) (Kurabo, Osaka, Japan) for cell culture.

**Cell Culture**—Freshly isolated or commercially obtained (Kurabo) endothelial cells were grown in HUMEDIA EG-2, containing 2% fetal calf serum, hydrocortisone, hVEGF, IGF-1, heparin, penicillin/streptomycin, and amphotericin B, as supplied by the manufacturer. Cells were cultured in 75-cm² cell culture dishes (Falcon) or 6-well plates (Nalge Nunc International, Rochester, NY). There were no differences in ER expression patterns or estrogen responsiveness between freshly isolated and commercially available endothelial cells (data not shown).

Unless otherwise indicated, experiments were performed on cells that had previously undergone three passages (i.e. 10 population doublings). To induce quiescence of human HUVECs by growth factor withdrawal, cultures were transferred 3 days after plating to a basal medium containing ascorbic acid, heparin, and serum-free or 1% charcoal-stripped serum (CSS) but lacking any other growth factor supplements and maintained under these conditions for 48 h.

**TRAP Assay**—Endothelial cells were harvested by trypsinization and lysed at 4 °C in cell lysis buffer. Aliquots of the cleared lysate equivalent to 1.5 μg of protein were assayed for telomerase activity by a modified telomeric repeat amplification protocol (TRAPeze), which is based on the method originally described by Kim et al. (19). Telomerase activity was calculated from the ratio of the intensity of the telomeric repeat ladder (starting at 50 bp) to that of the 36-bp internal control. Results, expressed in arbitrary units, were normalized to the signal obtained from an extract of 500 HeLa cells routinely assayed in parallel. Telomerase activity is expressed quantitatively, which reflects a ratio of the TRAP product ladder bands to internal control band and calculated according to the formula supplied in the manufacturer’s manual, by using Image J Imaging System Software Version 1.3 (National Institutes of Health, Bethesda, MD). The reliability and linearity of total product generated as a measure of telomerase activity have been confirmed by other investigators (20).

**siRNA Transfection**—Double-stranded RNA (dsRNA) was synthesized by Santa Cruz Biotechnology, Inc. Cells in 2-ml aliquots were plated in a 6-well plate at a concentration expected to provide 30–40% confluence 24 h later. At that time, hTERT or scramble (control) siRNA was diluted with 200 μl of Opti-MEM (Sigma). Three micrograms of PolyMag (OZ Biosciences) were placed in a microtube. Three micrograms of the siRNA solution were added to the PolyMag solution and mixed immediately by vigorous pipetting. After 20 min of incubation, 200 μl of serum- and supplement-free Opti-MEM were added, and the solution was added to the cell culture plate. The cell culture plate was placed on a magnetic stirring plate for 20 min. The medium was changed to HUMEDIA EG-2, and the culturing was continued for 24 h.

