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High glucose enhances mitogenic response to endothelin-1 in rabbit vascular smooth muscle cells

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KEY WORDS glucose; endothelin-1; Ca^{2+}-calmodulin dependent protein kinase; Western blotting; thoracic aorta; cultured cells; vascular smooth muscle

ABSTRACT

AIM: To examine the effects of high glucose on the mitogenic response of rabbit aortic vascular smooth muscle cells (VSMC) to endothelin-1 (ET-1). **METHODS**: VSMC were cultured in normal glucose (5.5 mmol·L^{-1}), high glucose (25 mmol·L^{-1}) or high osmolality (glucose 5.5 mmol·L^{-1}, plus mannitol 19.5 mmol·L^{-1}). DNA synthesis was measured by [^{3}H]thymidine incorporation. The expression of phospho-p44/42 MAPK was determined by Western blot. **RESULTS**: At a concentration range from 10^{-12} to 10^{-8} mol·L^{-1}, ET-1 stimulated [^{3}H]thymidine incorporation and phospho-p44/42 MAPK expression in VSMC in a concentration-dependent manner. From 10^{-11} to 10^{-8} mol·L^{-1}, the mitogenic effect of ET-1 was higher in VSMC cultured in high glucose at equivalent concentration than cells cultured in normal glucose or high osmolality ($P < 0.05$ or $P < 0.01$), but no marked difference was observed in the growth response between cells cultured under the latter two conditions. Similarly, ET-1 increased expression of phospho-p44/42 MAPK by 60 % - 65 % in VSMC cultured in high glucose, compared with cells in normal glucose or high osmolality. **CONCLUSION**: VSMC cultured in high glucose exhibited increased mitogenic response to ET-1, which seemed to be related to the enhanced expression of phospho-p44/42 MAPK.

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INTRODUCTION

Diabetes mellitus is associated with an increased prevalence of atherosclerotic disease[1]. Excessive vascular smooth muscle cell (VSMC) proliferation is considered to be a key feature in the development of diabetes-associated atherosclerosis[2]. However, the mechanism underlying this pathologic process is poorly understood at present. Hyperglycemia in diabetes has been suggested to be a contributing factor to its vascular complications, partially due to its mitogenic effects on VSMC[3]. Elevated circulating endothelin-1 (ET-1) levels were observed in patients with non-insulin-dependent diabetes mellitus (NIDDM)[4]. Being an endothelium-derived vasoactive peptide with mitogen properties, it is rational to speculate that elevated ET-1 levels might play a role in the pathogenesis of vascular disorders in diabetes mellitus. Up to date, no reports have been available in studying the effects of glucose on growth response of VSMC to ET-1.

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine protein kinases thought to play a central role in cell proliferation and differentiation. Two isoforms of MAPK, the p42 MAPK (Erk2) and p44 MAPK (Erk1) are expressed in most cell types and activated by many extracellular growth stimuli through phosphorylation of its threonine and tyrosine (Thr202/Tyr204)[5]. MAPK activity could be detected by Western blotting using phosphospecific anti-MAPK monoclonal antibody, and best of all, radioactivity is totally eliminated from the assay.

The aim of this study was to study the effect of high glucose on growth response to ET-1 in VSMC cultured in high glucose conditions mimicking diabetic hyperglycemia, and to study its possible molecular mechanism via examining the expression of phospho-p44/42 MAPK in above event.

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MATERIALS AND METHODS

Reagents ET-1 and medium 199 were purchased from Sigma Chemical Co. Phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody, HRP-conjugated anti-rabbit secondary antibody, Phototope-HRP Western Detection kit were purchased from New England Biolabs Inc. *D*-glucose was the product of the Chemical Reagent Factory of Hu-nan Normal University. [^{3}H]Thymidine was from China Institute of Atomic Energy, Beijing.

Cell culture VSMC were isolated by outgrowth from explants of thoracic aorta of New Zealand rabbit (1.5 – 2 kg), (supplied by the Animal Center of the Second Affiliated Hospital of Hu-nan Medical University), and cultured in M199 medium with 10% heat-inactivated fetal bovine serum (FBS), benzyl-penicillin 100 kU · L^{-1}, and streptomycin 100 mg · L^{-1}. After confluence, VSMC were randomly assigned to three different groups and subcultured in three different conditions at a ratio of 1:3 with 0.1 % trypsin; normal glucose (5.5 mmol · L^{-1}, NG-VSMC), high osmolality (glucose 5.5 mmol · L^{-1} + mannitol 19.5 mmol · L^{-1}, Mann-VSMC), high glucose (glucose 25 mmol · L^{-1}, HG-VSMC). Cells were characterized as VSMC by morphologic appearance of "valley and hill" and by immunohistochemical staining with monoclonal antibody to α-actin. Experiments were performed with these three different strains of VSMC from passage 4 – 10.

