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Estrogen prevents bovine aortic endothelial cells from TNF-α-induced apoptosis via opposing effects on p38 and p44/42 CCDPK

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KEY WORDS apoptosis; DNA fragmentation; Ca^{2+}-calmodulin dependent protein kinase; tumor necrosis factor; 17β-estradiol; Western blotting; vascular endothelium; cultured cells

ABSTRACT

AIM: To investigate the effect of 17β-estradiol (E_2) on TNF-α-induced apoptosis in cultured bovine aortic endothelial cells (BAEC) and the underlied mechanism. **METHODS**: BAEC were cultured and passaged in Dulbecco's modified Eagle's medium (DMEM). Morphologic changes and quantification of apoptotic cells were determined under fluorescence microscope with Hoechst 33258 staining. Cell viability was detected with MTT method. DNA fragmentation was visualized by agarose gel electrophoresis. The expression of phospho-p38 and phospho-p44/42 Ca^{2+}-calmodulin dependent protein kinase (CCDPK) was measured by Western blotting. **RESULTS**: TNF-α 5000 kU/L elicited typical apoptotic morphologic changes (chromatic condensation, nucleus fragmentation, and DNA fragmentation). E_2 0.1 pmol/L - 100 nmol/L enhanced the expression of phospho-p44/42 CCDPK induced by TNF-α, at the same time, inhibited TNF-α induced activation of p38 CCDPK. E_2 protected BAEC from apoptosis induced by TNF-α in a concentration-dependent manner. DNA fragmentation induced by TNF-α 5000 kU/L was also reduced by E_2 1 nmol/L. Both the E_2-induced upregulation of phospho-p44/42 CCDPK and its anti-apoptotic action were prevented by the specific p44/42 CCDPK inhibitor U0126. **CONCLUSION**: Activation of p44/42 CCDPK signaling together with inhibition of p38 CCDPK signaling by E_2 appears to be an important mechanism for its survival effect on endothelial cells.

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INTRODUCTION

Estrogen deficiency is associated with increased risk of developing coronary disease[1]. Estrogen replacement therapy markedly attenuates this risk in postmenopausal women[2]. Although part of the antiatherogenic action of estrogen appears to be due to its effects on systemic factors, direct effects of estrogen on vascular cells have been thought to be the main atheroprotective mechanism[3].

Endothelium impairment is a postulated initial step in atherogenesis[4]. Estrogen has previously been demonstrated to be a survival factor in toxin-induced endothelial cell death and such a protection of endothelial cells from toxic insults has been considered to represent an alternative mechanism for the inhibition of atherogenesis by estrogen[5]. However, the molecular mechanism responsible for this protective effect of estrogen has not been well defined up to date. TNF-α was the toxic interfering agent due to its important role in atherogenesis and restenosis and its well characterized cytotoxic action to endothelial cells[5]. In our recent study[6], opposing effects of p38- and p44/42-CCDPK signaling was demonstrated on TNF-α-induced apoptosis in endothelial cells. Estrogen has been demonstrated to activate p44/42 CCDPK in MCF-7 and Hella cells[7]. Considering the important modulatory roles of CCDPK cascades in control of cell apoptosis[8], it is rationale to speculate that activation of CCDPK signaling pathway may represent a potential mechanism underlying the anti-apoptotic effect of estrogen. This study is aimed to investigate the effect of estrogen on TNF-α-induced activation of p44/42- and p38 CCDPK in cultured BAEC.

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[9] and cultured in Dulbecco's modified Eagle's medium (DMEM) with 20 % heat-inactivated fetal bovine serum

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(FBS), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L. Experiments were performed with cells from passage 4 – 10.

Cell viability assay BAEC were seeded out in 24-well plates and grown to 80 % confluence in DMEM with 10 % FBS. The cultures were then rinsed in phenol free RPMI-1640 medium and incubated with respective test substances in phenol free and serum free RPMI-1640 for 24 h. Cell viability was measured by means of MTT (dimethylthiazol-diphenyltetrazolium bromide) assay[6].

Cell counting BAEC cultured in 24-well culture plates at 80 % confluency were rinsed in DMEM, then were treated with various concentrations of 17β-estradiol (E_2) in serum-free DMEM for 24 h. The cultures were subsequently trypsinized and cell numbers were measured using a hemocytometer.

