Sodium-Coupled Glucose Transporter as a Functional Glucose Sensor of Retinal Microvascular Circulation

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Abstract—To clarify the function of the Na\(^+\)-coupled glucose transporter in the regulation of cellular tone of cultured retinal pericytes, we investigated the effects of extracellular glucose concentration on cell size. The surface area and diameter of cultured bovine retinal pericytes under different glucose concentrations were measured by using a light microscope with a digital camera. We also examined the effects of extracellular Na\(^+\) and Ca\(^{2+}\), inhibitors of the Na\(^+\)-coupled glucose transporter and Na\(^+\)-Ca\(^{2+}\) exchanger, a Ca\(^{2+}\) channel blocker, and nonmetabolizable sugars on cell size. The surface area and diameter of the cells changed according to extracellular glucose concentrations. α-Methyl glucoside, which enters the cell through the Na\(^+\)-coupled glucose transporter, induced cellular contraction. However, the cells did not contract in response to 2-deoxyglucose, which enters the cell through a facilitated glucose transporter. Glucose-induced cellular contraction was abolished in the absence of extracellular Na\(^+\) and Ca\(^{2+}\). Moreover, phlorizin, an inhibitor of the Na\(^+\)-coupled glucose transporter, and 2′,4′-dichlorobenzamil-HCl, an inhibitor of the Na\(^+\)-Ca\(^{2+}\) exchanger, also abolished glucose-induced cellular contraction, whereas nicardipine, a Ca\(^{2+}\) channel blocker, did not. Our results indicate that high extracellular glucose concentrations induce contraction of bovine retinal pericytes via Na\(^+\) entry through a Na\(^+\)-coupled glucose transporter, suggesting that the Na\(^+\)-coupled glucose transporter may act as a functional glucose sensor of retinal microvascular circulation. (Circ Res. 2001;88:1183-1188.)

Key Words: pericytes ■ Na\(^+\)-coupled glucose transporter ■ microcirculation ■ Na\(^+\)-Ca\(^{2+}\) exchanger ■ Ca\(^{2+}\) channels

Pericytes are present in almost all capillaries and postcapillary vessels. These cells contain contractile proteins, and previous studies have shown that angiotensin II and endothelin-1 induce contraction of these cells.1,2 Thus, pericytes may play an important role in the regulation of the microvascular circulation, such as blood flow and transluminal filtration, by changing their cell tone.3

The Na\(^+\)-coupled glucose transporter (SGLT) is a membrane protein that transports glucose concomitantly with Na\(^+\).4 We have recently shown the presence of SGLT in cultured bovine retinal pericytes.5,7 The SGLT did not take up D-galactose, which enters the cell through SGLT1. The calculated \(K_m\) value of the transporter in the cells for D-glucose was 2.8 mmol/L, which was much higher than that for SGLT1.4 These findings suggest that SGLT present in bovine retinal pericytes is SGLT2. The ratio of concomitant uptake of glucose and Na\(^+\) in SGLT2 is reported to be 1:1.4 The \(K_m\) value of the SGLT in the cells seems to be sufficiently high for the transporter to act as a sensor of blood glucose levels in humans and animals.

Simultaneous Na\(^+\) transport through the transporter may influence intracellular Na\(^+\). Because Na\(^+\) can modulate the contractility of cells, it is possible that the activity of SGLT has a major influence on the contractility of retinal pericytes in response to changes in extracellular glucose concentrations. The present study was designed to investigate the role of SGLT in the regulation of cultured retinal pericyte contractility during exposure to different concentrations of extracellular glucose.

Materials and Methods

Cell Culture

Bovine retinal pericytes were prepared as reported previously.6 Briefly, bovine eyeballs were cut to remove the retinas. The retinas were then homogenized and filtered through a sieve of 85-μm pore size. The trapped microvessels were incubated at 37°C for 40 minutes in DMEM and nutrient Ham mixture F-12 (DMEM/F-12, 1:1; both from Sigma Chemical Co), containing 200 μg/mL of collagenase, and then filtered through a sieve of 53-μm pore size. After washing 3 times with DMEM/F-12, the cells were incubated at 37°C in an atmosphere of 95% air/5% CO\(_2\) in DMEM/F-12 containing 10% FBS (GIBCO-BRL) and 10% Na-serum (Collaborative Research) with penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma) on plastic plates (NUNC Brand Products). Retinal pericytes were identified on the basis of their morphological characteristics. The cells from the third to fifth passages were used in the present study.

