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Normalization of glucose entry under the high glucose condition by phlorizin attenuates the high glucose-induced morphological and functional changes of cultured bovine retinal pericytes

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Abstract

We previously reported that sodium-dependent glucose uptake is present in bovine retinal pericytes and that phlorizin normalizes its glucose consumption under high glucose conditions. To clarify the effect of phlorizin on morphological and functional change of retinal pericytes under high glucose conditions, retinal pericytes were incubated in media with 5 mM glucose, 30 mM glucose, and 30 mM glucose plus 0.2 mM phlorizin for 7 days. The diameter of cells in the concentrations of glucose more than 10 mM were significantly larger than those in 5 mM glucose and 30 mM glucose plus phlorizin. Glucose, sorbitol and fructose contents of the cells in 30 mM glucose were significantly increased compared with those in 5 mM glucose, and were normalized by phlorizin. Thymidine uptake in the concentrations of glucose more than 20 mM was significantly decreased compared with that in 5 mM glucose. Myoinositol uptake, and DNA in 30 mM glucose were significantly reduced, and were normalized with phlorizin. Myoinositol content in 30 mM glucose was the same as that in 5 mM glucose, but was significantly decreased by phlorizin. The ratios of glucose to sorbitol or fructose in 30 mM glucose were significantly decreased, compared with those in 5 mM glucose and 30 mM glucose plus phlorizin. Therefore, the cellular enlargement and decreased DNA synthesis in cultured bovine retinal pericytes with abnormal glucose metabolism under high glucose conditions are attenuated by phlorizin, independent of the cellular myoinositol content.

Keywords: Retinal pericyte; Phlorizin; Cell swelling; Sorbitol myoinositol

1. Introduction

Diabetic retinopathy is the most frequent diabetic complication resulting from the persistence of hyperglycemia [1]. It can be prevented by good glycemic control [2]. In the early stage of diabetic retinopathy, swelling and loss of retinal pericytes are important signs on its onset [3–5], since these changes precede the alteration of retinal endothelial cell function, which may lead to microvascular occlusion and neovascularization in the retina. One of the mechanisms of the swelling of retinal pericytes and their loss in diabetic retinopathy is the increased levels of sorbitol in the cells [6–8]. Sorbitol is formed by excessive

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glucose entry into the cells through the polyol pathway under high glucose conditions [6–9] in association with increased protein kinase C activity by redox state [10] and reduction of Na,K-ATPase activity [11,12]. Decreased Na,K-ATPase activity brings an increase of sodium ions in the cells, which in turn may decrease the myoinositol level since cellular uptake of myoinositol is reported to be sodium dependent [13]. From these observations, sodium metabolism seems to be very important for diabetic complications and may be one of the causes of intracellular edema in retinal pericytes.

We previously reported the presence of sodium-dependent glucose uptake in retinal pericytes [14], noting that phlorizin, an inhibitor for the sodium-coupled glucose transporter (SGLT) through which glucose and sodium enter the cell simultaneously [15–18], decreased glucose uptake by nearly 50% in bovine retinal pericytes. The use of phlorizin could normalize type IV and VI collagen synthesis, normalizing glucose consumption under a high glucose condition [14]. Phlorizin may attenuate cellular sorbitol content and sodium metabolism under high glucose conditions. This study was performed to determine the effects of phlorizin on cellular enlargement and functional alterations of retinal pericytes under high glucose conditions, measuring the intracellular levels of glucose, sorbitol, fructose and myoinositol, and the cellular uptake of myoinositol and thymidine.

2. Materials and methods

2.1. Pericyte preparation

Bovine eyeballs were purchased from a local slaughterhouse and were cut to remove the retinas. The retinas were placed in Dulbecco's modified Eagle's medium (DMEM) and Ham F-12 (1:1, both: Sigma, St. Louis, MO), and were then homogenized and filtered through a sieve of 85 μm pore size. The trapped microvessels were incubated at 37°C for 40 min in DMEM containing 200 μg/ml of collagenase, and then were filtered through a sieve of 53 μm pore size. After washing three times with DMEM-F12, the cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 in DMEM–F12 containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) and 10% Nu-serum (Collaborative Research, Bedford, MA) on plastic plates. The retinal pericytes were identified by their morphological characteristics. After overnight incubation in a medium containing 10 μg/ml of DiI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) (Biochemical Technologies, Stouton, MA), they did not incorporate DiI-Ac-LDL [19]. The retinal pericytes up to eight passages were used for this experiment.

