High Glucose Enhances Transient Receptor Potential Channel Canonical Type 6–Dependent Calcium Influx in Human Platelets via Phosphatidylinositol 3-Kinase–Dependent Pathway

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Background—Transient receptor potential canonical type 6 (TRPC6) channels mediating 1-oleoyl-2-acetyl-sn-glycerol (OAG)–induced calcium entry have been identified on human platelets. In the present study we tested the hypothesis that hyperglycemia increases the expression of TRPC6 channels.

Methods and Results—Platelets from healthy control subjects and patients with type 2 diabetes mellitus were incubated with glucose and calcium influx was measured using the fluorescent dye technique. TRPC channel protein expression was investigated using immunofluorescence and fluorescence microscopy of single platelets. Administration of 25 mmol/L glucose significantly enhanced the OAG-induced calcium influx, which was attenuated by inhibitors of the phosphatidylinositol 3-kinase, wortmannin or LY294002. The glucose-enhanced and OAG-induced calcium influx was concentration- and time-dependent. Glucose significantly increased the TRPC6 protein expression in platelets to 131 ± 12% (n=33; P<0.05), whereas the expression of TRPC1, TRPC3, TRPC4, or TRPC5 were unchanged. The glucose-induced TRPC6 expression was significantly attenuated in the presence of wortmannin or LY294002. Platelets from patients with type 2 diabetes mellitus showed increased TRPC6 expression compared to nondiabetic individuals (P<0.05).

Conclusion—The study indicates that high glucose increases TRPC6 channel protein expression on the platelet surface which is mediated by a phosphatidylinositol 3-kinase–dependent pathway. (Arterioscler Thromb Vasc Biol. 2008;28:746-751)

Key Words: platelet ▪ transient receptor potential channel canonical type 6 ▪ signal transduction ▪ diabetes ▪ phosphatidylinositol 3-kinase

Cardiovascular complications including microangiopathy, retinopathy, neuropathy, nephropathy, and macroangiopathy are a frequent cause of morbidity and mortality in patients with diabetes mellitus. It has been proposed that platelets contribute to diabetic microangiopathy and macroangiopathy.1,2 Platelets are an integral component of hemostasis and contribute to the events leading to thrombosis and atherosclerosis. Platelets in patients with type 2 diabetes mellitus adhere to vascular endothelium and aggregate more readily than those in healthy people. Complete activation of platelets by stimulatory agents leads to an increase of cytosolic calcium levels, which triggers many intracellular signaling processes important for the expression of functional responses.3 Recently, transient receptor potential canonical (TRPC) channels have been identified on human platelets.4,5 TRPC channels are nonselective cation channels mediating calcium influx including calcium influx into platelets. Recent studies suggest that TRPC6 can be activated by the diacylglycerol.6–8 For in vitro studies the membrane-permeant diacylglycerol-analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) is often used to stimulate cation entry.6–8

In the present study we tested the hypothesis that hyperglycemia increases the expression of TRPC6 channels. We observed a time- and concentration-dependent increase of TRPC6 channel expression on the platelet surface after administration of high glucose, which was accompanied by an increased OAG-induced calcium influx. The glucose-induced TRPC6 channel expression and the glucose-enhanced OAG-induced calcium influx in platelets could be blocked by inhibition of phosphatidylinositol 3-kinase. The study indicates that high glucose is associated with increased TRPC6 channel expression which is mediated by a phosphatidylinositol 3-kinase–dependent pathway.
Methods

