Problem Set 1 (**Due January 21st**)

1. Using the KEGG PATHWAY website:

    a. Sugar Metabolism:
        i. What metabolite can either continue through glycolysis or enter the Pentose phosphate pathway?
        ii. The conversion of Glucose-6-phosphate (G6P) to Gluconate-6-phosphate happens in two steps.
            1. Draw and name the intermediate.
            2. If cofactors are required in this process, draw the cofactor and explain its role.
            3. In humans, there are two possible paths from G6P to Gluconate-6-phosphate. Describe the difference in these two related paths.
            4. Consider the one step pathway. What is the name of the enzyme and the Gene ID in humans (HSA)?
            5. Is this enzyme important in any other pathways?
            6. What disease is linked to this enzyme in humans?
                a. A direct role in this disease seems unlikely, but the link is made in KEGG. Please describe how this enzyme is related to this disease.
                b. If you navigate to the Steroid hormone biosynthesis page directly from the Disease page, the relevant enzyme is highlighted in red. What is the name of this enzyme and what cofactor does it rely on? How might this be related to the oxidation of G6P?

    b. In Nitrogen Metabolism:
        i. What is the name of the enzyme that facilitates nitrogen fixation?
        ii. Click on the Taxonomy button. What are three species that can fix Nitrogen? (red highlights = genomes with this gene).
        iii. What is the net chemical reaction?
        iv. This enzyme is composed of two independent proteins. Name these proteins and the cofactors are involved with each?

2. What cofactors are important for energy storage? Draw each and explain why they are important.

3. Most transmembrane regions of transporters are composed of α–helices. Describe how multiple α–helices can combine to form a pore for aqueous molecules. Be specific.

4. Chemical potential (ΔG):

    a. If the sodium ion concentration inside the cell is 50mM and outside it is 560 mM, determine the chemical potential difference across the membrane if the membrane potential is 100 mV (inside negative).
    b. Calculate the maximum ratio $[glucose]_{in}/[glucose]_{out}$ that can be transported with this potential. Make sure to consider the Na/glucose stoichiometry for this symporter.

5. Clearly describe the affect that a sodium ionophore would have on the system described in 4.

6. You isolate a new strain of bacteria that has evolved to rely heavily on leucine and ethylene glycol for energy. Of course, these molecules need to get inside the cell to be useful. One of these molecules enter in a mediated fashion and the other through passive diffusion.
    a. Based on the experimental data below, determine which is which.
    b. For the passive diffuser, determine the permeability coefficient. Assume that $[A]_{in}$ is equal for all trials.
    c. For the mediated diffuser, determine K_t and J_{max}.

Leucine	
Concentration (μM)	Initial Uptake Rate ($\mu M\ s^{-1}$)
1	110
2	220
5	480
10	830
30	1700
100	2600
500	3100
1000	3200

Ethylene glycol	
Concentration (mM)	Initial Uptake Rate ($mM\ s^{-1}$)
0.5	50
1	110
5	220
10	480
50	830
100	1709.8

7. Familiarize yourself with the attached journal article (Wakisaka, M, *et. al. Biochim. Biophys. Acta* **1362**, 87-96.)
    a. How did the authors monitor glucose uptake?
    b. Carefully inspect Figure 2. Based on this data, what type of glucose transporter (GLUT or sodium symporter) is present in each cell type?
    c. Phlorizin is an inhibitor of sodium-coupled glucose transporters. Look up the structure of this molecule and propose a reason for this observation.
    d. Which figure confirms that phlorizin effectively inhibits sodium-dependent glucose transport in retinal pericytes?
    e. Why was choline chloride chosen as a control?
    f. Figure 7a shows us that phlorizin is not able to completely prevent glucose uptake. Propose a reason for this observation.

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Na^+-dependent glucose uptake and collagen synthesis by cultured bovine retinal pericytes

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Abstract

This study was performed to clarify the presence of sodium-dependent glucose uptake and its role in the synthesis of type IV and type VI collagen by cultured bovine retinal pericytes. The glucose uptake by retinal pericytes and retinal endothelial cells was measured using ^{3}H-D-glucose in the presence or absence of sodium. Glucose uptake in the presence of sodium was twice as high as that observed in the presence of phlorizin and sodium or in the absence of sodium. Sodium-dependent glucose uptake was observed at different sodium concentrations, and its half-maximal stimulation occurred at 48 mM. These findings were not observed in retinal endothelial cells. Levels of type IV and type VI collagen produced by retinal pericytes were significantly increased at glucose concentrations higher than 20 mM. Phlorizin decreased both collagen synthesis and glucose consumption by retinal pericytes incubated with 30 mM of glucose to the levels observed with 5 mM of glucose. These data suggest that sodium-dependent glucose uptake is present in retinal pericytes and that excessive glucose entry into the cell is an important factor for overproduction of collagen. Phlorizin normalized the synthesis of type IV and type VI collagen with decreasing glucose consumption under high glucose conditions. © 1997 Elsevier Science B.V.