DNA synthesis DNA synthesis in VSMC was evaluated by incorporation of [^{3}H]thymidine. After 80 % confluence, VSMC cultured in 24-well culture plates with differential M199 (high-glucose, high-osmolality, and normal-glucose conditions) were made quiescent for 24 h in M199 with 0.4 % FBS, then were stimulated with various concentrations of ET-1 for another 24 h, [^{3}H]thymidine 37 kBq · L^{-1} was added during the last 6 h. Cells were washed 3 times with cold phosphate buffered saline (PBS), detached with 0.1 % trypsin and 0.02 % edetic acid, then collected on GF/C filters, fixed with 10 % trichloroacetic acid (TCA). The TCA-precipitable material was dissolved in NaOH 0.2 mol · L^{-1}. Filter was dried and radioactivity was measured by a liquid scintillation counter (Beckman LS 3801, USA).

Preparation of lysates For MAPK detection, cells were seeded onto 12-well culture plates with NG-, Mann- or HG- M199 containing 10 % FBS. After 80 % confluence, cells were cultured under the low serum (0.4 % FBS) condition for 24 h, then all the culture mediums were changed to the NG-M199 without serum, and various concentrations of ET-1 were added. The treating time was chosen to be 10 min, since a peak expression of phospho-p44/42 MAPK was obtained at this time point in our experimental conditions. After being washed with ice-cold PBS for three times, cells were lysed with 60 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 · L^{-1} 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[b].

Western blot SDS sample buffer containing Tris-HCl 0.33 mol · L^{-1}, SDS 10 % (wt/vol), glycerol 40 % (vol/vol), and dithiothreitol 20 % (vol/vol) containing bromophenol blue 0.4 % of 1/4 volume were added to cell lysates. After being boiled for 5 min, the extracted protein 10 μg was electrophoresed on 10 % SDS-polyacrylamide gel (SDS-PAGE). Then the protein were transfered to nitrocellulose membrane, which was then blocked for 1 h at room temperature 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-p44/42 MAPK at a 1:10 000 dilution for 1 h, followed by incubation 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-p44/42 MAPK were quantitatively determined by thin-layer chromatography with Shimadzu Dual-Wavelength Chromato-Scanner (Japan, Model CS-930).

Statistical analysis Values were expressed as $\bar{x} \pm s$, and assessed by one-way ANOVA and Newman-Keuls test.

RESULTS

DNA synthesis At concentrations of 10^{-12} to

10^{-8} mol · L^{-1}, ET-I stimulated [^{3}H]thymidine incorporation into DNA in a concentration-dependent manner. A greater rate of DNA synthesis was observed in VSMC cultured in high glucose, compared with NG-or mann-VSMC. No significant difference was observed in [^{3}H]thymidine incorporation between cells cultured in normal glucose and high osmolality (Fig 1).

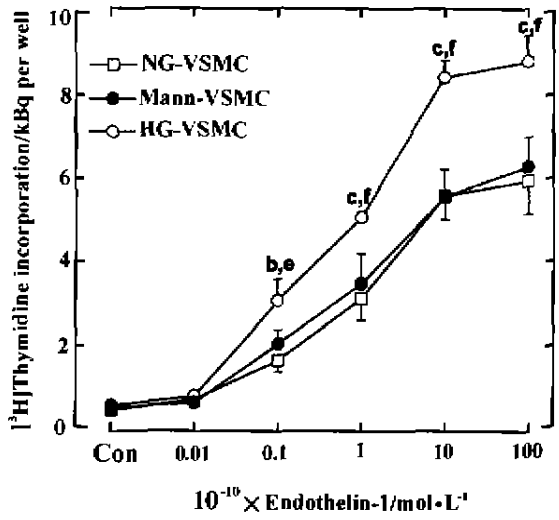

Fig 1. [^{3}H]Thymidine incorporation in NG-, Mann-, or HG-VSMC stimulated by ET-1. n = 4 experiments. Average of triplicates contributes one determination. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* NG-VSMC. $^eP < 0.05$, $^fP < 0.01$ *vs* Mann-VSMC.

MAPK expression ET-I, at a concentration range of 10^{-12} to 10^{-8} mol · L^{-1}, stimulated the expression of phospho-p44/42 MAPK in a concentration-dependent manner in NG-, HG-, and Mann-VSMC. The maximal effect was observed at a concentration of 10^{-9} mol·L^{-1}. A higher expression of phospho-p44/42 MAPK was observed in HG-VSMC at equivalent concentrations of ET-1, compared with cells in NG- or Mann-VSMC, but no difference was examined in the expression of phospho-p44/42 MAPK between cells in NG- and Mann-VSMC (Tab 1, Fig 2).

Tab 1. Effect of ET-1 on expression of phospho-p44/42 MAPK in NG-, HG-, and Mann-VSMC. n = 5 experiments. Average of duplicates constitutes one determination. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* NG-VSMC.
$^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* Mann-VSMC.

