Both estrogen and raloxifene protect against β -amyloid-induced neurotoxicity in estrogen receptor α -transfected PC12 cells by activation of telomerase activity via Akt cascade

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Abstract

Although estrogen is known to protect against β -amyloid (A β)-induced neurotoxicity, the mechanisms responsible for this effect are only beginning to be elucidated. In addition, the effect of raloxifene on A β -induced neurotoxicity remains unknown. Here we investigated whether raloxifene exhibits similar neuro-protective effects to estrogen against A β -induced neurotoxicity and the mechanism of the effects of these agents in PC12 cells transfected with the full-length human estrogen receptor (ER) α gene (PCER). Raloxifene, like 17 β -estradiol (E₂), significantly inhibited A β -induced apoptosis in PCER cells, but not in a control line of cells transfected with vector DNA alone (PCCON). Since telomerase activity, the level of which is modulated by regulation of telomerase catalytic subunit (TERT) at both the transcriptional and post-transcriptional levels, is known to be involved in suppressing apoptosis in neurons, we examined the effect of E₂ and raloxifene on telomerase activity. Although both E₂ and

raloxifene induced telomerase activity in PCER cells, but not in PCCON cells, treated with A β , they had no effect on the level of TERT expression. These results suggest that neither E₂ nor raloxifene affects the telomerase activity at the transcriptional level. We therefore studied the mechanism by which E₂ and raloxifene induce the telomerase activity at the post-transcriptional level. Both E₂ and raloxifene induced the phosphorylation of Akt, and pre-treatment with a phosphatidylinositol 3-kinase inhibitor, LY294002, attenuated both E₂- and raloxifene-induced activation of the telomerase activity. Moreover, both E₂ and raloxifene induced both the phosphorylation of TERT at a putative Akt phosphorylation site and the association of nuclear factor κ B with TERT. Our findings suggest that E₂ and raloxifene exert neuroprotective effects by telomerase activation via a post-transcriptional cascade in an experimental model relevant to Alzheimer's disease.

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Introduction

Alzheimer's disease (AD) is one of the most common neuro-degenerative diseases and is the most frequent cause of dementia in the elderly (Bondi *et al.* 1995). However, the cause of AD is still unknown and the treatment is therefore only palliative. Compared with men, women appear to be at an increased risk for AD after age 80 to 85 years (Miech *et al.* 2002, Zandi *et al.* 2002). Postmenopausal depletion of endogenous estrogens may contribute to this risk. Several clinical trials have shown that women who received estrogen replacement therapy had a reduced risk of developing AD (Tang *et al.* 1996, Kawas *et al.* 1997, Paganini-Hill & Henderson 1996). Estrogens may exert

several neuroprotective effects on the aging brain, including inhibition of β -amyloid (A β) formation, stimulation of cholinergic activity, reduction of oxidative stress-related cell damage, and protection against vascular risks (Skoog & Gustafson 1999). However, the precise mechanism of the neuroprotective mechanism of estrogen has not been elucidated.

Although estrogen replacement therapy is widely prescribed, it may have certain disadvantages because it has been shown to be associated with an increased risk of developing breast and uterine cancers. Thus, because of the need to circumvent the limitations of estrogen replacement therapy, there has been intense interest in the therapeutic use of nonsteroidal selective estrogen receptor

modulators (SERMs). Raloxifene, which has been classified as a SERM, produces both estrogen-agonistic effects on the bone and lipid metabolism and estrogen-antagonistic effects on the uterine endometrium and breast tissue (Delmas *et al.* 1997, Cummings *et al.* 1999, Ettinger *et al.* 1999). Although raloxifene is known to induce neurite outgrowth in estrogen receptor (ER)-positive PC12 cells (Nilsen *et al.* 1998), the mechanism of neuroprotection by raloxifene has also not been clearly resolved.

Telomerase is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA (Greider & Blackburn 1985, 1989). This enzyme is specifically activated in most malignant tumors but is usually inactive in normal somatic cells, with the result that the ends of chromosomes (telomeres) are progressively shortened during maturation and aging (Kim *et al.* 1994, Shay & Bacchetti 1997). Cells require a mechanism to maintain telomere stability in order to overcome replicative senescence, and telomerase activation may therefore be a rate-limiting or critical step in cellular immortality and oncogenesis (Harley & Villeponteau 1995). Telomerase consists of an RNA subunit and a protein called the catalytic subunit of telomerase (TERT). Although the RNA subunit of the telomerase complex is constitutively expressed in both tumor and normal somatic tissues, the expression of TERT correlates with telomerase activity during cellular differentiation and neoplastic transformation (Kilian *et al.* 1997, Meyerson *et al.* 1997, Nakamura *et al.* 1997). It was recently reported that TERT protects neurons against A β - (Zhu *et al.* 2000) or tropic factor withdrawal- and glutamate (Fu *et al.* 2000)-induced apoptosis.