Keywords: Retinal pericyte; Type IV collagen; Type VI collagen; Retinal endothelial cell; Phlorizin; Sodium-coupled glucose transporter

1. Introduction

In the retina, the ratio of endothelial cells to pericytes is 1:1. Pericytes are reported to prevent endothelial cell damage [1,2] and may play an important role in diabetic microangiopathy. The retinal microvascular pericyte loss and thickening of basement membrane that occur in the early stages of diabetic retinopathy may be important in predisposing the retinal circulation to the later development of microaneurysms, capillary nonperfusion, and the formation of new vessels [3]. One of the mechanisms for retinal pericyte loss is explained by sorbitol accumulation in the cells via the polyol pathway [4,5]. Sorbitol accumulation reportedly reduces myoinositol levels in the cells, which in turn decreases Na–K–ATPase activity [6,7]. Reduced Na–K–ATPase activity in the presence of diabetes induces an increase of sodium in the cells [8] and subsequently seems to accelerate swelling and damage of pericytes. For this reason, sodium metabolism in retinal pericytes seems to be very important.

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The retinal pericytes regulate blood flow in the retina and may modify the function and growth of retinal endothelial cells [9,10]; thus, their function resembles that of the mesangial cells in the glomerulus [11]. Wakisaka et al. [12] have reported the presence of a sodium-coupled glucose transporter (SGLT), through which glucose and sodium ions enter into the cell simultaneously, as well as the presence of facilitated glucose transporter 1 (GLUT1) and a small amount of facilitated glucose transporter 4 (GLUT4) in rat mesangial cells, by the method of demonstrating mRNA for these glucose transporters. Increases in the rate of type IV and type VI collagen synthesis by rat mesangial cells under conditions of high glucose have been also reported [13]. Overproduction of extracellular matrices seems to contribute to thickening of the basement membrane, which leads to the occlusion of retinal microvessels in diabetic retinopathy [14–16] or occlusion of glomerular microvessels and glomerular expansion in diabetic nephropathy [17,18]. Furthermore, overproduction of collagens is reported to correlate with glucose consumption by mesangial cells [13]. In light of these associations, the investigation of collagen synthesis and the kinetics of glucose uptake are important to clarify the mechanism of diabetic complications. The use of phlorizin, a specific inhibitor for SGLT [19–22], has been shown to reduce about 50% of the glucose uptake by rat mesangial cells when administered in the presence of 145 mM of NaCl [12]. It is unknown whether this reduction of glucose uptake by phlorizin decreases collagen synthesis.

This study was performed to determine whether sodium-dependent glucose uptake is present in retinal pericytes and to assess type IV and type VI collagen synthesis by the cells. The effect of phlorizin on type IV and type VI collagen synthesis by bovine retinal pericytes was also investigated.

2. Materials and methods

2.1. Pericyte and retinal endothelial cell preparation

Bovine eyeballs were purchased from a local slaughterhouse. The retinas were removed, cut into small pieces, and incubated at 37°C for 40 min in the Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 200 μg/ml of collagenase. The cells were obtained by centrifuging after washing three times with DMEM. The cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 in DMEM containing 10% fetal bovine serum (FBS) (GIBCO BRL, Grand island, NY). After small colonies of cells were formed on the collagen-coated plates, they were separated into retinal endothelial cells and pericytes by removal of other cells using small glass pipettes under light microscopy. After the cells were separated, the retinal endothelial cells were cultured in DMEM containing 10% FBS, and the retinal pericytes were cultured in DMEM–F12 (Sigma) (1:1) containing 10% FBS and 10% Nu-serum (Collaborative Research, Bedford, MA) as well as penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The retinal pericytes and retinal endothelial cells were identified by their morphological characteristics and DiI-labeled acetylated low-density lipoprotein (DiI–Ac–LDL) uptake [23] after overnight incubation in a medium containing 10 μg/ml of DiI–Ac–LDL (Biochemical Technologies, Stouton, MA). Less than 8th passage of retinal pericytes and retinal endothelial cells were used for this experiment.

2.2. Glucose uptake by retinal pericytes and retinal endothelial cells

Prior to the experiments, confluent retinal pericytes and retinal endothelial cells were cultured for 48 h in 12 well plates (23-mm diameter; Nunc, Roskide, Denmark) with either 1 ml of DMEM–F12 (1:1) (retinal pericytes) and DMEM (retinal endotherial cells) containing 5 mM of glucose and 5% FBS; the media were changed daily to maintain the glucose concentration. After the cells were washed twice with 20 mM of Tris/HEPES buffer, pH 7.4, containing 1.0 mM of $CaCl_2$, 5 mM of KCl and 2.5 mM of $MgSO_4$ and either 145 mM of NaCl or 145 mM of choline-Cl, they were incubated in 500 μl of media containing 5 μCi/ml of [2-^{3}H-]D-glucose (Dupont-NEN, Boston, MA), 0.1 mmol/l of D-glucose, and an appropriate concentration of NaCl with and without different inhibitors for 30 min. α-Methyl-D-glucoside (AMG) uptake was also determined using the same buffer containing 0.1 mM AMG (2 μ Ci/ml of [^{14}C]-α-methyl-D-glucoside). The incubations were termi-