2.2. Cell size determination

Confluent retinal pericytes were incubated in DMEM containing 5% FBS and 5% Nu-serum, and either 5, 10, 20, 30 mM glucose, or 30 mM glucose with 0.2 mM phlorizin on 6-well plates (Nunc, Roskilde, Denmark) for 7 days. The media were changed every 2 days. The cells were treated with PBS containing trypsin (Sigma) for 2 min to separate them into single cells, after washing three times with phosphate-buffered saline (PBS). This was followed by an incubation for 30 min at 37°C in Tris/Hepes buffer (pH 7.4) with 5 mM glucose and osmolarities of 230, 280 and 330 mosmol adjusted with mannitol in an atmosphere of 95% air and 5% CO_2. Diameters of all single cells in these conditions on light-microscopic photographs were measured.

2.3. Thymidine and D-myoinositol uptake by retinal pericytes

Prior to the experiments, confluent retinal pericytes in 12-well plates (23 mm diameter) (Nunc) were cultured in 1 ml of DMEM (for thymidine uptake) or DMEM–F12 (for myoinositol uptake) containing 5% FBS and 5% Nu-serum, and either 5, 10, 20, 30 mM glucose or 30 mM glucose plus 0.2 mM phlorizin for 5 days (thymidine uptake) or 7 days (myoinositol uptake). For the determination of thymidine uptake, the cells were incubated in DMEM containing 1% serum with the same conditions of glucose and phlorizin, under which the cells had been incubated before 5 days, for another 2 days to arrest the growth of pericytes. The media were changed every 2 days to maintain the glucose concentrations. To determine D-myoinositol uptake, the cells were incubated in 500 μl of 20 mM of Tris/

Hepes buffer (pH 7.4), with 145 mM of NaCl or 145 mM of choline-Cl, containing 1.0 mM of $CaCl_2$, 5 mM of KCl, 2.5 mM of $MgSO_4$, 5 μCi/ml of D-[2-^{3}H]myoinositol (Dupont-NEN, Boston, MA), 5 mmol/l of D-glucose, and 0.02 mmol/l of D-myo-inositol for 30 min, after three washings with the Tris/Hepes buffer. To determine thymidine uptake, the quiescent retinal pericytes were incubated in DMEM with 5 mM glucose containing 2 μCi/ml of thymidine (Dupont-NEN) for 4 h after three washings with DMEM. The incubations were terminated by rapid aspiration of the media followed by three washings with ice-cold PBS. Solubilization of the cells was then conducted at room temperature with 500 μl of 0.5 M NaOH. Four hundred-μl aliquots were subjected to a radioactive count with an Aloka liquid scintillation counter LSC 1000 (Aloka, Tokyo, Japan) following neutralization with acetic acid. The concentration of protein in the aliquots was determined by the Coomassie brilliant blue method using a Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as a standard. All of the incubations were carried out in quadruplicate. Thymidine and myoinositol uptake values were expressed as per mg of cell protein.

2.4. Sorbitol, myoinositol, glucose and fructose determination

Confluent retinal pericytes on 100-mm plates were incubated in DMEM containing 5% FBS and 5% Nu-serum, and either 5 mM glucose, 30 mM glucose, or 30 mM glucose plus 0.2 mM phlorizin for 7 days; the media were changed every 2 days. The cells were washed three times with ice-cold PBS containing 10 μg/ml of arabitol, were removed with 2 ml of water containing 10 μg/ml of arabitol, and then were sonicated using a Handy sonic model UR-20P (Tomy Seiko, Tokyo, Japan). The samples were lyophilized. The residues were dissolved in 1 ml of 5% $ZnSO_4$ and then were mixed with 1 ml of 0.3 M $Ba(OH)_2$. After centrifugation at $1700\times g$ for 10 min, the supernatants were lyophilized and silicated with 0.6 ml of a 3:2:1 mixture of pyrimidine/hexamethyldisilazane/trimethylchlorosilane at 80°C for 1 h. Then 1 ml of chloroform and 2 ml of distilled water were added to the solutions and were mixed well. The samples were centrifuged at $1700\times g$ for 5 min at 4°C. The lower layer was dried under a stream of N_2 gas, and the residue was dissolved in 0.1 ml CS_2. The trimethylsilyl ethers were assayed with a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan) on a DB-1 column, 60×0.32 mm i.d., with a 1 μm film thickness (J&W Scientific, CA) which was maintained at 190°C. Helium was used as a carrier gas at a flow rate of 3.5 ml/min. Detection was made with a flame ionizer at 320°C, and the area under the peak was calculated with a GC-Workstation (CLASS-GC10, Shimadzu). The hexose deliveries are expressed as nmol/100 μg DNA.