**Cell Proliferation Assay**—Cells were cultured in Medium 199 (Sigma) containing 1% CSS with vehicle or 10 nM raloxifene in the presence or absence of 100 nM AZT. The medium was changed every day throughout the culture period. The number of viable cells was counted by the trypan blue exclusion test at days 3 and 5 (5 wells in each group and for each time point). The cell condition and responsiveness are better when the cells are maintained in 1% serum than when they are starved for serum,
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FIGURE 1. Raloxifene up-regulates cell proliferation and telomerase activity. A, cells were cultured for 5 days with vehicle, vehicle + 100 nM AZT, 10 nM raloxifene (Ral), or 10 nM Ral + 100 nM AZT (Ral+AZT) by exchanging the culture medium containing these agent(s) with fresh medium every 24 h. The number of viable cells was counted by the trypan blue exclusion test. Data are shown as the means ± S.E. (n = 5 for each point). **p < 0.01 compared with vehicle, AZT, or raloxifene + AZT at day 5. B, cells treated with or without (0 h) vehicle, 10 nM raloxifene (Ral), or 10 nM estrogen (E2) for 24 h were harvested, and the telomerase activity in each preparation was detected by the TRAP assay. Relative telomerase activity, which was quantitative by using Image J Imaging System Software, calculated from the ratio of TRAP product band to the internal control band (IC). All the experiments were repeated three times with consistent results, and a representative result is shown. A representative example of an experiment that was repeated three times is shown. Significant differences are indicated by asterisks. **p < 0.01. C, cells were transfected with scramble (control) or hTRET-specific siRNAs as described under “Experimental Procedures.” Cell lysates were subjected to immunoprecipitation (IP) with anti-hTERT antibody followed by Western blotting (W.B.) using anti-hTERT antibody (A-hTERT, middle panel). To confirm that samples contained equal amounts of protein, cell lysates were subjected to Western blotting using anti-α-tubulin antibody (A-α-tubulin, bottom panel). The molecular masses are noted on the left. Relative densitometric units of the hTERT bands are shown in the top panel, with the density of the vehicle set arbitrarily at 1.0. The values shown represent the means ± S.E. from three separate experiments. Significant differences are indicated by asterisks. **p < 0.01. D, cells were transfected with scramble (control) or hTRET-specific (hTERT) siRNAs as described under “Experimental Procedures.” Transfected cells were treated with vehicle or 10 nM raloxifene (Ral) for 3 days, harvested, and subjected to the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay to assess cell proliferation (n = 12 in each group). Cell growth is shown with the absorbance of scramble siRNA-transfected cells cultured with vehicle set arbitrarily at 100%. The values shown represent the means ± S.E. (n = 12). Significant differences are indicated by asterisks. **p < 0.01.

as reported previously (21). However, even when HUVECs were maintained with serum starvation, similar data were obtained.

Cell Viability Assay—The growth of cells was arrested by incubation in phenol red-free Medium 199 containing 10% CSS for 48 h followed by culturing with serum-free phenol red-free Medium 199 containing 10% CSS with vehicle or 10 nM raloxifene for 3 days. The number of surviving cells was estimated by measuring the absorbance at 485 nm of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt for 3 h as described previously (22).

Reverse Transcriptase-Polymerase Chain Reaction Analysis of RNA—Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Inc.). The expression of hTERT mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA was analyzed by semiquantitative RT-PCR amplification as described previously (23). Briefly, hTERT mRNAs were amplified using the primer pair 5′-CGGAAGAGTTGCTGAGCAA-3′ and 5′-GGATGAAGCGGAGTCGGGA-3′. cDNA was synthesized from 1 μg of RNA using a RNA PCR kit version 2 (TaKaRa, Osaka, Japan) with random primers. Serially diluted cDNA reverse-transcribed from 1 μg of RNA was first amplified by RT-PCR to generate standard curves. The correlation between the band intensity and dose of cDNA template was linear under the conditions described below. Typically, 2-μl aliquots of the reverse-transcribed cDNA were amplified by 28 cycles of PCR in 50 μl of 1× buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2, and 50 mM KCl) containing 1 mM each dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq DNA polymerase (TaKaRa), and each specific primer at 0.2 μM. Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. PCR products were resolved by electrophoresis in a 1% agarose gel. The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers as described previously (23).

