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High glucose enhances H_2O_2-induced apoptosis in bovine aortic endothelial cells

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

ABSTRACT

AIM: To investigate the effect of high glucose on hydroperoxide (H_2O_2)-induced apoptosis in cultured bovine aortic endothelial cells (BAEC). **METHODS**: BAEC were cultured and passaged in normal glucose (5.5 mmol·L^{-1}, NG) and high glucose (25 mmol·L^{-1}, HG). Morphologic changes and quantification of apoptotic cells were determined under fluorescence microscope after H_2O_2-treated BAEC for 24 h with Hoechst 33258 staining. DNA fragmentation was visualized by agarose gel electrophoresis. The expression of phospho-p38 Ca^{2+}-calmodulin dependent protein kinase (CCDPK, formerly called MAPK) was measured by Western blotting. **RESULTS**: H_2O_2 elicited typical apoptotic morphologic changes (chromatic condensation, nucleus fragmentation). At 100 - 300 μmol·L^{-1}, both NG- and HG-BAEC incubated with H_2O_2 for 24 h increased cell apoptosis and phospho-p38 CCDPK expression in a concentration-dependent manner. In HG-BAEC, H_2O_2 induced DNA fragmentation at a lower concentration than that in NG-BAEC, and the apoptotic cell count in HG-BAEC was also higher than that of NG-BAEC ($P < 0.05$). Similarly, the expression of phospho-p38 CCDPK induced by H_2O_2 was up-regulated in HG-BAEC ($P < 0.05$). **CONCLUSION**: High glucose enhances H_2O_2-induced apoptosis in BAEC, which is related to high expression of phospho-p38 CCDPK.

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INTRODUCTION

Diabetes mellitus is associated with an increased prevalence of atherosclerotic disease[1]. Endothelium impairment has been thought to be the initial step in atherogenesis[2]. Increased oxidative stress due to raised free radical production and insufficient antioxidant potential have been postulated to play an important role in the development of diabetic vascular complications including atherosclerosis, which greatly raises the possibility that vascular endothelium is continuously exposed to oxidative damage[3]. Clinical trials have concluded that hyperglycemia is a primary cause of diabetic vascular complications[1]. There is also a clear link between hyperglycemia and increased oxidative stress[3]. It is rational to speculate that hyperglycemia is partially responsible for impaired vascular endothelium. Previous studies have demonstrated that high glucose could reduce endothelium-dependent relaxation[4] and delay endothelial cell proliferation[5]. However, up to date, few reports are available whether high glucose can enhance the toxic sensitivity to oxidative stress in endothelial cells.

The Ca^{2+}-calmodulin dependent protein kinases (CCDPK) are a family of serine/threonine protein kinases thought to mediate intracellular signal transduction[6]. Among them, the p38 CCDPK is activated by proinflammatory cytokines, heat shock, endotoxin, and other cellular stresses and has been thought to play an important role in cellular apoptosis[7]. CCDPK activity could be detected by Western blotting using phospho-specific anti-CCDPK monoclonal antibody, and best of all, radioactivity is totally eliminated from the assay.

Hydroperoxide (H_2O_2), an important free radical, has been demonstrated to be toxic to endothelial cells in previous study[8]. In the present study, we aimed to

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investigate the effect of high glucose on H_2O_2-induced apoptosis in cultured bovine endothelial cells and to study its possible molecular mechanism via examining the expression of phospho-p38 CCDPK in above event.

MATERIALS AND METHODS

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[9] and cultured in M199 medium with 20 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 kU·L^{-1}, and streptomycin 100 mg·L^{-1}. After confluence, BAEC were randomly assigned to two groups and subcultured in two different conditions at a ratio of 1:2 with 0.1 % trypsin: normal glucose (5.5 mmol·L^{-1}, NG-BAEC) and high glucose (25 mmol·L^{-1}, HG-BAEC). Cells were characterized as BAEC by morphologic appearance of "cobble stone" and by immunohistochemical staining with monoclonal antibody to factor Ⅷ-related antigen. Experiments were performed with cells from passage 4 – 10.