2.5. Other determinations

The DNA content of each well was determined by the Hoechst 2235 dye (Sigma) method using a TKO 100 (Hoeffer Scientific Instruments, San Francisco, CA) and calf thymus DNA (Clontech Laboratories, Palo Alto, CA) as a standard.

2.6. Statistical analysis

Analysis of variance (ANOVA) and Student's *t*-test (two-tailed) were used for statistical determination. A *P*-value of less than 0.05 was considered significant. Data were expressed as the mean ± S.E.M.

3. Results

Fig. 1 shows the cellular size of retinal pericytes after 7 days' incubation with different glucose concentration. The cellular size under the conditions of 5, 10, 20, 30 mM glucose and 30 mM glucose plus phlorizin were 18.4 ± 0.28, 20.4 ± 0.35, 20.4 ± 0.33, 20.5 ± 0.33 and 18.2 ± 0.29 μm, respectively. Those sizes in 10, 20 and 30 mM glucose were significantly larger than that in 5 mM glucose. By the addition of phlorizin in the media, the increased cellular size in 30 mM glucose was reduced to the size in 5 mM glucose.

Fig. 2 shows the size of retinal pericytes in the different osmolarities after 7 days' incubation with three different media: one with 5 mM glucose, one with 30 mM glucose, and one with 30 mM glucose plus 0.2 mM phlorizin. The cell sizes in 5 mM glucose and 30 mM glucose plus phlorizin were signifi-

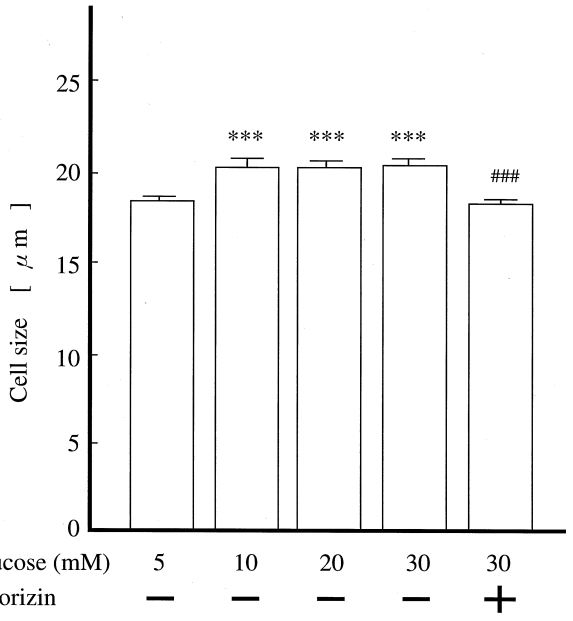

Fig. 1. Cellular size of pericytes 7 days after incubation in the different glucose concentration. The numbers of the cells measured in 5, 10, 20, 30 mM glucose and 30 mM glucose plus 0.2 mM phlorizin were 161, 140, 139, 153 and 128 cells, respectively. $^{***}P<0.0001$ vs. 5 mM glucose; $^{\#\#\#}P<0.0001$ vs. 30 mM glucose.

cantly increased in 230 mosmol, and were significantly decreased in 330 mosmol, compared with those in 280 mosmol. The cell size in 30 mM glucose in 280 mosmol was significantly increased compared with that in 330 mosmol. The cell size in 230 mosmol was slightly increased compared with that in 280 mosmol, but it was not significant. Although the cell sizes in 330 mosmol were not different among the three conditions, the cell size in 30 mM glucose was significantly larger than those in 5 mM glucose and 30 mM glucose plus phlorizin in 280 mosmol. In 230 mosmol, the cell size in 30 mM glucose plus phlorizin was significantly smaller than that in 5 mM glucose.