Platelet Preparation and Fluorescence Measurements

Platelets were obtained from healthy control subjects or patients with type 2 diabetes mellitus free from intercurrent illness. All donors gave informed consent. The study was approved by the local ethics committee. Subjects did not take antiplatelet drugs including aspirin for at least 2 weeks. 20 mL blood was anticoagulated with 3.2% trisodium citrate according to previously described methods. Blood was centrifuged at 240 x g for 10 minutes to obtain platelet rich plasma, which was centrifuged at 240 g for 15 minutes. Platelets were resuspended in Hanks balanced salt solution containing (in mmol/L) NaCl 136, KCl 5.40, CaCl2 1, KH2PO4 0.44, Na2HPO4 0.34, 2-ethanesulfonic acid 10, pH 7.4 and incubated with 1 µmol/L of the fluorescent dye 1-[2-(5-Carboxyoxazol-2-yl)-6-aminoenobenzofuran-5-oxo]-2-(2’-amino-5’-methylphenoxy)-ethane-N,N,N’-tetracetic acid acetoxymester (fura2-AM; Merck Biosciences) for 60 minutes. After centrifugation at 240 g for 15 minutes to remove extraneous dye, the platelet pellet was again resuspended in Hanks balanced salt solution. The platelet counts were adjusted to 1 x 10^9 platelets/mL. To exclude that the isolation procedure (including centrifugation and washing procedures) affects platelet action we performed additional experiments where platelets were isolated in the presence of 25 mmol/L glucose. Then platelets were exposed to 5.5 mmol/L glucose or 25 mmol/L glucose throughout all subsequent steps (including loading with fura 2-AM).

Fluorescence measurements were undertaken as previously described by our group with a 96-well fluorescent plate reader (Fluoroskan Ascent Fluorometer, Thermo LabSystems Oy) at 510 nm emission with excitation wavelengths of 340 nm and 380 nm, and the fluorescence ratio 340 nm/380 nm was calculated. Fluorescence measurements were performed after platelets had been incubated for 60 minutes with buffer for control, or glucose (25 mmol/L; final concentration) in the absence or presence of inhibitors of phosphatidylinositol 3-kinases, ie, wortmannin (final concentration 20 µmol/L) or 2-(4-Morpholino)-8-phenyl-4H-1-benzoazepan-4-one (LY294002, final concentration 10 µmol/L). Increasing concentrations of the cell-permeable diacylglycerol analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG), were used to activate the platelets. The sustained calcium increase after administration of OAG was determined. Some experiments were conducted in the presence of an inhibitor of TRP channels, 1-[(3-4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF-96365; Merck Biosciences; final concentration, 10 µmol/L) or in the presence of the L-type channel calcium blocker, 2-[(2-Aminoethoxy)-methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridined-carboxylic acid-3-ethyl-5-methyl-ester (amlodipine; Sigma-Aldrich; final concentration, 10 µmol/L).

Platelet expression of P-selectin was measured as previously described after platelets had been incubated for 60 minutes with buffer for control or 25 mmol/L glucose. After fixation in paraformaldehyde, platelets were washed and incubated with mouse IgG (nonspecific binding) and a monoclonal antibody against P-selectin (both from Coulter Immunotech), followed by a secondary fluorescent antibody (FITC)-labeled antibody (Sigma-Aldrich). Flow cytometry was thereafter performed (FACScan; Becton Dickinson). Data are given as the median immunofluorescence intensity (arbitrary units) after subtraction of the nonspecific mouse IgG binding.

Immunofluorescence of TRP Channels

For the identification of TRPC channels, quantitative immunofluorescence assays of human platelets were performed using the Odyssey infrared imaging system (Liceron Biosciences). Human platelets in 96-well plates were incubated with rabbit anti-human TRPC1, TRPC3, TRPC4, TRPC5, or TRPC6-antibodies (1:1000, Alomone Labs, Jerusalem, Israel) for 15 minutes to remove extraneous dye, the platelet pellet was again resuspended in Hanks balanced salt solution. The platelet counts were adjusted to 1 x 10^9 platelets/mL. After fixation in paraformaldehyde, platelets were washed and incubated with mouse IgG (nonspecific binding) and a monoclonal antibody against P-selectin (both from Coulter Immunotech), followed by a secondary fluorescent antibody (FITC)-labeled antibody (Sigma-Aldrich). Flow cytometry was thereafter performed (FACScan; Becton Dickinson). Data are given as the median immunofluorescence intensity (arbitrary units) after subtraction of the nonspecific mouse IgG binding.

