Effects of p38 and p42/p44 CCDPK signaling on H_2O_2-induced apoptosis in bovine aortic endothelial cells

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

ABSTRACT

AIM: To investigate the effects of p38 and p42/p44 Ca^{2+}-calmodulin dependent protein kinases (CCDPK) signaling on hydroperoxide (H_2O_2)-induced apoptosis in cultured bovine aortic endothelial cells (BAEC). **METHODS:** Morphologic changes and quantification of apoptotic cells were determined under fluorescence microscope after a 24-h treatment of BAEC by H_2O_2. Cell viability was determined 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:** H_2O_2 elicited typical apoptotic morphologic changes (chromatic condensation, nucleus fragmentation) and DNA fragmentation. At 100 - 500 $\mu mol \cdot L^{-1}$, incubation of BAEC with H_2O_2 for 24 h also induced phospho-p38 and phospho-p42/p44 CCDPK expression in a concentration-dependent manner. Interestingly, H_2O_2-induced apoptosis was markedly increased by preincubation with U0126, a specific p42/p44 CCDPK inhibitor. However, SB203580, a specific p38 CCDPK inhibitor, enhanced the expresssion of phospho-p42/p44 CCDPK induced by H_2O_2, but had no effect on BAEC survival. **CONCLUSION:** p42/p44 CCDPK signaling appears to play protective roles in H_2O_2-induced apoptosis in BAEC, whereas p38 CCDPK is not the main signaling pathway mediating H_2O_2-induced cellular apoptosis.

INTRODUCTION

Reactive oxygen species (ROS) are implicated in the pathophysiology of several vascular disorders including atherosclerosis[1]. Although the mechanism(s) of ROS-induced vascular damage remains unclear, there is increasing evidence for ROS-mediated modulation of signal transduction pathways[2,3]. Hydroperoxide (H_2O_2), an important free radical, has been demonstrated to be toxic to endothelial cells in the previous study[4]. Recent studies have demonstrated that H_2O_2 is capable of simultaneously activating different members of the Ca^{2+}-calmodulin dependent protein kinases (CCDPK) family in rat macrophages and human keratinocytes[5,6]. CCDPK are thought to regulate critical cellular functions including apoptosis[7]. However, up to now, little is known about the stimulating effects of H_2O_2 on the coexpression of CCDPK family members in endothelial cells and the cross talk between CCDPK cascades in modulating H_2O_2-induced endothelial cell survival or death. In the present study, we are interested in evaluating the modulatory roles of p42/p44 and p38 CCDPK in H_2O_2-induced apoptosis in endothelial cells.

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[8] and cultured in DMEM with 10 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 $kU \cdot L^{-1}$, and streptomycin 100 $mg \cdot L^{-1}$. 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 M1640 medium and incubated with respective test substances in phenol free and serum free M1640 for 24 h. Cell viability was measured by MTT (dimethylthiazol-diphenyltetrazolium bromide) assay. MTT was dissolved in phenol free M1640 at a concentration of 5 $g \cdot L^{-1}$. An amount of this solution equal to 10 % volume of the culture medium was added to cell cultures. After a 1-h incubation, cultures were removed from the incubator and the formazan crystals were solubilized by