10^{-10} × Endothelin-1/mol·L^{-1}	10^{-3} × Absolute peak area/mm^2		
	NG-VSMC	HG-VSMC	Mann-VSMC
Control	22 ± 3	23 ± 2^{ad}	24 ± 4^a
0.01	55 ± 6	68 ± 4^{be}	57 ± 7^a
0.1	81 ± 7	211 ± 21^{cf}	79 ± 7^a
1	113 ± 18	288 ± 26^{cf}	116 ± 16^a
10	154 ± 29	416 ± 23^{cf}	159 ± 15^a
100	147 ± 14	414 ± 17^{cf}	145 ± 11^a

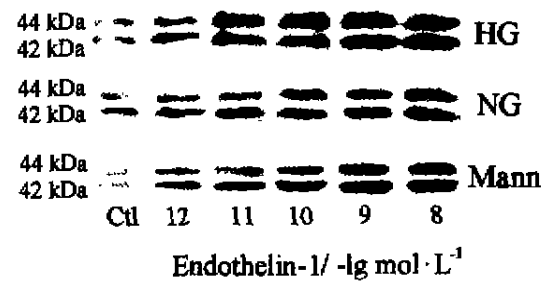

Fig 2. Effect of ET-1 on expression of HG-VSMC, NG-VSMC, and Mann-VSMC phospho-p44/42 MAPK proteins by Western blot.

DISCUSSION

The mitogenic response of VSMC to ET-1 was increased when the cells were cultured under a high glucose condition. In order to more closely simulate chronic hyperglycemia, VSMC were subcultured in high glucose for at least 4 passages rather than high glucose added acutely. The increased growth responsiveness in high glucose was not due to changes in osmolality since cells cultured in high mannitol grew as fast as those in normal glucose. These results suggested that hyperglycemia might be directly linked to accelerated vascular complications in diabetes mellitus by increasing the growth response of VSMC. Similar results were obtained in the previous study on porcine aortic VSMC treated with FCS[7].

Recently, Muniyappa *et al*[8] reported that interleukin-1-induced NO release and NO synthase expression were inhibited by high glucose in VSMC. Since NO has been known to inhibit VSMC tone, migration and proliferation, inhibition of NO was postulated to be one of the potential mechanisms for the growth-stimulating effect of high glucose. Recent

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evidence indicated that p44/42 MAPK were activated by many stimuli involved in cell growth and known as the common pathway to transmit extracellular signals into the nucleus regulating cell proliferation[9]. In the present study, we found that expression of phospho-p44/42 MAPK induced by ET-1 was higher in VSMC cultured in high glucose than that in normal glucose or in high osmolality, and it might be one of molecular mechanisms underlying the enhanced growth response in high glucose.

ET-1 is an endothelium-derived vasoactive peptide with mitogen properties and a potent vasoconstrictor. Elevated plasma ET-1 levels had been reported in patients with diabetes mellitus, which suggested to constitute one of the mechanisms involved in diabetic hypertension[10]. In our study, ET-1 stimulated DNA synthesis in VSMC in a concentration-dependent manner. Under diabetic conditions, elevated ET-1 levels, together with increased mitogenic response of VSMC, suggested an important role in excessive VSMC proliferation and could contribute one of the mechanisms involved in the high prevalence of atherosclerosis in diabetic patients.

In conclusion, the mitogenic response to ET-1 was increased in VSMC cultured in high glucose, which was not due to high osmolality. Enhanced expression of phospho-p44/42 MAPK by high glucose might be responsible for the changes in growth response.

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高糖增强兔血管平滑肌细胞对内皮素-1的增殖反应性

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关键词 葡萄糖; 内皮素-1; Ca^{2+}-钙调素依赖的蛋白激酶; 蛋白质印迹; 胸主动脉; 培养的细胞; 血管平滑肌

目的: 观察高糖对内皮素-1(ET-1)促兔主动脉血管平滑肌细胞(VSMC)增殖的影响. **方法**: VSMC 分别培养于含正常葡萄糖、高糖或高渗(5.5, 25, 葡萄糖 5.5 + 甘露醇 19.5 mmol·L^{-1}) 的培养基中. [^{3}H]胸腺嘧啶掺入法检测 DNA 合成速率, 蛋白质印迹法检测磷酸化 p44/42 MAPK 的表达. **结果**: 在 10^{-12}至 10^{-8} mol·L^{-1}浓度范围内, ET-1 以浓度依赖方式增加 VSMC 的[^{3}H]胸腺嘧啶掺入及磷酸化 p44/42 MAPK 的表达. 从 10^{-11}到 10^{-8} mol·L^{-1}, 培养于高糖的 VSMC 对相同浓度 ET-1 的增殖反应性高于正常糖或高渗培养条件下的 VSMC ($P < 0.05$, 或 $P < 0.01$), 而在后两种条件下, VSMC 对 ET-1 的增殖反应无显著差别. 同样, 在高糖条件下, ET-1 诱导的 VSMC 磷酸化 p44/42 MAPK 的表达较正常糖和高渗 VSMC 增加 60 % – 65 %. **结论**: 高糖增强 VSMC 对 ET-1 的增殖反应性, 可能与磷酸化的 p44/42 MAPK 高表达有关.

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