There is abundant evidence supporting the idea that the regulation of telomerase in mammalian cells is multifactorial. Telomerase activity can be regulated by modulating both the expression and phosphorylation of TERT. The region surrounding Ser-824 in human (h) TERT conforms to a consensus sequence for phosphorylation by Akt, and Akt kinase enhances the human telomerase activity through phosphorylation of hTERT (Kang *et al.* 1999). In addition, it was recently reported that estrogen protects against A β - (Zhang *et al.* 2001) or glutamate (Honda *et al.* 2000)-induced neurotoxicity via the activation of Akt. Moreover, pro-atherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism (Breitschopf *et al.* 2001) and we have reported that both estrogen (Hisamoto *et al.* 2001a) and raloxifene (Hisamoto *et al.* 2001b) induce endothelial nitric oxide synthase (eNOS) activation via an Akt cascade. Thus, it appears possible that estrogen and raloxifene exert neuroprotective effects by enhancing the human telomerase activity through an Akt cascade.

These findings led us to examine whether raloxifene has a neuroprotective function, as estrogen does, and whether the effects are induced by enhancing human telomerase activity through an Akt cascade.

Materials and Methods

Materials

Synthetic A β (human, 25–35), the cytotoxic sequence between amino acid residues 25 and 35 A β , was purchased from Peptide Institute (Osaka, Japan). LipofectAMINE Plus reagent and G418 (geneticin) were purchased from Invitrogen (Carlsbad, CA, USA). Raloxifene analog, LY117018, was a kind gift from Eli Lilly Research Laboratories (Indianapolis, IN, USA). 17 β -estradiol (E₂), dimethyl sulfoxide (DMSO), and rabbit IgG (immunoglobulin G) were purchased from Sigma. LY294002 was purchased from Calbiochem (San Diego, CA, USA). The anti-phospho-Akt, phospho-Akt substrate, and Akt were obtained from Cell Signaling (Beverly, MA, USA). The anti-nuclear factor kappaB (NF κ B) p65, TERT and ER α antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and experimental conditions

PC12 rat pheochromocytoma cells transfected with the full-length human ER α gene (PCER) or with vector DNA alone (PCCON) were a kind gift from Dr Monica M Oblinger (Chicago Medical School, North Chicago, IL, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μ g/ml streptomycin sulfate in the presence of 5% CO₂ at 37 °C.

Apoptosis assay

Five thousand cells/well were placed into extracellular matrix-coated chamber slides in DMEM with 10% FBS, and then starved for 48 h in phenol red-free DMEM with 10% charcoal-stripped serum (CSS). After starvation, some cells were treated with various materials. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using an Apoptosis *In Situ* Detection kit (Wako, Osaka, Japan), and the cells undergoing programmed cell death were counted in five separate fields per experiment.

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 (Kyo *et al.* 1999, Kawagoe *et al.* 2003, Kimura *et al.* 2004). Briefly, we resuspended the cell pellets in cell lysis buffer so that an aliquot of 20 μ l corresponded to 25 000 cells. After incubation for 60 min at 37 °C, the DNA product was isolated and 26 cycles of

