Pro-atherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism

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Abstract Advanced aging may contribute to impairment of angiogenesis and development of vascular diseases. Telomerase was shown to delay endothelial cell (EC) senescence. Therefore, we determined the regulation of telomerase activity in EC. Inhibition of phosphoinositol 3-kinase (PI3K) suppressed telomerase activity, whereas inhibitors directed against ERK1/2 or protein kinase C had no effect. Dominant negative Akt significantly reduced telomerase activity. Moreover, pro-atherogenic stimuli such as oxidized low density lipoprotein led to an inactivation of Akt and diminished telomerase activity. Thus, the PI3K/Akt pathway plays an important role in the regulation of telomerase activity. Pro-atherosclerotic factors impair telomerase activity and thereby may promote EC aging.

Key words: Aging; Endothelium; Atherosclerosis; Oxidized low density lipoprotein

1. Introduction

The integrity of endothelial cell (EC) function is compromised by advancing age and its impairment contributes to insufficient angiogenesis and age-dependent development of vascular diseases such as atherosclerosis [1–3]. At a cellular level, aging leads to an irreversible state of cell cycle arrest known as replicative senescence [4], which is mainly determined by telomere length [5]. Telomerase, a ribonucleoprotein with reverse transcriptase activity, synthesizes telomeric repeats at the linear ends of eukaryotic chromosomes [5,6]. Using the 3′-OH group at the DNA terminus as a primer, the catalytic component of telomerase, the telomerase reverse transcriptase (TERT), copies repeatedly only a very restricted region of the telomerase RNA component coding for the telomere repeats. This repeated reverse transcription of the template region can occur in a more or less processive manner, depending on the cellular system [6].

Stable overexpression of telomerase prevents EC senescence suggesting an important role for telomerase activity in the extension of the life span of somatic cells [7]. Although the molecular mechanisms involved in the tight regulation of telomerase expression in somatic cells are largely undefined, evidence is accumulating that post-transcriptional modification of telomerase by phosphorylation processes importantly modulates telomerase activity in a variety of cancer cells [8–11]. Specifically, it has been shown that the serine/threonine kinase Akt can enhance telomerase activity in melanoma cells [12]. Because Akt is a potent survival signaling kinase in EC [13,14] and importantly contributes to endothelial function [15], we investigated the potential involvement of Akt in the regulation of TERT activity in ECs. In addition, since the pro-atherosclerotic risk factor homocysteine has been shown to accelerate EC senescence [16], we tested the hypothesis that atherosclerotic risk factors might interfere with endothelial telomerase activity.

2. Materials and methods

2.1. Cell culture

Human umbilical cord endothelial cells (HUVEC) were purchased from Cell Systems/Clonetics (Solingen, Germany), and cultured in endothelial basal medium (EBM) supplemented with hydrocortisone (1 μg/ml), bovine brain extract (3 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 μg/ml), epidermal growth factor (10 μg/ml) and 10% fetal calf serum (FCS) until the third passage. COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% FCS and 2 mM L-glutamine.

2.2. Plasmids and transfection studies

Plasmids encoding the bovine Akt and the truncated dominant negative Akt mutant were kindly donated by Dr. J. Downward [17] and were subcloned into the pcDNA3.1(−)myc-his vector as described [18]. Human TERT cDNA was a gift from Dr. R.A. Weinberg [19] and was subcloned in the pcDNA3.1(−)myc-his vector. Clones with a verified sequence were transfected in HUVEC (4.0×10^5 cells/6 cm plate; 3 μg plasmid DNA; 25 μl Superfect) as described previously [18]. Transient transfections of COS-7 cells were performed as previously outlined [20].

2.3. Western blot analysis

Cells were lysed as described [18]. Western blots were performed with either anti-phospho Akt antibody (New England Biolabs, Schwalbach, Germany), anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Akt antibody (New England Biolabs).