Animal Study—Female Sprague-Dawley rats (8 weeks, 180–200 g) were ovariectomized via dorsal approach. One week after the operation, ovariectomized rats were divided into three groups: vehicle, 17β-E2 and raloxifene. Ovariectomized rats were treated with 17β-E2 (0.1 mg/kg/day) or raloxifene (1.0 mg/kg/day) by subcutaneous injection for 7 days. Animals were killed by an overdose of pentobarbital after the designated treatment period. Aortas were removed and were opened longitudinally and endothelium scraped with a scalpel blade. Total RNA was extracted from endothelium using Tri-Reagent, and then we performed RT-PCR and PCR to detect the change of rat TERT (rTERT) expression. The primers used in this study were as follows: rTERT forward primer, 5′-CAGGTAAGCGGGTCCA-3′; rTERT reverse primer, 5′-GAGGACCTGGCAGGACG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 5′-GGATGGTGTGAAACACGAGG-3′; and GAPDH reverse primer, 5′-CAGTGACCTTCC-
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**Figure 2. Raloxifene up-regulates telomerase activity via ER.** A, cells were treated with vehicle, 10 nM raloxifene (Ral), 10 nM raloxifene + 20 μM ICI 182780 (Ral+ICI) for 24 h. The telomerase activity in each preparation was detected by the TRAP assay. Relative telomerase activity, which was quantitative by using Image J Imaging System Software, was calculated from the ratio of TRAP product band to the internal control band (IC). A representative example of an experiment that was repeated three times is shown. Significant differences are indicated by asterisks. **, *p < 0.01. B, cells were transfected with scramble siRNA, ERα-specific (ERα) siRNA, or ERβ-specific (ERβ) siRNA as described under “Experimental Procedures.” Cell lysates were subjected to Western blotting (W.B.) using anti-ERα or anti-ERβ antibody (A-ERα or A-ERβ, upper panel). To confirm that samples contained equal amounts of protein, cell lysates were subjected to Western blotting using anti-α-tubulin antibody (A-α-tubulin, lower panel). Molecular masses are noted on the left. A representative example of an experiment that was repeated three times is shown. Significant differences are indicated by asterisks. **, *p < 0.01.

CGTTCAGCT-3’. The minimum of three animals was included in each group.

**Western Blot Analysis**—The cells were incubated in phenol red-free medium without serum for 16 h and then treated with various agents. They were then washed twice with phosphate-buffered saline and lysed in ice-cold HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 12,000 × g at 4 °C for 15 min, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blocking was done in 10% bovine serum albumin in 1× Tris-buffered saline. Western blot analyses were performed with various specific primary antibodies. For detection of phosphorylated hTERT or detection of the association of hTERT with NFκB p65, cell lysates were prepared using HNTG buffer. The lysates were incubated with anti-hTERT antibody overnight and then immunoprecipitated for 2 h with protein G-Sepharose. Immune complexes were washed with ice-cold HNTG buffer, electrophoresed, and analyzed by immunoblotting with anti-phospho-Akt substrate or anti-NFκB p65 antibody. Immunoreacted bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or antimouse immunoglobulin by using the enhanced chemiluminescence Western blotting system.

**Statistics**—Statistical analysis was performed by Student’s t test, and p < 0.05 was considered significant. The data are expressed as the means ± S.E.

**RESULTS**

**Raloxifene Induces Endothelial Cell Proliferation via the Regulation of Telomerase Activity and hTERT Expression**—We first examined whether raloxifene regulates the proliferation of HUVECs. The number of cells was significantly increased by incubation with 10 nM raloxifene (Fig. 1A). We then treated HUVECs with 10 nM raloxifene in the presence of AZT, an inhibitor of reverse transcriptase (24–27), to test whether telomerase activity was involved in the induction of endothelial cell proliferation by raloxifene. Although AZT alone did not affect the cell proliferation, AZT significantly inhibited the induction of proliferation by raloxifene (Fig. 1A).

Next, to examine whether raloxifene or estrogen induced the telomerase activity, HUVECs were treated with 10 nM raloxifene or 10 nM estrogen for 24 h and subjected to quantitative TRAP assays to assess the telomerase activity. Treatment with either raloxifene or estrogen significantly increased the telomerase activity (Fig. 1B). Moreover, we used hTERT-specific (hTERT) siRNA to examine whether the transcriptional activation of hTERT was involved in the induction of endothelial cell proliferation by raloxifene. We confirmed that the expression of hTERT in HUVECs transfected with hTERT siRNA was significantly lower than that in HUVECs transfected with scramble siRNA (Fig. 1C). Whereas transfection with scramble siRNA had no effect on the induction of cell proliferation by raloxifene, transfection with hTERT siRNA significantly inhibited the induction of cell proliferation by raloxifene (Fig. 1D).

