Effect of Glucose on Na, K-ATPase Activity in Cultured Bovine Aortic Endothelial Cells

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Abstract. The effect of high concentrations of glucose on Na,K-ATPase activity and the polyol pathway was studied using cultured bovine aortic endothelial cells. Na, K-ATPase activity was expressed as ouabain-sensitive K^+ uptake. A significant decrease in Na, K-ATPase activity with an intracellular accumulation of sorbitol was found in confluent endothelial cells incubated with 400 mg/dl glucose for 96 h. However, there was no significant change in the Na, K-ATPase activity or sorbitol content of the cells incubated with 100 mg/dl glucose plus 300 mg/dl mannitol. The decrease in Na, K-ATPase induced by the high glucose concentration was restored by the simultaneous addition of 10^{-4} M ponalrestat (ICI 128,436; Statil), an aldose reductase inhibitor. The addition of this agent also significantly reduced the increase in sorbitol induced by high glucose levels. These results suggest that the decrease in Na, K-ATPase activity induced in cultured aortic endothelial cells by high concentrations of glucose may be caused in part by the accumulation of sorbitol.

Key words: Na,K-ATPase, Glucose, Endothelial cells.

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HYPERGLYCEMIA plays an important role in the pathogenesis of diabetic macro- and microangiopathies. Vascular endothelial cells are believed to act as a barrier in maintaining vascular homeostasis. Therefore, a functional change in vascular endothelial cells induced by hyperglycemia would be a key event in the development of diabetic angiopathy. The mechanism underlying the altered metabolism of vascular endothelial cells induced by hyperglycemia is not clear. The possible metabolic abnormalities include an increase in nonenzymatic glycosylation of proteins or an alteration in the metabolism of polyol and myo-inositol [1, 2]. Such abnormalities may be responsible for diabetic vascular complications. It is considered that hyperglycemia and the subsequent

accumulation of intracellular sorbitol are closely related to the depletion of myo-inositol and a decrease in Na,K-ATPase activity, which may be an important cause of diabetic complications [3]. In the diabetic state, when circulating glucose levels are high, glucose can easily accumulate in insulin-independent tissue. The accumulated glucose is converted to sorbitol in the cells by activated aldose reductase. The increased sorbitol in the cells may then alter the activity of Na,K-ATPase. The decrease in Na,K-ATPase activity induced by polyol products further inhibits the cellular uptake of myo-inositol.

It has been also demonstrated that altered membrane Na,K-ATPase activity may be responsible for a change in the cellular receptors for hormones or growth factors, or in the physiological actions of these potent factors [4-6].

To evaluate these considerations, we studied the effect of glucose on Na, K-ATPase activity in the presence and absence of an aldose reductase

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inhibitor in cultured bovine aortic endothelial cells as they concern the pathogenesis of diabetic angiopathy.

Materials and Methods

Cell culture

Vascular endothelial cells were scraped from the intima of the bovine thoracic aorta obtained from young calves. As previously described by our laboratory [7, 8], the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 μ U/ml penicillin and 100 μ g/ml streptomycin at 37°C in an atmosphere of 95% air and 5% CO₂. The DMEM was replaced twice weekly. The cultured cells were identified as endothelial cells by phase contrast microscopy based on typical monolayer growth with "a cobblestone appearance" and by the detection of von Willebrand factor produced in the medium. Von Willebrand factor was determined with a von Willebrand reagent (Behring Werke AG, Marburg, FRG). When confluent, the cells were continuously subcultured by trypsinization with 0.05% (w/v) trypsin solution. Cells from the fifth passage were used in this study. When these cells reached confluence (approximately 5×10^4 cells/dish), they were further cultured in DMEM containing 10% FCS with 100 mg/dl glucose, 200 mg/dl glucose, 400 mg/dl glucose, or 100 mg/dl glucose plus 300 mg/dl mannitol for the required time.

