High glucose-induced oxidative stress increases transient receptor potential (TRP) channel expression in human monocytes

**Short title:** High glucose affects TRPC

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**Objective.** Transient receptor potential (TRP) channel induced cation influx activates human monocytes, which play an important role in the pathogenesis of atherosclerosis. In the present study we investigated the effects of high glucose-induced oxidative stress on TRP channel expression in human monocytes.

**Research design and methods.** Human monocytes were exposed to control conditions (5.6mmol/L D-glucose), high glucose (30mmol/L D-glucose or L-glucose), 100µmol/L peroxynitrite, or high glucose in the presence of the superoxiddismutase mimetic tempol (100µmol/L). TRP mRNA and TRP protein expression were measured using quantitative real-time RT-PCR and quantitative in-cell Western assay, respectively. Calcium influx and intracellular reactive oxygen species were measured using fluorescent dyes.

**Results.** Administration of high D-glucose significantly increased reactive oxygen species. High D-glucose or peroxinitrite significantly increased the expression of TRPC1, TRPC3, TRPC5, TRPC6, TRPM6 and TRPM7 mRNA and TRPC3 and TRPC6 proteins. High D-glucose plus tempol or high L-glucose did not affect TRP expression. Increased oxidative stress by lipopolysaccharide or tumor necrosis factor-α increased TRP mRNA expression, whereas the reduction of superoxide radicals using diphenylene iodonium significantly reduced TRP mRNA expression. Increased TRPC3 and TRPC6 protein expression was accompanied by increased 1-oleoyl-2-acetyl-sn-glycerol-induced calcium influx, which was blocked by the TRPC inhibitor 2-aminoethoxydiphenylborane. TRPC6 mRNA was significantly higher in monocytes from 18 patients with type 2 diabetes mellitus compared to 28 control subjects (p<0.05).

**Conclusion.** High D-glucose-induced oxidative stress increases TRP expression and calcium influx in human monocytes, pointing to a novel pathway for increased activation of monocytes and hence atherosclerosis in patients with diabetes mellitus.
Cardiovascular complications due to atherosclerotic disease are a frequent cause of morbidity and mortality in patients with diabetes mellitus (1). Epidemiological studies and preliminary intervention studies have shown that hyperglycemia is a direct and independent risk factor for cardiovascular disease (2). Atherogenesis has been considered to be an inflammatory disease with accumulation of monocytes within the artery wall (3). Monocytes are transitional cells, with a short half-life due to rapid differentiation into macrophages and they are rapidly recruited to sides of inflammation (4,5). Monocyte activation, adhesion to the endothelium, and transmigration into the subendothelial space are key events in early pathogenesis of atherosclerosis. The mechanisms by which high glucose supports monocyte-associated atherosclerosis are only partially known. Mononuclear blood cells from patients with diabetes mellitus show increased generation of reactive oxygen species because of chronic high glucose levels (6-8). An increased activation of monocytes from patients with diabetes mellitus is associated with elevated protein kinase C activity and increased cytosolic calcium concentrations (9-11). Elevated transmembrane calcium influx may be mediated by increased transient receptor potential canonical (TRPC) channels. Until now only few studies addressed TRP expression under diabetic high glucose conditions. One study observed TRPC1, TRPC4 and TRPC6 regulation and impaired capacitative calcium entry in vessels of diabetic patients compared to non-diabetic human vessels (12). As TRPC channels have been identified in several cell types including peripheral blood monocytes (13,14), the present study was aimed at elucidating the effects of high glucose and oxidative stress on TRP expression and their functional relevance in mediating calcium influx.

METHODS

Preparation of cells: Human monocytes were obtained from heparinized blood of healthy control subjects. All subjects gave written informed consent and the study was approved by the local ethics committee. Monocytes were separated using superparamagnetic polystyrene beads coated with a primary monoclonal antibody specific for the CD14 membrane antigen expressed on human monocytes (Invitrogen, Groningen, Germany) and resuspended in Hanks balanced solution containing (in mmol/L) NaCl 136, KCl 5.40, CaCl\textsubscript{2} 1, KH\textsubscript{2}PO\textsubscript{4} 0.44, Na\textsubscript{2}HPO\textsubscript{4} 0.34, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 10, pH 7.4. Monocytes were counted in Neubauer’s chamber and adjusted in each experiment.

