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Akt plays a central role in the anti-apoptotic effect of estrogen in endothelial cells☆

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Abstract

Estrogen has been reported to inhibit apoptosis in vascular endothelial cells. However, its precise mechanism still remains to be elucidated. Here we determined the role of Akt in the anti-apoptotic effect of estrogen. 17β-Estradiol prevented the apoptosis induced by TNF-α in bovine aortic endothelial cells, as evaluated by double staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide. Introducing a dominant negative mutant of Akt by using a cell-penetrating peptide of Tat protein inhibited the anti-apoptotic effect of estrogen in a concentration-dependent manner, and resulted in the complete inhibition of the anti-apoptotic effect of 17β-estradiol at 1 nM and higher concentrations. The dominant negative mutant without the cell-penetrating peptide and Tat peptide-conjugated protein A had no effect. The intracellular protein transduction was confirmed by immunoblot analysis. Our observations thus provide first direct evidence that Akt plays a central role in the anti-apoptotic effect of estrogen in vascular endothelial cells.

Keywords: Akt; Apoptosis; Endothelial cell; Estrogen; Protein transduction

Endothelial dysfunction plays a critical role in the early development of atherosclerotic lesions [1–3]. Estrogen has been shown to prevent endothelial apoptosis, and this ability may correlate with its anti-atherogenic effect seen in premenopausal women and in hormone replacement therapy [4,5]. However, the molecular mechanism of the anti-apoptotic effect of estrogen still remains to be determined. Estrogen has recently been shown to activate the phosphatidylinositol-3 kinase (PI3K)-Akt pathway [6–8]. PI3K is activated by the direct interaction of its regulatory subunit p85 with estrogen receptor α [8]. The following activation of Akt in turn phosphorylates endothelial NO synthase at Ser 1177 (in human), thereby activating NO production in a Ca^{2+}-independent manner [6,9,10]. On the other hand, Akt functions as a well-known survival factor and also prevents apoptosis in a variety of cells [11]. In endothelial cells, Akt has been shown to play an important role in the anti-apoptotic effect of angiotensin II [12]. However, it remains to be elucidated whether Akt is involved in the anti-apoptotic effect of estrogen in vascular endothelial cells.

In this report, we investigated the involvement of Akt in the anti-apoptotic effect of estrogen in bovine aortic endothelial cells (BAECs), by examining the effect of the dominant negative mutant of Akt on the estrogen-mediated prevention of apoptosis induced by TNF-α

☆ *Abbreviations:* BAECs, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; PI3K, phosphatidylinositol-3 kinase; PTD, protein transduction domain.

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in endothelial cells. The dominant negative mutant of Akt was introduced into endothelial cells as a fusion protein with a protein transduction domain (PTD) found in human immunodeficiency viral transcription factor Tat protein [13–16]. We herein provide, for the first time, direct evidence that Akt mediates the anti-apoptotic effect of estrogen in vascular endothelial cells.

Materials and methods

Cell culture. BAECs were cultured to confluence as previously described [17]. The cells then were plated at a density of 1.0×10^5 cells/60 mm culture dishes in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. After 24 h, the media were changed to serum-free, phenol red-free DMEM (day 0). TNF-α was applied on day 1, and then the degree of apoptosis was evaluated on day 5. 17β-Estradiol was applied on day 0, and recombinant proteins were applied on day 1.

Apoptosis assays. Apoptosis in BAECs was analyzed with the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain view, CA, USA), according to the manufacturer's instructions. The apoptotic cells were detected with a flow cytometer (BD Bioscience, San Jose, CA, USA).

Preparation of recombinant proteins. The vector, pQE30TATHA, used to express Tat PTD-tagged proteins with the hemagglutinin tag was as previously described [13]. pQE30 (Qiagen, Hilden, Germany) was used to express control $(His)_6$-tagged proteins without PTD. The cDNAs encoding the PH domain of Akt (amino acid residues 1–147 [18]) were obtained by PCR amplification from the human aorta cDNA library (Clontech, Palo Alto, CA, USA). The cDNA encoding the IgG-binding domain of protein A (amino acid residues 23–270 [19]) was obtained as previously described [14]. The recombinant proteins were expressed and purified through a Ni^{2+}-loaded Hi-Trap chelating column (Pharmacia Biotech, Japan) as previously described [13]. The protein concentration was determined using the Coomassie blue kit (Pierce, Rockford, USA).