Analytical methods

Na, K-ATPase activity was determined by ⁸⁶Rb uptake according to the method previously described [9, 10]. ⁸⁶Rb is a potassium analogue whose transport across the cell membrane is used as a marker for Na,K-ATPase activity. The fifth passage cells subcultured in 24-well culture dishes (Flow Laboratories Inc, Macheen, UA) were preincubated in DMEM with 10% FCS at 37°C for 30 min. The cells were incubated for 60 min at 37°C in 1 ml of DMEM containing 1×10^5 dpm/dish ⁸⁶Rb (NEN-Products, Boston, MA; 1–11 mCi/mg) without or with 1 mM ouabain (Sigma, St. Louis, MO, U.S.A.) to discriminate between ouabain-sensitive and insensitive ⁸⁶Rb uptake. Be-

cause the ouabain-sensitive K⁺ uptake values were too low to detect, we added 10% FCS or monensin (10^{-5} M) to the medium as a stimulator. Simultaneously, monensin (Sigma) or FCS was added to the various final concentrations to stimulate Na, K-ATPase activity. The assay was terminated by aspirating the medium and immediately washing the cells three times with ice-cold phosphate-buffered saline (PBS). The cells were then fixed at room temperature in 0.5 ml of 6% (w/v) trichloroacetic acid for 30 min and removed with a cell scraper. The incorporated ⁸⁶Rb radio-activity was counted in an LSC-700 liquid scintillation counter (Aloka Company, Tokyo, Japan). The method described provides a measure of potassium influx into endothelial cells via Na, K-ATPase expressed in this study as Na, K-ATPase activity or as ouabain-sensitive K⁺ uptake. A fluorescent enzymatic assay was used to measure the sorbitol concentration in cultured endothelial cells as described previously [11]. Briefly, cultured endothelial cells in dishes were scraped off with a rubber spatula into 1 ml of PBS after washing three times with PBS. The suspensions were precipitated with perchloric acid and neutralized with 3 M K₂CO₃. The samples were then centrifuged at 3000 rpm for 15 min. The supernatant was collected and 50 mM glycine buffer (pH 9.4), 1.25 mg/ml nicotinamide adenine dinucleotide (NAD, Sigma) and 0.6 U of sorbitol dehydrogenase (Sigma, from sheep liver) were added. The mixture was incubated for 45 min at 37°C and the fluorescence was measured with a spectrofluorometer (RF-5000, Shimadzu Company, Japan) with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The protein concentration in 50 μ l aliquots of the samples was measured by the method previously described and a BSA standard [12]. The activity of lactate dehydrogenase (LDH), released only on severe cellular damage, was measured at several different times during cell cultures in medium as previously described [13].

Statistical methods

Statistical analysis was carried out by Student's *t*-test. Data are shown as the mean $\pm$ SEM and *P* values less than 0.05 were accepted as statistically significant.

Results

Figure 1 shows the time-dependent uptake of K^+ into cultured endothelial cells stimulated by 10% FCS during incubation. The total and ouabain sensitive K^+ uptake increased in a linear fashion until 90 min. Therefore, ouabain-sensitive K^+ uptake with incubation for 60 min was used in subsequent experiments. We could not measure ouabain-sensitive K^+ uptake in the absence of stimulator of Na,K-ATPase because of its low value, and subsequent experiments were performed in the presence of a stimulator of Na,

Fig. 1. Time course of 10% FCS-stimulated K^+ uptake into cultured bovine aortic endothelial cells. Each point represents the mean $\pm$ SEM for 5 different cell lines. Closed square shows total K^+ uptake, closed circle ouabain-insensitive K^+ uptake, and open circle ouabain-sensitive K^+ uptake.

Fig. 2. Effect of FCS on ouabain-sensitive K^+ uptake of aortic endothelial cells. Bars indicate the mean $\pm$ SEM for 5 different cell lines. Statistically significance vs. ouabain-sensitive K^+ uptake without FCS. *, $P < 0.01$; **, $P < 0.001$.