Determination of apoptosis After treatment, BAEC were observed under fluorescence microscope after Hoechst 33258 staining. Quantification of apoptosis was routinely determined by counting the number of cells with condensed or fragmented chromatin as described previously[10]. Briefly, cells from different treatment were cytospun onto glass slides and fixed with a 50 % solution of fixative (3∶1 methanol/acetic acid). The preparations were stained with Hoechst 33258 5 mg/L for 10 min, rinsed, and dried. The preparations were examined using fluorescence microscope. Condensed and fragmented nuclei, typical morphologic changes of apoptosis, were easily distinguishable from intact nuclei and percentages were calculated by counting. Six randomly chosen fields of view were observed after exposure to the conditions indicated.

DNA electrophoresis At the end of each incubation, DNA was extracted with the standard method using phenol and chloroform. DNA were electrophoretically fractionated on 1.5 % agarose gel and visualized by ethidium bromide.

Western blot For CCDPK detection, BAEC cultured in 6-well culture plates were grown to 80 % – 90 % confluence and then starved for 24 h in serum free DMEM, then various concentrations of E_2 and TNF-α (5000 kU/L) were added. The treating time was chosen to be 10 min, since a peak expression of phospho-p44/42 and phospho-p38 CCDPK was obtained at this time point in our experimental conditions. For inhibitor studies, cells were pretreated for 10 min with U0126 before the addition of TNF-α and E_2. Total cell protein was determined by the dye method[11]. Phospho-p38, phospho-p44/42 CCDPK or total p38, p44/42 CCDPK were detected with same Western blot method as described in our previous study[6]. Bands of CCDPK were quantitatively determined by thin-layer chromatography with Shimadza Dual Wavelength Chromato-Scanner (Japan, Model SC-930).

Reagents 17β-Estradiol, BSA, DMEM medium, and Hoechst 33258 were purchased from Sigma Chemical Co. CCDPK monoclonal antibodies, HRP-conjugated anti-rabbit secondary antibody, and phototope-HRP Western detection kit were purchased from New England Biolabs Inc. TNF-α was the product of Beijing Biotinge-Tech Co Ltd. U0126 was a kind gift from Dr TRZASKOS JM.

Statistics Values were expressed as $x \pm s$, and assessed by one-way ANOVA and t-test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Morphologic changes After exposure to TNF-α 5000 kU/L for 24 h, BAEC showed typical morphologic changes of apoptosis. The cell volume was reduced, the chromatin became condensed, and nucleus fragmented. A marked decrease in TNF-α-induced apoptotic cells was observed in the presence of E_2 1 nmol/L (Fig 1).

DNA electrophoresis Incubation of BAEC with TNF-α 5000 kU/L for 24 h elicited a characteristic "ladder" of DNA fragments representing integer multiples of the internucleosomal DNA length (about 180 – 200 base pair). When BAEC were treated with E_2 1 nmol/L simultaneously with TNF-α, DNA fragmentation was reduced. Pretreatment of BAEC with U0126 5 μmol/L for 10 min abolished the apoptotic protective effect of E_2 (Fig 2).

Quantification of apoptosis E_2 0.1 pmol/L – 100 nmol/L protected BAEC from TNF-α-induced apoptosis in a concentration-dependent manner. After incubation with TNF-α 5000 kU/L for 24 h, apoptosis occurred in 27 % ± 4 % of endothelial cells. When E_2 1 nmol/L was applied simultaneously with TNF-α, BAEC death reduced to 14 % ± 3 %. U0126 5 μmol/L completely abolished the protective effect of E_2 on endothelial cells, and apoptotic rate recovered to 28 % ± 4 % (Tab 1).