**Raloxifene Induces Telomerase Activity via ERα and ERβ**—We next examined whether the ER is involved in the raloxifene-induced telomerase activation. HUVECs were treated with 10 nM raloxifene in the presence or absence of ICI 182780 and
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subjected to quantitative TRAP assays to assess the telomerase activity. Treatment with ICI 182780 clearly attenuated the raloxifene-induced telomerase activation, suggesting that the ER is involved in the induction of telomerase activity by raloxifene (Fig. 2A). Next we examined which subtype of ER is involved in the raloxifene-induced telomerase activation. HUVECs express both ERα and ERβ (Fig. 2B, lane 1). We confirmed that the expression of ERα and ERβ in HUVECs transfected with ERα-specific (ERα) siRNA and ERβ-specific (ERβ) siRNA were significantly lower than that in HUVECs transfected with scramble siRNA (Fig. 2B). Moreover, transfection with either ERα siRNA or ERβ siRNA significantly reduced the E2 induced ERE-tk109-luc (ERE-tk-luc) reporter activity (28), suggesting that these constructs blocked the functional estrogen receptors (data not shown). Raloxifene-induced telomerase activity was attenuated by transfection with ERα siRNA or ERβ siRNA (Fig. 2C). These data suggest that both ERα and ERβ are involved in the induction of telomerase activity by raloxifene.

Raloxifene Induces hTERT mRNA Expression in Endothelial Cells and in Vivo—Semiquantitative RT-PCR assays were performed to examine whether or not the raloxifene-induced increase in the telomerase activity was due to an increase in the expression of hTERT mRNA. Treatment of HUVECs with 10 nM raloxifene or 10 nM estrogen for 24 h increased the amount of hTERT mRNA (Fig. 3A). Moreover, raloxifene as well as estrogen upregulated the expression of rTERT mRNA in endothelium of ovariectomized rat (Fig. 3B). These results suggest that the up-regulation of telomerase activity by raloxifene and estrogen was, at least in part, due to an increase in the expression of hTERT mRNA.

Raloxifene Induces Telomerase Activity via the PI3K/Akt Cascade—Telomerase activity may also be regulated by post-translational modifications of the enzyme. It has been reported that the region surrounding Ser-824 in hTERT conforms to a consensus sequence for phosphorylation by Akt and that Akt kinase enhances human telomerase activity through the phosphorylation of hTERT (18). Therefore, we next examined whether raloxifene induces the phosphorylation of Akt. Cells were treated with 10 nM raloxifene for various times, and the cell lysates were resolved by SDS-PAGE and then subjected to Western blotting with anti-phospho-Akt or anti-Akt antibody. Although raloxifene did not affect the total amount of Akt (Fig. 4A, bottom panel), it induced transient phosphorylation of Akt (Fig. 4A, top and middle panels), with a maximum effect at 30 min. Next, we examined whether raloxifene induces the phosphorylation of hTERT at the putative Akt phosphorylation site. For this, cells were treated with 10 nM raloxifene for the indicated times and the cell lysates were immunoprecipitated with anti-hTERT antibody and then subjected to Western blotting with anti-phospho-Akt substrate antibody (Fig. 4B). Although raloxifene did not affect the expression of α-tubulin (Fig. 4B, lower panel), the increase by raloxifene of the phosphorylation of hTERT at the putative Akt phosphorylation site reached a peak at 30 min and declined thereafter (Fig. 4B, top and middle panels). To examine whether the PI3K/Akt cascade is involved in the raloxifene- and estrogen-induced telomerase activation, HUVECs were treated with 10 nM raloxifene or 10 nM estrogen in the presence or absence of LY294002 and subjected to quantitative TRAP assays to assess the telomerase activity. LY294002 clearly attenuated both the raloxifene- and estrogen-induced telomerase activation (Fig. 4C), suggesting that the PI3K/Akt cascade is involved in the induction of telomerase activity by raloxifene and estrogen.

