Raloxifene Inhibits Estrogen-induced Up-regulation of Telomerase Activity in a Human Breast Cancer Cell Line*

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The mechanism by which raloxifene acts in the chemoprevention of breast cancer remains unclear. Because telomerase activity is involved in estrogen-induced carcinogenesis, we examined the effect of raloxifene on estrogen-induced up-regulation of telomerase activity in MCF-7 human breast cancer cell line. Raloxifene inhibited the induction of cell growth and telomerase activity by 17β-estradiol (E2). Raloxifene inhibited the E2-induced expression of the human telomerase catalytic subunit (hTERT), and transient expression assays using luciferase reporter plasmids containing various fragments of the hTERT promoter showed that the estrogen-responsive element appeared to be partially responsible for the action of raloxifene. E2 induced the phosphorylation of Akt, and pretreatment with a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, attenuated the E2-induced increases of the telomerase activity and hTERT promoter activity. Raloxifene inhibited the E2-induced Akt phosphorylation. In addition, raloxifene also inhibited the E2-induced hTERT expression via the PI3K/Akt/NFκB cascade. Moreover, raloxifene also inhibited the E2-induced phosphorylation of hTERT, association of NFκB with hTERT, and nuclear accumulation of hTERT. These results show that raloxifene inhibited the E2-induced up-regulation of telomerase activity not only by transcriptional regulation of hTERT via an estrogen-responsive element-dependent mechanism and the PI3K/Akt/NFκB cascade but also by post-translational regulation via phosphorylation of hTERT and association with NFκB.

Chemoprevention, defined as the prevention of cancer by the administration of chemical compounds, is a new approach for the management of cancer. Breast cancer remains a significant health problem for women. The large chemoprevention clinical trial with the selective estrogen receptor modulator tamoxifen showed a 38% reduction in breast cancer incidence (1–4). However, rates of uterine endometrial cancer were increased in all tamoxifen prevention trials (1–4). Ideal chemopreventive agents are nontoxic. Therefore, tamoxifen cannot yet be recommended as a preventive agent except for women at high risk for breast cancer (5). Raloxifene is a nonsteroidal benzothiophene that has also been classified as a selective estrogen receptor modulator (6) on the basis of studies in which it produced both estrogen-agonistic effects on bone (7) and lipid metabolism (8) and estrogen-antagonistic effects on uterine endometrium (9) and breast tissue (10, 11). Because of its ideal tissue selectivity, raloxifene may have fewer side effects than tamoxifen. The MORE (Multiple Outcomes of Raloxifene Evaluation) trial was a randomized study designed to determine whether raloxifene would reduce the risk of fracture in postmenopausal women with osteoporosis (12). The development of breast cancer was a secondary end point of the trial. At a median 48-month follow-up, raloxifene treatment resulted in a 72% reduction in breast cancer incidence without association with an increased risk of uterine endometrial cancer. However, the mechanism by which raloxifene acts to prevent breast cancer remains unclear.

Telomerase is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA (13, 14). This enzyme is specifically activated in most malignant tumors but is usually inactive in normal somatic cells, with the result that telomeres are progressively shortened with cell division in normal cells (15, 16). Cells require a mechanism to maintain telomere stability to overcome replicative senescence, and telomerase activation may therefore be a rate-limiting or critical step in cellular immortalization and oncogenesis (17). For example, telomerase activity is known to be involved in estrogen-induced carcinogenesis (18). The level of telomerase activity in cells can be regulated by modulating both the expression and phosphorylation of the catalytic subunit (hTERT).1 The hTERT promoter contains an imperfect palindromic estrogen-responsive element (ERE), and it was reported that estrogen activates telomerase via direct and indirect effects on hTERT in MCF-7 cells (18). However, the mechanism of the indirect effects remains unclear. It was reported that the hTERT promoter contains two putative NFκB-binding motifs (19) and that IGF-1 and IL-6 activate the PI3K/Akt/NFκB cascade in a human multiple myeloma cell line (20). Thus, it is

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1 The abbreviations used are: hTERT, human telomerase reverse transcriptase subunit; E2, 17β-estradiol; ERE, estrogen-responsive element; ER, estrogen receptor; RT, reverse transcription; PI3K, phosphatidylinositol 3-kinase; NFκB, nuclear factor κB; IκB, inhibitor of NFκB; TPA, 12-O-tetradecanoylphorbol-13-acetate; CSS, charcoal-stripped serum; HA, hemagglutinin; NLS, nuclear localization signal; URR, upstream regulatory region.
possible that estrogen enhances the transcription of hTERT via the PI3K/Akt/NFκB cascade.