Immunoblot detection of the intracellular protein transduction. The protein transduction was evaluated by an immunoblot analysis as previously described [13,14]. In brief, BAECs were mechanically harvested from the dishes and suspended in DMEM at approximately 5×10^6 cells/ml. The cells were incubated in DMEM containing 3 μM recombinant proteins for 24 h at 37 °C. After being thoroughly washed in ice-cold PBS, the cells were homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μM of 4-aminidophenylmethane sulfonyl fluoride), and 50 μg total protein was subjected to immunoblot detection with anti-$(His)_6$ antibody. The immune complex was detected by an enhanced chemiluminescence technique (Amersham–Pharmacia Biotech, Piscataway, NJ, USA).

Statistics. All data were expressed as means ± SD. The unpaired Student's *t* test was used to evaluate statistical significance. A value of $P < 0.05$ was considered to be significant.

Results

Anti-apoptotic effect of 17β-estradiol on TNF-α-induced apoptosis in BAECs

When BAECs were incubated in the serum-free media for 5 days, only a small fraction of the cells (3.8 ± 0.5%, $n = 3$) showed positive staining with fluorescein isothiocyanate-labeled annexin V (Fig. 1A, control). On the other hand, when 100 ng/ml TNF-α was applied on day 1, a significant fraction of the cells showed positive staining for annexin V on day 5. However, most of the annexin V-positive cells showed negative staining for propidium iodide (PI) (Fig. 1A, TNF-α). The annexin V-positive and PI-negative population was defined as the cells in apoptosis. The 4-day incubation with TNF-α increased the percentages of apoptotic cells at concentrations higher than 20 ng/ml (data not shown). The maximal effect was obtained at 100 ng/ml, thus causing apoptosis in 27.0 ± 2.2% ($n = 3$) of the cells (Fig. 1B). When 17β-estradiol was applied 24 h before and during the TNF-α treatment, the percentage of apoptotic cells decreased (Fig. 1A, TNF-α + E2). 17β-Estradiol significantly inhibited TNF-α-induced apoptosis at 1 nM and higher concentrations, and 16.3 ± 1.2% of the cells were found to demonstrate

