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Opposing effect of p38 CCDPK and p44/42 CCDPK signaling on TNF-α-induced apoptosis in bovine aortic endothelial cells

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

AIM: To investigate the pro-apoptotic role of tumor necrosis factor α (TNF-α) in cultured bovine aortic endothelial cells (BAEC) and its underlied apoptotic signaling pathways. **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 after TNF-α treated BAEC for 24 h with Hoechst 33258 staining. Cell viability was determined 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, formerly called MAPK) was measured by Western blotting. **RESULTS**: TNF-α elicited typical apoptotic morphologic changes (chromatic condensation, nucleus fragmentation) and DNA fragmentation. At 1000-5000 kU/L, incubation with TNF-α for 24 h induced BAEC apoptosis and both of phospho-p38 and phospho-p44/42 CCDPK expression in a concentration-dependent manner. Interestingly, TNF-α-stimulated activation of p44/42 CCDPK was completely blocked, TNF-α-induced apoptosis was markedly increased by preincubation with U0126, a specific p44/42 CCDPK inhibitor. However, SB203580, a specific p38 CCDPK inhibitor, completely blocked TNF-α-stimulated activation of p38 CCDPK, and enhanced the expresssion of phospho-p44/42 CCDPK induced by TNF-α, substantially inhibited the pro-apoptotic effect of TNF-α. **CONCLUSION**: TNF-α simultaneously activates p38 CCDPK and p44/42 CCDPK, and these two CCDPK signaling pathways appeared to play opposing roles in TNF-α-induced apoptosis in BAEC.

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INTRODUCTION

Signal transduction pathways controlled by kinase modules regulate critical cellular functions such as cell growth, differentiation, and apoptosis. The Ca^{2+}-calmodulin dependent protein kinases (CCDPK) are a family of serine/threonine protein kinases thought to mediate intracellular signal transduction[1]. Among them, the p44/42 CCDPK activated by mitogens and survival factors is considered to be closely associated with cell growth and survival. In contrast, the p38 CCDPK is activated by pro-inflammatory cytokines, heat shock, endotoxin, and other cellular stresses and has been thought to play an important role in cellular apoptosis[2]. Recently, considerable attention has been focused on the balance between CCDPK pathways in modulating cell survival or death[3-5].

Tumor necross factor α (TNF-α) has been considered to be a very important mediator of atherogenesis and restenosis due to its pluripotent actions on vascular cells, such as inflammatory, proliferative, chemotatic, and cytotoxic effects[6]. Previous studies have demonstrated that TNF-α was capable of activating different members of CCDPK family depending on cell type to mediate its chemotactic, DNA synthesis inhibition or pro-apoptotic effects[7-9]. However, up to date, little is known about the co-modulatory effects of CCDPK family members in TNF-α-induced endothelial cell survival or death and the cross talk between CCDPK cascades. In the present study, we are interested in evaluating the cross-talk between p44/42 and p38 CCDPK in TNF-α induced apoptosis in endothelial cells.

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[10] and cultured in Dulbecco's modified Eagle's medium

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(DMEM) with 10 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L. Confluent cells were subcultured by trypsin digestion. 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. MTT was dissolved in phenol free M1640 at a concentration of 5 g/L. An amount of this solution equal to 10 % of the culture medium volume was added to cell cultures. After 1 h incubation, cultures were removed from the incubator and the formazan crystals were solubilized by adding solubilization solution including 10 % (v/v) Triton X-100 and HCl 0.1 mol/L in isopropanol equal to the original culture medium volume. Metabolic activity was quantified by measuring light absorbance at 570 nm.

Morphologic determination of apoptosis After treatment, BAEC were observed under fluorescence microscope after Hoechst 33258 staining.