nated by rapid aspiration of the media followed by three washings with ice-cold phosphate buffered saline (PBS). Solubilization of the cells was then achieved at room temperature with 500 μl of 0.5 M NaOH. Following neutralization with 2 M acetic acid, 400 μl of aliquots were taken to measure radioactivity using a Aloka liquid scintillation counter LSC 1000 (Aloka, Tokyo, Japan). Protein concentrations of the aliquots were determined by the Coomassie brilliant blue method using the Bio-Rad protein assay (Bio-Rad) and bovine serum albumin as a standard. All of the incubations were carried out in quadruplicate for each of the experiments. D-Glucose and AMG uptake values were calculated per mg of cell protein.

2.3. SDS–PAGE and immunoblot for type IV and type VI collagen

The confluent retinal pericytes were cultured for 48 h in six well plates (35 mm diameter, NUNC) with DMEM–F12 (1:1) containing 5 mM glucose, 5% FBS and 5% Nu-serum; the media were changed daily. The cells were subsequently incubated for 48 h with 2 ml of DMEM containing 1% FBS and 1% Nu-serum, 0.28 μmol/l of ascorbic acid, 0.5 mmol/l of 1-proline, 0.2 mmol/l of β-amino propionitrile, and either 5, 10, 20, 30 mM of glucose. The effect of phlorizin on collagen synthesis and glucose consumption was determined by adding 0.2 mmol/l of phlorizin to the same media with 5 or 30 mM of glucose. The media recovered from the second 24 h were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [24], using an ATTO apparatus (ATTO, Tokyo, Japan); the non-gradient 7% acryl amide separating gel overlaid by 3.5% stacking gel was used to determine type IV and type VI collagen synthesis. The proteins were transferred to a nitrocellulose membrane using a Bio-Rad Transblot Cell (both supplied by Bio-Rad Laboratories, Hercules, CA). The membrane was washed with PBS containing 0.5% Tween-20, and then treated with polyclonal antibodies against bovine type IV and type VI collagen (both supplied by LSL, Tokyo, Japan) (1:300 dilution) followed by treatment with peroxidase-labeled protein A (Amersham, Arlington, IL). The antigen–Ig–G– protein A complexes were visualized by ECL Western blotting detection reagents (Amersham) using X-omatic AR film (Eastman–Kodak, Rochester, NY). Densitometric analysis was performed using two-dimensional scanning with an EPSON image scanner GT8000 (Epson, Tokyo, Japan) on a Macintosh Quadra 800 computer using the public domain NIH image program (written by Wayne Rasband at the US National Institute of Health).

2.4. Other determinations

The glucose concentrations of the media were determined by the glucose oxidase method using a Beckmann glucose autoanalyzer 2 (Beckman, Fullerton, CA). The DNA content of each well was determined by the Hoechst 2235 dye (Sigma) method using a TKO 100 (Hoeffer, San Francisco, CA) and calf thymus DNA (Clontech Laboratories, Palo Alto, CA) as a standard. The data were expressed as rela-

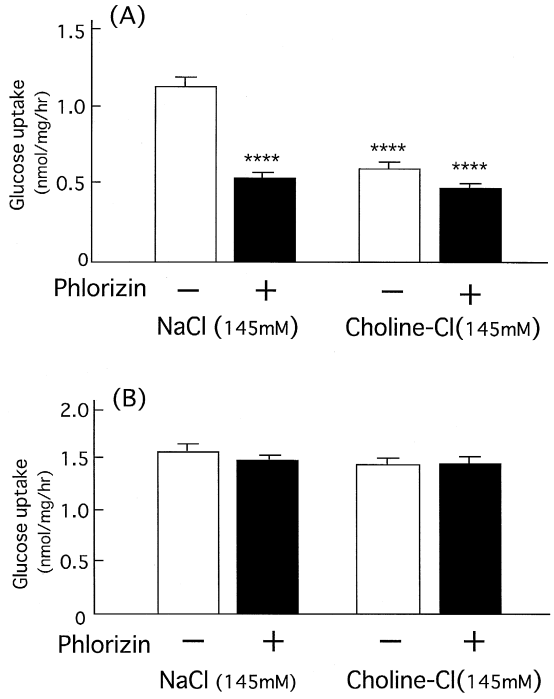

Fig. 1. Effect of phlorizin on glucose uptake by retinal pericytes (A) and retinal endothelial cells (B) in the presence and absence of sodium. The cells were incubated with [2-^{3}H]-D-glucose (0.1 mM, 5 μCi/ml) in the presence of 145 mM of NaCl or 145 mM of Choline-Cl with (+) or without (−) 0.2 mM of phlorizin. Glucose uptake is expressed as nmol/mg cell protein/h. □ without phlorizin; ■: with phlorizin; ＊＊＊＊: $p < 0.001$ vs. NaCl without phlorizin.