Figure 1 Both E_2 and raloxifene attenuate the $A\beta$ -induced apoptosis. (A) Lysates (250 μ g protein) of PC12 cells transfected with control vector (PCCON) (lane 1), ER α vector (PCER) (lane 2), or MCF-7 (lane 3) cells were resolved by 8% SDS-PAGE and subjected to Western blotting (W.B.) with anti-ER α (upper panel) or anti- α -tubulin (lower panel) antibody. The positions of molecular weight markers are noted on the left. Experiments were repeated three times with essentially similar results. (B) PCCON (left) and PCER (right) cells were grown on a chamber slide. Cells were pretreated with (lower panels) or without (upper panels) 20 μ M $A\beta$ followed by treatment with vehicle control (i), 10^{-8} M E_2 (ii), or 10^{-8} M raloxifene (iii) for 24 h. Cells were stained by the TUNEL method using a kit for detection of apoptosis *in situ*. Apoptotic cells were stained darker and were counted by visual inspection under a light microscope. Methyl green solution (0.5%) was also used for counterstaining. Magnification: $\times 200$. Bar = 100 μ m. (C) The percentage of apoptotic cells in 100 examined cells is shown as the proportion of apoptotic cells in the cultures treated with vehicle control set arbitrarily at 100%. The ratio of apoptotic cells is shown as a percentage of that of the vehicle control group. Values shown represent the mean $\pm$ S.E. from five separate experiments. Significant differences are indicated by asterisks, $**P < 0.01$.

PCR amplification were performed at 95 $^{\circ}$ C for 30 s, at 68 $^{\circ}$ C for 30 s and at 72 $^{\circ}$ C for 45 s. The PCR products were electrophoresed on a 7% polyacrylamide gel and visualized with SYBR Green I Nucleic Acid Gel Stain (FMC BioProducts, Rockland, ME, USA). To monitor the efficiency of PCR amplification, 10 ng of an 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 developed at the US National Institute of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>.

RT-PCR analysis

Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The expression of rat TERT (rTERT) mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by semiquantitative RT-PCR amplification as described previously (Kyo *et al.* 1999). Briefly, rTERT mRNAs were amplified using the primer pair 5'-GGCTCTTCTTCTACCGTAAG-3' and 5'-TGATGCTTGACCTCCTCTTG-3'. cDNA was synthesized from 1 μ g RNA using an RNA PCR kit version 2

Figure 2 Both E₂ and raloxifene increase the telomerase activity. PCCON (left panel) and PCER (right panel) cells pretreated with 20 μM Aβ were incubated with vehicle control (lanes 1 and 4), 10⁻⁸ M E₂ (lanes 2 and 5), or 10⁻⁸ M raloxifene (lanes 3 and 6) 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.

(TaKaRa, Ohtsu, Japan) with random primers. Serially diluted cDNA reverse-transcribed from 1 μg RNA was first amplified by RT-PCR to generate standard curves. The correlation between 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 29 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 of dATP, dCTP, dGTP and dTTP, 2.5 units Taq DNA polymerase (TaKaRa), and each specific primer at 0.2 μM. Each cycle consisted of denaturation at 94 °C for 60 s, annealing at 52 °C for 60 s, and extension at 72 °C for 90 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 GAPDH-specific primers as described previously (Kyo *et al.* 1999).

Western blot analysis

Cells were incubated in phenol red-free medium without serum for 48 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) (Mabuchi *et al.* 2002). 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-PAGE and transferred to nitrocellulose membranes. Blocking was done in 5% skim milk in 1× Tris-buffered saline (TBS). Western blot analyses were performed with various specific primary antibodies. For detection of phosphorylated rTERT or association of rTERT with Akt, and the association of TERT with NFκB, cell lysates were prepared using HNTG buffer. Lysates were incubated with anti-TERT 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 using the enhanced chemiluminescence Western blotting system.

Statistics

Statistical analysis was performed by Student's *t*-test, and *P* < 0.01 was considered significant. Data are expressed as the mean ± S.E.

Results

Both E₂ and raloxifene attenuate Aβ-induced apoptosis

Since it has been reported that both estrogen (Bonnefont *et al.* 1998) and raloxifene (Nilsen *et al.* 1998) induce neurite outgrowth in ER-positive PC12 cells, we examined whether estrogen or raloxifene attenuates Aβ-induced apoptosis using ERα-transfected PC12 cells (PCER), whose parent cells are known to express ERβ but not ERα (Bonnefont *et al.* 1998, Gollapudi & Oblinger 2001). The MCF-7 human breast cancer cell line was used as a positive control to detect ERα. Western blotting analysis confirmed that PCER and MCF-7 cells expressed ERα, while PCCON cells (cells transfected with vector alone) did not (Fig. 1A). Cultured cells were grown in the presence of 10% CSS (Fig. 1B, upper panel) or 10% CSS + Aβ (Fig. 1B, lower panel) with or without 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene. Cultured cells were stained by the TUNEL method. Apoptotic cells were stained brown (Fig. 1B), and were counted under a light microscope. Although Aβ induced apoptosis in both PCCON and PCER cells, 10⁻⁸ M E₂ or 10⁻⁸ M