Quantification of apoptosis Apoptosis was routinely determined by counting the number of cells with condensed or fragmented chromatin as described previously[11]. Briefly, cells from different treatment were cytospun onto glass slides, and then fixed with fixative solution (3 : 1 methanol/acetic acid). The preparations were stained with Hoechst 33258 5 mg/L for 10 min, rinsed, and dried. Under 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, with a minimum number of 500 cells scored in each condition.

DNA electrophoresis At the end of each incubation, cells were harvested and incubated with a nuclear lysis buffer (Tris-HCl 10 mmol/L, pH 8.0, edetic acid 10 mmol/L, NaCl 0.1 mol/L, 2 % SDS, proteinase K 60 mg/L, DTT 0.6 g/L) at 55 ℃ for 16 h, then the lysates were gently extracted thrice with an equal volume of phenol followed by chloroform. DNA was pelleted with ice-cold ethanol and dissolved in Tris-HCl 10 mmol/L, pH 8.0, and edetic acid 10 mmol/L. RNA was removed with DNAase-free RNase 100 mg/L at 37 ℃ for 3 h. DNA were electrophoretically fractionated on 1.5 % agarose gel and visualized by ethidium bromide.

Preparation of lysates 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 TNF-α were added. For inhibitor studies, cells were pretreated for 10 min with U0126 or SB203580 before the addition of TNF-α. After being washed with ice-cold PBS thrice, cells were lysed with 60 μL of ice-cold lysis buffer containing (mmol/L) NaCl 50, Na_3VO_4 2, phenylmethylsulfonyl fluoride 0.5, and HEPES 10 at pH 7.4, along with 0.01 % Triton X-100 and leupeptin 10 mg was added. The lysates were obtained by centrifugation at 18 000 × g at 4 ℃ for 15 min. Total cell protein was determined by the dye method[12].

Western blot SDS sample buffer 1/4 volume containing Tris-HCl 0.33 mol/L, SDS 10 % (w/v), glycerol 40 % (v/v), and bromophenol blue 0.4 % were added to cell lysates. After being boiled for 5 min, the extracted protein 10 μg was electrophoresed on SDS-polyacrylamide gel (SDS-PAGE). The protein were transferred to nitrocellulose membrane, which was then blocked for 1 h at 25 ℃ with 5 % BSA in TBST (Tris-HCl 50 mmol/L, pH 7.4, NaCl 150 mmol/L containing 0.1 % Tween-20). The blots were incubated with the primary antibodies against phospho-p38, phospho-p44/42 CCDPK or total p38, p44/42 CCDPK at a 1:1 000 dilution overnight at 4 ℃, followed by incubation at room temperature for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1:2000 dilution. Immunoreactive signals were visualized by the Phototope Western Detection System. To control for equal protein concentrations in CCDPK experiments, 2 gels for each group were loaded in parallel with the same protein samples and blotted for activated, phosphorylated CCDPK or total CCDPK. Bands of CCDPK were quantitatively determined by thin-layer chromatography with Shimadza Dual-Wavelength Chromato-Scanner (Japan, Model CS-930).

Reagents BSA, DMEM medium, SB203580, and Hoechst 33258 were purchased from Sigma Chemical Co. CCDPK monoclonal antibodies, HRP-conjugated anti-rabbit secondary antibody, 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 $\bar{x} \pm s$, 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 (Fig 1).