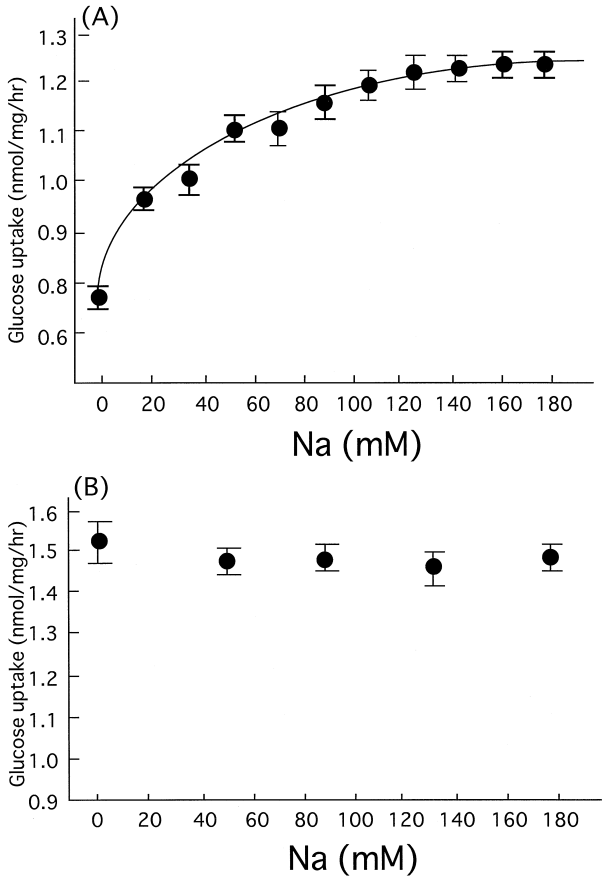

Fig. 2. Effect of sodium on glucose uptake by retinal pericytes and retinal endothelial cells. The cells were incubated with [2-^{3}H]-D-glucose (0.1 mM, 5 μCi/ml) at different NaCl concentrations up to 180 mM with substitution of 180 mM of Choline-Cl to maintain constant osmolarity. (A) Glucose uptake by retinal pericytes was increased with increasing sodium concentrations. The r^2 value for a logarithmic regression curve of the data was 0.93 ($p < 0.001$). Half-maximal stimulation occurred at 48 mM of sodium. (B) Glucose uptake by retinal endothelial cells did not change at different sodium concentrations.

tive density compared with 5 mM of glucose adjusted for DNA. Neither type IV nor type VI collagen was detected by Western blot in the media containing 1% FBS and 1% Nu-serum without cells.

2.5. Statistical analysis

Student's *t*-test (two-tailed) and analysis of variance (ANOVA) were used for statistical determination. For the kinetics of glucose uptake, a logarithmic regression analysis was used. Data were expressed as the mean ± SEM.

3. Results

Fig. 1 shows D-glucose uptake by retinal pericytes and retinal endothelial cells at 0.1 mM glucose in the presence (NaCl) and absence (choline-Cl) of sodium. Glucose uptake by retinal pericytes (Fig. 1(A)) was 1.18 ± 0.07 nmol/mg/h and was significantly reduced to 0.61 ± 0.04 nmol/mg/h in the presence of phlorizin in the medium containing sodium. Glucose uptake in the absence of sodium was 0.67 ± 0.07 nmol/mg/h and was reduced to 0.58 ± 0.02 nmol/mg/h after phlorizin was added; neither value was significantly different from those observed in the presence of sodium and phlorizin. In contrast, glucose uptake by bovine retinal endothelial cells (Fig. 1(B)) was comparable in the presence and absence of sodium (1.58 ± 0.05 and 1.37 ± 0.06 nmol/mg/h, respectively) and was not changed by the addition of phlorizin (1.46 ± 0.06 and 1.38 ± 0.08 nmol/mg/h, respectively).

Fig. 2shows sodium-dependent glucose uptake by retinal pericytes and retinal endothelial cells. Glucose

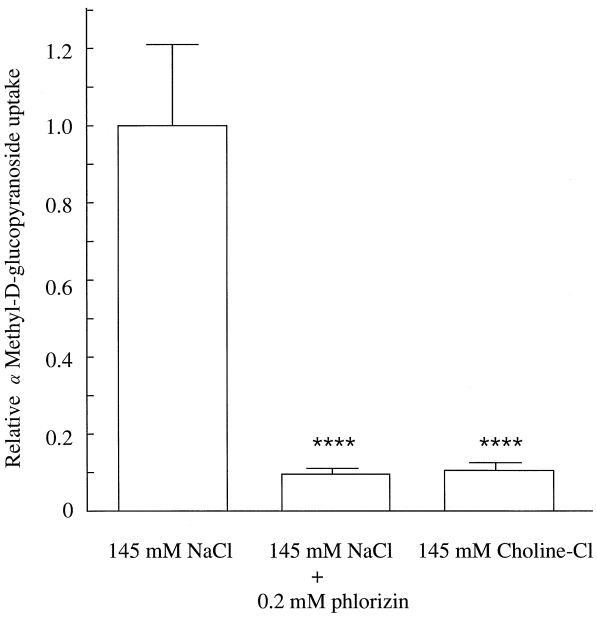

Fig. 3. α-Methyl-D-glucoside (AMG) uptake by retinal pericytes. The cells were incubated with [^{14}C]-α-methyl-D-glucoside (AMG)(0.1 mM, 2 μCi/ml) in the presence of 145 mM of NaCl with or without 0.2 mM of phlorizin or 145 mM of choline chloride. AMG uptake in 145 mM NaCl was 10-fold higher than that in 145 mM Choline-Cl, and was decreased to the level observed in 145 mM Choline-Cl by the addition of phlorizin. ****: $p < 0.001$ vs. NaCl without phlorizin.