Fig. 3. Effect of ouabain on 10% FCS-stimulated K^+ uptake into cultured bovine aortic endothelial cells. Bars indicate the mean $\pm$ SEM for 5 different cell lines. Statistically significant vs. K^+ uptake without ouabain. *, $P > 0.01$; **, $P < 0.001$.

Fig. 4. Effect of monensin on ouabain-sensitive K^+ uptake into cultured bovine aortic endothelial cells. Bars indicate the mean $\pm$ SEM for 5 different cell lines. Statistically significant vs. ouabain-sensitive K^+ uptake without monensin. *, $P < 0.01$; **, $P < 0.001$.

K-ATPase such as FCS or monensin. FCS stimulated the ouabain-sensitive K^+ uptake in a dose-dependent manner. Maximal stimulation was found at a final concentration of 10% FCS (Fig. 2). Ouabain significantly inhibited the total K^+ uptake stimulated by 10% FCS in a dose dependent manner. The maximal inhibition of approximately 42.7% was found at a final concentration of 10^{-2} M ouabain (Fig. 3). Total K^+ uptake was inhibited from 24.1 ± 1.1 nmol/dish/60 min (mean $\pm$ SEM, $n=5$) without ouabain to 13.8 ± 1.3 nmol/dish/60 min with 10^{-2} M ouabain. On the other hand, monensin as well as FCS significantly stimulated the ouabain-sensitive K^+ uptake in a dose-

dependent manner up to 10^{-5} M (Fig. 4). The maximal stimulation by 10^{-5} M monensin was 38.2 ± 1.9 nmol/dish/60min (mean $\pm$ SEM, $n=5$). The monensin-stimulated ouabain-sensitive K^+ uptake was inhibited by the simultaneous addition of ouabain similar to that stimulated by FCS (data not shown).

The effect of high glucose concentrations on ouabain-sensitive K^+ uptake was studied. Confluent endothelial cells were incubated with various concentrations of glucose or mannitol for 96 h. As shown in Fig. 5, by increasing the final concentrations of glucose from 100 mg/dl to 400 mg/dl, both the FCS- and monensin-stimulated ouabain-sensitive K^+ uptake were significantly decreased from 15.2 ± 1.0 nmol/dish/60min (mean $\pm$ SEM) to 9.8 ± 1.0 nmol/dish/60min and from 40.0 ± 2.0 nmol/dish/60min to 29.2 ± 1.9 nmol/dish/60 min after 96 h incubation, respectively. However, there was no significant change in ouabain-sensitive K^+ uptake in the cells incubated with 200 mg/dl glucose or with 100 mg/dl glucose plus 300 mg/dl mannitol as compared to those incubated with 100 mg/dl glucose (Fig. 5). In evaluating the effect of ponalrestat (ICI 128,436;

Fig. 5. Time course of monensin- and FCS-stimulated ouabain-sensitive K^+ uptake into cultured endothelial cells with various concentrations of glucose or mannitol. Each point represents the mean $\pm$ SEM for 5 different cell lines. Open circle shows ouabain-sensitive K^+ uptake into the cells with 100 mg/dl glucose; open square with 200 mg/dl glucose; closed circle with 400 mg/dl glucose; closed triangle 100 mg/dl glucose plus 300 mg/dl mannitol. *: $p < 0.01$ vs. a; ouabain-sensitive K^+ uptake of the cells cultured with 100 mg/dl glucose by monensin stimulation, **: $p < 0.01$ vs. b; ouabain-sensitive K^+ uptake of the cells cultured with 100 mg/dl glucose by 10% FCS stimulation.