Cell viability At concentrations of 0.1 pmol/L – 100 nmol/L, E_2 protected BAEC from the decrease

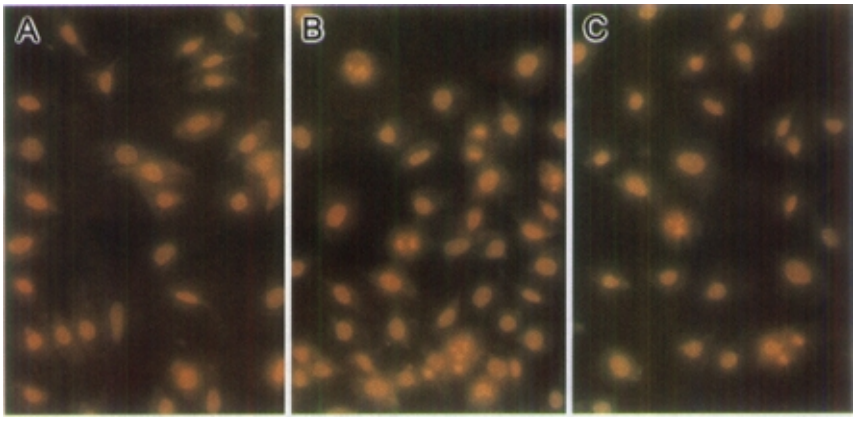

Fig 1. Fluorescence photomicrograph of BAEC stained with Hoechst 33258. ×400. A) Nuclei of control BAEC were stained uniformly. B) TNF-α-treated BAEC showing apoptotic nuclei (condensed or fragmented). C) A marked decrease of apoptotic cells after exposure to TNF-α in the presence of E_2.

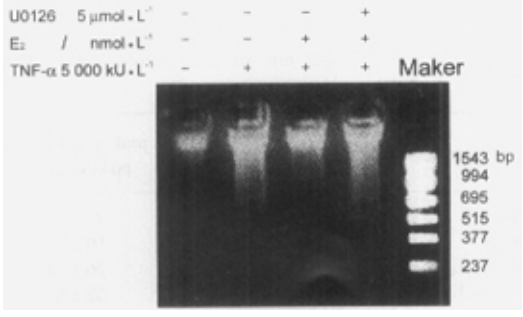

Fig 2. Agarose gel electrophoresis of TNF-α-induced DNA fragmentation in BAEC.

Tab 1. Concentration-dependent effect of 17β-estradiol on TNF-α 5000 kU/L-induced apoptosis in BAEC and the effect of U0126 5 μmol/L. $n = 4$ experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^fP < 0.01$ *vs* TNF-α.

Treatment	Apoptotic rate/%
Control	4.2 ± 1.1
TNF-α	27 ± 4[c]
TNF-α + E_2 0.1 pmol/L	26 ± 6[d]
TNF-α + E_2 10 pmol/L	20 ± 4[d]
TNF-α + E_2 1 nmol/L	14 ± 3[f]
TNF-α + E_2 100 nmol/L	13 ± 3[f]
TNF-α + E_2 1 nmol/L + U0126	28 ± 4[d]

in cell viability induced by TNF-α 5000 kU/L in a concentration-dependent manner. After incubation with TNF-α 5000 kU/L for 24 h, endothelial cell death occurred in 25.4 % of cells (absorbance: 0.423 ± 0.025 *vs* 0.567 ± 0.015 of control). When E_2 1 nmol/L was applied simultaneously with TNF-α, BAEC death rate was only 15.2 % (absorbance: 0.481 ± 0.027 *vs* 0.567 ± 0.015 of control). Preincubation of BAEC with U0126 5 μmol/L completely abolished the protective effect of E_2 on endothelial cells (Fig 3). Treatment with E_2 (1 pmol/L – 100 nmol/L) for 24 h did not result in increase in cell number ($P > 0.05$) (Tab 2), which suggested that the increase in cell viability was not the result of E_2-induced endothelial cell proliferation.

Tab 2. Effect of 17β-estradiol on cell number in BAEC. $n = 4$ experiments. $\bar{x} \pm s$. $^aP > 0.05$ *vs* control.

Concentration	10^{-5} × Cell number/well
0	2.15 ± 0.14
0.1 pmol/L	2.22 ± 0.27[a]
10 pmol/L	2.27 ± 0.17[a]
1 nmol/L	2.19 ± 0.16[a]
100 nmol/L	2.17 ± 0.19[a]

Phospho-p44/42 and phospho-p38 CCDPK expression E_2 activated p44/42 CCDPK in BAEC, but had no effect on p38 CCDPK activation (Fig 4). E_2 (1 nmol/L – 100 nmol/L) enhanced TNF-α-stimulated expression of phospho-p44/42, while reduced the expression of phospho-p38 CCDPK in a concentration-dependent manner. Treatment with U0126 5 μmol/L completely blocked TNF-α and E_2-induced p44/42 CCDPK activation (Fig 5, Tab 3).

