ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jan; 23 (1): 45-49
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VEGF protects bovine aortic endothelial cells from TNF-α- and H_2O_2-induced apoptosis via co-modulatory effects on p38- and p42/p44-CCDPK signaling

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

ABSTRACT

AIM: To investigate the effect of VEGF on TNF-α- or H_2O_2- induced apoptosis in cultured bovine aortic endothelial cells (BAEC) and the underlied signal transduction mechanisms related to Ca^{2+}-calmodulin dependent protein kinase (CCDPK). **METHODS**: BAEC were cultured and passaged in 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-p42/p44 CCDPK was measured by Western blotting. **RESULTS**: TNF-α 5000 kU/L and H_2O_2 300 μmol/L elicited DNA fragmentation in BAEC. Vascular endothelial growth factor (VEGF) 100 μg/L significantly protected BAEC from apoptosis induced by TNF-α or H_2O_2, as shown in cell viability assay and apoptotic cell counting. DNA fragmentation induced by TNF-α or H_2O_2 was also reduced by VEGF 100 μg/L. VEGF enhanced TNF-α and H_2O_2 stimulated expression of phospho-p42/p44 CCDPK, simultaneously inhibited TNF-α- and H_2O_2-induced activation of phospho-p38 CCDPK. Both the VEGF-induced up-regulation of phospho-p42/p44 CCDPK and its anti-apoptotic action were prevented by the specific p42/p44 CCDPK inhibitor U0126. **CONCLUSION**: VEGF protects BAEC from apoptosis induced by TNF-α and H_2O_2, and its co-modulatory effects by activation of p42/p44 CCDPK signaling together with inhibition of p38 CCDPK signaling appear to be an important mechanism for its survival effect on endothelial cells.

INTRODUCTION

Vascular endothelial cell injury is a postulated initial step in the pathogenesis of atherosclerosis[1]. TNF-α and free radical damage have been demonstrated to be relevant causative factors of vascular endothelial cell apoptosis[2-4]. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that has been shown to act as an endothelial cell mitogen as well as a vascular permeability factor[5]. Several recent reports have also implicated VEGF as a major survival factor for endothelial cells exposed stress stimuli such as TNF-α or serum starvation[6,7]. Various growth factors have been shown to prevent H_2O_2-induced apoptosis in differenct cell types[8,9]. VEGF has also been shown to increase endothelial resistance to H_2O_2[10], so it-is rationale for us to speculate VEGF may prevent H_2O_2-induced endothelial cell death. TNF-α and oxidants have recently been demonstrated to trigger the activation of multiple signaling pathways that influence the cytotoxicity observed in affected cells, including the activation of different members of Ca^{2+}-calmodulin dependent protein kinases (CCDPK)[3,4,11,12]. In our recent findings, different modulatory actions of p38 and p44/p42 CCDPK were observed in H_2O_2- and TNF-α induced apoptosis in cultured bovine aortic endothelial cells, suggesting considerable variation in response to different stimuli[3,4].

The present study is aimed to examine p38- and p44/p42 CCDPK coupling signal transduction mechanisms mediating the anti-apoptotic effect of VEGF on TNF-α- and H_2O_2-induced apoptosis in cultured bovine aortic endothelial cells.

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Received 2001-06-18 Accepted 2001-09-11

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jan; 23 (1)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested and subcultured as previously described[13]. Experiments were performed with cells from passage 4 - 10. In order to exclude the possibility that mitogenic effect of VEGF on endothelial cells might mask the ongoing apoptosis induced by TNF-α and H_2O_2, all the experiments were performed in medium with 10 % FBS. Under such an experimental condition, the proliferative effect of VEGF was completely covered up by FBS.

Cell viability assay BAEC were seeded out in 24-well plates (6×10^4 cells per well) and grown to 70 % confluence in DMEM with 10 % FBS. The cultures were then rinsed in phenol free RPMI-1640 medium and incubated in phenol free RPMI-1640 with 10 % FBS for 24 h with or without TNF-α 5000 kU/L, H_2O_2 300 μmol/L, and /or VEGF 100 μg/L. The concentrations of TNF-α and H_2O_2 were chosen according to our recent experimental results, while VEGF 100 μg/L exhibited maximal protective effect on endothelial cell survival in a recent study[14]. Cell viability was measured by means of MTT assay[3].

Quantification of apoptosis Apoptosis was routinely determined by counting the number of cells with condensed or fragmented chromatin after Hoechst 33258 staining[15]. Condensed and fragmented nuclei, typical morphologic changes of apoptosis, were easily distinguishable from intact nuclei under fluorescence microscope 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.