Table 1 shows DNA, protein, glucose, fructose, sorbitol and myoinositol contents, and the ratios of protein to DNA, glucose to sorbitol and glucose to fructose in retinal pericytes incubated with 5 mM glucose, 30 mM glucose and 30 mM glucose plus 0.2 mM phlorizin. The DNA content of cells in 30 mM glucose was significantly decreased compared with those in 5 mM glucose and 30 mM glucose plus phlorizin. The protein contents were not different among these three conditions. The glucose, fructose and sorbitol contents of cells in 30 mM glucose were significantly increased compared with those in

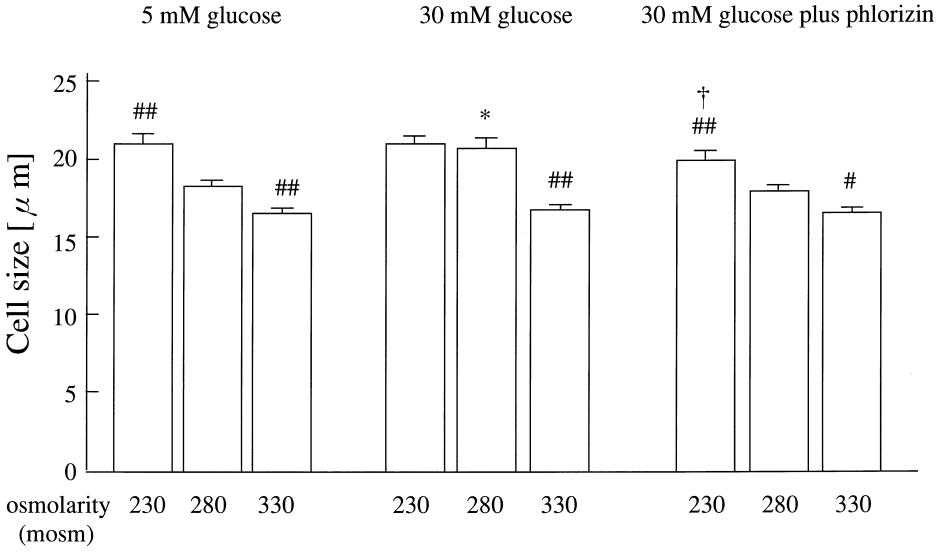

Fig. 2. The effect of osmolarities on the cellular size of pericytes cultured in the different glucose concentrations in the presence of phlorizin. The cell sizes were determined with microscopic scale after 7 days of incubation in three different osmolarities. Determination of cell sizes was repeated in different pericyte preparations, measuring more than 100 cells on photographs from 6-well plates. Data are the mean ± S.E.M. $^{\dagger}P<0.05$ vs. 5 mM and 30 mM glucose in 230 mosmol; $^{*}P<0.001$ vs. 5 mM glucose in 280 mosmol; $^{\#}P<0.01$ vs. 280 mosmol in each group; $^{\#\#}P<0.001$ vs. 280 mosmol in each group.

Table 1
Cellular DNA, glucose, sorbitol fructose and myoinositol contents of bovine retinal pericytes after 7 days of incubation with 5 mM and 30 mM glucose with or without phlorizin

	5 mM	30 mM	30 mM plus phlorizin
DNA (μg/well)	42.1 ± 1.5	33.2 ± 0.9$^{**\#}$	39.9 ± 2.7
Protein (μg/well)	500 ± 27	478 ± 50	487 ± 20
Protein/DNA ratio	11.9 ± 0.9	14.5 ± 1.3	12.4 ± 0.9
Glucose (nmol/100 μgDNA)	44.5 ± 6.7	164.0 ± 19.0$^{**\#\#}$	55.8 ± 20.8
Sorbitol (nmol/100 μgDNA)	3.0 ± 0.8	21.9 ± 3.4$^{*\#\#}$	4.7 ± 1.5
Fructose (nmol/100 μgDNA)	5.2 ± 1.2	38.0 ± 6.4$^{**\#\#}$	8.8 ± 0.4
Myoinositol (nmol/100 μgDNA)	6.7 ± 0.9	6.0 ± 0.7	2.9 ± 0.6†
Glucose/sorbitol	16.0 ± 2.1	7.6 ± 0.6$^{**\#}$	11.3 ± 1.4
Glucose/fructose	9.3 ± 0.9	4.0 ± 0.2$^{**\#}$	6.4 ± 0.9
Fructose/sorbitol	1.74 ± 0.21	1.73 ± 0.07	1.63 ± 0.25

The ratio of glucose to sorbitol was calculated from the molar concentration of glucose divided by that of sorbitol in the same individual well. The other ratios of various carbohydrates were calculated in the same fashion.
$^{*}P < 0.005$ vs. 5 mM glucose; $^{**}P < 0.001$ vs. 5 mM glucose.
$^{\#}P < 0.05$ vs. 30 mM glucose plus phlorizin; $^{\#\#}P < 0.01$ vs. 30 mM glucose plus phlorizin.
$^{\dagger}P < 0.01$ vs. 5 mM and 30 mM glucose.