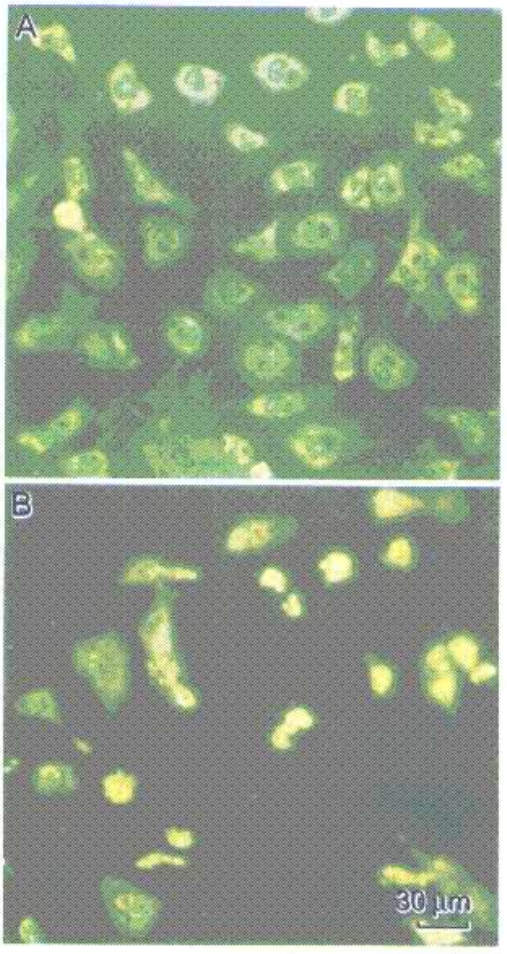

Fig 1. Fluorescence photomicrograph of BAEC stained with Hoechst 33258. × 300. A) Nuclei of control BAEC were stained uniformly. B) TNF-α-treated BAEC showing apoptotic nuclei (condensed or fragmented).

Cell viability TNF-α was found cytotoxic to BAEC in a concentration-dependent manner. Exposure of BAEC to TNF-α for 24 h in a concentration of 1000 kU/L had only minor effect on cell viability, whereas exposure of the cells to TNF-α 5000 kU/L decreased viability by 23.2 % (absorbance: 0.43 ± 0.03 *vs* 0.564 ± 0.016 of control) as assessed by the MTT test. Preincubation with the specific p44/42 CCDPK inhibitor U0126 5 μmol/L for 10 min markedly enhanced the toxicity of TNF-α to BAEC, cell death increased to 39.3 % (absorbance: 0.34 ± 0.04 *vs* 0.43 ± 0.03 of TNF-α 5000 kU/L, $P < 0.01$). However, the specific p38 CCDPK inhibitor SB203580 10 μmol/L decreased endothelial cell death to 10.7 %. Under our experimental conditions, no cytotoxic effect to BAEC was found after exposure to SB203580 10 μmol/L alone, while U0126 5 μmol/L caused cell death (absorbance: 0.532 ± 0.022 *vs* 0.564 ± 0.016, $P < 0.05$) (Fig 2).

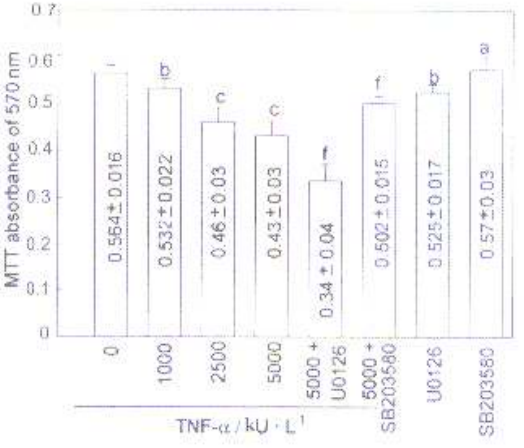

Fig 2. The effect of TNF-α 5000 kU/L, U0126 5 μmol/L, and SB203580 10 μmol/L on BAEC viability. Cell viability was analyzed by the MTT assay. $n = 6$ experiments. $\bar{x} \pm s$. Average of triplicates constitutes one determination. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* TNF-α 5000 kU/L.

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). Pretreatment with U0126 5 μmol/L for 10 min, strengthened the apoptosis-induced by TNF-α, while SB203580 10 μmol/L, greatly reduced the DNA fragmentation induced by TNF-α (Fig 3).