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[10]. Briefly, cells from different treatment 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 with a 50 % solution of fixative (3:1 methanol/acetic acid) for 5 min. And then fixed with neat fixative for 10 min. The preparations were stained with Hoechst 33258 (5 mg·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 at each concentration of H_2O_2-treated NG- and HG-BAEC, with a minimum number of 500 cells scored in each condition.

DNA electrophoresis At the end of each incubation, DNA fragments were selectively extracted. Cells were harvested and incubated with a hypo-osmotic solution (Tris-HCl 5 mmol·L^{-1}, pH 8.0, edetic acid 5 mmol·L^{-1}, 1 % Triton X-100) at 4 ℃ for 30 min. After centrifugation at 12 000 × g for 15 min, the supernatant was incubated with 1/10 volume of SDS and proteinase K 100 mg·L^{-1} at 55 ℃ for 16 h, then the lysates were gently extracted three times with an equal volume of phenol followed by chloroform. After centrifugation, the upper layer containing DNA was transferred to a new tube, and 0.5 volume of NaCl 5 mol·L^{-1} and 2.5 volumes of ethanol were added. The tube was kept overnight at −20 ℃. After centrifugation at 12 000 × g for 15 min, the resulting DNA pellet was dissolved in 20 μL of Tris-HCl 10 mmol·L^{-1}, pH 8.0, and edetic acid 10 mmol·L^{-1}, and DNAase-free RNase 100 mg·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, cells were seeded onto 12-well culture plates with NG- or HG-M199 containing 20 % FBS. After confluence, the two culture mediums were changed to the NG-M199 containing 10 % FBS, and various concentrations of H_2O_2 were added. The treating time was chosen to be 30 min, since a peak expression of phospho-p38 CCDPK was obtained at this time point in our experimental conditions. After being washed with ice-cold PBS for three times, cells were lysed with 30 μL of ice-cold lysis buffer containing (mmol·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[11].

Western blot A 1/4 volume of SDS sample buffer containing Tris-HCl 0.33 mol·L^{-1}, 10 % SDS (w/v), 40 % glycerol (v/v), and 0.4 % bromophenol blue was 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 for 1 h at 25 ℃ with 5 % BSA in PBST (Na_2HPO_4 80, NaH_2PO_4 20, and NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated at 25 ℃ with the primary antibodies against phospho-p38 CCDPK at a 1:10 000 dilution for 1 h with secondary antibody (horseradish peroxidase

conjugated) at a 1:1000 dilution. Immunoreactive signals were visualized by the Phototope Western Detection System. Bands of phospho-p38 CCDPK were quantitatively determined by thin-layer chromatography with Shimadza Dual-Wavelength Chromato-Scanner (Japan, Model CS-930).

Reagents BSA, M199 medium, and Hoechst 33258 were purchased from Sigma Chemical Co. Phospho-p38 CCDPK monoclonal antibody, HRP-conjugated anti-rabbit secondary antibody, and Phototope-HRP Western Detection kit were purchased from New England Biolabs Inc.

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

Morphologic changes After exposure to H_2O_2 200 μmol for 24 h, BAEC showed typical morphologic changes of apoptosis. The cell volume was reduced, the chromatin became condensed and nucleus fragmented (Fig 1).

DNA electrophoresis Incubation of NG- and HG-BAEC with H_2O_2 for 24 h elicited a characteristic "ladder" of DNA fragments representing integer multiples of the internucleosomal DNA length (about 180 – 200 base pair). H_2O_2 100 μmol·L^{-1} elicited a "DNA ladder" in HG-BAEC, while in NG-BAEC, DNA "ladder" could not be observed until incubation with H_2O_2 at a concentration up to 200 μmol·L^{-1}. These changes were furthered at H_2O_2 300 μmol·L^{-1} in both NG- and HG-BAEC (Fig 2).