2.4. Telomeric repeat amplification protocol (TRAP) assay

A PCR–ELISA-based assay (Roche Molecular Biochemicals, Mannheim, Germany) was used to measure the telomerase activity as previously described [21]. The levels of telomerase activity were within the linear range of the TRAP assay. For visualizing, the telomerase-synthesized DNA ladder was transferred to nylon membranes and the Biotin Luminescent Detection Kit was subsequently applied according to the manufacturer's instructions (Roche Molecular Biochemicals).

2.5. In vitro kinase assay

For phosphorylation of TERT by Akt kinase in vitro, myc-tagged TERT and Akt were overexpressed in COS-7 cells and isolated by

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immunoprecipitation with an anti-myc antibody (Santa Cruz Biotechnology). In vitro Akt kinase assays were then carried out as described [20]. The reactions were terminated by addition of sodium dodecyl sulfate (SDS) loading dye and samples were subjected to 8% SDS–PAGE and analyzed by a PhosphorImager or autoradiography.

2.6. Statistics

Data are expressed as mean ± S.E.M. from at least three independent experiments. Statistical analysis was performed with ANOVA followed by a modified LSD test (SPSS Software).

3. Results

3.1. Regulation of telomerase activity by protein kinases

HUVEC starved with serum-free medium for 18 h revealed a 70% reduction of telomerase activity (Fig. 1A). To determine which protein kinase is responsible for the maintenance of telomerase activity under serum-containing conditions, we assayed the telomerase activity of HUVEC that were treated with several protein kinase inhibitors against phosphoinositol 3-kinase (PI3K), mitogen-activated protein kinases ERK1/2 and protein kinase C (PKC). The PI3K inhibitor Ly294002 significantly reduced telomerase activity (Fig. 1B,C). The treatment of cells with PD98059, an inhibitor of the ERK1/2 pathway, slightly affected telomerase activity, whereas bisindolylmaleimide I, an inhibitor highly specific for PKC, did not alter telomerase activity (Fig. 1D). Furthermore, incubation of HUVEC with a combination of PD98059 and Ly294002 did not result in an additive decrease of TERT activity, indicating a dominant PI3K-dependent signal transduction pathway in altering telomerase activity (Fig. 1D).

3.2. Akt regulates telomerase activity in EC

Since Akt is an important downstream mediator of PI3K signaling, HUVEC were transiently transfected with either empty vector, myc epitope-tagged wild-type Akt, constitutively active Akt or dominant negative Akt expression constructs in order to assess the direct influence of Akt on telomerase activity. As illustrated in Fig. 2, the expression of dominant negative Akt resulted in a significant reduction of telomerase activity, whereas expression of a constitutively active Akt construct led to an enhancement of telomerase activity. These data indicate that Akt importantly contributes to the regulation of telomerase activity in HUVEC.

3.3. Phosphorylation of TERT by Akt kinase

To determine the phosphorylation of TERT by Akt, myc epitope-tagged Akt and the myc-tagged TERT construct were overexpressed in COS-7 cells followed by immunoprecipitation of the respective proteins. An in vitro kinase assay demonstrates that TERT is phosphorylated in the presence of Akt kinase (Fig. 3).

3.4. Pro-atherogenic stimuli inhibit telomerase activity

Pro-atherogenic stimuli were shown to accelerate EC senescence [16]. Therefore, we investigated the effect of tumor necrosis factor α (TNFα), oxidized low density lipoprotein (oxLDL) and H_2O_2 on telomerase activity. All stimuli significantly inhibited telomerase activity in ECs (Fig. 4A). Concentrations as low as 10 ng/ml TNFα were sufficient to significantly reduce telomerase activity (data not shown). The effect of oxLDL on telomerase activity was dose-dependent with a maximal efficacy at a concentration of 10 μg/ml oxLDL (Fig. 4A, data not shown). Incubation of whole cell extract with H_2O_2 or oxLDL in vitro did not affect telomerase activity (data not shown) indicating that inactivation of TERT is not due to direct oxidation.