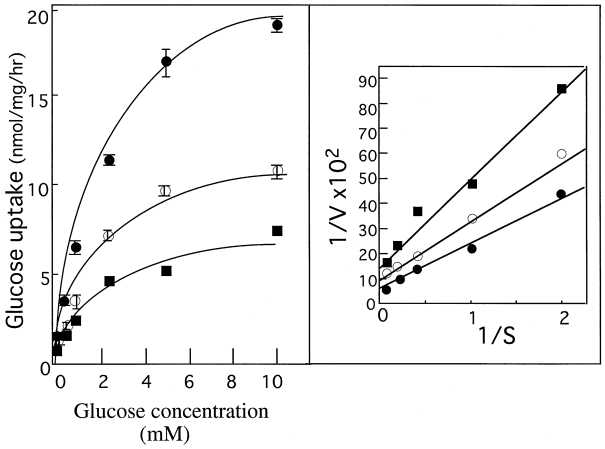

Fig. 4. Kinetics of glucose uptake by retinal pericytes in the presence and absence of sodium. The cells were incubated with [2-^{3}H]-D-glucose (0.1 mM, 5 μCi/ml) at different glucose concentrations ranging from 0.5 to 10 mM. Glucose uptake at 145 mM of NaCl (●) and 145 mM of Choline-Cl (○) is expressed as nmol/mg cell protein/h. Sodium-dependent glucose uptake (■) was calculated as the net increase in glucose uptake (glucose uptake in NaCl − glucose uptake in Choline-Cl). The inset shows the Lineweaver–Burk plot of the data. The r^2 values for logarithmic regression curve of the data from glucose uptake at 145 mM of NaCl or 145 mM of Choline-Cl and sodium-dependent glucose uptake were 0.94 ($p < 0.001$), 0.93 ($p < 0.001$), and 0.91 ($p < 0.001$), respectively.

uptake by retinal pericytes increased with increasing sodium concentrations of up to 180 mM; the half-maximal stimulation occurred at 48 mM sodium ion (Fig. 2(A)). In contrast, glucose uptake by retinal endothelial cells did not change at different sodium concentrations (Fig. 2(B)).

Fig. 3 shows α-methyl-D-glucoside (AMG) uptake by retinal pericytes in 145 mM NaCl in the presence and absence of 0.2 mM phlorizin or in 145 mM Choline-Cl. AMG uptake in 145 mM NaCl was 10-fold higher than in 145 mM Choline-Cl, and was decreased to the level observed in 145 mM Choline-Cl by the addition of phlorizin.

Effects of sodium ions on glucose uptake by retinal pericytes at different glucose concentrations with and without sodium are shown in Fig. 4. Glucose uptake increased with increasing glucose concentrations. The K_m values determined from Lineweaver–Burk plots in the presence and absence of sodium were 2.54 and 2.39 mM, respectively, while the V_{max} values were 19.30 and 11.85 nmol/mg/h, respectively. The K_m and V_{max} values of the calculated sodium-dependent glucose uptake (net increase of glucose uptake between the presence or the absence of sodium [15]) were 7.68 nmol/mg/h and 2.84 mM, respectively.

Effect of inhibitors on D-glucose uptake by retinal pericytes in 145 mM Choline-Cl and 145 mM NaCl with 0.1 mM glucose are summarized in Table 1. D-Glucose uptake was decreased both in the presence and absence of sodium ($p < 0.001$, respectively) by the additions of D-glucose of 0.5 and 5 mM. Both 0.5

Table 1
Effect of inhibitors on D-glucose uptake by bovine retinal pericytes in the presence (145 mM NaCl) or absence (145 mM Choline-Cl)of sodium

Inhibitor	*n*	Relatvive uptake		
		Choline-Cl	NaCl	Sodium dependent
None	4	1.00 ± 0.07	2.26 ± 0.06	1.26 (100%)
0.5 mM D-glucose	4	0.67 ± 0.02	1.26 ± 0.10	0.59 (47%)
5 mM D-glucose	4	0.37 ± 0.01	0.51 ± 0.08	0.14 (11%)
0.5 mM α-methyl-D-glucoside	3	0.99 ± 0.12	1.94 ± 0.19	0.95 (75%)
5 mM α-methyl-D-glucoside	3	0.98 ± 0.14	1.76 ± 0.08	0.78 (62%)
0.5 mM D-galactose	3	0.96 ± 0.07	2.15 ± 0.27	1.19 (94%)
5 mM D-galactose	3	0.90 ± 0.09	1.98 ± 0.10	1.08 (86%)
5 mM 2-deoxy-glucose	3	0.77 ± 0.09	1.91 ± 0.09	1.14 (90%)
0.2 mM phlorizon	4	0.95 ± 0.16	1.08 ± 0.05	0.13 (10%)