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 (21). In addition, it was reported that an Akt cascade mediates the estrogen-induced S phase entry and cyclin D1 promoter activity in MCF-7 cells (22). Thus, it is possible that estrogen enhances human telomerase activity through an Akt cascade. Another possible mechanism for post-translational modulation of telomerase activity is via the interaction of hTERT with accessory proteins. Recently, it was reported that 14-3-3 proteins (23, 24) and NFκB (25) are post-translational modifiers of telomerase that function by controlling the intracellular localization of hTERT.

These findings led us to examine whether estrogen induces up-regulation of telomerase activity not only by transcriptional regulation of hTERT via an ERE-dependent mechanism and a PI3K/Akt/NFκB cascade but also by post-translational regulation via Akt-dependent phosphorylation of hTERT in MCF-7 cells. In addition, we attempted to clarify the mechanism by which raloxifene inhibits the induction of telomerase activity by estrogen.

**ExPERIMENTAL PROCEDURES**

*Materials—* Raloxifene analog LY117018 was a kind gift from Eli Lilly Research Laboratories (Indianapolis, IN). 17β-Estradiol, TPA, and rabbit IgG were purchased from Sigma. ICI 182,780 was obtained from TOCRIS (Ballwin, MO). LY294002 was purchased from Calbiochem. The anti-phospho-Akt, phospho-Akt substrate, and Akt antibodies were obtained from Cell Signaling (Beverly, MA). The anti-HA, IκBα, IκBα-M, and hTERT antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The IκBα phosphorylation inhibitor BAY-11-7082 was purchased from Alexis Biochemicals (San Diego, CA). The specific NFκB nuclear translocation inhibitor SN-50 was purchased from BIOMOL (Plymouth Meeting, PA). Hoechst 33258 was obtained from Molecular Probes (Eugene, OR).

*Constructs—* pCR3 vector and pCR3-hTERT were kind gifts from Dr. Takashi Tsuruo (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan) (24). The pCR-FLAG-p50 and pCR-FLAG-p50NLS constructs were kind gifts from Dr. Gourisankar Ghosh (Department of Chemistry and Biochemistry, University of California, San Diego, CA) (26).

*Cell Culture—* MCF-7 human breast cancer cells were obtained from the American Type Culture Collection. The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in the presence of 5% CO2 at 37°C.

*Cell Proliferation Assay—* The cells were plated at a density of 10^4 cells/well in 12-well plates and allowed to attach overnight. The cells were growth-arrested by phenol red-free Dulbecco’s modified Eagle’s medium with 10% charcoal-stripped serum (CSS) for 48 h and were then treated with vehicle, E2, raloxifene, or E2 + raloxifene by exchanging the culture medium containing these agent(s) with fresh medium every 48 h for 8 days. A Neubauer chamber was used to count the cell number, and a trypan blue exclusion test was carried out to determine the cell viability. All of the experiments were carried out in quadruplicate. The values shown are the means ± S.E. of three independent experiments performed in quadruplicate at three different passages of the cell lines.

*Stretch PCR Assay—* For quantitative analysis of telomerase activity, stretch PCR assays were performed using the Telochaser system according to the manufacturer’s protocol (Toyobo, Tokyo, Japan) as described previously (18). The PCR products were electrophoresed on a 7% polyacrylamide gel and visualized with SYBR green I nucleic acid gel stain (FMC BioProducts, Rockland, ME). To monitor the efficacy of PCR amplification, 10 ng of internal control consisting of phage DNA (Toyobo) together with 50 pmol of specific primers (Toyobo) were added to the PCR mixture per reaction. Band intensity was measured using NIH Image software.