To address whether inactivation of the PI3K/Akt pathway is involved in pro-atherogenic stimulus-induced telomerase inactivation, HUVEC were treated with TNFα, oxLDL and H_2O_2, and Akt activity was determined by Western blot analysis with an anti-phospho Akt antibody directed against serine residue 473 [13]. As illustrated in Fig. 4B, incubation with oxLDL, TNFα or H_2O_2 resulted in dephosphorylation of Akt. The direct involvement of an Akt kinase-mediated mech-

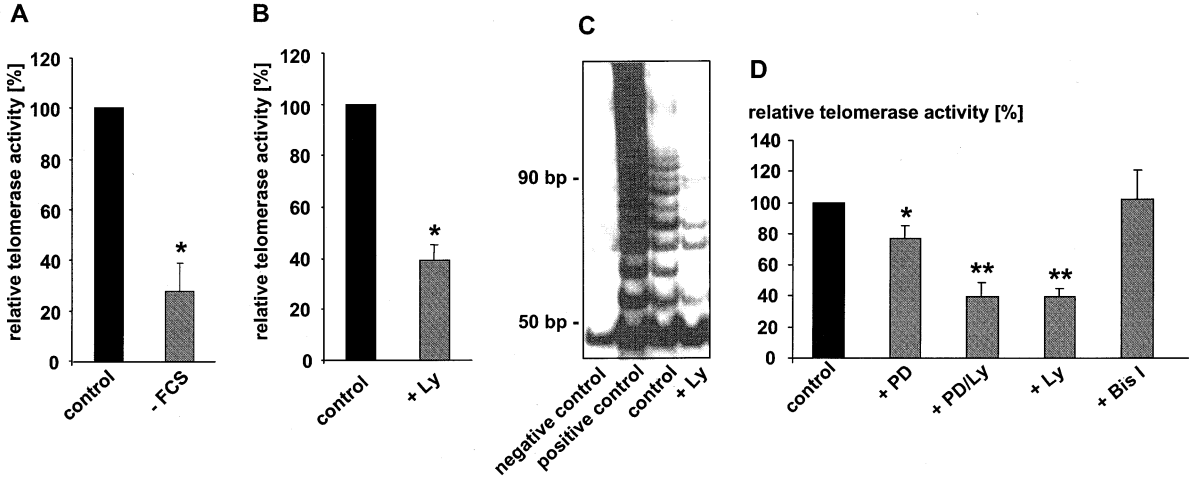

Fig. 1. Telomerase activity is dependent on phosphorylation processes. A: HUVEC were incubated in EBM containing 1% bovine serum albumin (BSA) for 18 h (without FCS) and telomerase activity was determined by the TRAP assay. Data are mean ± S.E.M., $*P < 0.0001$ versus control, $n = 10$. B,C: HUVEC were incubated in EBM containing 1% BSA for 8 h, then EBM supplemented with growth factors and 10% FCS was added. 10 μM Ly294002 was then added for 18 h and telomerase activity was quantitatively measured (B) and visualized as described in Section 2 (C). EC lysate with heat-inactivated telomerase activity served as negative control. HeLa cell extracts were used as positive control. Data are mean ± S.E.M., $*P < 0.0005$ versus control, $n = 4$. D: HUVEC were incubated as described for B with PD98059 (PD; 10 μM), Ly294002 (Ly; 10 μM) or bisindolylmaleimide (Bis I; 10 nM). Data are mean ± S.E.M., $*P < 0.05$ versus control, $n = 4$; $**P < 0.005$ versus +PD, $n = 4$.