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adding solubilization solution including 10 % (v/v) Triton X-100 and HCl 0.1 $mol \cdot L^{-1}$ in isopropanol equal to the volume of original culture medium. 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[9]. Briefly, cells from different treatments were cytospun onto glass slides, followed by a mild hypotonic treatment (1:1 ratio distilled water/growth medium) for 10 min. The cells were prefixed for 5 min with a 50 % fixative solution (3:1 methanol/acetic acid), and then fixed with neat fixative solution for 10 min. The preparations were stained with Hoechst 33258 5 $mg \cdot L^{-1}$ 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 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 $mmol \cdot L^{-1}$, pH 8.0, edetic acid 10 $mmol \cdot L^{-1}$, NaCl 0.1 $mol \cdot L^{-1}$, 2 % SDS, proteinase K 60 $mg \cdot L^{-1}$, DTT 0.6 $g \cdot L^{-1}$) at 55 ℃ for 16 h, then the lysates were gently extracted thrice with an equal volume of phenol followed by chloroform. After centrifugation, the upper layer containing DNA was transferred to a new tube, and 2.5 volumes of ice-cold ethanol were added. After centrifugation at 12 000 × g for 15 min, the resulting DNA pellet was dissolved in Tris-HCl 10 $mmol \cdot L^{-1}$, pH 8.0, and edetic acid 10 $mmol \cdot L^{-1}$ and DNAase-free RNase 100 $mg \cdot L^{-1}$ was added and incubated 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 H_2O_2 were added. For inhibitory studies, cells were pretreated for 10 min with U0126 or SB203580 before the addition of H_2O_2. After being washed with ice-cold PBS thrice, cells were lysed with 60 μL ice-cold lysis buffer containing ($mmol \cdot L^{-1}$) 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[10].

Western blot SDS sample buffer 1/4 volume containing Tris-HCl 0.33 $mol \cdot L^{-1}$, 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 was transferred to nitrocellulose membrane, which was then blocked at 25 ℃ for 1 h with 5 % BSA in TBST (Tris-HCl 50, pH 7.4, NaCl 150 $mmol \cdot L^{-1}$ containing 0.1 % Tween-20). The blots were incubated with the primary antibodies against phospho-p38, phospho-p42/p44 CCDPK or total p38, p42/p44 CCDPK at a 1:1000 dilution at 4 ℃ overnight , 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 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.

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. 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 Student's t-test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Morphologic changes After exposure to H_2O_2 300 $\mu mol \cdot L^{-1}$ for 24 h, BAEC showed typical morphologic changes of apoptosis. The cell volume was reduced, the chromatin became condensed, and nucleus became fragmented (Fig 1).

DNA electrophoresis Incubation of BAEC with H_2O_2 300 $\mu mol \cdot L^{-1}$ for 24 h elicited a characteristic "ladder" of DNA fragments representing integer multiples of the internucleosomal DNA length (about 180 – 200

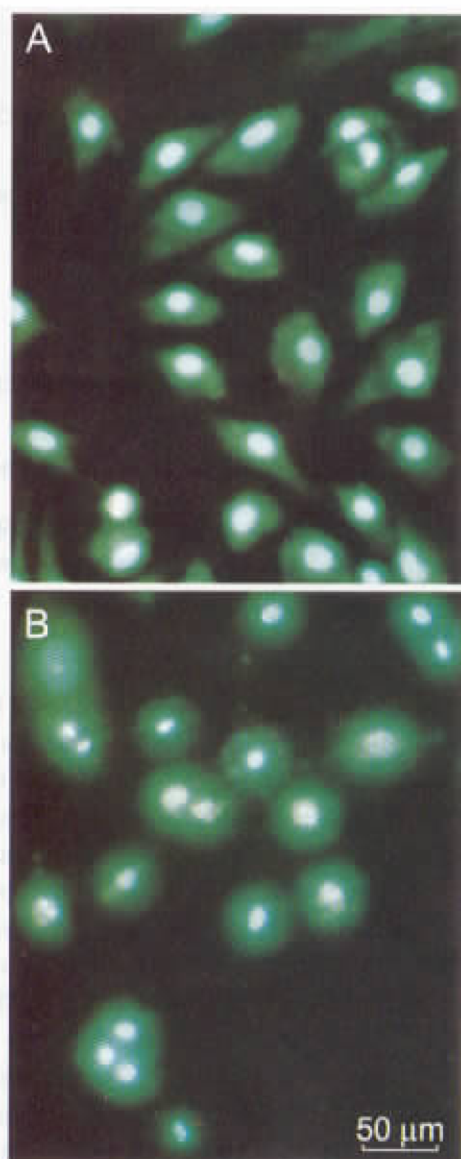

Fig 1. Fluorescence photomicrograph of BAEC stained with Hoechst 33258, ×200. A) Nuclei of control BAEC were stained uniformly. B) H_2O_2 treated BAEC showing apoptotic nuclei (condensed or fragmented).

base pair). Pretreatment with U0126 5 μmol · L^{-1} strengthened H_2O_2-induced apoptosis. While SB203580 10 μmol·L^{-1} had no effect on H_2O_2-induced DNA fragmentation (Fig 2).