*Rt-PCR Analysis—* 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 (18). Briefly, hTERT mRNA was amplified using the primer pair 5’-CGGAAGAGTGCTGGAGCAA-3’ and 5’-GGAAGACGGAGGATCTTGAGA-3’ cDNA was synthesized from 1 μg of RNA using an RNA PCR kit version 2 (TaKaRa, Ohtsu, Japan) with random primers. Serially diluted cDNA reverse-transcribed from 1 μg of RNA was first amplified.

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**FIG. 1.** Raloxifene attenuates the E2-induced cell growth and telomerase activity. A, MCF-7 cells were growth-arrested for 48 h and were then cultured with 10 nM E2, 10 nM raloxifene, or 10 nM E2 + 10 nM raloxifene by exchanging the culture medium containing these agent(s) with fresh medium every 48 h for 8 days. The number of viable cells was counted by the trypan blue exclusion test. The data are shown as the means ± S.E. from at least three separate experiments. B, MCF-7 cells were treated with 10 nM E2 (lane 2), 10 nM raloxifene (RAL, lane 3), 10 nM E2 + 10 nM raloxifene (E2+RAL, lane 4), or 10 nM E2 + 1 μM ICI 182,780 (E2+ICI, lane 5) for 24 h. The telomerase activity in each preparation was detected by the stretch PCR assay. A representative example of an experiment that was repeated three times is shown.
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 μM 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 gapped PCR (HindIII digestion of RT-PCR product) using a gap-specific primer as described previously (18).

Luciferase Assay—Plasmids pGL3-3328 and pGL3-2000 are hTERT promoter-luciferase reporters in which full-length or 5′-deleted promoters including a 77-bp 5′-untranslated region are cloned upstream of the luciferase gene in pGL3-Basic at MluI and BglII sites (18). pGL3-ERE-promoter was constructed by inserting head-to-tail tetramers of ERE located at −2677 in the hTERT promoter, into the enhancer-less vector pGL3-promoter (18). pGL2-HPV31URR-luc vector is an HPV-31 enhancer and promoter-luciferase reporter containing the whole upstream regulatory region (URR) of HPV-31 cloned upstream of the luciferase gene in pGL2-Basic (27). These reporter plasmids were transiently transfected into cells for 24 h using LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol. The cells were harvested and subjected to luciferase assays using a luciferase assay system (Promega) according to the manufacturer's protocol. The luciferase activity was normalized to β-galactosidase activity, and the activity in cells treated with vehicle was set at 1.0. The data are expressed as the mean fold activation ± S.E. of six transfections.

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. The lysates were centrifuged at 12,000 × g 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 association of hTERT with NFκB p65, cell lysates were prepared using HNTG buffer. The lysates were incubated with
Fig. 3. Raloxifene inhibits E2-induced Akt phosphorylation. A and B, MCF-7 cells were treated with 10 nM E2 for various times (A) or treated with 10 nM E2 (lane 2), 10 nM raloxifene (lane 3), 10 nM E2 + 10 nM raloxifene (lane 4), 10 nM E2 + 1 μM ICI 182,780 (lane 5), or 10 nM E2 + 20 μM LY294002 (lane 6) for 60 min (B) and then harvested and used to prepare cell lysates. The lysates were subjected to SDS-PAGE and blotted with anti-phospho-Akt substrate antibody or anti-Akt (middle panels) or anti-Akt (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-Akt bands are shown in the top panels, with the density of the vehicle bands (0 min) set arbitrarily at 1.0. The values shown represent the mean ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks. **, p < 0.01. C, MCF-7 cells were treated with 10 nM E2 (lane 2) or 10 nM E2 + 20 μM LY294002 (lane 3) for 24 h. Telomerase activity in each preparation was detected by the stretch PCR assay. A representative example of an experiment that was repeated three times is shown. W.B., Western blot; RAL, raloxifene; LY, LY294002.