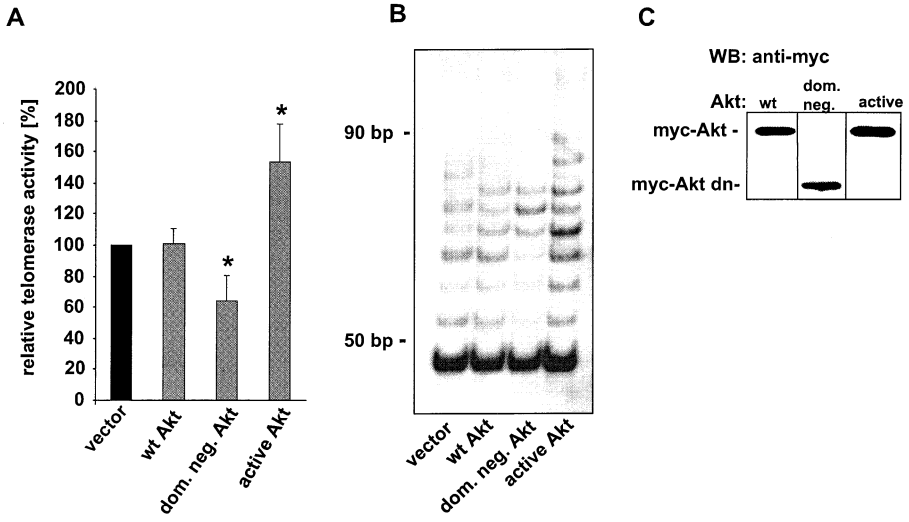

Fig. 2. Direct influence of Akt on telomerase activity. HUVEC were transiently transfected with either empty vector, wild-type Akt, dominant negative Akt or constitutively active Akt constructs. Following transfection, cells were first incubated with EBM containing 1% BSA for 6 h and then with EBM supplemented with growth factors and 10% FCS for an additional 18 h. Cells were lysed and applied to the TRAP assay, quantified (A) and visualized (B) as described in Section 2. Data are mean ± S.E.M., $*P < 0.005$ versus wild-type Akt, $n \geq 6$. C: Expression of the various Akt constructs was verified by Western blot analysis using an anti-myc antibody.

anism on telomerase inactivation by pro-atherogenic stimuli was further demonstrated with TNFα. For this purpose, HUVEC were transiently transfected with either empty vector or constitutively active myc-tagged Akt expression constructs. Following 6 h transfection, cells were treated with TNFα for 18 h and telomerase activity was monitored by a TRAP assay. Fig. 4C demonstrates that telomerase activity in cells expressing active Akt was only slightly reduced by TNFα to basal levels. In contrast, cells that were transfected with empty vector revealed a dramatic decrease in telomerase activity in the presence of TNFα (Fig. 4C). These data suggest that TNFα-triggered reduction of telomerase activity involved inactivation of the PI3K/Akt signal transduction pathway.

Since Akt kinase is known to activate the endothelial nitric oxide (NO) synthase [18] and NO prevents age-related downregulation of telomerase activity [21], we tested the effect of the NO synthase inhibitor N^G-monomethyl-L-arginine (LNMMA). As illustrated in Fig. 4A, LNMMA did not affect telomerase activity.

4. Discussion

The data of the present study indicate an important regulatory role of the cell survival kinase Akt for the maintenance of telomerase activity by possible phosphorylation of its catalytic subunit TERT. Pro-inflammatory and pro-atherosclerotic factors such as TNFα, oxLDL or H_2O_2 reduce telomerase activity in human ECs, and might thereby contribute to accelerated senescence of ECs.