Raloxifene Induces Telomerase Activity by Increasing the Association of NFκB with hTERT—It has been reported that IGF-1 and IL-6 activate telomerase activity via PI3K/Akt/NFκB in a human multiple myeloma cell line (27), and NFκB was reported to be a post-translational modifier of telomerase that functions by controlling the intracellular localization of hTERT (29). NFκB is regulated through its association with an inhibitory cofactor, IκB, which sequesters NFκB in the cytoplasm. Phosphorylation of IκB by upstream kinases promotes its degradation, allowing NFκB to translocate to the nucleus with hTERT (30–32). We therefore examined whether or not raloxifene induces the phosphorylation of IκB. For this, cells were treated with raloxifene and then analyzed by Western blotting with anti-Akt or anti-phospho-IκB antibody. Although raloxifene did not affect the expression of Akt (Fig. 5A, bottom panel), it increased the level of phosphorylated IκB, with a maximum effect at 30 min (Fig. 5A, top and middle panels). Moreover, we examined whether raloxifene induces the association of NFκB p65 with hTERT. Cells were treated with raloxifene and used to prepare cell lysates that were immunoprecipitated with anti-TERT antibody and then subjected to Western blotting with anti-NFκB p65 antibody (Fig. 5B, middle panel). Raloxifene did not affect the expression of α-tubulin (Fig. 5B, bottom panel), but the association of hTERT with NFκB p65 was up-regulated by raloxifene with a maximum effect at 30
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A

B

C

FIGURE 4. Raloxifene increases the telomerase activity via the phosphorylation of Akt and hTERT. A, cells were treated with 10 nM raloxifene (Ral) for the indicated times. The lysates were subjected to Western blotting using anti-phospho-Akt (A-p-Akt, middle panel) or anti-Akt (A-Akt, bottom panel) antibody. The molecular masses are noted on the left. Relative densitometric units of the p-Akt bands are shown in the top panel, with the density of the control band (0 min) set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01.

B

C

DISCUSSION

One of the two novel findings in this study was that telomerase activity was induced by raloxifene and the telomerase activation was involved in the raloxifene-induced cell proliferation of HUVECs. The other novel finding was that raloxifene induced the up-regulation of telomerase activity by three mechanisms in HUVECs, i.e. by increasing the amount of hTERT mRNA (Fig. 3), increasing hTERT phosphorylation through the PI3K/Akt cascade (Fig. 4), and increasing the association of hTERT with NFκB (Fig. 5). We have reported that the increase of the phosphorylation of Akt induced by raloxifene is necessary for the raloxifene-induced synthesis of NO in endothelial cells (9). Thus, since the phosphorylation of Akt is involved in both NO synthesis and cell proliferation in vascular endothelial cells, Akt may have an important role in maintaining the functions of vascular endothelial cells.

It appeared that raloxifene-induced hTERT phosphorylation occurred sooner than raloxifene-induced expression of hTERT mRNA, as we detected previously (33–35). Since telomerase activity is regulated not only by transcriptional regulation of hTERT but also by post-transcriptional regulation via Akt-dependent phosphorylation of hTERT, the time frame of raloxifene-induced telomerase activity might be different from that of raloxifene-induced expression of hTERT mRNA. Whether transcriptional and post-transcriptional regulation occurs in parallel or at the same time still remained unclear in this experiment, and additional experiments will be required to answer these questions.