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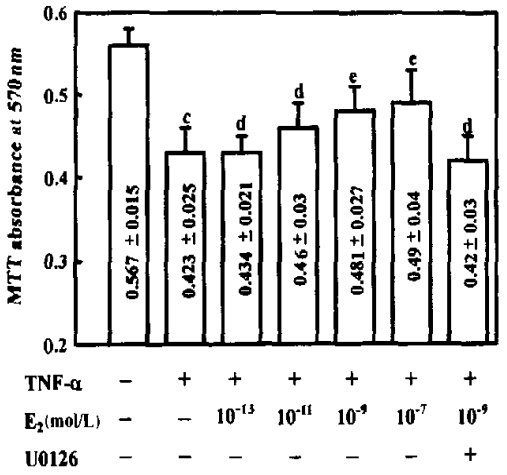

Fig 3. Concentration-dependent effect of 17β-estradiol (E_2) on TNF-α 5000 kU/L-induced decrease in BAEC viability and the effect of U0126 5 μmol/L. n = 4 experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$ *vs* TNF-α.

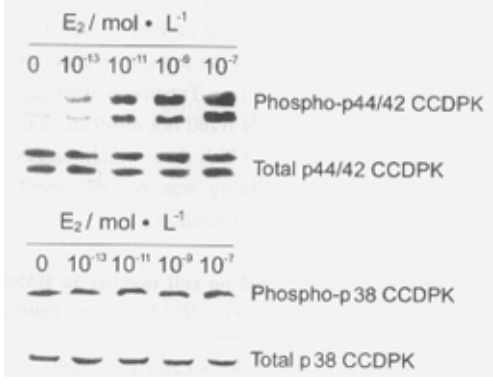

Fig 4. Effect of E_2 on expression of phospho-p44/42 and phospho-p38 CCDPK proteins in BAEC by Western blot.

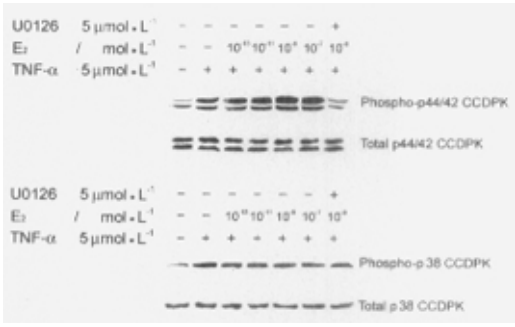

Fig 5. Effect of 17β-estradiol (E_2) on TNF-α 5000 kU/L-induced expression of phospho-p44/42 and phospho-p38 CCDPK proteins in BAEC by Western blot and the effect of U0126 5 μmol/L.

Tab 3. Concentration-dependent effect of 17β-estradiol (E_2) on TNF-α 5000 kU/L-induced expression of phospho-p44/42 and phospho-p38 CCDPK and the effect of U0126 5 μmol/L in BAEC. n = 3 experiments. $x \pm s$. Average of duplicates constitutes one determination. $^aP > 0.05$, $^bP < 0.05$ *vs* TNF-α.

Treatment	10^{-3} × Absolute peak area/mm² pp44/42 CCDPK	pp38 CCDPK
Control	44 ± 8	24 ± 7
TNF-α	105 ± 19	60 ± 9
TNF-α + E_2 0.1 pmol/L	116 ± 18[a]	56 ± 11[a]
TNF-α + E_2 10 pmol/L	131 ± 21[a]	52 ± 8[a]
TNF-α + E_2 1 nmol/L	175 ± 21[b]	42 ± 6[b]
TNF-α + E_2 100 nmol/L	164 ± 14[b]	40 ± 7[b]
TNF-α + E_2 1 nmol/L + U0126	54 ± 10[b]	55 ± 11[a]

DISCUSSION

Estrogen has been assumed to protect against cardiovascular diseases by an anti-atherogenic action on the lipid profile and by direct effects on arterial wall[13,3]. The present study demonstrates that E_2 exerts a protective effect, inhibiting endothelial cell apoptosis induced by TNF-α, suggesting an alternative mechanism for the atheroprotective effect of estrogen.