5 mM glucose. Levels of DNA, glucose, fructose and sorbitol contents of the cells in 30 mM glucose plus phlorizin were the same as those in 5 mM glucose. On the other hand, the myoinositol content of cells in 30 mM glucose plus phlorizin was significantly decreased compared with those in 5 mM and 30 mM glucose. Protein to DNA ratios were not different among these conditions. The ratios of glucose to

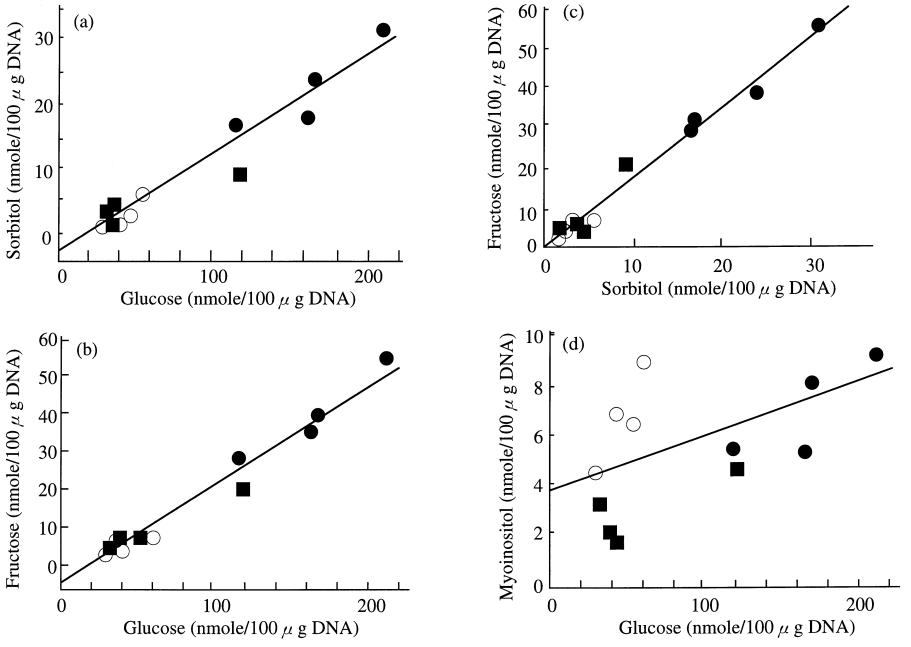

Fig. 3. Cellular contents of glucose, sorbitol, fructose and myoinositol in retinal pericytes cultured on 100-mm plates with 5 mM and 30 mM glucose with or without 0.2 mM phlorizin. There were significant correlations among glucose content and sorbitol (a, $r = 0.966$, $P < 0.001$), glucose and fructose (b, $r = 0.977$, $P < 0.001$), sorbitol and fructose (c, $r = 0.99$, $P < 0.001$). There was no significant correlation between glucose and myoinositol (d). $n = 4$ in each condition. ○, 5 mM glucose; ●, 30 mM glucose; ■, 30 mM glucose plus phlorizin.