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Cellular Contraction Experiment
Changes in the tonus of bovine retinal pericytes were examined by measuring two different parameters of the cells, ie, surface area and diameter. The cellular surface area was measured by using cells cultured on 6-well plates. The cells were preincubated in DMEM/F-12 with 5 mmol/L glucose containing 1% FBS and 1% Nu-serum for 1 hour at 37°C in a 95% air/5% CO2 gas mixture. The cell diameter was measured by using the cells treated with trypsin. Cultured pericytes were treated with PBS containing 0.25% trypsin and washed with PBS. The treated cells were collected and centrifuged in 10-mL polystyrene tubes and then washed with DMEM/F-12. This was followed by preincubation with DMEM/F-12 containing 5 mmol/L glucose and 1% FBS and 1% Nu-serum for 1 hour at 37°C in a 95% air/5% CO2 gas mixture. Cells were examined by a light microscope equipped with a digital camera (Nikon). Serial changes in the surface area and diameter were calculated by using NIH image software (written by W. Rasband). More than 30 cells and >100 cells were used for determination of surface area and diameter, respectively.

To determine the effect of Na+, cells were incubated in 20 mmol/L Tris/HEPES (pH 7.4) buffer containing 5 mmol/L KCl, 2.5 mmol/L MgSO4, and 1 mmol/L CaCl2 with different concentrations of glucose (2.5, 5, 10, 20, and 30 mmol/L) in the presence (145 mmol/L NaCl) or absence (145 mmol/L choline chloride) of Na+ after they were washed 3 times with the same buffer. To determine the effect of extracellular Ca2+ ion, cells were incubated in 20 mmol/L Tris/HEPES (pH 7.4) buffer containing 5 mmol/L KCl, 2.5 mmol/L MgSO4, and 1 mmol/L CaCl2-free EGTA. An SGLT inhibitor, phlorizin (10−4, 10−5, 10−6, and 10−7 mol/L, Sigma), a Na+−Ca2+ exchanger inhibitor, 50 μmol/L 2,4-dichlorobenzenzilam-HCl or 50 μmol/L benzamil-HCl (Molecular Probes), and a voltage-sensitive Ca2+ channel, 100 nmol/L nicardipine were also added to 20 mmol/L Tris/HEPES buffer (pH 7.4) containing 1 mmol/L CaCl2, 5 mmol/L KCl, and 2.5 mmol/L MgSO4 with 5 mmol/L or 30 mmol/L glucose in the presence of Na+. We also tested the effects of nonmetabolizable sugars, 25 mmol/L α-methyl glucoside (AMG) and 25 mmol/L 2-deoxy-glucose (2DOG), and angiotensin II (Bachem) on the contraction of retinal pericytes.

Glucose Uptake by Retinal Pericytes
Confluent retinal pericytes on 12-well plates (Nunc) were used to determine D-glucose uptake. The cells were preincubated with DMEM/F-12 and then incubated with glucose or 5 mmol/L glucose combined with 25 mmol/L mannitol. Higher glucose concentrations in the buffer decreased the cellular surface area in a concentration-dependent manner, whereas 2.5 mmol/L glucose increased the surface area.

Effects of Glucose and Angiotensin II on Cell Diameter
Figure 2 shows changes in the diameter of bovine retinal pericytes in the presence of different concentrations of glucose. The cellular diameter remained stable in the presence of 5 mmol/L glucose or 5 mmol/L glucose combined with 25 mmol/L mannitol.

Statistical Analysis
All data were expressed as mean±SD. Differences between groups examined by the cells were measured by using ANOVA and the Welch t test (2-tailed). A value of P<0.05 denoted the presence of a statistically significant difference.

Results
Effects of Angiotensin II and Glucose on the Surface Area of Pericytes
Figure 1A shows the effects of angiotensin II on contraction of bovine retinal pericytes. At >1 μmol/L, angiotensin II significantly reduced the surface area of the cells at 30 and 60 minutes in a dose-dependent manner. Figure 1B shows the serial changes in surface area of bovine retinal pericytes in the presence of different glucose concentrations. The surface area of the cells did not change in response to either 5 mmol/L glucose or 5 mmol/L glucose combined with 25 mmol/L mannitol. Higher glucose concentrations in the buffer decreased the cellular surface area in a concentration-dependent manner, whereas 2.5 mmol/L glucose increased the surface area.
ence of 5 mmol/L glucose for up to 30 minutes. Changes in the cell diameter in response to extracellular glucose were similar to those in the cell surface area. However, the cell diameter decreased significantly in the presence of 5 mmol/L glucose with 25 mmol/L D-mannitol, which was similar to that in 10 mmol/L glucose. Changes in the cell diameter were maximal at 10 minutes under all experimental conditions and were constant up to 30 minutes (Figure 2A). Changes in the cell diameter began at the initiation of exposure, and maximum response was noted at 8 minutes after exposure to 0.5 and 30 mmol/L (Figure 2B). The mean diameter of pericytes exposed to 30 mmol/L glucose remained small for at least 24 hours, returned to the basal level after 2 days, and significantly increased after 4 days compared with that in 5 mmol/L (data not shown). Angiotensin II also decreased the cell diameter (data are not shown).