Anti-hTERT antibody overnight and then immunoprecipitated for 2 h with protein A-Sepharose. Immune complexes were washed with ice-cold HNTG buffer, electrophoresed, and analyzed by immunoblotting with anti-phospho-Akt substrate antibody or anti-NFκB p65 antibody. Immunoreacted bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system.

Fluorescence Microscopy—MCF-7 cells were grown on glass coverslips in six-well dishes. The cells were transfected with the pCR3-hTERT plasmid for 24 h and then incubated with various reagents. The cells were fixed with 10% formalin for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 3% bovine serum albumin for 1 h. Anti-HA antibody and Alexa Fluor secondary antibody were used at 2 μg/μl in blocking solution. The cells were counterstained with 10 mMol/liter Hoechst 33258 to visualize the nucleus. The samples were mounted on glass slides with Vectashield (Vector Laboratories), and the cells were examined using fluorescence microscopy. For quantification experiments, 100 cells were scored according to whether hTERT was higher in the nucleus (N), evenly distributed between nucleus and cytoplasm (N+C), or higher in the cytoplasm (C). The data represent the mean of three independent experiments.

Statistics—Statistical analysis was performed by Student’s t test, and p < 0.01 was considered significant. The data are expressed as the means ± S.E.

RESULTS

Raloxifene Attenuates the E2-induced Up-regulation of Telomerase Activity and hTERT Expression—We first examined whether or not raloxifene regulates the proliferation of MCF-7 human breast cancer cells (Fig. 1A). E2 significantly induced cell growth at 10 nM. Although 10 nM raloxifene had no effect on the basal cell growth, it did significantly inhibit the E2-induced cell growth. To examine the effects of raloxifene on the estrogen-induced telomerase activity, MCF-7 human breast cancer cells were treated with 10 nM E2, 10 nM raloxifene, 10 nM E2 + 10 nM raloxifene, or 10 nM E2 + 1 μM ICI 182,780 (a highly selective ER antagonist) for 24 h (Fig. 1B). ICI 182,780 (positive control) attenuated the E2-induced telomerase activity (Fig. 1B, lane 5). Although raloxifene had no effect on basal telomerase activity (Fig. 1B, lane 3), it attenuated the E2-induced increase in telomerase activity (Fig. 1B, lane 4). Semi-quantitative RT-PCR assays were performed to examine whether the attenuation of estrogen-induced telomerase activity by raloxifene was due to the attenuation by raloxifene of the estrogen-induced up-regulation of the expression of hTERT (Fig. 2A). As we previously reported (29), treatment of MCF-7 cells with 1 μM tamoxifen for 24 h attenuated the up-regulation of hTERT mRNA induced by 10 nM E2 (Fig. 2A, lane 5). Treatment of MCF-7 cells with 10 nM raloxifene for 24 h also attenuated the up-regulation of hTERT mRNA induced by 10 nM E2 (Fig. 2A, lane 6).

To examine the effect of raloxifene on the estrogen-induced transcriptional activity of the hTERT promoter, luciferase assays of cells into which hTERT-promoter reporter plasmids were transfected were performed (Fig. 2B). We have previously
reported that an imperfect palindromic ERE is located at -2677 in the hTERT promoter and is capable of direct association with ER (18). Luciferase reporter plasmids containing the full-length hTERT 5′ regulatory region (pGL3–3328) or a deletion mutant lacking the imperfect palindromic ERE (pGL3–2000) were transfected into MCF-7 cells. ICI 182,780 attenuated the E2-induced transcriptional activation of pGL3–3328 (Fig. 2B, lane 5). Raloxifene also attenuated the E2-induced transcriptional activation of pGL3–3328 (Fig. 2B, lane 4). To examine whether the ERE at -2677 is involved in the E2-induced up-regulation of the hTERT promoter activity and the inhibitory effect of raloxifene, this putative ERE was cloned upstream of the SV40 promoter in a luciferase reporter plasmid (pGL3-ERE-promoter) and used for transfection. Although E2 had no effect on transcriptional activation of enhancer-less pGL3-promoter containing only SV40 promoter (data not shown), E2 treatment caused transcriptional activation of pGL3-ERE-promoter (Fig. 2C, lane 2), as we reported previously (28). Treatment with raloxifene (Fig. 2C, lane 4) or ICI 182,780 (Fig. 2C, lane 5) attenuated the E2-induced transcriptional activation of the pGL3-ERE-promoter. E2 did activate the transcriptional activity of pGL3–2000 to some extent (Fig. 2B, lane 2), and raloxifene (Fig. 2B, lane 4) or ICI (Fig. 2B, lane 5) attenuated the E2-induced transcriptional activation of pGL3–2000. These results suggest that the ERE at -2677 is partially responsible for the E2-induced activation of the hTERT promoter and the inhibitory effect of raloxifene.