Quantification of apoptosis In order to verify the decrease in cell viability was mainly due to cell apoptosis rather than necorsis induced by TNF-α, direct apoptotic cell counting was performed. A similar concentration-dependent manner for TNF-α to induce BAEC apop-

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tosis was observed in apoptotic cell counting. Under control condition, 4.1 % ± 0.9 % of BAEC were apoptotic by direct apoptotic cell counting. After incubation with TNF-α (5000 kU/L) for 24 h, 28 % ± 3 % of cells were apoptotic, which was consistent with the results in cell viability assay. A greater or a lower rate of cell apoptosis were also observed respectively for preincubation with U0126 5 μmol/L or SB203580 10 μmol/L as compared with the treatment with TNF-α alone (Tab 1).

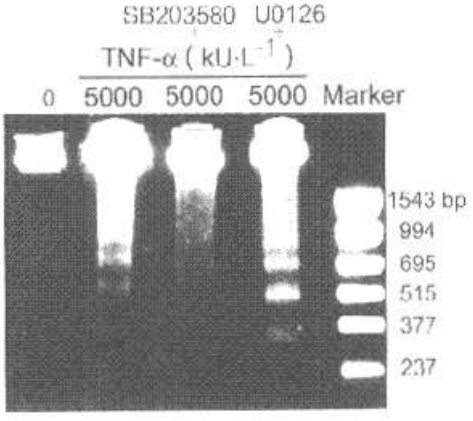

Fig 3. Agarose gel electrophoresis of TNF-α-induced DNA fragmentation in BAEC.

Tab 1. Concentration-dependent effect of TNF-α on apoptosis in BAEC and the effect of U0126 5 μmol/L and SB203580 10 μmol/L on TNF-α-induced apoptosis in BAEC. $n = 4$ experiments. $x \pm s$. $^{c}P < 0.01$ vs control. $^{a}P < 0.05$, $^{b}P < 0.01$ vs TNF-α 5000 kU/L.

TNF-α/kU·L⁻¹	Apoptotic BAEC/ % of total BAEC
0	4.1 ± 0.9
01000	9.7 ± 2.4[c]
02500	18.6 ± 2.9[c]
05000	28 ± 3[c]
05000 + U 0126	39 ± 5[b]
05000 + SB203580	12.2 ± 2.1[b]

Phospho-p44/42, phospho-p38 CCDPK expression TNF-α 5000 kU/L stimulated the activation of p44/42 CCDPK and p38 CCDPK in a similar time course with a maximal induction at 10 min after stimulation that returned near to baseline levels by 2 h after treatment, but the peak expression of phospho-p38 CCDPK maintained a longer period of plateau than phospho-p44/42 CCDPK (Fig 4). At concentrations of 1000, 2500, 5000 kU/L, treatment with TNF-α for 10 min stimulated the expression of phospho-p44/42 and phspho-p38 CCDPK in a concentration-dependent manner (Fig 5).