The cells were incubated in Tris/HEPES buffer containing 1.0 mM $CaCl_2$, 5 mM KCl, 2.5 mM $MgSO_4$, 0.1 mM D-glucose (5 μCi/ml of [2-^{3}H-]D-glucose) and 145 mM NaCl or 145 mM Choline-Cl with inhibitors for 30 min.
The data were normalized to the uptake observed in Choline-Cl in the absence of inhibitors. Sodium-dependent D-glucose uptake was estimated by the calculation that the uptake in NaCl minus that in Choline-Cl.

and 5 mM AMG inhibited glucose uptake only in the presence of sodium ($p < 0.0005$, $p < 0.001$, respectively). Inhibitory effects of D-galactose and 2-deoxy glucose (2DOG) on D-glucose uptake were also observed in the presence and absence of sodium. Five mM D-galactose and 2DOG inhibited D-glucose uptake to the level with 0.5 mM AMG in the presence of sodium. Among these inhibitors, phlorizin and D-glucose were most effective on the calculated sodium-dependent glucose uptake followed by AMG. The inhibitory effect of D-galactose and 2DOG was less.

Fig. 5 shows immunoblots for type IV and type VI collagen from the medium cultured with retinal pericytes. Immunoblots for type IV collagen demonstrated a single band (α1; M_r 200 kD) and those for type VI collagen demonstrated 2 bands (α3: M_r 205 kD; and α1, α2: M_r 150 kD).

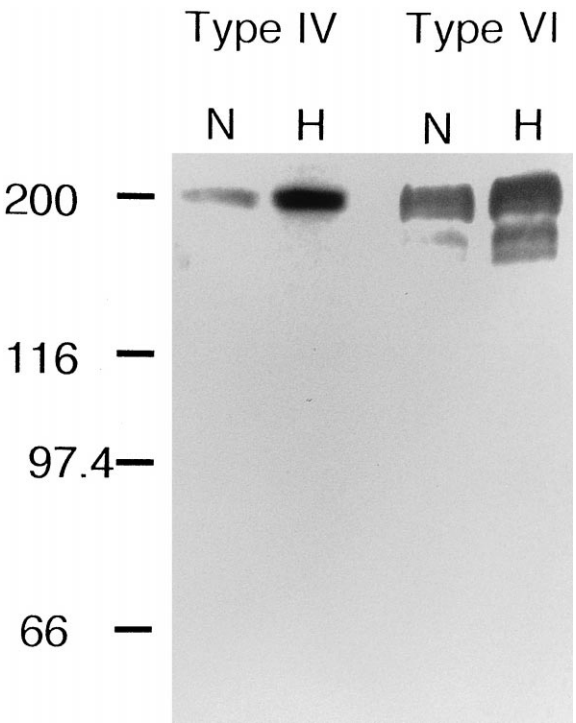

Fig. 5. Immunoblots for type IV and type VI collagen from the medium cultured with retinal pericytes. The cells were incubated with DMEM–F12 (1:1) containing 1% FBS and 1% Nu-serum with ascorbic acid, β-aminopropionitril, l-proline and 5 or 30 mM of glucose in the presence or absence of 0.2 mM phlorizin; the medium was changed daily. Media from the second 24 h incubation were used for determination of type IV and type VI collagen. Immunoblots for type IV collagen revealed 1 band (α1; M_r 200 kD) and those for type VI collagen revealed 2 bands (α3; M_r 205 kD and α1, α2; M_r 150 kD). N: 5 mM glucose, H: 30 mM glucose.