Figure 3 The effect of E₂ and raloxifene on the mRNA expression of TERT. PCCON (A) and PCER (B) cells were treated with vehicle control (lane 1), 10⁻⁸ M E₂ (lane 2), 10⁻⁸ M E₂ + 20 μM Aβ (lane 3), 10⁻⁸ M raloxifene (lane 4), 10⁻⁸ M raloxifene + 20 μM Aβ (lane 5), or 20 μM Aβ (lane 6) for 24 h. MCF-7 cells were treated with vehicle control (lane 7) or 10⁻⁸ M E₂ (lane 8) for 24 h. RNA was extracted and RT-PCR assays were performed to detect TERT mRNA expression. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

raloxifene significantly inhibited the Aβ-induced apoptosis in PCER cells treated with Aβ, but not in PCCON cells treated with Aβ (Fig. 1B, C). These data suggest the possibility that both E₂ and raloxifene might attenuate the Aβ-induced apoptosis in the presence of ERα.

Both E₂ and raloxifene induce telomerase activity

Since TERT has been reported to protect neurons against Aβ-induced apoptosis (Zhu *et al.* 2000), we examined the effects of estrogen and raloxifene on telomerase activity. Aβ-treated PCCON and PCER cells were treated with 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene and were subjected to quantitative stretch PCR assays to assess the telomerase activity. It appeared that both E₂ and raloxifene induced the telomerase activity in PCER cells treated with Aβ, but not in PCCON cells treated with Aβ (Fig. 2).

The effects of E₂ and raloxifene on the expression of TERT

Since the telomerase activity is known to be modulated by regulation of the level of telomerase catalytic subunit (TERT) at both the transcriptional and post-transcriptional levels, semiquantitative RT-PCR assays were performed to examine whether the activation of telomerase by estrogen or by raloxifene was due to up-regulation of the expression of TERT mRNA. Although treatment of MCF-7 cells with 10⁻⁸ M E₂ for

Figure 4 Both E₂ and raloxifene induce the phosphorylation of Akt. PCER cells were treated with 10⁻⁸ M E₂ (A) or 10⁻⁸ M raloxifene (B) for the indicated times, and then harvested and used to prepare cell lysates. The lysates were subjected to SDS-PAGE and blotted with anti-phospho-Akt (A-p-Akt; middle panels) or anti-Akt (A-Akt; lower panels) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the p-Akt bands are shown in the upper panel, with the density of the vehicle 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. W.B., Western blotting.

Figure 5 Both E₂ and raloxifene induce the telomerase activity via a PI3K/Akt cascade. PCER cells pretreated with 20 μM Aβ were treated with 10⁻⁸ M E₂ (lanes 2 and 5) or 10⁻⁸ M raloxifene (lanes 3 and 6) for 24 h in the presence (lanes 4–6) or absence (lanes 1–3) of LY294002 and were subjected to quantitative stretch PCR assays to assess the telomerase activity.

24 h induced the expression of TERT mRNA (Fig. 3A and B, lanes 7 and 8), treatment of PCCON (Fig. 3A) or PCER (Fig. 3B) cells with 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene in the presence or absence of Aβ for 24 h seemed to exhibit no change in the expression of TERT mRNA, suggesting the possibility that these agents might induce the telomerase activity by a mechanism without influencing the transcriptional level, as shown previously for the cytokine-induced telomerase activity (Akiyama *et al.* 2002).

Both E₂ and raloxifene induce the phosphorylation of Akt

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 phosphorylation of hTERT (Kang *et al.* 1999). In addition, it was reported that estrogen protects against Aβ-induced neurotoxicity by activation of Akt (Zhang *et al.* 2001). Therefore, we next examined whether E₂ or raloxifene induces the phosphorylation of Akt. The cells were treated with E₂ or raloxifene for various times, and the cell lysates were resolved by SDS-PAGE and then subjected to Western blotting

with anti-phospho-Akt antibody or anti-Akt antibody. Although E₂ and raloxifene did not affect the expression of Akt (Fig. 4A, B, bottom panel), they induced transient phosphorylation of Akt (Fig. 4A, B, top and middle panels).