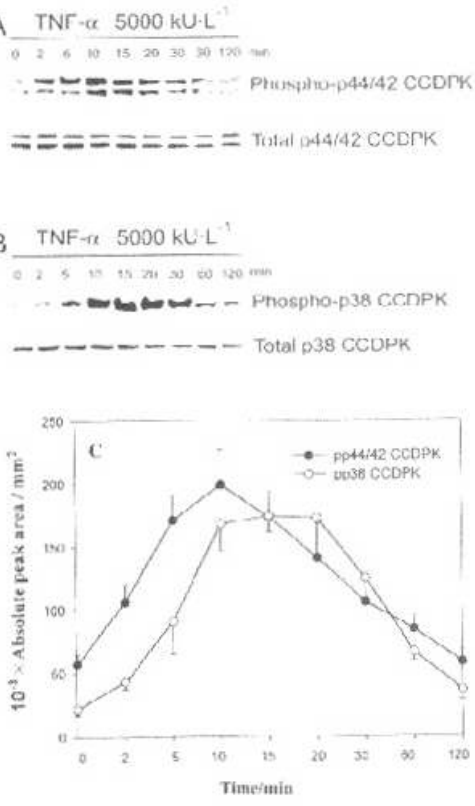

Fig 4. Time course of TNF-α induced expression of phospho-p44/42 and p38 CCDPK in BAEC by Western blot. A) Phospho-p44/42 CCDPK and total p44/42 expression. B) Phospho-p38 CCDPK and total p38 CCDPK expression. C) Absolute peak area of phospho-p44/42 and phospho-p38 CCDPK determined by thin-layer chromatography. $n = 3$ experiments. $x \pm s$. Average of duplicates constitutes one determination.

Treatment with U0126 5 μmol/L or SB203580 10 μmol/L completely blocked TNF-α-induced p44/42 or p38 CCDPK activation, respectively. Interestingly, blockade of p38 CCDPK activation with SB203580 enhanced TNF α induced phospho-p44/42 CCDPK expression (Fig 6, Tab 2). In parallel experiments, the amount of total p44/42 or p38 CCDPK was determined in the same cell extracts with the use of an antibody that recognized all p44/42 or p38 CCDPK independent of their phosphorylation state.

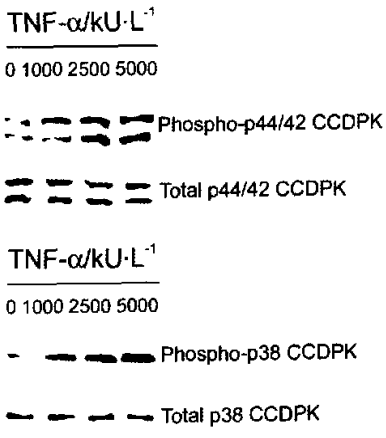

Fig 5. TNF-α induced expression of phospho-p44/42 and phospho-p38 CCDPK in BAEC in a concentration-dependent manner.

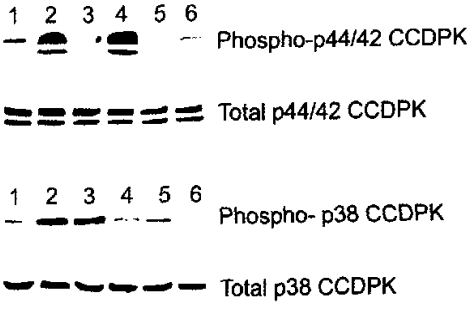

Fig 6. Effect of U0126 and SB203580 on TNF-α induced expression of phospho-p44/42 and phospho-p38 CCDPK in BAEC. Lane 1: control; 2: TNF-α (5000 kU/L); 3: TNF-α + U0126; 4: TNF-α + SB203580; 5: U0126; 6: SB203580.

Tab 2. Effect of U0126 (5 μmol/L) and SB203580 (10 μmol/L) on TNF-α (5000 kU/L)-induced expression of phospho-p44/42 and phospho-p38 CCDPK in BAEC. n = 3 experiments. $\bar{x} \pm s$. Average of duplicates constitutes one determination. $^bP<0.05$, $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* TNF-α.

TNF-α/kU·L⁻¹	10^{-3} × Absolute peak area/mm² pp44/42 CCDPK	pp38 CCDPK
Ctl	43 ± 3	28 ± 9
TNF-α 5000	97 ± 16^c	67 ± 7^c
TNF-α + U0126	29 ± 4^f	69 ± 5
TNF-α + SB203580	138 ± 13^e	29 ± 8^f
U0126	19 ± 3^c	27 ± 8
SB203580	45 ± 7	10 ± 2^b