Estrogen has been shown to exhibit antiapoptotic functions on several cell lines. Estrogen safeguarded neurons from death via preservation of autoregulatory function, an antioxidant effect, increased expression of the antiapoptotic factor *bcl*-2, and the activation of mitogen activated protein kinase pathways[14]. Antiapoptotic effect of E_2 on ovine follicles was recently reported via an antioxidant mechanism[15]. In endothelial cells, E_2 exhibited an antiapoptotic effects via enhancing endothelial cell adhesion to matrix[5]. Recently, estrogen has been demonstrated to activate CCDPK cascade[16], however, the physiological significance of estrogen-induced CCDPK activation remains to be elucidated. Our present results show that E_2 also activates p44/42 CCDPK in BAEC, but had no effect on p38 CCDPK activation, consistent with recent findings in rat cardiomyocytes[17]. Considering the important roles of CCDPK cascades in modulating growth and death in various cell species[8,18], the present study further investigated the potential mechanism underlying

the protective effects of E_2. Co-modulatory roles in mediating cell apoptosis between CCDPK subfamily members have been investigated in our recent work and by others[6,19-21]. Generally, p38 CCDPK cascade is activated by stress stimuli and has been thought to mediate cell apoptosis, while p44/42 CCDPK is activated by growth stimuli and promotes cell survival, a similar result was also observed in TNF-α-induced BAEC apoptosis[6]. The data of this investigation that E_2 enhances TNF-α-induced expression of phospho-p44/42 CCDPK, at the same time, downregulates the expression of phospho-p38 CCDPK suggest an alternative molecular mechanism underlying the protective effect of E_2 on endothelial cells. The fact that p44/42 CCDPK inhibitor U0126 prevented both p44/42 CCDPK activation and the anti-apoptotic effect of E_2 further indicates a role of CCDPK cascade in modulating the protective effect of E_2.

In conclusion, E_2 protects BAEC apoptosis induced by TNF-α and activation of p44/42 CCDPK signaling together with inhibition of p38 CCDPK signaling by E_2 appears to be an important mechanism for its survival effect on endothelial cells. Our study also suggests a novel possible mechanism of the atheroprotective effect of estrogen.

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雌激素通过对 p38 和 p44/42 CCDPK 的相反作用阻止 TNF-α 诱导牛主动脉内皮细胞凋亡

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关键词 细胞凋亡；DNA 断片；Ca^{2+}-钙调蛋白依赖性蛋白激酶；肿瘤坏死因子；17β-雌二醇；蛋白质印迹；血管内皮；培养的细胞

目的：研究雌二醇(17β-estradiol，E_2)对肿瘤坏死因子α(TNF-α)诱导牛主动脉内皮细胞(BAEC)凋亡的影响及机制. **方法**：BAEC 培养并传代于 DMEM. BAEC 经 Hoechst 33258 染色后用荧光显微镜观察形态学变化及计数凋亡细胞；用 MTT 法测定细胞活性；用琼脂糖凝胶电泳分析 DNA 降解；用 Western blot 法检测磷酸化 p38 和 p44/42 CCDPK 表达. **结果**：TNF-α 诱导 BAEC 产生典型的凋亡细胞形态学变化(核浓染，核碎裂)和 DNA 断片. E_2 (0.1 pmol/L－100 nmol/L)能增强 TNF-α 诱导的磷酸化 p44/42 表达，同时抑制 p38 CCDPK 的活化而浓度依赖性阻止 TNF-α 诱导的 BAEC 凋亡；E_2 1 nmol/L 明显减弱 TNF-α 所致 DNA 断裂；p44/42 CCDPK 抑制剂 U0126 可拮抗 E_2 的作用. **结论**：雌激素通过激活 p44/42 CCDPK，同时抑制 p38 CCDPK 而产生的抗凋亡效应，是其使内皮细胞存活的重要机制.

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