Cell viability MTT assay, which measures mitochondrial function, is capable of detecting cell death earlier than other techniques. H_2O_2 was found to be cytotoxic to BAEC in a concentration-dependent manner. Exposure of BAEC to H_2O_2 at a concentration of 100 μmol·L^{-1} for 24 h decreased cell viability only by about 9 %, whereas H_2O_2 300 μmol·L^{-1} decreased viability by 30 % (absorbance: 0.38 ± 0.03 *vs* 0.54 ± 0.02 of control, $P < 0.01$) as assessed by the MTT test. Preincubation with the specific p42/p44 CCDPK inhibitor U0126 5 μmol·L^{-1} for 10 min markedly enhanced the toxicity of H_2O_2 to BAEC, cell death increased by 47.6 % (absorbance: 0.28 ± 0.02 *vs* 0.38 ± 0.03 of H_2O_2 300 μmol·L^{-1}, $P < 0.01$). However, the specific p38 CCDPK inhibitor SB203580 10 μmol·L^{-1} exhibited no effect on H_2O_2-induced decrease in cell viability (absorbance: 0.38 ± 0.03 *vs* 0.38 ± 0.03 of H_2O_2 300 μmol·L^{-1}, $P > 0.05$ (Tab 1).

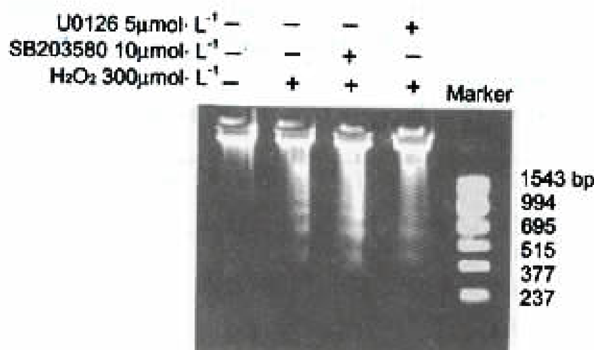

Fig 2. Agarose gel electrophoresis of H_2O_2-induced DNA fragmentation in BAEC.

Quantification of apoptosis A similar concentration-dependent manner for H_2O_2 to induce BAEC apoptosis was observed in apoptotic cell counting. Under control conditions, 3.9 % ± 1.4 % of BAEC were apoptotic by direct apoptotic cell counting. After incubation with H_2O_2 300 μmol·L^{-1} for 24 h, 31.2 % ± 4.8 % of cells were apoptotic, which was consistent with the results in cell viability assay. A greater rate of cell apoptosis was also observed for preincubation with U0126 5 μmol·L^{-1} as compared with the treatment with H_2O_2 300 μmol·L^{-1}. No effect was observed for SB203580 10 μmol·L^{-1}(Tab 1).

Phospho-p42/p44 and phospho-p38 CCDPK expression H_2O_2 300 μmol·L^{-1} stimulated the activation of p42/p44 CCDPK and p38 CCDPK in a similar time course with a maximal induction at 10 min after stimulation, which returned nearly to baseline levels 1 h after treatment. At concentrations of 100-500 μmol·L^{-1}, treatment with H_2O_2 for 10 min stimulated the expression of phospho-p42/p44 and phospho-p38 CCDPK in a concentration-dependent manner (Fig 3). Treatment with U0126 5 μmol·L^{-1} or SB203580 11 μmol·L^{-1} completely blocked H_2O_2-induced p42/p44 or p38 CCDPK activation, respectively. Interestingly, blockade of p38 CCDPK activation with SB203580 enhanced H_2O_2-

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Tab 1. Effect of H_2O_2, U0126 5 $\mu mol \cdot L^{-1}$, and SB203580 10 $\mu mol \cdot L^{-1}$ on cell viability and apoptosis in BAEC. $n = 4$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ vs control. $^dP > 0.05$, $^fP < 0.01$ vs H_2O_2 300 $\mu mol \cdot L^{-1}$.