Statistics

All data are presented as mean ± SEM of at least 3 to 10 independent experiments, and were compared using a 2-tailed Student’s t test or ANOVA as appropriate. A 2-sided probability value less than 0.05 was used to indicate statistical significance.

Results

In the present study we measured the effects of glucose on TRPC channel expression and furthermore investigated OAG-induced calcium influx (ie, channel activation by OAG) in platelets.

Glucose Enhances the 1-oleoyl-2-acetyl-sn-glycerol (OAG)-Induced Calcium Influx

Cytosolic calcium was measured in fura2-loaded platelets that had been incubated in the absence and presence of 25 mmol/L glucose for 60 minutes (Figure 1A). Resting cytosolic calcium in platelets was not significantly different after incubation in the absence or presence of 25 mmol/L glucose (F340 nm/F380 nm fluorescence ratio, 1.8 ± 0.1 U

Figure 1. 1-oleoyl-2-acetyl-sn-glycerol (OAG)-induced calcium influx in platelets treated with 25 mmol/L glucose (filled circles or bars) or control buffer (open circles or bars) under control conditions (A), in the presence of amlodipine (Amlo; B), or SKF-96365 (SKF; C). Dose-response curve for glucose (D) and for OAG (E). *P < 0.05.

Monoclonal anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000; Serotec, Düsseldorf, Germany) antibodies were used as internal reference after permeabilization. After incubation with Alexa Fluor680-allophycocyanin-fluorescence-labeled goat anti-rat antibodies (1:1000, MoBiTec, Göttingen, Germany) imaging was performed at 700 nm emission with an excitation wavelength of 680 nm. TRPC6 expression was analyzed under control conditions and after administration of 25 mmol/L glucose. To exclude osmotic effects platelets were also exposed to 25 mmol/L urea or 25 mmol/L taurine.

Fluorescence staining of platelets from healthy subjects was performed with specific TRPC6 antibodies and fluorescent dye–labeled secondary antibodies and analyzed with a TE2000 Nikon microscopy. The fluorescence intensities obtained from measurements of single platelets were averaged.

All substances were obtained from Sigma-Aldrich or Merck Biosciences if not indicated otherwise.
[n=37] versus 1.7±0.1 U [n=40]; P>0.05). Platelets were incubated under control conditions and with 25 mmol/L glucose for 60 minutes. The administration of 60 μmol/L OAG increased the F340 nm/F380 nm fluorescence ratio in glucose-treated platelets by 0.76±0.10 U and in control platelets by 0.40±0.06 U (each n=13). The OAG-induced calcium influx was significantly higher in glucose-treated platelets (P<0.05). Next, we used the calcium channel blocker amlodipine. In the presence of the L-type calcium channel blocker amlodipine the OAG-induced calcium influx was also higher in glucose-treated platelets compared to control conditions (0.61±0.03 U versus 0.51±0.02 U; n=10, P<0.05; Figure 1B). Furthermore, we used SKF-96365, an inhibitor of TRP channels.11,13,14 In the presence of SKF-96365 the OAG-induced calcium influx in glucose-treated platelets was significantly reduced from 0.76±0.10 U (n=13) to 0.43±0.02 U (n=10; P<0.05). In the presence of SKF-96365 the OAG-induced calcium influx was not significantly different in glucose-treated platelets compared to control conditions (0.43±0.02 U versus 0.38±0.04 U; each n=10, P>0.05; Figure 1C). The glucose-enhanced OAG-induced calcium influx was concentration-dependent (Figure 1D). As shown in Figure 1E the effect of glucose on calcium influx could be observed with different OAG concentrations.

To exclude that the isolation procedure affects platelet action, the platelets were isolated in the presence of 25 mmol/L glucose. Then platelets were exposed to 5.5 mmol/L glucose or 25 mmol/L glucose throughout all subsequent steps. These experiments confirmed that exposure to 25 mmol/L glucose as compared to 5.5 mmol/L glucose increased OAG-induced calcium influx into platelets (0.86±0.05 U versus 0.48±0.03 U, each n=15, P<0.05).