Assay for DNA fragmentation DNA was prepared by standard phenol-chloroform extraction. DNA were electrophoretically fractionated on 1.5 % agarose gel and visualized by ethidium bromide.

Western blot analysis For CCDPK detection, BAEC cultured in 6-well culture plates (2×10^5 cells per well) were grown to 80 % - 90 % confluence, then TNF-α 5000 kU/L or H_2O_2 300 μmol/L and / or VEGF 100 μg/L were added. The treating time was chosen to be 10 min, since a peak expression of phospho-p42/p44 and phospho-p38 CCDPK was obtained at this time point in our experimental conditions[3,4]. For inhibitor studies, cells were pretreated for 10 min with U0126 before the above treatments. Total cell protein was determined by the dye method[16]. 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[3]. Bands of CCDPK were quantitatively determined by thin-layer chromatography with Shimadzu Dual Wavelength Chromato-Scanner (Model SC-930 Japan).

Reagents BSA and Hoechst 33258 were purchased from Sigma Chemical Co. VEGF was the product of R&D Systems. CCDPK mono-clonal anti-bodies, 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 TRZAKOS JM.

Statistics Values were expressed as $x \pm s$ and assessed by one-way ANOVA and Student's t-test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of VEGF on apoptosis of BAEC The characteristic features of apoptosis exhibited after being cultured in TNF-α 5000 kU/L or in H_2O_2 300 μmol/L for 24 h including chromatin condensation and nucleus fragmentation. Direct apoptotic cell counting was used to confirm the protective effect of VEGF against apoptosis. After incubation with TNF-α 5000 kU/L or H_2O_2 300 μmol/L for 24 h, endothelial cell apoptosis occurred in 17.9 % ± 2.9 % or 21 % ± 6 % of cells, respectively. When VEGF 100 μg/L applied simultaneously with TNF-α or H_2O_2, BAEC death reduced to 10.3 % ± 2.6 % or 12 % ± 4 %, respectively ($P < 0.05$). U0126 5 μmol/L completely abolished the protective effect of VEGF on TNF-α- or H_2O_2-induced apoptosis, BACE death increased to 18 % ± 7 % or 20 % ± 4 %, respectively ($P < 0.05$, Tab 1).

Anti-apoptotic effect of VEGF was further examined with the DNA fragmentation assay. TNF-α and H_2O_2 elicited a characteristic "ladder" of DNA fragments in BAEC. When VEGF 100 μg/L was added simultaneously to TNF-α or H_2O_2, DNA fragmentation was significantly reduced. Pretreatment of BAEC with U0126 5 μmol/L for 10 min abolished the anti-apoptotic effect of VEGF (Fig 1).

Tab 1. The effect of VEGF (100 μg/L) and U0126 (5 μmol/L) on TNF-α (5000 kU/L)- or H_2O_2 (300 μmol/L)-induced apoptosis in BAEC. $n = 4$ **experiments.** $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^eP < 0.05$ *vs* TNF-α. $^hP < 0.05$ *vs* TNF-α + U0126. $^kP < 0.05$ *vs* H_2O_2. $^nP < 0.05$ *vs* H_2O_2 + U0126.

Treatment	MTT absorbance at 570 nm	Apoptotic BAEC/ % of total BAEC
Control	0.603 ± 0.017	1.6 ± 0.5
TNF-α	0.47 ± 0.04^c	17.9 ± 2.9^c
TNF-α + VEGF	0.53 ± 0.03^e	10.3 ± 2.6^e
TNF-α + VEGF + U0126	0.46 ± 0.04^h	18 ± 7^h
H_2O_2	0.45 ± 0.04^c	21 ± 6^c
H_2O_2 + VEGF	0.532 ± 0.028^k	12 ± 4^k
H_2O_2 + VEGF + U0126	0.46 ± 0.04^n	20 ± 4^n
VEGF	0.605 ± 0.019^a	

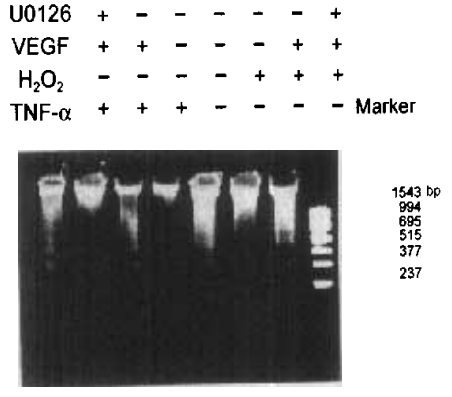

Fig 1. Effect of VEGF 100 μg/L and U0126 5 μmol/L on TNF-α (5000 kU/L)- and H_2O_2 (300 μmol/L)-induced DNA fragmentation in BAEC visualized on agarose gel electrophoresis.