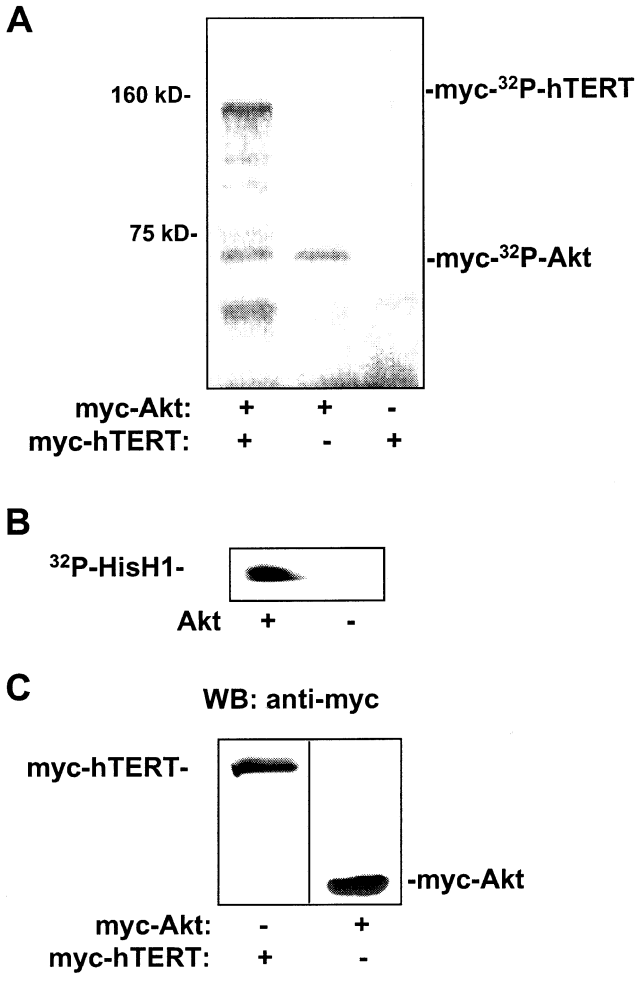

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Fig. 3. Phosphorylation of TERT by Akt in vitro. A: Myc-tagged full-length TERT was transiently transfected into COS-7 cells and TERT was isolated via immunoprecipitation with an anti-myc antibody. The kinase assay was carried out as described Section 2. B: Histone H1 was used as a positive control for Akt activity. C: Western blot analysis with anti-myc antibody confirms Akt and TERT expression in COS-7 cells.