We previously reported that whereas raloxifene inhibited the E2-induced up-regulation of telomerase activity in human breast cancer cells (33), raloxifene, like estrogen, induced the telomerase activity in PC12 cells (34). Raloxifene also induced the telomerase activity in HUVECs like estrogen (Figs. 1B, 2B, and 3B).
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FIGURE 5. Involvement of NFκB cascade in raloxifene-induced telomerase activity. A, cells were treated with 10 nM raloxifene (Ral) for the indicated times. The lysates were subjected to Western blotting using anti-phospho-IκB (A-p-IκB, middle panel) or anti-Akt (A-Akt, bottom panel) antibody. The molecular masses are noted on the left. Relative densitometric units of the p-IκB bands are shown in the top panel, with the density of the control bands (0 min) set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01. B, cells were treated with 10 nM raloxifene (Ral) for the indicated times. The lysates were subjected to immunoprecipitation (I.P.) with anti-IκB antibody (A-IκB, middle panel), followed by Western blotting using anti-NFκB p65 antibody (A-NFκB p65, middle panel). To confirm that samples contained equal amounts of protein, cell lysates were subjected to Western blotting using anti-α-tubulin antibody (A-α-tubulin, bottom panel). The molecular masses are noted on the left. Relative densitometric units of the NFκB p65 bands are shown in the top panel, with the density of the control bands (0 min) set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01. C, cells were treated with vehicle (V), 10 nM raloxifene (Ral), or 10 nM raloxifene + 50 μM BAY (Ral+BAY) for 30 min. The lysates were subjected to immunoprecipitation (I.P.) with anti-IκB antibody, followed by Western blotting using anti-NFκB p65 antibody (A-NFκB p65, middle panel). To confirm that samples contained equal amounts of protein, cell lysates were subjected to Western blotting using anti-α-tubulin antibody (A-α-tubulin, bottom panel). The molecular masses are noted on the left. Relative densitometric units of the NFκB p65 bands are shown in the top panel, with the density of the density of the vehicle bands set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01; *, p < 0.05. D, cells were treated with vehicle (lane 1, vehicle), 10 nM raloxifene (lane 2, Ral), 10 nM raloxifene + 50 μM SN50 (lane 3, Ral+SN50), 10 nM raloxifene + 50 μM BAY-11–7082 (lane 4, Ral+BAY), 50 μM SN50 (lane 5, SN50), or 50 μM BAY-11–7082 (lane 6, BAY) for 24 h. The telomerase activity in each preparation was detected by the TRAP assay. All the experiments were repeated three times with consistent results, and a representative result is shown. E, cells were treated with vehicle (V), 10 nM raloxifene (Ral), 10 nM raloxifene + 20 μM LY294002 (Ral+LY), 10 nM raloxifene + 50 μM SN50 (Ral+SN50), or 10 nM raloxifene + 50 μM BAY-11–7082 (Ral+BAY) for 15 min. The lysates were subjected to Western blotting using anti-phospho-ERK (A-p-ERK) antibody or anti-ERK (A-ERK) antibody. The molecular masses are noted on the left. All the experiments were repeated three times with consistent results, and a representative result is shown. Relative telomerase activity, which was quantitative by using Image J Imaging System Software, was calculated from the ratio of TRAP product band to the internal control band (IC).

and 4C). Thus, whereas raloxifene shows an anti-estrogenic effect in human breast cancer cells, it shows an estrogenic effect in PC12 and HUVECs. We previously demonstrated that the hTERT promoter contains an imperfect palindromic estrogen-responsive element, and transient expression assays using luciferase reporter plasmids containing various fragments of the hTERT promoter showed that this imperfect palindromic estrogen-responsive element is responsible for the transcriptional activation by estrogen (35), indicating that hTERT is a target gene of estrogen. Recently, coregulators were reported to participate in the tissue specificity of selective estrogen receptor modulators (36). Corepressors are involved in the anti-estrogenic activity of raloxifene in breast cancer cells. However, the mechanism by which raloxifene exerts its estrogenic effect in vascular endothelial cells remains unknown. We are currently investigating the involvement of coactivators in the raloxifene-induced telomerase activity in vascular endothelial cells.