Both E₂ and raloxifene induce telomerase activity via the phosphatidylinositol 3-kinase (PI3K)/Akt cascade

To examine whether the PI3K/Akt cascade is involved in the E₂- and raloxifene-induced telomerase activation, Aβ-treated PCER cells were treated with 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene in the presence or absence of LY294002 and subjected to quantitative stretch PCR assays to assess the telomerase activity. LY294002 apparently attenuated both E₂- and raloxifene-induced telomerase activation in Aβ-treated PCER cells (Fig. 5), suggesting the possibility that the PI3K/Akt cascade might be involved in the induction of telomerase activity by E₂ and raloxifene.

Both E₂ and raloxifene induce the phosphorylation of TERT at a putative Akt phosphorylation site

We examined whether E₂ or raloxifene induces the phosphorylation of TERT at a putative Akt phosphorylation site. Cells were treated with E₂ or raloxifene for various times and then used to prepare lysates that were immunoprecipitated with anti-TERT antibody and then subjected to Western blotting with anti-phospho-Akt substrate antibody or anti-TERT antibody (Fig. 6). The increase in TERT phosphorylation induced by E₂ or raloxifene at a putative Akt phosphorylation site reached a plateau at 30 min and declined thereafter (Fig. 6, top and middle panels). The anti-TERT Western blot analysis showed equal precipitation of TERT protein in the various lysates (Fig. 6, bottom panel).

Both E₂ and raloxifene induce the association of NFκB with TERT

One possible mechanism for the post-translational modification of telomerase is via the interaction of TERT with accessory proteins. Recently, NFκB was reported to be a post-translational modifier of telomerase that functions by controlling the intracellular localization of hTERT (Akiyama *et al.* 2003). Therefore, we examined whether E₂ or raloxifene induces the association of NFκB p65 with TERT. Cells were treated with E₂ or raloxifene for the indicated times and used to prepare cell lysates that were immunoprecipitated with anti-TERT antibody and then subjected to Western blotting with anti-NFκB p65 antibody or anti-TERT antibody. E₂ and raloxifene did not affect the expression of hTERT (Fig. 7, bottom panel), but

Figure 6 Both E₂ and raloxifene induce the phosphorylation of TERT at a putative Akt phosphorylation site. PCER cells were treated with 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene for the indicated times, and then harvested and lysed. The lysates were subjected to immunoprecipitation with anti-hTERT antibody (A-TERT). The immunoprecipitates were subjected to SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane and blotted with anti-phospho-Akt substrate (A-p-Akt; middle panel) or anti-hTERT (lower panel) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the p-hTERT bands are shown in the upper panel, with the density of the vehicle 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. I.P., immunoprecipitation; W.B., Western blotting.

the association of TERT with NFκB p65 was up-regulated by E₂ and raloxifene (Fig. 7, top and middle panels).

Discussion

One of the two novel findings in this study was that raloxifene, like estrogen, protects neurons against Aβ-induced apoptosis by activation of Akt in PC12 cells transfected with ERα. Correlative studies with raloxifene and Akt in the brain have not been performed, but we previously demonstrated that raloxifene rapidly activates Akt in vascular endothelial cells (Hisamoto *et al.* 2001b), suggesting that a similar regulation in the brain by raloxifene is possible. Another finding we made here was that the up-regulation of telomerase activity induced by both estrogen and raloxifene via phosphorylation of hTERT at a putative Akt phosphorylation site and association of hTERT with NFκB might be involved in

their neuroprotective functions. Thus, Akt may have an important role in the neuroprotective functions of both estrogen and raloxifene (Fig. 8).

The Women's Health Initiative Memory Study (WHIMS) has recently shown that estrogen plus progestin therapy did not prevent cognitive impairment and did not improve cognitive function (Rapp *et al.* 2003, Shumaker *et al.* 2003). It was also reported that raloxifene treatment for 3 years did not affect overall cognitive scores in postmenopausal women with osteoporosis (Yaffe *et al.* 2001). Since the participants in these studies were elderly postmenopausal women aged 65 years or older, it remains possible that either estrogen or raloxifene may be useful in younger postmenopausal women who are in the latent pathogenetic stages of AD before extensive damage to the integrity of the brain occurs.

We examined the mechanism of the estrogen- and raloxifene-induced post-transcriptional up-regulation of telomerase activity in this study. Phosphorylation of TERT protein is one mechanism of telomerase activation.