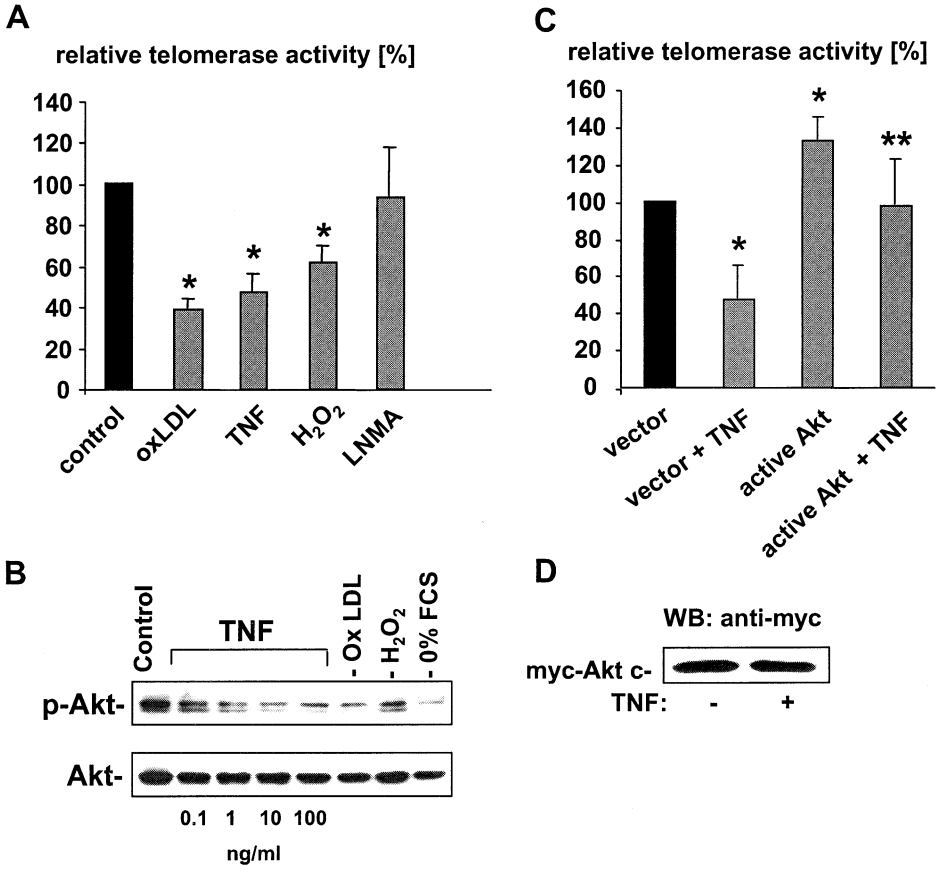

Fig. 4. Pro-atherogenic stimuli significantly affect telomerase activity. A: HUVEC were incubated with a series of pro-atherogenic stimuli (oxLDL (10 μg/ml), TNFα (100 ng/ml), H_2O_2 (200 μM), or LNMMA (1 mM)) for 18 h. B: oxLDL, TNFα and H_2O_2 induce dephosphorylation of Akt at serine residue 473. HUVEC were treated as described above followed by Western blot analysis with an anti-phospho Akt antibody. Reprobing of the blot with total Akt antibodies verifies equal loading of proteins. A representative blot out of $n=3$ individual experiments is shown. C,D: HUVEC were transiently transfected with either empty vector or constitutively active Akt. After transfection, cells were first incubated for 6 h in EBM containing 1% BSA and then in EBM supplemented with growth factors and 10% FCS. Following 1 h incubation, TNFα was added at a concentration of 100 ng/ml for 18 h. Data are mean ± S.E.M., $*P<0.01$ versus vector, $**P<0.05$ versus vector+TNF, $n=5$. Western blot analysis with anti-myc antibody to control for Akt expression is shown in D.

The reduction of TERT activity in ECs by inhibition of the PI3K/Akt pathway extends the results of a recent study in a human melanoma cell line, which demonstrated that Akt enhances telomerase activity through phosphorylation of TERT [12]. Indeed, the present study disclosed a direct phosphorylation of full-length TERT by Akt in vitro. In contrast, PKCα and the mitogen-activated protein kinase ERK2 did not phosphorylate TERT (data not shown), in accordance with the lack of pharmacological inhibition of ERK1/2 or PKC to significantly alter telomerase activity in HUVEC. Thus, the PI3K/Akt pathway seems to play a predominant role in telomerase activation within ECs.

Beside the direct phosphorylation of TERT, Akt might also act by increasing the activity of the endothelial NO synthase [18,22]. This has to be taken into account because we recently demonstrated that long-term NO addition can prevent age-associated down-regulation of EC TERT activity [21]. However, a sole effect of Akt via NO was ruled out by the finding that acute inhibition of the NO synthase by LNMMA did not impair EC TERT activity. Taken together, although the overall organization of telomerase activity is still enigmatic, our data indicate an important regulatory role of Akt in the maintenance and enhancement of telomerase activity in ECs with Akt acting directly in an acute fashion most likely via phosphorylation of the regulatory subunit of the telomerase, whereas NO has additional long-term effects by an unknown mechanism.

The data of the present study demonstrated that pro-atherosclerotic factors impair telomerase activity. Mechanistically, the inhibition of telomerase activity seems to be mediated by reduction of the Akt phosphorylation resulting in deactivation of the kinase. The reduction of Akt phosphorylation by TNFα and oxLDL is in accordance with previous findings in ECs [23,24] and might be due to activation of the ceramide/sphingomyelinase pathway, which is known to promote Akt dephosphorylation [23,25]. However, other studies have reported that TNFα can also stimulate Akt [26,27]. Similarly, H_2O_2 was shown to augment Akt phosphorylation in vascular smooth muscle cells [28]. One may speculate that the discrepancies of these studies might be due to cell type specificities. Although the mechanism by which Akt phosphorylation is reduced by TNFα, oxLDL and H_2O_2 remains to be elucidated, a causal role for Akt deactivation in the reduction of telomerase activity is evidenced by the finding that overexpression of a constitutively active Akt construct prevents TNFα-induced telomerase inactivation.

In conclusion, the prevention of telomerase inactivation by pro-atherosclerotic factors by Akt might be of substantial

importance in the protection against replicative senescence. Moreover, Akt-mediated regulation of telomerase activity might contribute to the maintenance of the integrity of the endothelium and, therefore, affect the development of age-related diseases such as atherosclerosis.

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