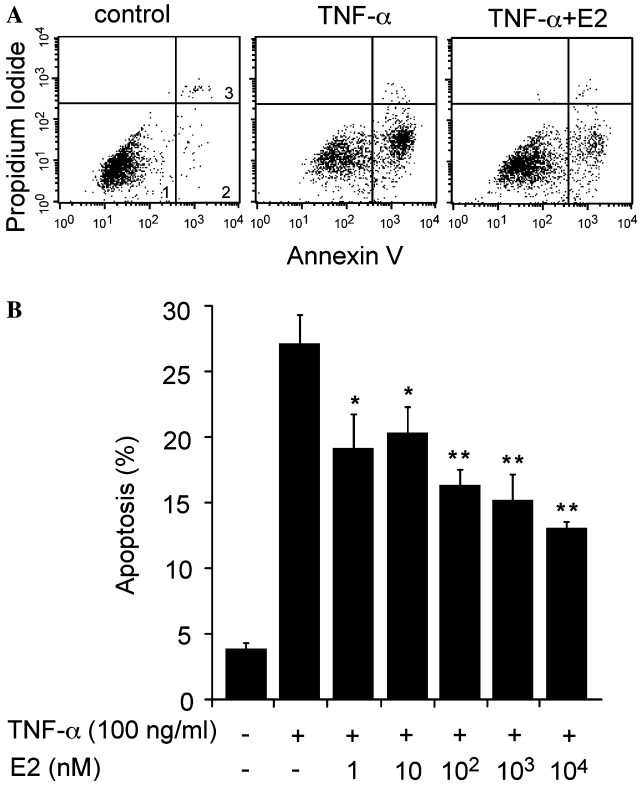

Fig. 1. The anti-apoptotic effects of 17β-estradiol (E2) on TNF-α-induced apoptosis in bovine aortic endothelial cells. (A) Representative dot-plots of a flow cytometric analysis of staining with annexin V and propidium iodide (PI). The endothelial cells were untreated (control) or treated by 100 ng/ml TNF-α without (TNF-α) and with (TNF-α + E2) 100 nM E2 in serum-free media for 4 days. E2 was applied 24 h before and during treatment with TNF-α. Quadrant 1, non-apoptotic, non-necrotic cells; quadrant 2, apoptotic cells; and quadrant 3, necrotic cells. (B) The concentration-dependent effects of E2 on apoptosis induced by 100 ng/ml TNF-α. The apoptotic cells were expressed as a percentage of the total cell count (10,000 cells). The data are means ± SD ($n = 3$). *$P < 0.05$; **$P < 0.01$ vs. 100 ng/ml TNF-α alone.

apoptosis after the application of 100 nM of 17β-estradiol (Fig. 1B).

The dominant negative mutant of Akt inhibited the anti-apoptotic effects of 17β-estradiol in BAECs

We examined the involvement of Akt in the anti-apoptotic effect of 17β-estradiol, using the Tat PTD-mediated protein transduction technique (Fig. 2). The pleckstrin homology domain (PH) of Akt was used as the dominant negative mutant (Fig. 2A), as previously described [11,20]. This domain was introduced into BAECs as a fusion protein with Tat PTD (TAT-Akt) (Fig. 2A). BAECs were exposed to 100 ng/ml TNF-α for 4 days in serum-free media with and without 100 nM of 17β-estradiol, in the presence of TAT-Akt at the indicated concentrations (Fig. 2B). 17β-Estradiol was applied 24 h before and during the treatment with TNF-α, while TAT-Akt was simultaneously applied with TNF-α. In the absence of TAT-Akt, TNF-α-induced apoptosis in 45.5 ± 3.1% ($n = 3$) of the cells, while the addition of 17β-estradiol decreased apoptosis to 32.8 ± 3.6% ($n = 3$) (Fig. 2B). The addition of TAT-Akt (0.01–10 nM) per se had no significant effect on the TNF-α-induced apoptosis seen in the absence of 17β-estradiol. However, TAT-Akt inhibited the anti-apoptotic effect of 17β-estradiol in a concentration-dependent manner, thus resulting in the complete inhibition of the anti-apoptotic effect of 17β-estradiol at 1 nM and higher concentrations (Fig. 2B).