Cell viability After incubation with TNF-α 5000 kU/L or H_2O_2 300 μmol/L for 24 h, BAEC death occurred in 22.5 % or 25.0 %, respectively. VEGF 100 μg/L decreased TNF-α or H_2O_2 induced cell death by 10.9 % or 13.2 %, respectively. Preincubation of BAEC with U0126 5 μmol/L completely abolished the protective effect of VEGF (Tab 1). Under our experimental conditions, the proliferative effect of VEGF was completely covered up by 10 % FBS (absorbance at 570 nm: 0.605 ± 0.019 *vs* 0.603 ± 0.017 of control, $P > 0.05$). U0126 itself had no effect on cell viability (data not shown).

Phospho-p42/p44, phospho-p38 CCDPK expression VEGF 100 μg/L activated phospho-p42/p44 CCDPK, but had no effect on the expression of phospho-p38 CCDPK. At the same time, VEGF enhanced TNF-α- or H_2O_2-stimulated expression of phospho-p42/p44, while reduced the expression of phospho-p38 CCDPK. However, there was no marked change in the total p42/p44 CCDPK and p38 CCDPK protein, as seen by parallel blots using p42/44 CCDPK-specific or p38 CCDPK-specific antibody, which dectects total CCDPK (Fig 2). Treatment with U0126 5 μmol/L completely blocked p42/p44 CCDPK activation induced by TNF-α or H_2O_2 and VEGF, interestingly, it also abolished the inhibitory effect of VEGF on the expression of phospho-p38 CCDPK stimulated by TNF-α or H_2O_2 (Fig 2, Tab 2).

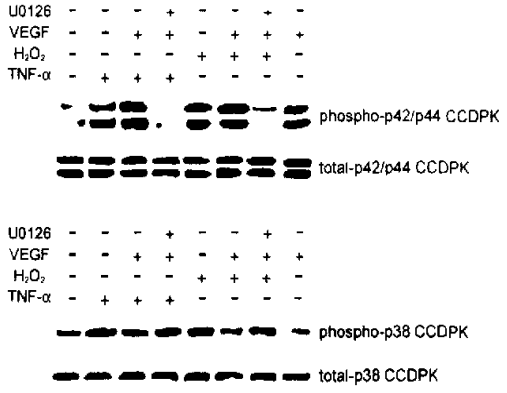

Fig 2. Effect of VEGF 100 μg/L and U0126 5 μmol/L on TNF-α (5000 kU/L)- and H_2O_2 (300 μmol/L)-induced expression of phospho-p42/p44 and phospho-p38 CCDPK proteins in BAEC by Western blot.

Tab 2. Effect of VEGF (100 μg/L) and U0126 (5 μmol/L) on TNF-α (5000 kU/L)- or H_2O_2 (300 μmol/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.** $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* TNF-α. $^gP > 0.05$, $^hP < 0.05$, $^iP < 0.01$ *vs* H_2O_2.

Treatments	10^{-3} × Absolute peak area/mm^2 pp42/p44 CCDPK	pp38 CCDPK
Control	41 ± 10	39 ± 8
TNF-α	147 ± 20^c	101 ± 19^c
TNF-α + VEGF	210 ± 31^e	68 ± 8^e
TNF-α + VEGF + U0126	35 ± 8^f	96 ± 19^d
H_2O_2	129 ± 28^c	97 ± 18^c
H_2O_2 + VEGF	215 ± 29^h	48 ± 7^h
H_2O_2 + VEGF + U0126	45 ± 7^i	85 ± 10^g
VEGF	132 ± 7^c	32 ± 5^a

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jan; 23 (1)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

DISCUSSION

VEGF has been considered to be the most potent and specific endogenous angiogenic factor due to its abilities to stimulate endothelial cell proliferation as well as inhibit endothelial cell apoptosis[5,6]. Over the past decade, the mitogenic effect of VEGF and its mechanisms have been extensively investigated. However, its protective effect on endothelial cell has been noticed only recently, and the related mechanisms are not well defined. Our present study demonstrated that protective effect of VEGF on TNF-α- and H_2O_2-induced apoptosis in bovine aortic endothelial cells and the mechanism underlying the survival effect of VEGF. We found that VEGF enhanced TNF-α-and H_2O_2-stimulated activation of phospho-p42/p44 CCDPK, at the same time, inhibited the activation of phospho-p38 CCDPK. In agreement with the general view, p42/p44 CCDPK has been shown to be a survival signaling in TNF-α- and H_2O_2-induced endothelial cell apoptosis in our recent work[3,4], so upregulation of the expression of phospho-p42/44 CCDPK may represent at least one part of the mechanisms for the survival effect of VEGF, which was verified by the fact that both VEGF-induced activation of phospho-p42/p44 CCDPK and its anti-apoptotic effect were abolished by specific p42/p44 CCDPK inhibitor U0126.