We examined whether raloxifene has a general inhibitory effect on transcription. Raloxifene did not have an inhibitory effect on 10% CSS-induced hTERT promoter activity using a deletion mutant of the imperfect palindromic ERE (pGL3–2000) (Fig. 2D). In addition, we used HPV31URR-luc, which is a well characterized reporter for examining the AP-1 activity (27). Raloxifene did not have an inhibitory effect on TPA-induced HPV31 promoter activity (Fig. 2E). These results suggest that raloxifene does not have an inhibitory effect on the induction of AP-1 or SRE activity in promoters that lack an ERE.

Raloxifene Inhibits E2-induced Akt Phosphorylation—Because it was previously reported that hTERT expression is induced via an Akt cascade (21), we first examined whether E2 induces Akt phosphorylation in MCF-7 cells. The cells were treated with E2 for various times and then used to prepare lysates that were subjected to Western blotting with anti-phospho-Akt (panel ii), or anti-Akt (panel iv) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-IκB bands are shown in panel i with the density of the vehicle bands set arbitrarily at 1.0. The values shown represent the means ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks.

**p < 0.01.

**p < 0.001.

M LY249002 for 30 min (Fig. 3A, lane 2), or LY294002 for 30 min (Fig. 3A, lane 3), or 10 nM E2 + 10 nM raloxifene for 30 min (lane 4) and then harvested and used to prepare cell lysates that were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-phospho-IκB (panel ii), anti-IκB (panel iii), or anti-Akt (panel iv) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-IκB bands are shown in panel i with the density of the vehicle bands set arbitrarily at 1.0. The values shown represent the means ± S.E. from at least three separate experiments. Significant differences are indicated by asterisks.

**p < 0.01.
cascade is involved in the E2-induced up-regulation of telomerase activity and hTERT expression.

Next, we examined whether raloxifene attenuates the E2-induced Akt phosphorylation. Although raloxifene at 10 nM did not affect the expression of Akt (Fig. 3B, bottom panel, lane 3), it inhibited the E2-induced Akt phosphorylation (Fig. 3B, middle and top panels, lane 4).

**Raloxifene Inhibits E2-induced hTERT Expression via PI3K/Akt Cascade**—It has been reported that the hTERT promoter contains two putative NFκB-binding motifs (19) and that IGF-1 and IL-6 activate PI3K/Akt/NFκB in a human multiple myeloma cell line (20). Therefore, we examined whether NFκB is a nuclear target in the E2-induced up-regulation of hTERT expression via the Akt cascade. 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 and induce target genes (30, 31). We first examined whether E2 induces the phosphorylation and degradation of IκB (Fig. 4A). The cells were treated with E2 for 30 min and used to prepare lysates that were analyzed by Western blotting with anti-phospho-IκB, anti-IκB, or anti-Akt antibody. Although the each expression of Akt was not changed (Fig. 4A, panel iv), E2-simulated MCF-7 cells showed an increase in phosphorylated IκB (Fig. 4A, panel ii, lane 2) and subsequent degradation of IκB (Fig. 4A, panel iii, lane 2), and treatment with LY294002 attenuated the E2-induced phosphorylation (Fig. 4A, panel ii, lane 4) and degradation of IκB (Fig. 4A, panel iii, lane 4), suggesting that E2 induces the activation of NFκB through phosphorylation and degradation of IκB in a PI3K-dependent manner. Moreover, we examined the effect of raloxifene on the E2-induced phosphorylation and degradation of IκB. Raloxifene inhibited the E2-induced IκB phosphorylation (Fig. 4A, panel ii, lane 3) and degradation (Fig. 4A, panel iii, lane 3).