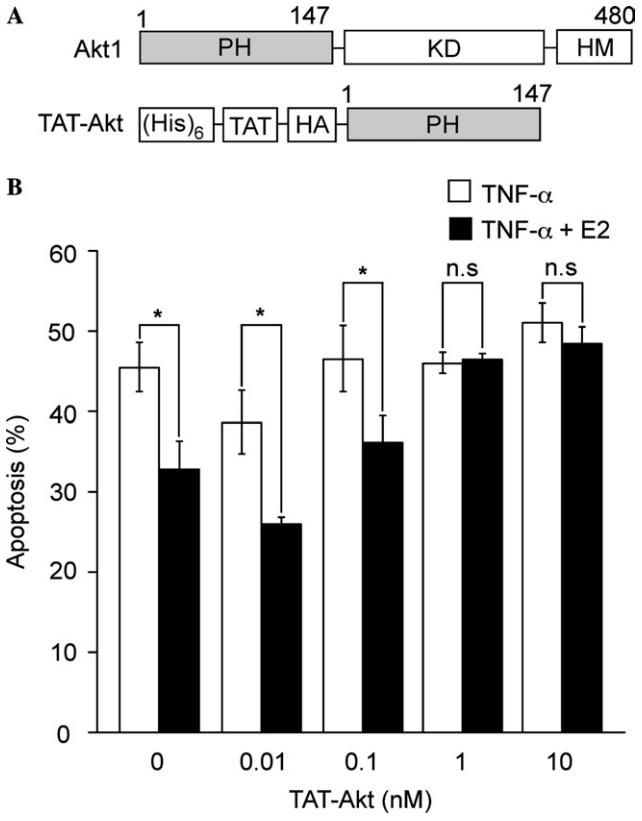

Fig. 2. The dominant negative mutant of Akt inhibited the anti-apoptotic effects of 17β-estradiol (E2) in the bovine aortic endothelial cells. (A) The schematic presentations of the protein structure of Akt1 (Accession No. M63167) and TAT-Akt1. PH, pleckstrin homology domain; KD, kinase domain; HM, hydrophobic motif; $(His)_6$, a hexahistidine tag; TAT, Tat protein transduction domain; and HA, a hemagglutinin tag. (B) The concentration-dependent inhibition of the anti-apoptotic effects of E2 by TAT-Akt. The cells were treated by 100 ng/ml TNF-α with and without 100 nM E2 in serum-free media for 4 days, in the presence of TAT-Akt at the indicated concentrations. E2 was applied 24 h before and during treatment with TNF-α. TAT-Akt was applied when TNF-α was applied. The data are means ± SD ($n = 3$). *$P < 0.05$; ns, not significantly different.

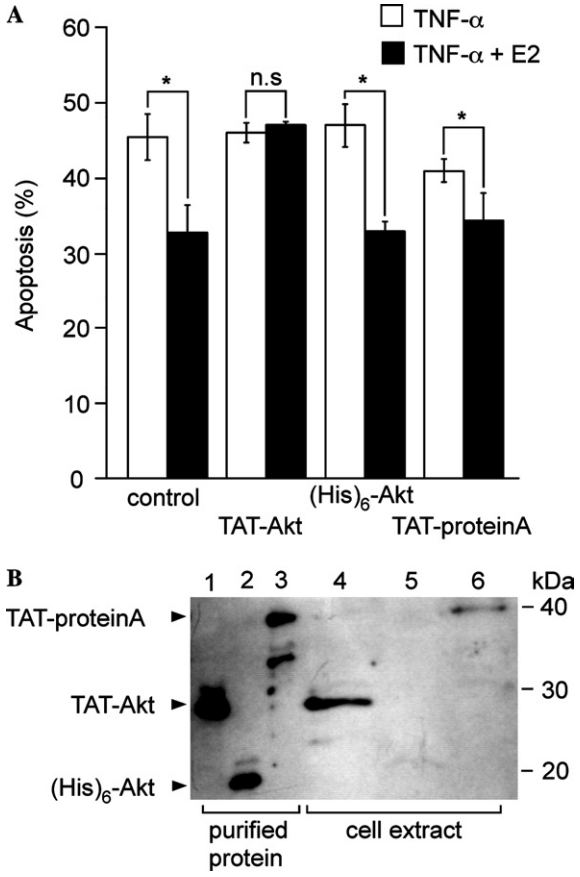

Fig. 3. The specific inhibition of anti-apoptotic effect of 17β-estradiol (E2) by TAT-Akt in the bovine aortic endothelial cells. (A) The effects of TAT-Akt, $(His)_6$-Akt, and TAT-protein A on the anti-apoptotic effects of E2. The cells were treated by 100 ng/ml TNF-α with and without 100 nM E2 in serum-free medium for 4 days, in the absence (control) and presence of 1 nM recombinant proteins. E2 was applied 24 h before and during treatment with TNF-α. Recombinant proteins were applied when TNF-α was applied. The data are means ± SD ($n = 3$). *$P < 0.05$; ns, not significantly different. (B) Immunoblot detection of recombinant proteins with anti-$(His)_6$ antibody. Lanes 1–3, 50 ng purified proteins as positive control; TAT-Akt (lane 1), $(His)_6$-Akt (lane 2), and TAT-protein A (lane 3). Lanes 4–5, the extract (50 μg protein) of the cells exposed to 3 μM TAT-Akt (lane 4), 3 μM $(His)_6$-Akt (lane 5), and 3 μM TAT-protein A (lane 6) for 24 h.