H_2O_2/ $\mu mol \cdot L^{-1}$	MTT absorbance/ 570 nm	Apoptotic BAEC/ total BAEC
0	0.54 ± 0.02	3.9 ± 1.4
100	0.50 ± 0.03^b	9.0 ± 2.9^b
200	0.45 ± 0.03^c	20.3 ± 2.9^c
300	0.38 ± 0.03^c	31.2 ± 4.8^c
400	0.29 ± 0.02^c	42.8 ± 2.5^c
500	0.23 ± 0.03^c	58.7 ± 6.3^c
300 + U 0126	0.28 ± 0.02^f	44.5 ± 3.5^f
300 + SB203580	0.38 ± 0.03^d	30.5 ± 3.7^d

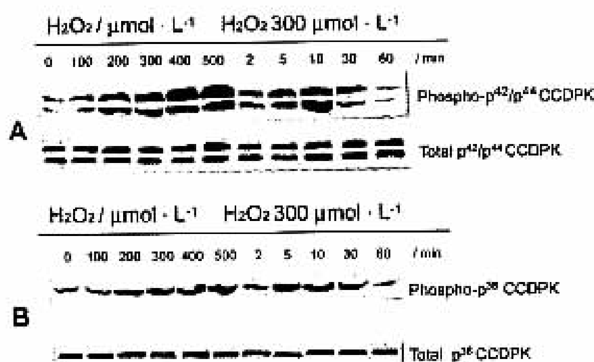

Fig 3. H_2O_2-induced expression of phospho-p42/p44 and phospho-p38 CCDPK in BAEC in a concentration-dependent manner and in a similar time course by Western blot.

induced phospho-p42/p44 CCDPK expression (Fig 4). In parallel experiments, the amount of total p42/p44 or p38 CCDPK was determined in the same cell extracts with an antibody that recognized all p42/p44 or p38 CCDPK independent of their phosphorylation state.

DISCUSSION

Oxidative damage to vascular endothelium is an important cause of many vascular diseases including atherosclerosis[11]. Despite the importance of oxyradicals in the pathophysiology of the vascular endothelium, the mechanisms regulating the oxidative response of endothelial cells are poorly understood. The present study demonstrated that H_2O_2 induced BAEC apoptosis, at the same time, H_2O_2 activated both p42/p44 and p38 CCDPK in a concentration-dependent manner. In the

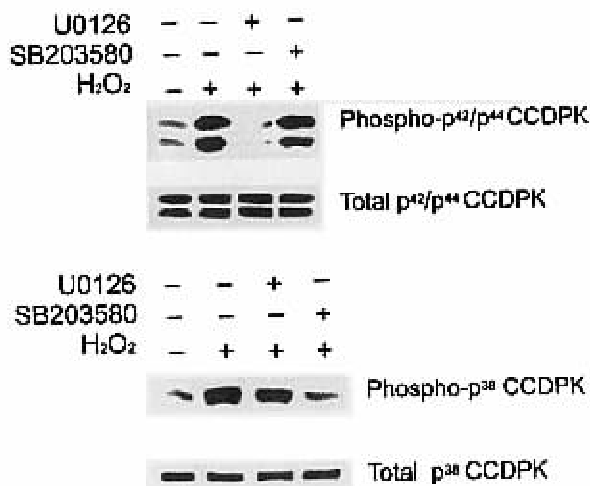

Fig 4. Effect of U0126 (5 $\mu mol \cdot L^{-1}$) and SB203580 (10 $\mu mol \cdot L^{-1}$) on H_2O_2 (300 $\mu mol \cdot L^{-1}$)-induced expression of phospho-p42/p44 and phospho-p38 CCDPK in BAEC by Western blot.