Effects of Na$^+$ and Ca$^{2+}$ on Contraction of Pericytes
The effects of high glucose concentrations on the cell surface area (Figure 3A) and diameter (Figure 3B) were observed only in the presence of both Na$^+$ and Ca$^{2+}$. However, the lack of either extracellular Na$^+$ or Ca$^{2+}$ did not reduce the cell surface area and diameter even in the presence of high glucose concentrations. The effects of nonmetabolizable sugars, AMG (which enters the cell through SGLT) and 2DOG (which enters through facilitated glucose transporter), on the cell surface area (Figure 4A) and diameter (Figure 4B) were tested. Both the cell surface area and diameter decreased in the presence of AMG, but not 2DOG. High glucose–induced cellular contraction was abolished by the addition of an inhibitor of SGLT, phlorizin, at concentrations $>10^{-7}$ mol/L (Figures 5A and 5B). Benzamil-HCl and 2',4'-dichlorobenzamil-HCl, inhibitors of the Na$^+$.Ca$^{2+}$ exchanger, also inhibited the contractile responses (Figures 6A and 6B). We also examined the contribution of the voltage-dependent Ca$^{2+}$ channel to the high glucose–induced cell contraction (Figure 7). Nicardipine, a Ca$^{2+}$ channel blocker, did not inhibit the contraction, whereas nicardipine inhibited angiotensin II–induced cell contraction.
Correlation Between Glucose Uptake and Cell Tonicity

The relationship between cellular tonicity and extracellular glucose concentration followed a Michaelis-Menten equation and was a mirror image of the relationship between the calculated glucose uptake through SGLT and extracellular glucose concentration (Figure 8).

Discussion

To maintain a stable glucose supply to peripheral tissues, peripheral blood flow should increase in response to a fall in blood glucose levels. On the other hand, an excessive supply of glucose in the tissues may be harmful to peripheral cells when the glucose level increases, because high glucose overload is thought to exert toxic effects on cells.9–12 Regulation of cellular tone of pericytes by extracellular glucose concentration, with cells contracting at high glucose levels and relaxing at low glucose levels, may be beneficial to maintain a stable glucose supply to peripheral tissues. Such a type of metabolic regulation of retinal pericyte contraction has also been reported. Chen and Anderson13 have suggested that CO₂ regulates the cell tone and may control retinal blood flow in response to local metabolic conditions.

Cellular contraction had been studied previously by measuring the decrease in the cell surface area or increase in wrinkling of the silicon membrane around cells.14,15 The present study demonstrated glucose concentration-dependent changes in the cell diameter and surface area. Because angiotensin II also decreased these parameters, the extracellular glucose concentration seems to regulate the tone of pericytes. Our results showed that the concentration of angiotensin II necessary to induce cellular contraction in retinal pericytes was 100 μmol/L to 1 μmol/L, which is higher than that required to induce contraction of smooth muscle cells and mesangial cells.2,16,17 Changes in the cell surface area in response to changes in extracellular glucose concentration were slow compared with changes affecting the cell diameter, which began on application and terminated within 8 minutes. This difference may be due to the contact of cells with the walls of plastic plates. The response of pericytes overlying endothelial cells to glucose may be fast in microvessels. Although the cell diameter decreased after the addition of 25 mmol/L mannitol combined with 5 mmol/L glucose, the surface area of the cells did not respond to the same condition. Thus, changes in the cell diameter may be...
exaggerated because of the effect of osmotic pressure. Glucose regulation of the cell tone observed in the present study was also dependent on the presence of extracellular Na\(^+\) ion. Combined together, these results suggest that the simultaneous entry of Na\(^+\) and glucose into the cells seems to be important for the glucose-dependent regulation of cellular tone.