Raloixifene inhibited the E2-induced cell proliferation by inhibition of E2-induced up-regulation of telomerase activity via post-translational regulation of Akt-dependent phosphorylation of hTERT in human breast cancer cells (33). Raloxifene also inhibited the platelet-derived growth factor-induced cell proliferation in vascular smooth muscle cells, in part through inhibition of the gene expression of cyclin D1 followed by dephosphorylation of Rb mediated by ERα via a transcription-dependent mechanism, a so-called genomic mechanism (11), and in part by inducing apoptosis through a p38 cascade whose activation is mediated by ERα via a nongenomic mechanism (12). In contrast, raloxifene, like estrogen (36–38), induced cell proliferation in vascular endothelial cells by the up-regulation of telomerase activity via post-translational regulation of Akt-dependent phosphorylation of hTERT. Thus, the
tissue specificity of selective estrogen receptor modulators may also involve “nongenomic” actions, as reported previously for the “genomic” actions. It has been reported that estrogen receptor may be located in caveolae, and intracellular Ca²⁺ and caveolin-1 are key molecules for the regulation of NO synthesis in vascular endothelial cells (40). However, what kinds of molecules participate in the tissue-specific induction of cell proliferation by selective estrogen receptor modulators via the nongenomic mechanism remains unknown.

Two ER isoforms, ERα and ERβ, are expressed in HUVECs (Fig. 2B), as reported previously (42). Which ER is involved in the raloxifene-induced telomerase activity? We have reported that estrogen (10) and raloxifene (9) induced the activation of eNOS via binding to non-nuclear ERα but not ERβ in vascular endothelial cells. Moreover, we have shown that estrogen and raloxifene induced telomerase activity in PC12 cells transfected with the full-length ERα gene (34) and estrogen induced the activation of Akt via ERα, but not via ERβ, in human ovarian cancer cells (41). However, estrogen treatment reduces the vascular injury in the wild-type, ERα knock-out (ERαKO), and ERβKO mice (42–44) but does not reduce the injury in ERαERβ double KO mice (45), suggesting that ERα and ERβ may play redundant roles in vascular protection. Similarly, our observations suggest that raloxifene induced telomerase activity in HUVECs via binding to ERα and ERβ (Fig. 2).

Both estrogen and raloxifene induce NO production (9, 10) and telomerase activity via the Akt cascade in vascular endothelial cells, suggesting that both agents can improve the vascular endothelial function. In hormone replacement therapy, the addition of progestin is required to protect the uterine endometrium. We reported that medroxyprogesterone acetate attenuates the induction of both eNOS activity and NO production by estrogen in HUVECs (5). Since raloxifene exerts estrogen-antagonistic effects on the uterine endometrium (6), the addition of progestin is not required. In addition, although estrogen induces the levels of CRP and triglyceride, raloxifene has no such adverse effects on the levels of CRP and triglyceride (46). The multiple outcomes of raloxifene evaluation (MORE) trial was a randomized study designed to determine whether raloxifene would reduce the risk of fracture in postmenopausal women with osteoporosis (47). The development of cardiovascular disease was a secondary end point of the trial. In post hoc analyses, raloxifene had no effect on the incidence of cardiovascular events in the overall trial population; however, in a subgroup of women at increased risk for cardiovascular events, those assigned to raloxifene compared with placebo experienced significantly fewer cardiovascular events. Thus there is a possibility that the effects of raloxifene will be more pronounced in endothelial cells from blood vessels more prone to atherosclerosis and more relevant to the aging process, and additional experiments will be required to answer these questions. Currently, the RUTH (raloxifene use for the heart) trial, which is a randomized study designed to determine whether raloxifene reduces the incidence of cardiovascular disease in postmenopausal women, is ongoing (48), and we are now awaiting the results.

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