Specific inhibition of the anti-apoptotic effect of 17β-estradiol by TAT-Akt in BAECs

The specificity of the inhibitory effect of TAT-Akt on the anti-apoptotic effect of 17β-estradiol was examined by comparing it with the effect of two control constructs (Fig. 3). The addition of 1 nM $(His)_6$-Akt, a PH domain of Akt tagged only with a $(His)_6$ tag, had no significant effect on TNF-α-induced apoptosis. 17β-Estradiol prevented TNF-α-induced apoptosis in the presence of 1 nM $(His)_6$-Akt as that seen in the control. TAT-protein A (1 nM) per se had no significant effect on TNF-α-induced apoptosis, and 17β-estradiol prevented TNF-α-induced apoptosis in the presence of TAT-protein A.

The Tat PTD-mediated transduction of recombinant proteins was evaluated by an immunoblot analysis using anti-$(His)_6$ antibody (Fig. 3B). The cells were exposed to 3 μM TAT-Akt, $(His)_6$-Akt, and TAT-protein A for 24 h, and then the cellular proteins were extracted. TAT-Akt (Fig. 3B, lane 4) and TAT-protein A (Fig. 3B, lane 6), but not $(His)_6$-Akt, were detected in the cell extracts (Fig. 3B, lane 5). All purified proteins (Fig. 3B, lanes 1–3) were detected with anti-$(His)_6$ antibody.

Discussion

A body of literature has suggested that Akt plays an important role in the anti-apoptotic signal transduction in many types of cells including vascular endothelial cells [11,12,21]. Estrogen has been shown to prevent apoptosis in vascular endothelial cells [4,5,22,23]. Recently, estrogen has been shown to activate Akt by activating PI3K in endothelial cells [6–8,24]. The activation of Akt in turn phosphorylates and activates endothelial NO synthase, thereby inducing NO production in the endothelial cells [6,7]. It is thus speculated that Akt also contributes to anti-apoptosis seen with estrogen. However, the role of Akt in the anti-apoptotic effect of estrogen remains to be elucidated. The present study provides, for the first time, direct evidence that Akt plays a central role in the anti-apoptotic effect of estrogen.

The inhibition of survival factor by overexpressing a dominant negative mutant could per se induce apoptosis, thereby hindering an analysis of its role in anti-apoptotic signaling. On the other hand, endothelial cells are known to be resistant to the conventional transfection of plasmid DNA. In order to overcome these obstacles, we utilized the Tat PTD-mediated protein transduction technique, which thus made it possible to introduce proteins effectively into endothelial cells in a quantitative manner. By this transduction technique, the effect of the dominant negative mutant of Akt on the anti-apoptotic effect of estrogen was specifically investigated without any interference by the direct apoptotic effect of the dominant negative mutant. The introduction of proteins into the cells was proven by an immunoblot analysis. Furthermore, the specificity of the effect of the dominant negative mutant of Akt was also supported by observations with $(His)_6$-Akt and TAT-protein A.

The full activation of Akt results from the recruitment of Akt to the plasma membrane due to interaction of the PH domain with lipid products of PI3K and the phosphorylation at T308 and S473 by upstream kinases referred to as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2 [11,20,25]. Either the PH domain of Akt, the kinase-dead Akt or Akt with mutations of both activation phosphorylation sites (T308 and S473) have been successfully used as dominant negative mutants of Akt [12,18,25]. Our observation that the PH domain inhibited the anti-apoptotic effect of estrogen is thus consistent with the previously demonstrated PI3K-dependent activation of Akt by estrogen [6–8]. Survivin has been suggested to serve as a downstream effector in the Akt-mediated anti-apoptotic effect of angiotensin II [12]. However, the downstream effectors of Akt involved in the anti-apoptotic effect of estrogen remain to be elucidated in the future study.

In conclusion, our findings suggest that Akt plays a central role in the anti-apoptotic effect of estrogen in vascular endothelial cells. This protective effect of estrogen may thus correlate with the lower incidence of atherosclerosis in premenopausal women. The Tat PTD-mediated protein transduction could be a powerful tool to investigate the quantitative role of signaling proteins in the cell function.

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