Quantification of apoptosis At concentrations of 100, 200, 300 μmol·L^{-1}, incubation with H_2O_2 for 24 h induced BAEC apoptosis in a concentration-dependent manner, and a greater rate of cell apoptosis was measured in HG-BAEC, compared with NG-BAEC (Tab 1).

Phospho-p38 CCDPK expression H_2O_2 100, 200, 300 μmol·L^{-1} stimulated the expression of phospho-p38 CCDPK in a concentration-dependent manner both in NG- and HG-BAEC. A higher expression of phospho-p38 CCDPK was measured in HG-BAEC at equivalent concentrations of H_2O_2, when compared with NG-BAEC (Fig 3, Tab 2).

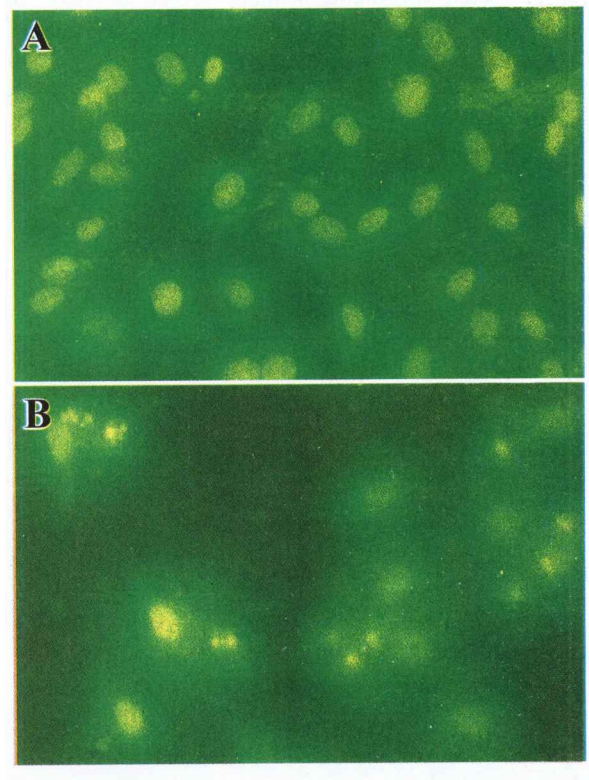

Fig 1. Fluorescence photomicrograph of BAEC stained with Hoechst 33258. × 200. A) Nuclei of control BAEC were stained uniformly. B) H_2O_2-treated BAEC showing apoptotic nuclei (condensed or fragmented).

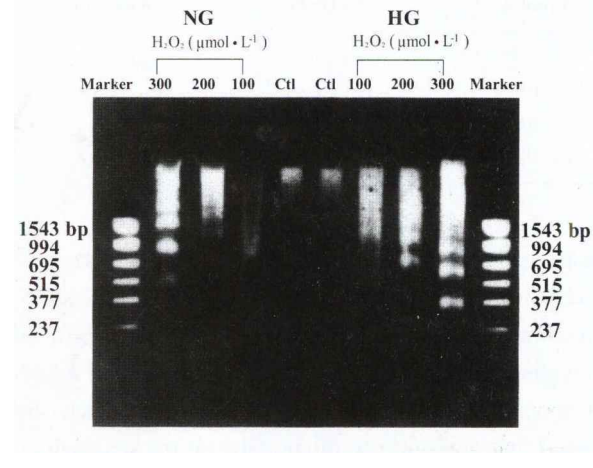

Fig 2. Agarose gel electrophoresis of H_2O_2-induced DNA fragmentation in NG- and HG-BAEC.

DISCUSSION

It is well known that most patients with diabetes, died from macrovascular complications, were closely

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Tab 1. Concentration-dependent effect of H_2O_2 on apoptosis in NG- and HG-BAEC. $n = 4$ experiments. $x \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* NG-BAEC.