Table 1. Effect of glucose and ponalrestat on ouabain-sensitive K^+ uptake of cultured endothelial cells

Stimulant	Glucose (mg/dl)	Ponalrestat (10^{-4} M)	Ouabain-sensitive K^+ uptake (nmol/60 min/dish)
FCS (10%)	100	—	14.9 ± 1.0^a
	100	+	15.9 ± 1.3^b
	400	—	9.8 ± 0.9^c
	400	+	12.4 ± 0.6^d
Monensin (10^{-5} M)	100	—	36.0 ± 1.8^e
	100	+	37.8 ± 2.2^f
	400	—	24.1 ± 1.8^g
	400	+	30.8 ± 1.6^h

Values are expressed as the mean $\pm$ SEM ($n=5$).

a, $P < 0.01$ vs. c; c, $P < 0.05$ vs. d; d, $P < 0.05$ vs. b; e, $P < 0.01$ vs. g; g, $P < 0.05$ vs. h; h, $P < 0.05$ vs. f.

Statil), an aldose reductase inhibitor, on the decreased ouabain-sensitive K^+ uptake by the high concentration of glucose, we observed that the decreased ouabain-sensitive K^+ uptake stimulated by both FCS and monensin after 96 h-incubation with 400 mg/dl glucose was significantly restored by the addition of 10^{-4} M ponalrestat with 400 mg/dl glucose (Table 1). In contrast, no significant changes were obtained in the ouabain-sensitive K^+ uptake stimulated by both FCS and monensin when ponalrestat was added to the cells incubated with 100 mg/dl glucose (Table 1). In fact, the intracellular sorbitol contents of cultured endothelial cells following incubation for 96 h with 400 mg/dl glucose was increased significantly as compared to those incubated with 100 mg/dl, but no statistically significant change was observed following 24 and 48 h-incubation with each concentration of glucose. Ponalrestat significantly reduced the increased sorbitol content of the cells incubated with 400 mg/dl glucose but not with 100 mg/dl glucose (Table 2). It was clear that ponalrestat reduced the increased sorbitol content and restored the decreased Na,K-ATPase activity of cultured endothelial cells exposed to high concentrations of glucose.

LDH release from the endothelial monolayer was not significantly increased after incubation with 400 mg/dl glucose for up to 96 h compared with 100 mg/dl glucose (data not shown).

Table 2. Effect of glucose and ponalrestat on intracellular sorbitol content of bovine aortic endothelial cells cultured for 24, 48 and 96 h

	Glucose (mg/dl)	Ponalrestat (10^{-4} M)	Sorbitol (nmol/mg prot)
24 h	100	(-)	15.9±1.0
	100	(+)	14.8±1.3
	400	(-)	16.6±0.9
	400	(+)	16.0±1.3
48 h	100	(-)	16.5±1.4
	100	(+)	15.4±1.0
	400	(-)	18.7±1.2
	400	(+)	17.7±1.2
96 h	100	(-)	16.2±1.3 ^a
	100	(+)	14.1±1.1 ^b
	400	(-)	24.1±1.2 ^c
	400	(+)	18.9±1.1 ^d

Values are expressed as the mean±SE (n=5).

a, $P<0.01$ vs. c; c, $P<0.05$ vs. d; d, $P<0.05$ vs. b.

Discussion

Recent investigations [13, 14] have demonstrated that high concentrations of glucose induce metabolic changes in vascular endothelial cells *in vitro*; some of these changes may contribute to the pathogenesis of the vascular complications seen in diabetes. Na, K-ATPase plays a physiologically significant role in cellular function [15–18]. Na, K-ATPase activity may also be altered in various other conditions such as obesity [19], essential hypertension [20], uremia [21], thyroid dysfunction [22], and aging [23].

In this study, Na,K-ATPase activity was measured as indicated by ouabain-sensitive ^{86}Rb uptake, a marker for K^+ uptake [9, 10], in cultured bovine aortic endothelial cells. Since the uptake of K^+ was very low when cells were incubated with the DME medium alone, we added FCS or monensin to the medium to stimulate Na, K-ATPase activity. However, the exact mechanism of FCS-stimulation in aortic endothelial cells remains unclear. Concerning other types of cells, previous reports have shown that the serum contains factors that stimulate Na, K-ATPase activity [5, 9, 10, 24–26], but the characterization and purification of the various factors have not been accomplished. Additionally, the sodium ionophore monensin stimulates ouabain-sensitive K^+ uptake due to the activation of Na, K-ATPase to counteract the increase in the concentration of intracellular sodium. Ouabain-sensitive K^+ uptake stimulated