Inhibition of Phosphatidylinositol 3-Kinase Attenuates the Glucose-Enhanced OAG-Induced Calcium Influx in Human Platelets Because activation of phosphatidylinositol 3-kinase is thought to be necessary for the translocation of TRPC channels to the surface we examined the effects of specific inhibitors of the phosphatidylinositol 3-kinase, wortmannin or LY294002. In the presence of wortmannin or LY294002 the OAG-induced calcium increase after incubation with 25 mmol/L glucose was significantly attenuated (Figure 2A). Compared to control conditions, wortmannin significantly reduced the glucose-enhanced OAG-induced calcium increase to 42±7% (P<0.05; n=6) and LY294002 significantly reduced it to 36±1% (P<0.05; n=6; Figure 2B). As indicated in Figure 2C the inhibitory effect of wortmannin on glucose-enhanced OAG-induced calcium increase was dose-dependent. These data indicate that high glucose concentrations enhance OAG-induced calcium influx by a phosphatidylinositol 3-kinase-dependent mechanism.

Glucose Increases TRPC6 Channel Expression in Platelets It is well-known that OAG-induced calcium influx is at least in part mediated through TRPC channels. Therefore we evaluated whether glucose enhances the expression of TRPC channels on human platelets. Using quantitative immunofluorescence we observed an increased TRPC6 protein surface expression after incubation of platelets with 25 mmol/L glucose (Figure 3). The effects of glucose on TRPC6 expression were measured in 33 experiments. Glucose significantly increased the TRPC6 expression to 131±12% (n=33; P<0.05). On the other hand glucose did not significantly change the protein expression of TRPC1, TRPC3, TRPC4, or TRPC5 (Figure 3B). The administration of 25 mmol/L taurine did not significantly affect TRPC6 protein surface expression (100±1%; n=4; P>0.05 compared to control conditions). The administration of 25 mmol/L urea did not significantly affect TRPC6 expression (102±10%; n=5; P>0.05 compared to control), further excluding osmotic effects.

We also evaluated whether glucose increased the expression of P-selectin on human platelets using immunofluorescence and FACScan flow cytometry. Compared to control conditions, the administration of 25 mmol/L glucose did not significantly change the expression of P-selectin in platelets (4.2±0.2 arbitrary units versus 4.3±0.4 arbitrary units; each n=8; P>0.05).

To evaluate the effects of phosphatidylinositol 3-kinases on TRPC6 expression we examined TRPC6 expression after administration of glucose in the presence of inhibitors of the phosphatidylinositol 3-kinase, wortmannin or LY294002. As
shown in Figure 4, glucose significantly increased the TRPC6 expression on the platelet surface to 151±9% (n=10; P<0.01). The increased TRPC6 expression after incubation with 25 mmol/L glucose was significantly attenuated in the presence of wortmannin to 99±6% (n=4), or in the presence of LY294002 it was significantly attenuated to 76±2% (n=4; each P<0.01 compared to incubation with 25 mmol/L glucose alone; Figure 4B). These results were also observed using immunofluorescence of single platelets which were analyzed by fluorescence microscopy (Figure 4C). Compared to control conditions the administration of 25 mmol/L glucose significantly increased the TRPC6 expression on the platelet surface (152±2%, n=102; versus 100±3%, n=70; P<0.001).

As shown in Figure 5 the glucose-induced increase of TRPC6 protein surface expression in platelets was time-dependent. TRPC6 protein expression was significantly increased after incubation with high glucose concentration for at least 40 minutes.

Furthermore, we examined platelets from patients with type 2 diabetes mellitus (mean age, 61±3 years; fasting blood glucose 212±32 mg/dL) and age-matched nondiabetic individuals (mean age, 62±4 years; fasting blood glucose 98±9 mg/dL). Platelets from patients with type 2 diabetes mellitus showed increased TRPC6 expression compared to nondiabetic individuals (273±40% versus 100±36%, each n=5, P<0.05; Figure 5C).