H_2O_2/ $\mu mol \cdot L^{-1}$	Apoptotic BAEC/% of attached BAEC NG	HG
0	3.5 ± 0.6	4.0 ± 0.8[a]
100	5.0 ± 1.4	12.5 ± 3.1[b]
200	17.8 ± 4.3	25.8 ± 4.1[b]
300	34.3 ± 5.1	43.3 ± 3.9[b]

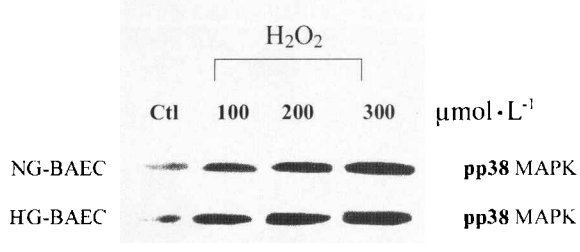

Fig 3. Effect of H_2O_2 on the expression of phospho-p38 CCDPK proteins in NG- and HG-BAEC by Western blot.

Tab 2. Quantification of effect of H_2O_2 on expression of phospho-p38 CCDPK in NG- and HG- BAEC. $n = 4$ experiments. $x \pm s$. Average of duplicates constitutes one determination. $^aP > 0.05$, $^bP < 0.05$ *vs* NG-BAEC.

H_2O_2/ $\mu mol \cdot L^{-1}$	10^{-3} × Absolute peak area/mm^2 NG-BAEC	HG-BAEC
0	39 ± 3	46 ± 5[a]
100	122 ± 14	193 ± 23[b]
200	205 ± 30	279 ± 20[b]
300	274 ± 29	364 ± 26[b]

associated with atherosclerosis[12], but its underlying mechanisms are still unknown. In the present study, we demonstrated for the first time that bovine aortic endothelial cells cultured in high glucose exhibited a higher apoptotic rate when treated with H_2O_2, which suggested that high glucose might enhance the sensitivity of endothelial cells to oxidative damage. This may partially account for the mechanisms underlying the increased prevalence of atherosclerosis disease in diabetes.

Endothelial injury and loss of normal endothelial function is a key event for the development of atherosclerosis[2]. Prospective randomized long-term clinical studies have demonstrated that a clear link between diabetic hyperglycemia and endothelial dysfunction[13]. However, the defined mechanism through which excess glucose results in endothelium damage has not been well elucidated. There is a substantial evidence that vasodilatation mediated by endothelium-derived nitric oxide is impaired in animal models of diabetes[14] and in patients with diabetes mellitus[15]. *In vivo* studies have demonstrated that prolonged exposure to elevated glucose reduced endothelial dependent relaxation in normal blood vessels[1] and delayed cell replication in cultured endothelial cells[5], which was also observed in our recent study. Furthermore, these adverse effects of high glucose on endothelial functions could be reversed by antioxidant. Hyperglycemia has been demonstrated to increase the production of free radicals through glucose oxidation, polyol pathway, prostanoid synthesis, and protein glycation, and increased oxidative damage was considered to accelerate the development of diabetic vascular complications[3]. Recently, high glucose has been shown to stimulate the generation of O_2^- in cultured human aortic endothelial cells[16]. In the present study, prolonged exposure of BAEC to high glucose exhibited a higher rate of cell apoptosis induced by H_2O_2, which further indicated that high glucose could enhance the oxidative injury to blood vessels due to increased toxic sensitivity to free radicals. Consistent with our results, Kashiwagi observed that exposure to high glucose increased the release of lactic dehydrogenase of human endothelial cells into the culture medium when treated with H_2O_2, and abnormal glutathione metabolism was considered to be responsible for increased toxicity of H_2O_2 to endothelial cells[17].

p38 CCDPK cascade is activated by many cellular stresses, including oxidative stress, and has been considered to transduce growth inhibitory or apoptotic signals in various cell types[18]. The fact that the expression of phospho-p38 CCDPK induced by H_2O_2 was upregulated in BAEC cultured in high glucose which was positively related to the higher apoptotic rate in HG-BAEC, suggested that enhanced activity of p38 CCDPK might be responsible for the increased toxic sensitivity of BAEC to H_2O_2.