We previously reported the presence of SGLT in cultured bovine retinal pericytes. Interestingly, phlorizin abolished the high glucose–induced cellular contraction in our experiment. AMG, which enters the cells mainly through SGLT, induced cell contraction. On the other hand, 2DOG, which enters the cells through facilitated glucose transporters, did not induce cell contraction. Although increased ATP production is reported to induce cell contraction\(^{19}\) and although more glucose entry at higher glucose concentrations may induce ATP production, this mechanism cannot explain the high glucose–induced cellular contraction, because AMG is a nonmetabolizable sugar.\(^{19,20}\) Moreover, the responses of both the calculated glucose uptake through SGLT and cell tone to extracellular glucose levels were mirror images. These findings suggest that Na\(^+\) entry with glucose through SGLT, according to extracellular glucose levels, is essential for the glucose regulation of cellular tone.

The high glucose–induced cellular contraction was also abolished in the absence of extracellular Ca\(^{2+}\) ion. Ca\(^{2+}\) entry into cells is important for cellular tone in contractile cells. Although Ca\(^{2+}\) entry occurs mainly through Ca\(^{2+}\) channels, some enters the cell through the Na\(^+\)-Ca\(^{2+}\) exchanger.\(^{21,22}\) The Na\(^+\)-Ca\(^{2+}\) exchanger has 2 modes of Ca\(^{2+}\) to Na\(^+\) exchange, namely, the Ca\(^{2+}\) exit mode and the Ca\(^{2+}\) entry mode, depending on Na\(^+\) and Ca\(^{2+}\) concentration gradients, and its exchange ratio of Na\(^+\) to Ca\(^{2+}\) is almost 3:1.\(^{22}\) The Na\(^+\)-Ca\(^{2+}\) exchanger is also known to contribute to the contraction of cardiomyocytes and smooth muscle cells.\(^{23–25}\) Our results showed that the high glucose–induced contraction of retinal pericytes was abrogated by Na\(^+\)-Ca\(^{2+}\) exchanger inhibitors, benzamil-HCl and 2',4'-dichlorobenzamil-HCl. However, nicardipine, a Ca\(^{2+}\) channel blocker, did not inhibit the high glucose–induced contraction of pericytes. Ca\(^{2+}\) channel blockers are also known to inhibit the Na\(^+\)-Ca\(^{2+}\) exchanger, whereas their inhibitory effect is very weak.\(^{26}\) On the basis of these observations, it seems that glucose regulation of cellular tone is independent of the Ca\(^{2+}\) channel. Thus, action of the Ca\(^{2+}\) entry mode through the Na\(^+\)-Ca\(^{2+}\) exchanger, depending on intracellular Na\(^+\), which is influenced by Na\(^+\) entry through SGLT and extracellular glucose levels, seems to regulate cellular tone.

Activation of protein kinase C (PKC) is known to play an important role in cellular contraction.\(^{27}\) High glucose levels activate PKC via de novo synthesis of diacylglycerol.\(^{28}\) Previous studies have shown reduced retinal blood flow rates in diabetic rats and have indicated that treatment by a PKC inhibitor or a diacylglycerol kinase inhibitor improves flow rates.\(^{29}\) However, this PKC-related mechanism is unlikely to be involved in the cell contraction noted in the present study, inasmuch as a nonmetabolizable sugar, AMG, also induced cellular contraction.

It is well known that the contractile response of pericytes is reduced under long-term high glucose conditions.\(^{30,31}\) We also reported swelling of cultured retinal pericytes that were incubated in 30 mmol/L glucose for 7 days, and we noted that this response was attenuated by the addition of phlorizin.\(^{6}\) On the basis of these findings, we speculate on the presence of abnormal intracellular Na\(^+\) metabolism via SGLT under high glucose conditions. The reduced contractile response under long-term high glucose conditions may also be derived from high glucose–induced abnormal intracellular Na\(^+\) metabolism. In such conditions, SGLT of cultured bovine retinal pericytes may act as a water channel and significantly influence cell volume, as reported for SGLT1.\(^{19}\) On the other hand, the loss of pericytes in the early stages of diabetic retinopathy is an important pathological process.\(^{32,33}\) The pathological implications of abnormal pericyte function under hyperglycemic conditions on the microcirculation in diabetic retinopathy remain unknown. Further experiments are necessary to clarify the pathological mechanisms of pericyte contraction under high glucose levels.

In conclusion, we have demonstrated in the present study that the cellular tone of bovine retinal pericytes is regulated by extracellular glucose concentration, and this regulation depends on Na\(^+\) ion entry through SGLT. Thus, SGLT may act as a functional glucose sensor of retinal microvascular circulation.

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