Enhanced secretion of endothelin-1 by elevated glucose levels from cultured bovine aortic endothelial cells

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We have investigated the effect of glucose on the release of endothelin-1-like immunoreactivity (ET-1-LI) from cultured bovine aortic endothelial cells. Elevation of glucose concentrations in cultured media from 5.5 to 11.1 or 22.2 mM significantly stimulated ET-1-LI release from cultured endothelial cells. An aldose reductase inhibitor did not affect the high glucose-induced ET-1-LI release. These findings suggest the possibility that hyperglycemia in diabetic patients enhances ET-1-LI release at the local site of vascular endothelium, which might be involved in the developments of vascular complications and atherosclerosis.

Endothelin, Endothelial cell, Glucose, Diabetes mellitus

1. INTRODUCTION

Hyperglycemia is generally accepted as the major cause of vascular complications in diabetic patients. Toxic effects of excess glucose including the hyperosmotic action, nonenzymatic glycosylation of various proteins, or increase in an intracellular sorbitol concentration by activating aldose reductase have been thought to be responsible for alteration of the functions of vascular endothelial cells and the subsequent development of atherosclerosis, although the precise mechanism is still unknown. Endothelin (ET), which is a peptide hormone with 21 amino acid residues recently isolated from the culture medium of porcine aortic endothelial cells [1], exerts a wide spectrum of effects on vasculature such as a potent vasoconstriction [1] and mitogenesis [2,3], suggesting a possible involvement of ET in the pathogenesis of atherosclerosis. In the present study, to evaluate the possibility that moderately elevated levels of blood glucose seen commonly in diabetic patients can affect the metabolism of ET, we have examined the effects of the elevated concentrations of glucose on the secretion of ET-1-like immunoreactivity (ET-1-LI) from cultured bovine aortic endothelial cells.

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Abbreviations: ET, endothelin; ET-1-LI, endothelin-1-like immunoreactivity

2. MATERIALS AND METHODS

2.1. *Cell culture*

Endothelial cells were scraped off from the intima of bovine thoracic aorta, subcultured and characterized as described previously [4,5]. The cells from 4th to 7th passage were harvested by trypsinization (0.05%, w/v) and further seeded into Costar 6-well plates. About 12 h later, the medium was removed and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) containing various concentrations of glucose, mannitol and ICI 128436 (statil; ICI Pharm., Macclesfield, UK) [6] usually for 7–8 days until confluent. When the cells reached confluency (2.0×10^5 cells/well), they were washed twice with phosphate-buffered saline (PBS; pH 7.4) and the medium was changed to FCS-free medium containing various concentrations of glucose, mannitol and statil. The media were then collected for measurement of the concentration of ET-1-LI at various time intervals.

2.2. *Measurement of ET-1-LI*

ET-1-LI released into the media was measured by direct radioimmunoassay (RIA) as previously described [7]. Briefly, synthetic ET-1 (Peptide Institute, Osaka, Japan) and monoiodinated ET-1 ([^{125}I]ET-1, 2.10–2.15 μCi, pmol) prepared as previously described [8] were used as standard and tracer. The antiserum raised against ET-1(His^{16}-Trp^{21}), 'As-N7' [7] was used in the present study. The cross-reactivities of ET-1, ET-2, ET-3 and human big ET-1 were 100, 100, 100 and 0.001%, respectively, on a molar basis. The minimal detectable quantity and the 50% inhibition of binding were 2 fmol/tube and 20 fmol/tube, respectively, for ET-1 using the antiserum at a final dilution of 1:250000 (Fig. 1). The intra- and inter-assay coefficients of variance (n = 10) were 6.3% and 8.0%, respectively.