by monensin directly indicates enzymatic activity of Na, K-ATPase and that stimulated by FCS can be thought to be a mediated hormonal receptor or an indirect expression of the activity of Na, K-ATPase. In our study, the inhibitory effect of glucose on Na, K-ATPase activities was shown during stimulation by both FCS and monensin. So it was suggested that the decrease in ouabain-sensitive K^+ uptake caused by high glucose was due to the direct inhibition of enzymatic activity of Na, K-ATPase, probably mediated by a polyol pathway.

In the present investigation, we demonstrated that a high glucose concentration reduced the Na, K-ATPase activity of cultured endothelial cells following incubation for 96 h. The mechanism of this effect of glucose is not fully understood but it appears to be related to the increase in intracellular sorbitol content. Furthermore, ponalrestat, an aldose reductase inhibitor (ARI), partially reduced the sorbitol content and prevented the inhibitory effect of glucose on Na, K-ATPase activity. In this study, we used aortic vascular endothelial cells relevant to the study of diabetic macroangiopathy. There are few reports on Na, K-ATPase activity and the polyol pathway in aortic tissue. Similar to our observations, it has been reported that high glucose concentrations decreased the ouabain-sensitive uptake of K^+ into rabbit aortic intima-media tissues; the addition of myo-inositol was indispensable for this inhibitory effect of glucose [27]. It has also been shown that in cultured

neuroblastoma cells galactose inhibited Na, K-ATPase activity and that sorbinil, an ARI, partially restored the inhibitory effect of galactose [28]. A decrease in Na, K-ATPase activity has also been shown in the retinal layers of diabetic subjects [29].

Following exposure to high concentrations of glucose for 5 days, cultured retinal endothelial cells showed an increase in intracellular sorbitol content and a decrease in their Na, K-ATPase activity. The addition of sorbinil or the activation of protein kinase C prevented a decrease in Na, K-ATPase activity [30]. In agreement with this finding, our study demonstrated a decrease in Na, K-ATPase activity by glucose in aortic endothelial cells that were incubated for longer periods (96 h) but not for shorter periods such as 24 or 48 h. In addition the effect of ponalrestat on the sorbitol content and Na, K-ATPase activity was found only after incubation for 96 h. In contrast, it has been reported that a decrease in Na, K-ATPase activity of the rabbit aortic wall was found after incubation for only 30 min with a high concentration of glucose [27] and an increase in intracellular sorbitol content of human endothelial cells caused by a high concentrations of glucose was detected after incubation for only 6 h [31]. This discrepancy in incubation time is thought to be caused by differences in cell type, culture conditions and by methods of analyzing Na, K-ATPase and in-

tracellular sorbitol.

In our study, neither the sorbitol content nor the Na, K-ATPase activity changed in the cells incubated with physiological concentrations of glucose (100 mg/dl). However, some reports have shown the stimulation of Na, K-ATPase activity by ARI in other tissues through other mechanisms. It was reported that the treatment of isolated rat renal glomeruli with sorbinil increased Na, K-ATPase activity in physiological conditions [32]. Moreover, another report showed a direct stimulation by ARI of the Na, K-ATPase of bovine kidney [33]. Our last report also suggested a direct stimulation by ponalrestat of Na, K-ATPase activity of washed erythrocytes [34]. In view of these facts, a direct effect of ponalrestat on Na, K-ATPase activity in cultured aortic endothelial cells cannot be ruled out. The decrease in Na, K-ATPase activity of vascular endothelial cells induced by a high glucose concentration may be related to the other metabolic changes; for example, cell growth [17, 18, 35, 36], prostacyclin generation [37] and endothelin production to mention but a few possibilities [38]. These functional alterations, including the decrease in Na, K-ATPase activity in vascular endothelial cells, may be involved in the pathogenesis of diabetic angiopathy.

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