general conception, p42/p44 CCDPK serves as a survival signaling pathway and its activity is suppressed in conditions of stress leading to apoptosis, while p38 CCDPK and SAPK/JNK are activated by stress stimuli and serve as a death signal. However, recently, the attention has been drawn on researching the balance between p42/p44 and p38 CCDPK or SAPK/JNK signaling pathways in determining the fate of a cell. Several reports are available demonstrating the coexpression of different CCDPK family members in response to a common stress stimulus[12-14]. In our recent work, TNF-α simultaneously activated p42/p44 and p38 CCDPK signaling pathway, and these two cascades played opposing roles in modulating TNF-α-induced apoptosis in BAEC (to be published). Our present data showed that p42/p44 CCDPK inhibitor U0126 completely blocked H_2O_2-stimulated activation of p42/p44 CCDPK, significantly enhanced H_2O_2-induced apoptosis, suggesting a relationship between p42/p44 CCDPK activation and cell survival after oxidant injury, which was consistent with our recent results for TNF-α as well as the recent findings of others[12,13,15]. However, contrast results were reported in recent studies showing that p42/p44 CCDPK was associated with H_2O_2-induced apoptosis[16,17]. In this study, p38 CCDPK inhibitor SB203580 completely blocked the activation of p38 CCDPK, but unexpectedly, had no effect on cell survival in response to oxidative stress, suggesting that H_2O_2-induced apoptosis may be independent of p38 CCDPK signaling. Compared to our recent finding that p38 CCDPK mediated TNF-α-induced apoptosis in

BAEC, it is worth noting that there is considerable variation in the roles of CCDPK family members in regulating cell apoptosis in response to different stimuli. The fact that the inhibition of p38 CCDPK activation by SB203580 was concomitant with the upregulation of the expression of phospho-p42/p44 CCDPK suggests a dynamic balance between these two kinase cascades, but further work is needed to quantitatively identify the relationship between this dynamic balance and its influence on cells. In the present study, the simple inhibition of p42/p44 CCDPK with U0126 triggered apoptosis in BAEC, which was consistent with the previous observation in Hela cells[18]. In conclusion, we proposed that H_2O_2 simultaneously activated p38 CCDPK and p42/p44 CCDPK, and p42/p44 CCDPK acted as a survival factor in H_2O_2-induced apoptosis in BAEC, while p38 CCDPK had no effect on regulating apoptosis in response to oxidative injury.

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p38和p42/p44 Ca^{2+}-钙调蛋白依赖性蛋白激酶信号在过氧化氢诱导牛主动脉内皮细胞凋亡中的作用

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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；过氧化氢；蛋白质印迹；血管内皮；培养的细胞

目的：研究p38和p42/p44 Ca^{2+}-钙调蛋白依赖性蛋白激酶(CCDPK)信号通路对过氧化氢(H_2O_2)诱导牛主动脉内皮细胞(BAEC)凋亡的调节作用．**方法**：H_2O_2处理BAEC 24 h后，荧光显微镜下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，蛋白质印迹法检测磷酸化p38和p42/p44 CCDPK表达．**结果**：H_2O_2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．H_2O_2($100-500\ \mu mol \cdot L^{-1}$)浓度依赖性刺激磷酸化p42/p44和p38 CCDPK的表达．p42/p44 CCDPK抑制剂U0126显著增强H_2O_2致凋亡作用；然而p38 CCDPK抑制剂SB203580可增强H_2O_2诱导的磷酸化p42/p44 CCDPK的表达，但不影响BAEC的存活．**结论**：p42/p44 CCDPK对H_2O_2诱导的BAEC凋亡起保护作用，而p38 CCDPK不是介导H_2O_2所致细胞凋亡的主要信号通路．

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