2.3. *Reverse-phase HPLC*

The media collected were lyophilized, reconstituted with the initiating solvent and injected onto an ODS-120T column (0.46 cm × 25 cm, Toyo-Soda, Tokyo, Japan). A linear gradient of acetonitrile from 10% to 60% in 0.01% trifluoroacetic acid was applied over a period of 60 min at a flow rate of 1 ml/min. Fractions were collected

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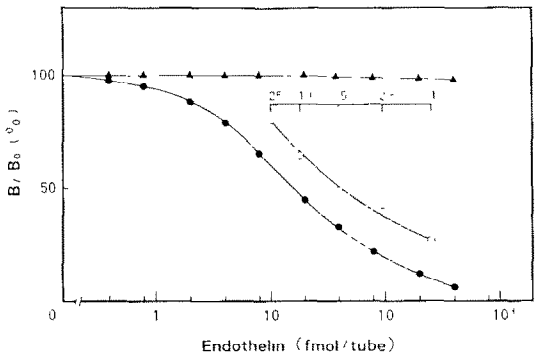

Fig. 1. A standard curve of ET-1 (●) with the antiserum 'As-N7', the cross-reactivity of human big ET-1(1–38) (▲), and the dilution curve of the conditioned medium from cultured bovine aortic endothelial cells (○).

every 36 s and subjected to RIA for ET-1. The recovery of ET-1-LI during the chromatography was more than 90%.

2.4. *Other analytical procedures*

Sorbitol levels were determined by a fluorescent enzymatic assay according to the method of Malone et al. [9]. Endothelial cells in Costar 6-well plates were washed twice and scraped off. The cells collected in 1 ml of PBS, were sonicated for 1 min and centrifuged at 12000 × *g* for 30 min. After centrifugation, the resulting supernatant was subjected to the measurement of sorbitol. Single and multiple comparisons were performed by a Student's *t*-test, and by analysis of variance followed by a Newman-Keuls test, respectively.

3. RESULTS

When confluent bovine aortic endothelial cells were incubated with FCS-free DMEM over a 24 h period, the content of ET-1-LI in the culture media increased linearly with time, and the release of ET-1-LI from the cultured cells was significantly enhanced by elevating the concentration of glucose in DMEM from 5.5 to 22.2 mM (Fig. 2). The dilution curve of the culture

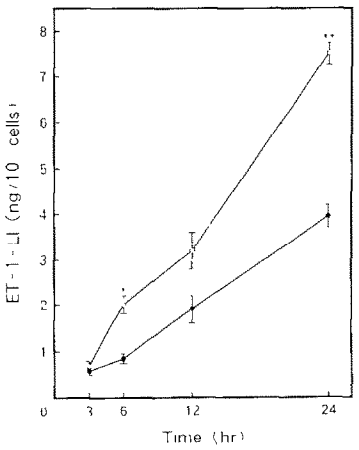

Fig. 2. Time course of ET-1-LI release by bovine aortic endothelial cells cultured in DMEM containing 5.5 mM (●) and 22.2 mM (○) glucose. Values are expressed as means ± SE of 3 experiments. * $P < 0.05$, ** $P < 0.001$ vs 5.5 mM glucose; Student's *t*-test.

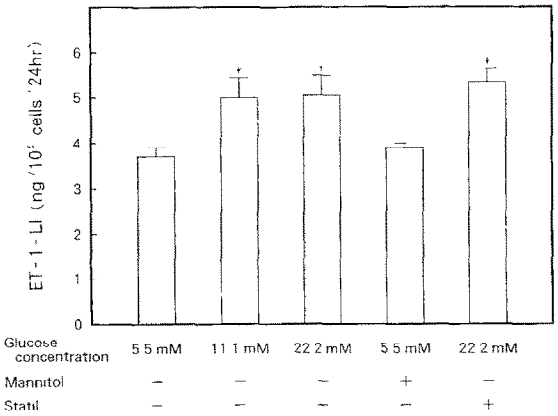

Fig. 3. Effects of glucose, mannitol and statil on ET-1-LI release for 24 h by cultured bovine aortic endothelial cells. Values are expressed as means ± SE of 4 different experiments. Comparisons were done by a Newman-Keuls test. * $P < 0.01$.

media was parallel to that of standard ET-1 (Fig. 1). The elevation of glucose level from 5.5 to 11.1 mM also significantly increased the ET-1-LI release (3.7 ± 0.2 vs 5.0 ± 0.4 ng/10^5 cells/24 h, mean ± SE, $n = 4$, $P < 0.01$), but simple elevation of osmotic pressure by inclusion of 16.7 mM mannitol in the media containing 5.5 mM glucose did not affect the ET-1-LI release (3.7 ± 0.2 vs 3.9 ± 0.1 ng/10^5 cells/24 h; $n = 4$) (Fig. 3). The addition of an aldose reductase inhibitor of statil in the incubation media containing 22.2 mM glucose did not suppress the hyperglycemia-induced increase of ET-1-LI release (5.4 ± 0.3 vs 5.1 ± 0.4 ng/10^5 cells/24 h; $n = 4$), although the hyperglycemia-induced increase of sorbitol content in the cells was corrected by the addition of the same concentration of statil (100 μM) (Table I).