We next examined whether NFκB is involved in the induction of hTERT promoter activity by E2. Five homologous polypeptides, p50, p65, c-Rel, RelB, and p52, comprise the mammalian Rel/NFκB transcription factor family. The subunits associate in a combinatorial fashion to form transcriptionally active homo- and hetero-dimers. The best characterized species of NFκB dimer is the p50/p65 hetero-dimer (32). A previous report demonstrated that the nuclear localization signal (NLS) polypeptide of p50 is required for its translocation to the nucleus (33) and that p50ANLS lacking the NLS domain inhibits the nucleocytoplasmic shuttling of NFκB dimers. Therefore, we examined the effect of p50ANLS on the induction of hTERT promoter activity by E2. Cotransfection of p50ANLS resulted in a significantly weaker transactivation by E2 of pGL3–2000, a deletion mutant of the imperfect palindromic ERE, compared with the induction in cells expressing wild-type p50 (Fig. 4B). The result suggests that NFκB plays a pivotal role in E2-induced transcriptional activation of pGL3–2000.

**Raloxifene Inhibits E2-induced Phosphorylation of hTERT at a Putative Akt Phosphorylation Site**—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 phospho-
hTERT and Nuclear Accumulation of hTERT

The positions of molecular mass markers are noted on the lane 4.

We examined whether E2 induces the association of NFκB with hTERT at a putative Akt phosphorylation site. The cells were treated with E2 for the indicated times and then used to prepare lysates that were immunoprecipitated with anti-hTERT antibody and then subjected to Western blotting with anti-NFκB p65 antibody (Fig. 5A, middle panel) or anti-hTERT antibody (Fig. 5A, bottom panel). Although E2 did not affect the expression of hTERT (Fig. 5A, bottom panel), an increase in hTERT phosphorylation at a putative Akt phosphorylation site was induced by E2, reached a peak at 30 min, and declined thereafter (Fig. 5A, middle and top panels, lanes 2 and 3). Although raloxifene did not affect the expression of hTERT (Fig. 5B, bottom panel, lane 3), it inhibited the E2-induced hTERT phosphorylation (Fig. 5B, middle and top panels, lane 4).

**Raloxifene Inhibits E2-induced Association of NFκB with hTERT**

One possible mechanism for the post-translational modulation of telomerase activity is via the interaction of hTERT with accessory proteins. Recently, it was reported that NFκB is a post-translational modifier of telomerase that functions by controlling the intracellular localization of hTERT (25). Therefore, we examined whether E2 induces the association of NFκB p65 with hTERT. The cells were treated with E2 for the indicated times, used to prepare cell lysates that were immunoprecipitated with anti-hTERT antibody or anti-HA antibody (nonrelevant control antibody), and then subjected to Western blotting with anti-NFκB p65 antibody (Fig. 6A, middle panel) or anti-hTERT antibody (Fig. 6A, bottom panel). Neither hTERT nor NFκB p65 expression was detected in E2 treatment in immunoprecipitates with anti-HA antibody (Fig. 6B, lane 5). E2 did not affect the expression of hTERT (Fig. 6A, bottom panel), but the association of hTERT with NFκB p65 was transiently up-regulated by E2 (Fig. 6A, middle and top panels). Although raloxifene did not affect the expression of hTERT (Fig. 6B, bottom panel, lane 3), it inhibited the E2-induced association of NFκB p65 with hTERT (Fig. 6B, middle and top panels, lane 4).