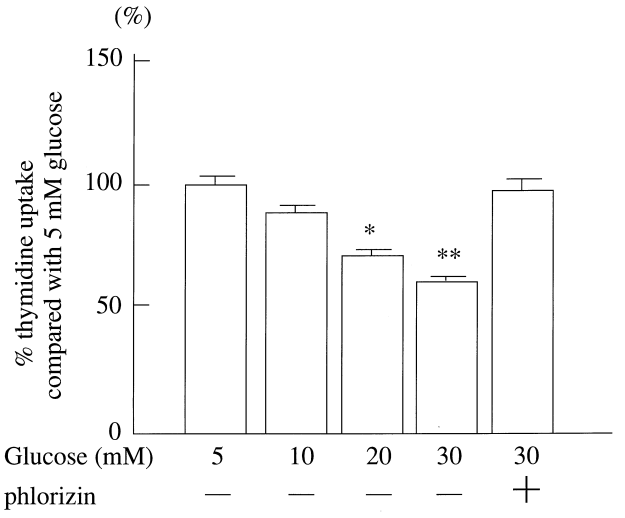

Fig. 4. Thymidine uptake by pericytes after 7 days of incubation with either 5, 10, 20, 30 mM glucose or 30 mM glucose plus 0.2 mM phlorizin. Confluent and quiescent retinal pericytes on 12-well plates after incubation with DMEM containing 1% serum were used. This experiment was repeated and the same results were obtained. $n=4$ in each condition. Data are the mean ± S.E.M. $^{*}P<0.05$ vs. 5 mM glucose; $^{**}P<0.01$ vs. 5 mM glucose.

sorbitol and glucose to fructose in 30 mM glucose were significantly decreased compared with those in 5 mM glucose or 30 mM glucose plus phlorizin. There were no significant differences of fructose to sorbitol ratio among the three conditions.

The correlations among glucose, sorbitol, fructose and myoinositol contents in retinal pericytes are shown in Fig. 3. There were significant positive correlations of glucose content with sorbitol ($r=0.966$, $P<0.0001$, Fig. 3a), and fructose ($r=0.977$, $P<0.0001$, Fig. 3b) and of sorbitol content with fructose ($r=0.99$, $P<0.001$, Fig. 3c). However, there was no significant correlation of myoinositol with glucose (Fig. 3d), sorbitol, or fructose (data from the latter two are not shown).

Thymidine uptake by retinal pericytes after 7 days' incubation with different glucose concentrations are shown in Fig. 4. Thymidine uptake significantly decreased at the concentration of glucose more than 20 mM. The reduced thymidine uptake at 30 mM glucose was returned to the level at 5 mM glucose by adding phlorizin.

Myoinositol uptake in the presence of sodium (NaCl) by retinal pericytes 7 days after incubation with 30 mM glucose was significantly decreased compared with that in 5 mM glucose. The reduced myoinositol uptake in 30 mM glucose was normalized by adding phlorizin. Myoinositol uptakes were very small in the absence of sodium (choline-Cl), and there were no differences among the three groups (Fig. 5).

4. Discussion

The increased cell size with the decreased thymidine uptake and DNA content under higher glucose

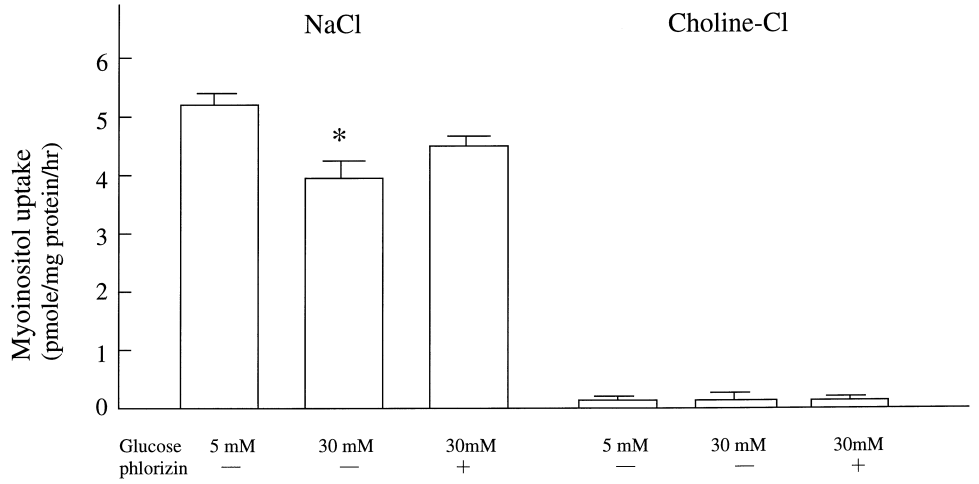

Fig. 5. Myoinositol uptake by pericytes after 7 days of incubation in 5 and 30 mM glucose and 30 mM glucose plus 0.2 mM phlorizin in the presence (NaCl) or absence of sodium (choline-Cl). Four wells from 12-well plates in each condition were used. Data are the mean ± S.E.M. $^{*}P<0.05$ vs. 5 mM glucose.