Figure 7 Both E₂ and raloxifene induce the association of NFκB with TERT. PCER cells were treated with 10⁻⁸ M E₂ or 10⁻⁸ M raloxifene for the indicated times, and then harvested and lysed. The lysates were subjected to immunoprecipitation with anti-hTERT (A-TERT) antibody. The immunoprecipitates were subjected to SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane and blotted with anti-NFκB p65 antibody (A-NFκB p65; middle panel) or anti-hTERT (lower panel) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the NFκB p65 bands are shown in the upper panel, with the density of the vehicle 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. I.P., immunoprecipitation; W.B., Western blotting.

Telomerase activity in human breast cancer cells is markedly inhibited by treatment with protein phosphatase 2A (Li *et al.* 1997). Some protein kinases, such as Akt kinase and protein kinase C, have been reported to mediate the phosphorylation of TERT protein, leading to telomerase activation (Li *et al.* 1998, Kang *et al.* 1999). The region surrounding Ser-824 in hTERT conforms to a consensus sequence for phosphorylation by Akt (Kang *et al.* 1999). In the present study, both estrogen and raloxifene-induced telomerase activity seems to be dependent on the phosphorylation of TERT at a putative Akt phosphorylation site (Fig. 5) and independent of the amount of TERT expression (Fig. 3), as shown previously in the case of cytokine-induced telomerase activity (Akiyama *et al.* 2002).

Moreover, NFκB p65 was recently reported to be a post-translational modifier of telomerase that functions by controlling the intracellular localization of hTERT (Akiyama *et al.* 2003, Kawagoe *et al.* 2003). We found that the association of TERT with NFκB p65 was up-regulated by both E₂ and raloxifene in PCER cells (Fig. 7). This result leads us to speculate that NFκB p65 in neural

cells may play a pivotal role in regulating telomerase activity by modulating the nuclear translocation of TERT. As we have demonstrated that nuclear translocation of TERT is necessary for E₂-induced telomerase activity in MCF-7 cells (Kawagoe *et al.* 2003), we are also examining whether E₂ and raloxifene induce nuclear translocation of TERT in PCER cells.

Two ER isoforms, ERα and ERβ, are expressed in the adult brain. Which ER is involved in the neuroprotection by estrogen and raloxifene? To elucidate the individual ER subtype involved in E₂-mediated neuroprotection *in vivo*, specific ER knockout mice models have been used, including ERα knockout mice (ERKO) and ERβ knockout mice (βERKO). E₂ failed to protect the brain of ovariectomized ERKO mice (Dubal *et al.* 2001). In contrast, E₂ protected the brain of ovariectomized βERKO mice in a manner similar to that observed in ovariectomized wild-type mice (Dubal *et al.* 2001). Moreover, we have shown that estrogen and raloxifene induce the activation of Akt via ERα, but not ERβ, in vascular endothelial (Hisamoto *et al.* 2001a,b) and human ovarian

Figure 8 Molecular mechanisms of both E₂ and raloxifene increase the telomerase activity in ERα-transfected PC12 cells. The up-regulation of telomerase activity via phosphorylation of hTERT at a putative Akt phosphorylation site and association of hTERT with NFκB might play an important role in the neuroprotective functions of both estrogen and raloxifene. Solid and dashed lines indicate cellular and nuclear membranes respectively.

cancer (Mabuchi *et al.* 2004) cells. In addition, all membrane forms described to date are related to ERα, and not to ERβ (Flouriot *et al.* 2000, Norfleet *et al.* 2000, Marquez & Pietras 2001). However, further studies are needed to clarify the role(s) of the individual ER isoforms in estrogen- or raloxifene-mediated neuroprotection.

Induction of telomerase activity via Akt might not be the only cascade involved in the neuroprotection by estrogen and raloxifene against Aβ-induced apoptosis. Much of the cellular damage caused by Aβ can be attributed to dysregulation of calcium homeostasis. Because Bcl-2 plays a key role in mitochondrial Ca²⁺ regulation (Murphy *et al.* 1996), E₂-induced attenuation of the increased mitochondrial sequestration of Ca²⁺ in response to excitotoxic glutamate is reported to be correlated with an increase in the expression of the anti-apoptotic gene *bcl-2* (Nilsen & Brinton 2003), which is also increased by E₂ *in vivo* (Alkayed *et al.* 2001) and *in vitro* (Singer *et al.* 1998). These findings suggest the involvement of a genomic mechanism of the actions for estrogen in its neuroprotective effects.

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