**Discussion**

In the present study we investigated the effects of glucose on TRPC channel expression, and furthermore we investigated OAG-induced calcium influx (ie, channel activation by OAG) in platelets. The study indicates that high glucose increases TRPC6 channel protein expression which is mediated by a phosphatidylinositol 3-kinase pathway. Furthermore, the increased TRPC6 channel expression was associated with an increased OAG-induced calcium influx. Altered platelet function in patients with diabetes mellitus and hyperglycemia which causes accelerated atherosclerosis and increases the risk of thrombotic events has been attributed to several mechanisms including altered calcium homeostasis. Several reports show increased agonist-induced calcium influx in patients with diabetes mellitus. In accordance with these reports the present study shows that high glucose significantly enhances the OAG-induced calcium influx.

Which channels are responsible for increased OAG-induced calcium influx in platelets exposed to high glucose? It has been known for a long time that OAG induces calcium influx and thereby activates platelets. TRPC channels are likely candidates for mediating OAG-induced calcium influx. First, Brownlow & Sage and Hassock et al reported the presence of TRPC channels in human platelets. It is well established that TRPC channels are nonselective cation chan-
nels that mediate calcium influx. TRPC6 channels have been shown to be activated by the diacylglycerol analogue OAG and thus to generate calcium entry. Second, using different techniques (immunofluorescence and fluorescence microscopy of single platelets) we showed that the incubation of platelets with high glucose concentrations significantly increased the TRPC6 protein expression on the platelet surface in a dose- and time-dependent manner. When platelets were incubated with high glucose the TRPC6 protein expression on the platelet surface increased within 60 minutes. We verified that high glucose concentrations did not significantly change the protein expression of TRPC1, TRPC3, TRPC4, or TRPC5 channels in platelets, indicating a selective mechanism. From these experiments we concluded that prolonged exposure to high glucose enhances TRPC6 expression on the platelet surface and enhances agonist-induced calcium influx.

Which mechanisms are involved mediating the increased TRPC6 protein expression in platelets exposed to high glucose? An increased protein expression on the cell surface in the presence of high glucose has been described in several cell types, including human cortical collecting duct and human umbilical vein endothelial cells. Furthermore, high glucose has been associated with increased number of glycoprotein GPIIb/IIIa molecules on platelets. However, the underlying mechanisms are only partly known. We may speculate that glucose stimulation of platelets initiates the rapid translocation of TRPC channels from vesicles held in reserve just under the plasma membrane. A similar mechanism had been postulated in human embryonic kidney cells and neurons. It should be noted that P-selectin was not significantly changed after administration of high glucose. These findings are similar to those reported by Gresele et al who showed that P-selectin expression on platelets did not change significantly after hyperglycemic clamp. These results indicated that the glucose-induced elevation of TRPC6 expression mainly predisposes to increased agonist-induced calcium influx into platelets.

We found that the glucose-induced TRPC6 protein expression was significantly attenuated by inhibitors of the phosphatidylinositol 3-kinase, wortmannin or LY294002. Phosphatidylinositol 3-kinases are lipid kinases that phosphorylate phosphoinositides on position 3 of their inositol head group. The 2 main phosphoinositide products are phosphatidylinositol-3,4-phosphate and phosphatidylinositol-3,4,5-phosphate. It has been recently shown that the activation of phosphatidylinositol 3-kinase is necessary for the translocation of TRPC channels to the surface. Furthermore it has been reported that high glucose directly activates the phosphatidylinositol 3-kinase activity. These data indicate that high glucose increases phosphatidylinositol 3-kinase activity which enhances the translocation of TRPC6 channels to the platelet surface, and finally enhances platelet activation.

In summary the study shows that high glucose causes increased TRPC6 channel protein expression on the platelet surface which is mediated by a phosphatidylinositol 3-kinase pathway.

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Disclosures
None.

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