Our results were in agreement with a recent report that VEGF prevented endothelial cell from apoptosis induced by serum starvation or ceramide treatment, in which activation of p42/p44 CCDPK together with inhibition of SAPK/JNK activity was considered to account for the anti-apoptotic effect of VEGF[7]. Considering that p38 CCDPK and SAPK/JNK, two different CCDPK family members, have been demonstrated to mediate apoptotic signal pathway in many different experimental model systems[17], a very similar mechanism underlying the protective effect of VEGF was shown in these two studies with endothelial cells in response to different stress stimuli. However, under our experimental conditions, VEGF only partially inhibited the apoptosis-inducing effect of TNF-α and H_2O_2, while completely abolished ceramide-induced apoptosis in Cupta's study[7]. The observed quantitative differences in the anti-apoptotic effect of VEGF may be the consequence of different properties of human microvascular endothelial cells and bovine large vessel endothelial cells. Another possibility is that this difference reflects a different quantitative shift caused by VEGF in the dynamic balance between survival signal and apoptotic signal in different experimental models. Further studies are under way to quantitatively determine the relationship between the p42/p44 CCDPK-to-p38 CCDPK and/or SAPK/JNK ratio and the fate of cell survival or death. P38 CCDPK activation, demonstrated in our recent studies, is an apoptotic-mediating signal for TNF-α, but is not involved in H_2O_2-induced apoptosis. However unexpectedly, in the present study, VEGF inhibited both TNF-α- and H_2O_2-stimulated activation of phospho-p38 CCDPK, independent of its role in apoptotic process, suggesting activation of phospho-p42/p44 CCDPK and inhibition of phospho-p38 CCDPK may be a common pathway by which growth factors prevent apoptosis. Contrast changes have been previously shown for the activation of different members of CCDPK family in modulating apoptosis of different cell types in response to different stress stimuli[7,18,19]. But further studies are needed to explore the detailed dynamic changes for upregulation or downregulation of CCDPK family members in modulating cell apoptosis. Our findings support the general hypothesis, put forth by Xia *et al*, that the dynamic balance between p42/p44 CCDPK and p38 CCDPK or SAPK/JNK pathways is important in determining whether a cell survives or undergoes apoptosis[18].

Recently, VEGF has drawn the attention of cardiologists interested in angiogenic therapies for ischemic atherosclerotic diseases[20]. The focus here on TNF-α and H_2O_2 as endothelial stresses is perhaps pertinent to atherosclerosis, as they directly cause endothelium injury and play important roles in the pathogenesis and development of atherosclerosis[1]. Our present results further confirm the protective effect of VEGF on endothelial cells apoptosis.

In summary, VEGF protects BAEC from apoptosis induced by TNF-α and H_2O_2, and its co-modulatory effects by activation of p42/p44 CCDPK together with inhibition of p38 CCDPK signaling appear to be an important mechanism for its survival effect on endothelial cells.

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VEGF 通过对 p38 和 p42/p44 CCDPK 信号的共调节作用抑制 TNF-α 和 H_2O_2 诱导的牛主动脉内皮细胞凋亡

R96 A

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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；过氧化氢；内皮生长因子；蛋白质印迹；血管内皮；培养的细胞

目的：研究血管内皮细胞生长因子（VEGF）对肿瘤坏死因子（TNF-α）和过氧化氢（H_2O_2）诱导牛主动脉内皮细胞（BAEC）凋亡的影响及信号机制。**方法**：BAEC 培养并传代于 DMEM。经 TNF-α 或 H_2O_2 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 p42/p44 CCDPK 表达。**结果**：TNF-α 5000 kU/L 和 H_2O_2 300 μmol/L 均可诱导 BAEC 产生 DNA 断片。VEGF 100 μg/L 显著增强 TNF-α 和 H_2O_2 诱导的磷酸化 p42/p44 CCDPK 表达，而明显抑制磷酸化 p38 CCDPK 的活化。对二者所致 BAEC 凋亡起明显的抑制作用。P42/p44 CCDPK 抑制剂 U0126 可取消 VEGF 引起的磷酸化 p42/p44 CCDPK 表达上调和其抗凋亡作用。**结论**：VEGF 通过其共调节作用激活 p42/p44 CCDPK，抑制 p38 CCDPK 信号途径而对 TNF-α 和 H_2O_2 所致凋亡产生的抑制效应，是内皮细胞存活的重要机制。

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