DISCUSSION

Previous studies demonstrated that TNF-α could trigger apoptosis in a variety of cell types, such as endothelial cells[13]. The mechanisms by which TNF-α induced apoptosis have been the subject of intense investigation. Recent evidence has indicated a dynamic balance among different CCDPK members in determining cell death or survival[3-5]. In the present study, we demonstrated that TNF-α triggered apoptosis in BAEC, at the same time, TNF-α activated both p44/42 and p38 CCDPK in a concentration-dependent manner and a similar time course. Inconsistent with Laird's study with BAEC[8] that the activation of p38 CCDPK induced by TNF-α was much stronger than that of p44/p42 CCDPK, our present findings indicated that TNF-α strongly activated both p44/p42 and p38 CCDPK. In addition, the dynamic courses of CCDPK activation were also not completely identical in these two studies. Although we did not further investigate the cause of this difference, it may be partly interpreted by different concentrations of TNF-α used in these two studies.

p44/42 CCDPK inhibitor U0126 completely blocked TNF-α-stimulated activation of p44/42 CCDPK, at the same time, markedly enhanced TNF-α-induced apoptosis in BAEC. p38 CCDPK inhibitor SB203580 completely blocked the activation of p38 CCDPK, but not completely, and reduced the apoptosis-inducing effect of TNF-α. These data demonstrate that there are at least two CCDPK signal transduction pathways simultaneously involved in mediating TNF-α-induced apoptosis. Consistent with the general conception, in our study, p44/42 CCDPK acted as a survival factor, while p38 CCDPK played important roles in mediating apoptosis. An interesting finding is that the inhibition of p38 CCDPK activation by SB203580 was concomitant with the upregulation of the expression of phospho-p44/42 CCDPK suggests a dynamic balance between these two kinase cascades in modulating apoptosis, which is consistent with our recent study with H_2O_2[14]. The complete blockade of p38 CCDPK activation by SB203580 did not completely abolish the apoptosis-inducing effect of TNF-α, suggesting that there might be other different signal trasduction pathways in mediating TNF-α-induced apoptosis. Among them, SAPK/JNK signal pathway has been demonstrated to mediate the apoptotic effect of TNF-α in recent studies[15,16]. In the present study, the simple inhibition of p44/42 CCDPK with U0126 triggered apoptosis in BAEC, which was consistent with the previous observation in Hella

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cells[3]. In the latter study, the activation of p38 CCDPK was considered to play a decisive role in Hella cell apoptosis. In conclusion, we propose that TNF-α simultaneously activates p38 CCDPK and p44/42 CCDPK, and these two CCDPK signaling pathway appears to play opposing roles in TNF-α-induced apoptosis in BAEC. Our data suggest that cross-talk between p44/42 and p38 CCDPK is important in control of cell apoptosis.

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p38 和 p44/42 CCDPK 信号在 TNF-α 诱导牛主动脉内皮细胞凋亡中的相反作用

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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；蛋白质印迹；血管内皮；培养的细胞

目的：研究肿瘤坏死因子(TNF-α)诱导牛主动脉内皮细胞(BAEC)凋亡及其信号途径. **方法**：BAEC 培养并传代于 DMEM. 经 TNF-α 处理 24 h 后，Hoechst 33258 染色，荧光显微镜观察形态学变化及凋亡细胞计数. MTT 法测定细胞活性，琼脂糖凝胶电泳分析 DNA 降解，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. TNF-α (1000 – 5000 kU/L)浓度依赖性诱导 BAEC 凋亡，并同时刺激磷酸化 p44/42 和 p38 CCDPK 的表达. p44/42 CCDPK 抑制剂 U0126 可完全阻断 TNF-α 诱导的 p44/42 CCDPK 的活化，显著增强 TNF-α 致凋亡作用；而 p38 CCDPK 抑制剂 SB203580 可完全阻断 TNF-α 诱导的 p38 CCDPK 的活化，还可增强 TNF-α 诱导的磷酸化 p44/42 CCDPK 的表达，明显抑制 TNF-α 促凋亡作用. **结论**：TNF-α 同时激活 p38 和 p44/42 CCDPK，这两种 CCDPK 信号通路在 TNF-α 诱导 BAEC 凋亡中起相反作用.

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