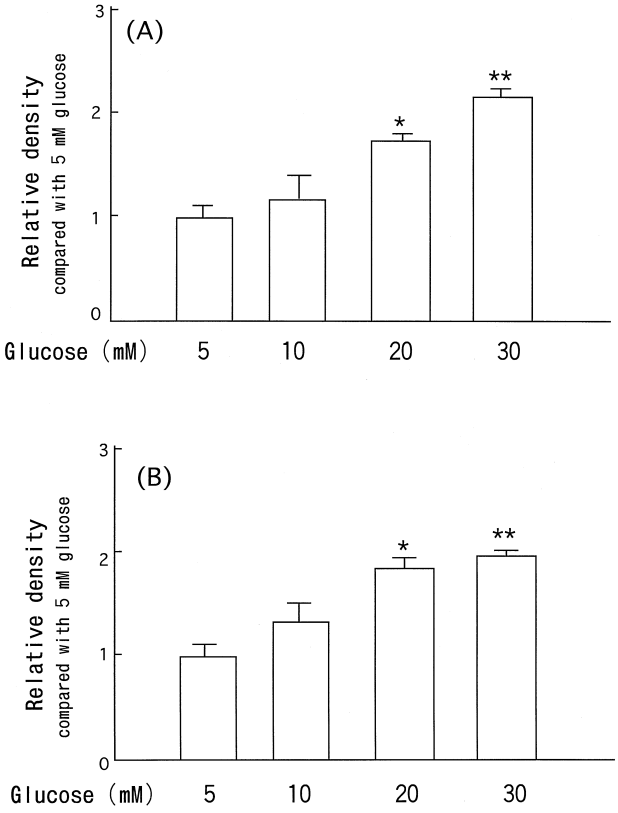

Fig. 6. Effect of different glucose concentration on type IV (A) and type VI (B) collagen synthesis by bovine retinal pericytes. The cells were incubated with DMEM–F12 (1:1) containing 1% FBS and 1% Nu-serum with ascorbic acid, β-aminopropionitril, l-proline, and 5 to 30 mM of glucose; the medium was changed daily. Media from the second 24 h incubation were used for determination of type IV and type VI collagen by immunoblot in quadruplicate. The data are expressed as relative density compared with 5 mM of glucose after correction for the DNA content. *: $p < 0.05$; **: $p < 0.01$ vs. 5 mM of glucose.

Fig. 6 shows type IV (Fig. 6(A)) and type VI collagen synthesis (Fig. 6(B)) by retinal pericytes at different glucose concentrations. Both type IV and type VI collagen were synthesized by retinal pericytes. Collagen synthesis increased significantly with increasing glucose concentrations, to a maximum 2-fold increase at more than 20 mmol/l of glucose.

Fig. 7 shows the effects of phlorizin on net glucose utilization (Fig. 7(A)) and type IV and type VI collagen synthesis (Fig. 7(B) and (C)) by bovine

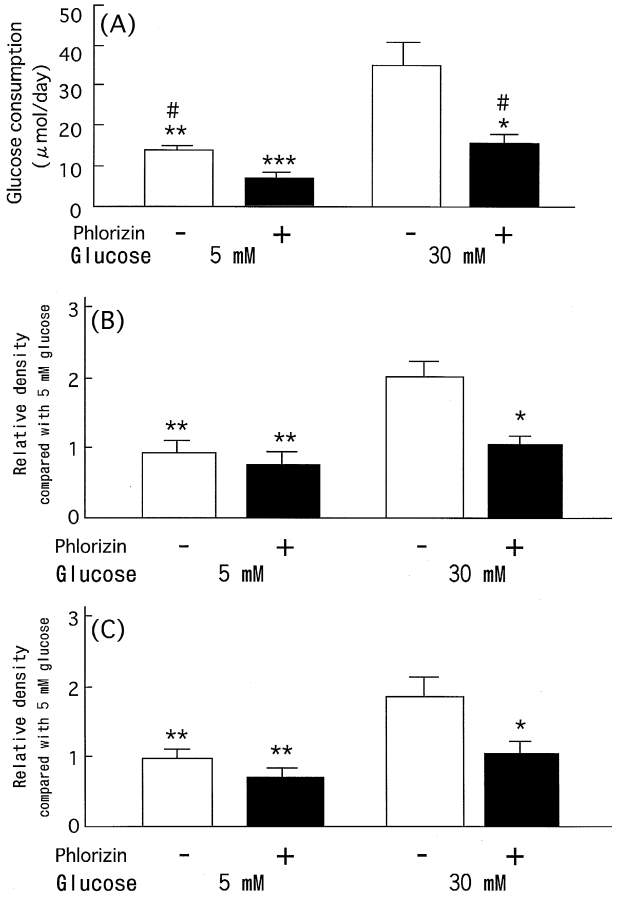

Fig. 7. The effect of phlorizin on glucose consumption (A) and synthesis of type IV (B) and type VI (C) at 5 mM or 30 mM of glucose by bovine retinal pericytes. The cells and media were treated as described in Fig. 4. □: without phlorizin, ■: with phlorizin Glucose consumption is expressed as nmol/100 μg DNA/24 h. ∗: $p < 0.05$; ∗∗: $p < 0.01$; ∗∗∗: $p < 0.001$ vs. 30 mM of glucose; #: $p < 0.05$ vs. 5 mM glucose with phlorizin.

retinal pericytes. Net glucose consumption by retinal pericytes in the media with 30 mM of glucose was significantly higher than with 5 mM of glucose, and was significantly lower with phlorizin than without. Glucose consumption at 5 mM of glucose was comparable to that observed with 30 mM of glucose and phlorizin. Synthesis of type IV collagen and type VI collagen at 30 mM of glucose with phlorizin was comparable to that seen with 5 mM of glucose. Collagen synthesis in the medium containing 5 mM of glucose and phlorizin was not reduced and was comparable to that observed with 5 mM of glucose.