conditions seem to reflect the swellings and loss of pericytes that are observed in the early stage of diabetic retinopathy. The increase of cell size in 30 mM glucose is not cellular hypertrophy but seems to be attributable to an intracellular edema, since the protein to DNA ratios in 5 mM and 30 mM glucose were similar, and the cell size in 30 mM glucose changed according to the osmolarities. These morphological and functional changes in 30 mM glucose were normalized by phlorizin which also normalized the 3.7-fold increase of glucose contents in the cells. It is worth noting that the excess glucose entry in GLUT1-overexpressed mesangial cells under the normal glucose condition induced overproduction of type IV collagen [20] as observed in normal rat mesangial cells under a high glucose condition [21], and that the normalization of glucose entry in retinal pericytes under the high glucose condition with phlorizin normalized the collagen synthesis [14]. An increase of glucose entry may be important for the early stage of diabetic complications.

Predominant metabolic alterations of the excess glucose entry into cells under high glucose conditions is shown to be the sorbitol accumulation [6–8,22] and the increase of protein kinase C (PKC) activity [23]. The increase of cellular sorbitol in the polyol pathway induces increase of cytosolic redox potential (increased NADH/NAD^+ ratio). In this study, significant increases of glucose and sorbitol contents and significant decrease of the ratios of glucose/sorbitol and glucose/fructose, which may reflect the presence of increased cytosolic redox potential, were present in retinal pericytes incubated in 30 mM glucose; these abnormalities were normalized by the addition of phlorizin. Normalization of cytosolic NADH/NAD^+ by an aldose reductase inhibitor, which is reported to attenuate high glucose-induced intracellular increase of sorbitol and decrease of myoinositol [24], has been found to protect both diabetic and nondiabetic hearts from ischemic injury [25]. Apoptosis in retinal pericytes under high glucose conditions is explained the loss of retinal pericytes in the early stage of diabetic retinopathy [26,27]. The attenuation of high glucose-induced intracellular metabolic alterations by phlorizin may protect pericytes from the apoptosis.

Increased sorbitol content and decreased myoinositol content under high glucose conditions are reported to decrease Na,K-ATPase activity [12,28], on which the uptake of myoinositol by cells depends [29]. The reduction of Na,K-ATPase activity by high glucose conditions more than 10 mM is reported to occur in 60 min and not to be prevented by the addition of myoinositol [30]. This study showed that the myoinositol uptake by the cells in 30 mM glucose after 7 days of incubation was significantly decreased in spite of the absence of a decrease in the cellular myoinositol content. It may take longer time than 7 days to decrease cellular myoinositol content. The decrease of myoinositol in cerebral microvascular pericytes under 22.2 mM glucose condition is not observed after 3 days of incubation but is observed after 18 days of incubation [31]. Decreased Na,K-ATPase activity under high glucose conditions induces an increase of sodium content in cells, which brings intracellular edema and altered function [11]. This may be the reason why the morphological change of retinal pericytes occurs in the glucose concentration more than 10 mM. Although the effect of myoinositol on diabetic complications is controversial [32,33], a decrease of thymidine uptake in GLUT1-overexpressed mesangial cells with increased cellular sorbitol and myoinositol contents is reported [20]. The morphological and functional changes of pericytes in 30 mM glucose seems to be independent of intracellular myoinositol levels, since these changes were attenuated by phlorizin, which inhibits sodium dependent myoinositol transporter [34].

It is interesting that retinal pericytes and mesangial cells, which seems to play an important role in diabetic retinopathy and nephropathy, possess SGLT [14,35]. SGLT is also reported to have a water permeable pore [36]. Although the role of SGLT for diabetic complications is unknown at present, the sodium entry through SGLT may contribute to cellular swellings, since swellings of SGLT1 transfected Sf9 cells has been reported [37]. The cell size in 30 mM glucose with phlorizin at 230 mosmol was significantly smaller than that in 5 mM glucose, which may be explained by an inhibition of entering sodium and water through SGLT by phlorizin. Moreover, SGLT may contribute to apoptosis in retinal pericytes, since sodium ionophore is reported to cause apoptosis in HL-60 cells [38]. Further experiments are needed to clarify the role of SGLT concerning

sodium-ion concentration in cells and its relation to diabetic complications.

In conclusion, phlorizin attenuates high glucose-induced morphological and functional changes of retinal pericytes in relation to the ameliorations of cellular contents of glucose and its metabolites, sorbitol and fructose.

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