We also examined whether E2 induces nuclear accumulation of hTERT. The cells were transfected with pcR3-hTERT-HA and subjected to indirect immunofluorescence staining to evaluate the expression of hTERT-HA. The cells were also counterstained with Hoechst 33258 to visualize the nucleus. hTERT-expressing cells were evaluated for nuclear (N), nuclear-cytoplasmic (N+C), and cytoplasmic (C) fluorescence (Fig. 7A). E2 induced nuclear accumulation of hTERT (Fig. 7B). Cotreatment with LY294002, ICI 182,780, an IκBα phosphorylation inhibitor (BAY-11-7082) (34), or a specific NFκB nuclear translocation inhibitor (SN-50) attenuated the E2-induced nuclear accumulation of hTERT (Fig. 7B), suggesting that E2-induced nuclear accumulation of hTERT is mediated by ER via the PI3K/Akt/NFκB cascade. Raloxifene also inhibited the E2-induced nuclear accumulation of hTERT (Fig. 7B).
One of the two novel findings in this study was that the induction of telomerase activity by estrogen is due not only to transcriptional regulation of hTERT via an ERE-dependent mechanism and a PI3K/Akt/NFκB cascade but also to post-translational regulation via phosphorylation of hTERT and association with NFκB in MCF-7 cells, which is also true of the mechanism by which cytokines modulate telomerase activity (20, 25). The other novel finding we made was that raloxifene inhibited the E2-induced up-regulation of telomerase activity via not only transcriptional regulation but also post-translational regulation of hTERT. It thus seems that the inhibition of Akt phosphorylation by raloxifene is one of the key events in the inhibition of estrogen-induced up-regulation of telomerase activity. This finding about Akt phosphorylation is consistent with a large body of evidence reported in the following publications. Estrogen-induced Akt activation is blocked by the antiestrogens ICI 182,780 and 4-hydroxytamoxifen (35). It also has been reported that constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, and tamoxifen in breast cancer cells (36), that activation of Akt in breast cancer predicts a worse outcome among endocrine-treated patients (37), and that Akt protects breast cancer cells from tamoxifen-induced apoptosis (38). Thus, Akt may have an important role in the chemoprevention of breast cancer.

**DISCUSSION**

One of the two novel findings in this study was that the induction of telomerase activity by estrogen is due not only to transcriptional regulation of hTERT via an ERE-dependent mechanism and a PI3K/Akt/NFκB cascade but also to post-translational regulation via phosphorylation of hTERT and association with NFκB in MCF-7 cells, which is also true of the mechanism by which cytokines modulate telomerase activity (20, 25). The other novel finding we made was that raloxifene inhibited the E2-induced up-regulation of telomerase activity via not only transcriptional regulation but also post-translational regulation of hTERT. It thus seems that the inhibition of Akt phosphorylation by raloxifene is one of the key events in the inhibition of estrogen-induced up-regulation of telomerase activity. This finding about Akt phosphorylation is consistent with a large body of evidence reported in the following publications. Estrogen-induced Akt activation is blocked by the antiestrogens ICI 182,780 and 4-hydroxytamoxifen (35). It also has been reported that constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, and tamoxifen in breast cancer cells (36), that activation of Akt in breast cancer predicts a worse outcome among endocrine-treated patients (37), and that Akt protects breast cancer cells from tamoxifen-induced apoptosis (38). Thus, Akt may have an important role in the chemoprevention of breast cancer.
ing in breast cancer cells, because we have reported that estrogen induces the activation of endothelial nitric-oxide synthase via a PI3K/Akt cascade in a nongenomic manner in vascular endothelial cells (39). Moreover, we have reported that raloxifene induces the activation of endothelial nitric-oxide synthase via binding to non-nuclear ERα in vascular endothelial cells (40). Thus, raloxifene might also bind to the non-nuclear ER in breast cancer cells and competitively block the action of estrogen. Because raloxifene also inhibited the E2-induced up-regulation of telomerase activity by transcriptional regulation of hTERT via an ERE-dependent mechanism and a PI3K/Akt/NFκB cascade but also by post-translational regulation via phosphorylation of hTERT and association with NFκB in MCF-7 cells (Fig. 8).

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