4. Discussion

This study revealed that (1) sodium dependent D-glucose uptake occurs in bovine retinal pericytes; (2) in the presence of sodium and phlorizin, this uptake is suppressed to 50%. These findings does not seem to be derived from the secondary effects of sodium and phlorizin, since neither the sodium dependent uptake nor the inhibitory effect of phlorizin were observed in retinal endothelial cells. These findings resemble previously reported results regarding sodium-dependent glucose uptake by rat mesangial cells [12]. Sodium-dependent AMG uptake into cells via SGLT has also been observed in retinal pericytes and has been suppressed about 90% by the addition of phlorizin, as reported by Loike et al. [25]. The half-maximal sodium concentration for glucose uptake by retinal pericytes was 48 mM, which was nearly the same as that of rat mesangial cells (K_m = 35 mM) [12]. The V_{max} values for sodium-dependent and independent glucose uptake in retinal pericytes (11.9 and 7.68 nmol/mg/h, respectively) were lower than those in mesangial cells (29.5 and 18.0 nmol/mg/h, respectively) [12], which indicates that sodium-dependent and independent glucose uptake occurred at a ratio of nearly 1:1 in both cells.

The sodium-coupled glucose transporter (SGLT), through which glucose entry into the cell is sodium dependent, is known to be localized in the epithelial cells of the small intestine and renal tubules [19–22]. In rat mesangial cells, mRNA for SGLT has been identified together with GLUT 1 and a small amount of GLUT 4 [12]. The presence of SGLT has also been detected in bovine brain cortical vessels by demonstrating SGLT-like protein and gene [26]. To our knowledge, the present study is the first to demonstrate sodium-dependent glucose uptake by retinal pericytes.

At least two sodium-coupled glucose transporters have been reported. One has high-affinity (K_m = 0.3 mM) and relatively low-capacity for D-glucose, and is present in the distal tubules of the kidney and the intestine (SGLT 1). Another has low-affinity (K_m = 2 to 6 mM) and high-capacity, and is present primarily in the proximal tubules of the kidney (SGLT 2) [27,28]. The calculated K_m of sodium-dependent glucose uptake by retinal pericytes was 2.84 mM, which was similar to that of rat mesangial cells

(1.93 mM) [12]. D-Galactose is reported to enter into cells through SGLT 1 but not through SGLT 2 [29,30], while D-galactose enters into cells through GLUT 1 which has a higher K_m value for D-galactose (30 mM) than for D-glucose (2.6 mM) [31]. The inhibitory effect of D-galactose on D-glucose uptake by retinal pericytes in this study was less compared with those of D-glucose and AMG, which is similar to the previous report of D-glucose uptake in proximal tubules of the kidney [32]. These data suggest that SGLT 2 may be present in bovine retinal pericytes.

Immunohistological studies have shown that type I–V and IX collagens are present in the retina [14,15]. Here, we have demonstrated that bovine retinal pericytes produce type VI and type IV collagens as do rat and bovine mesangial cells, bovine glomerular endothelial cells [13,33], rat cardiac endothelial cells [34,35], and rat cardiac pericytes [36]. These collagens increased significantly with high glucose concentrations (more than 20 mM) in the medium. Since an increase of mRNA for type IV collagen is also reported in human diabetic retinopathy [16], overproduction of these collagens by bovine retinal pericytes at high glucose concentrations seems to lead to basement-membrane thickening and microvessel occlusion, which lead in turn to diabetic retinopathy.

Interestingly, addition of phlorizin to the media with high-glucose served to directly attenuate type IV and type VI collagen synthesis by normalizing glucose consumption, although phlorizin is often used to decrease blood glucose levels in animals with streptozotocin-induced diabetes via inhibition of glucose absorption from the small intestine and glucose reabsorption from the kidney [37,38]. As reported previously, glucose consumption by rat mesangial cells under high-glucose conditions increased during 48 h observation, and the collagen synthesis increased with increasing glucose consumption [13]. Recently, Heilig et al. [39] reported that the increased glucose utilization by mesangial cells with overexpressed GLUT 1 induced overproduction of type IV collagen synthesis under normal glucose conditions. These observations suggest that increased glucose entry into the cells may be an important factor for over-production of collagens.

Excessive entry of glucose into the cells under high-glucose conditions increases cellular sorbitol, which is mediated by aldose reductase [7], and protein kinase C (PKC) through de novo synthesis of diacylglycerol [40]. The aldose reductase activities in the retinal pericytes [4,5] and mesangial cells [41] are reported to be one of the important factors in diabetic retinopathy, as well as in nephropathy. Abnormal PKC activity in the diabetic state is found in a variety of vessels. Increased PKC activity induces vascular dysfunction, such as overproduction of extracellular matrices [42]. Although we did not examine intracellular sorbitol contents and PKC activity, the normalization of glucose entry into the retinal pericytes, in which sodium-dependent and independent glucose uptake are functioning at similar levels, under high-glucose conditions by phlorizin may improve increased sorbitol concentrations and PKC activity, and these improvements normalize collagen synthesis even under high-glucose conditions.

In conclusion, sodium-dependent glucose uptake is present in retinal pericytes. Excess entry of glucose into the cell via this glucose transporter may be an important factor for overproduction of type IV and type VI collagen.

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