When the media obtained after incubation in the presence of 5.5 and 22.2 mM glucose were analyzed by reverse-phase HPLC followed by RIA for ET-1, it was shown that only an immunoreactive peak corresponding to authentic ET-1 was increased by elevating glucose level, and that the contribution of big ET-1 to the ET-1 immunoreactivities determined by the RIA present was negligible (Fig. 4).

Table I

Effects of elevated glucose levels and statil on intracellular sorbitol contents

Conditions	Sorbitol contents (nmol/mg protein)
Control (glucose 5.5 mM)	10.6 ± 0.8
Glucose 22.2 mM	14.9 ± 0.7*
Glucose 22.2 mM + statil	12.2 ± 0.7**

Statil was added at a final concentration of 100 μM. Results are shown as means ± SE of 4 experiments. * $P < 0.01$ vs control; ** $P < 0.05$ vs 22.2 mM glucose; Student's *t*-test

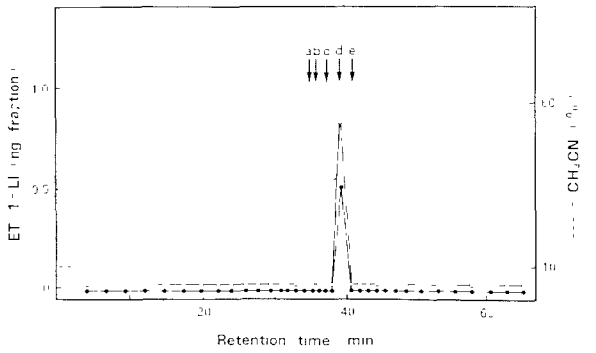

Fig. 4. Reverse-phase HPLC analysis of cultured media incubated with bovine endothelial cells in the presence of 5.5 mM (●) and 22.2 mM (○) glucose. Arrows indicate the elution positions of synthetic standards of (a) ET-3, (b) human big ET-1, (c) Met^7-sulfoxide-ET-1, (d) ET-1, and (e) ET-2.

4. DISCUSSION

The epidemiologic studies have indicated that high blood glucose levels in diabetic patients are associated with increased risk of vascular complications [10]. Our previous work demonstrated that a long-term exposure to high glucose is capable of altering the metabolism of endothelial cells [11], and the present study clearly demonstrated that the elevation of glucose levels can stimulate ET-1-LI release from cultured bovine aortic endothelial cells. Takahashi et al. [12] have recently reported that plasma-immunoreactive ET-1 concentrations are greatly raised in the diabetic patients, which is compatible with our in vitro observations. Various chemical stimuli such as thrombin [1], epinephrine [1], calcium ionophore A23187 [1], phorbol esters [13], angiotensin II [14], vasopressin [14], and transforming growth factor-β (TGF-β) [15] have been shown to augment the secretion of ET-1. These agonists, except for TGF-β, stimulate ET-1 release by activating protein kinase C (PKC). The mechanism of the stimulation of ET-1-LI release by elevated concentrations of glucose is unknown. Our results indicated that neither the high osmotic pressure of the media nor the high concentrations of sorbitol in the cells enhanced the ET-1-LI release. Lee et al. [16,17] reported that the membranous pool of PKC activities in cultured bovine retinal capillary endothelial cells was increased after exposure to elevated glucose levels for 5 days, and the addition of an aldose reductase inhibitor did not change PKC activity. The stimulating effect of glucose on ET-1 secretion was observed after incubation with elevated levels of glucose for 5–6 days (data not shown). Those reported observations, together with the present findings, might suggest that hyperglycemia-stimulated ET-1-LI release is mediated by the activation of PKC activity.

The present study, to our knowledge, is the first report demonstrating that the elevation of glucose levels to those usually encountered in diabetic patients can enhance the secretion of ET-1, and thus might propose the possibility that endothelial damage and the subsequent acceleration of ET-1 production by high blood glucose are involved in the developments of vascular complications and atherosclerosis by the mitogenic action of ET-1 [2,3].

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