In conclusion, our study provides new data in approval of the hypothesis that increased oxidative damage induced by hyperglycemia may partially account for the development of diabetic vascular diseases.

REFERENCES

1 The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993; 329: 977 - 86.
2 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. Nature 1993; 362: 801 - 9.
3 Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care 1996; 19: 257 - 67.
4 Tesfamariam B, Brown ML Cohen RA. Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. J Clin Invest 1991; 87: 1643 - 8.
5 Curcio F, Ceriello A. Decreased cultured endothelial cell proliferation in high glucose medium is reversed by antioxidants: new insights on the pathophysiological mechanisms of diabetic vascular complications. In Vitro Cell Dev Biol 1992; 28A: 787 - 90.
6 Seger R, Krebs EG. The CCDPK signaling cascade. FASEB J 1995; 9: 726 - 35.
7 Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. J Biol Chem 1997; 272: 20490 - 4.
8 Hermann C, Zeiher AM, Dimmeler S. Shear stress inhibits H_2O_2-induced apoptosis of human endothelial cells by modulation of the glutathione redox cycle and nitric oxide synthase. Arterioscler Thromb Vasc Biol 1997; 17: 3588 - 92.
9 Su Z, Ling Q, Guo ZG. Effects of lysophosphatidylcholine on bovine aortic endothelial cells in culture. Cardioscience 1995; 6: 31 - 7.
10 Bates RC, Buret A, van Helden DF, Horton MA, Burns GF. Apoptosis induced by inhibition of intercellular contact. J Cell Biol 1994; 125: 403 - 15.
11 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 - 54.
12 Pyorala K, Laakso M, Uusitupa M. Diabetes and atheroslcerosis: an epidemiologic view. Diabetes Metab Rev 1987; 3: 463 - 524.
13 Haller H, Drab M, Luft FC. The role of hyperglycemia and hyperinsulinemia in the pathogenesis of diabetic angiopathy. Clin Nephrol 1996; 46: 246 - 55.
14 Taylor PD, Oon BB, Thomas CR, Poston L. Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats. Br J Pharmacol 1994; 111: 35 - 41.
15 Elliott TG, Cockroft JR, Groop PH, Viberti GC, Ritter JM. Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. Clin Sci 1993; 85: 687 - 93.
16 Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aoritc endothelial cells. Circulation 1997; 96: 25 - 8.
17 Force T, Bonventre JV. Growth factors and mitogen-activated protein kinases. Hypertension 1998; 31: 152 - 61.
18 Kashiwagi A, Asahina T, Ikebuchi M, Tanaka Y, Takagi Y, Nishio Y, *et al*. Abnormal glutathione metabolism and increased cytotoxicity caused by H_2O_2 in human umbilical vein endothelial cells cultured in high glucose medium. Diabetologia 1994; 37: 264 - 9.

高糖增强过氧化氢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；过氧化氢；葡萄糖；蛋白质印迹；血管内皮；培养的细胞

目的：研究高糖对过氧化氢（H_2O_2）诱导牛主动脉内皮细胞（BAEC）凋亡作用。**方法**：BAEC 培养并传代于正常葡萄糖（5.5 mmol·L^{-1}）和高糖（25 mmol·L^{-1}）中，经 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；琼脂糖凝胶电泳分析 DNA 降解，Western blot 法检测磷酸化 p38 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）。在 100 - 300 μmol·L^{-1} 范围内，正常糖和高糖 BAEC 经 H_2O_2 处理后，浓度依赖性诱导细胞凋亡和磷酸化 p38 CCDPK 表达。高糖条件下诱导 BAEC DNA 降解浓度低于正常糖 BAEC，细胞凋亡率和磷酸化 p38 CCDPK 表达均显著高于正常糖组（$P < 0.05$）。**结论**：高糖促进 H_2O_2 诱导 BAEC 凋亡，可能与其增强磷酸化 p38 CCDPK 的表达相关。

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