To evaluate the effects of high glucose and oxidative stress on TRP channel expression monocytes were exposed to 5.6mmol/L D-glucose (Control), 30mmol/L D-glucose, or 100µmol/L peroxinitrite for 4 hours. Additional experiments were performed using 30mmol/L L-glucose or 30mmol/L D-glucose in the presence of the superoxidismutase mimetic tempol (100µmol/L). We also evaluated the effects of lipopolysaccharide-induced oxidative stress, tumor necrosis factor-α (TNF-α)-induced activation of NAD(P)H oxidase and production of superoxide radicals as well as reduction of superoxide radicals using diphenylene iodonium. Next, the effects of selective inhibition of phosphatidylcholine-specific phospholipase C by tricyclodecan-9-yl xanthogenate (D609) or the inhibition of phospholipase C activation by 1-[6-]((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino[hexyl]-1H-pyrrole-2,5-dione (U73122) on TRP expression in monocytes
RNA isolation and reverse transcription: Total RNA was isolated from monocytes using the RNeasy mini kit including RNase-free DNase set (Qiagen, Hilden, Germany). Using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany), cDNA was synthesized from 2µg of total RNA using oligo dT (12-18) and 5 U AMV reverse transcriptase at 50°C for 60 min, followed by heating to 85°C for 5 min.

Quantitative real-time reverse transcriptase polymerase chain reactions: Quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCR) for transient receptor potential canonical type 1 (TRPC1), type 3 (TRPC3), type 5 (TRPC5), type 6 (TRPC6), transient receptor potential melastatin type 6 (TRPM6), type 7 (TRPM7), tumor necrosis factor alpha (TNF-α) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were performed using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). The primers were as follows:

TRPC1 (Reference Sequence (RefSeq) database accession number: NM_003304.4), forward, 5’TGCTTACCAAACGTGGGTG3’; reverse, 5’AACTGTTTTGCCGTTTGACC3’;

TRPC3 (NM_003305), forward, 5’GACTTTGGGATGCTGTCCAT3’; reverse, 5’GTACGCAATCCGAGAGAAGC3’;

TRPC5 (NM_012471.2), forward, 5’CCACCAGCTATCAGATAAGG3’; reverse, 5’GGAAACAAGCCACTTATAACC3’;

TRPC6 (NM_004621), forward, 5’GCCAATGAGCATCTGGAAT3’; reverse, 5’TGGAGTCACATCTGGGAGA3’;

TRPM6 (NM_017662.3), forward, 5’TTATGATTGCCACCTGGAGTCTCGTG3’; reverse, 5’ATGACCTTGGCACCAGCTTT3’;

TNF-α (NM_000594.2), forward, 5’CCCAGGGACCTCTCTCTAATC3’; reverse, 5’CATCTTGCTGAGAAGACTC3’;

GAPDH (NM_002046), forward, 5’AACTGCTTAGACCCCTGGC3’; reverse, 5’TTATGATTGCCACCTGGAGTCTCGTG3’.

The expected and observed sizes of the PCR products were 243bp for TRPC1, 249bp for TRPC3, 161bp for TRPC5, 243bp for TRPC6, 347bp for TRPM6; 214bp for TRPM7, 84bp for TNF-α, and 200bp for GAPDH.

qRT-PCR was performed using 2µg RNA. LightCycler-Fast Start DNA SYBR Green I Kit (Roche Diagnostics) and 500nmol/L of each primer were used in a final volume of 20µL. The reaction was initiated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 10 s at 68°C (TRPM6), at 60°C (TRPC3, TRPM7, TNF-α), at 57°C (TRPC3, TRPM6) or at 54°C (TRPC5, TRPC6) or at 54°C (TRPC5, TRPC6) and extension at 72°C for 15 s. Melting curve analysis were performed from 65°C to 95°C with a heating rate of 0.1°C/s. Data were recorded on a LightCycler 2.0 Instrument using LightCycler Software Version 4.0 (Roche Diagnostics).

The relative quantification method was used whereby the change in expression of the target genes (TRPC1, TRPC3, TRPC5, TRPC6, TRPM6, TRPM7, TNF-α) relative to the housekeeping gene (GAPDH) was calculated.

Immunofluorescence assay of TRPC channel protein: For the identification of TRPC channel proteins, quantitative in-cell Western assays of human monocytes were performed using the Odyssey infrared imaging system (Licor Biosciences, Bad Homburg, Germany). Human monocytes in
96-well plates were incubated with rabbit anti-human TRPC3 or TRPC6-antibodies (1:1000, alomone labs, Jerusalem, Israel) for 2 hours, washed, incubated with IRDye800-infrared fluorescent dye-conjugated sheep anti-rabbit antibodies (1:1000, Biomol, Hamburg, Germany) overnight at 4°C. Imaging was performed at 810nm emission with an excitation wavelength of 780nm. Control experiments were performed with omission of primary or secondary antibodies. Conventional immunoblotting was performed as described previously by our group (13).

**Measurements of cytosolic calcium using fluorescent dye technique:** For ratiometric imaging experiments monocytes were loaded with 2µmol/L of the calcium-sensitive, cell-permeable, intracellular fluorescence dye 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methyl-phenoxy)-ethane-N,N,N′,N′-tetra acetic acid penta acetoxymethyl ester (fura-2/AM, Merck Biosciences, Darmstadt, Germany) at room temperature for 60 min and washed to remove extraneous dye. Fluorescence measurements were performed in a temperature-controlled 96-well-fluorescent plate reader at 37°C (Fluoroskan Ascent Fluorometer, Thermo LabSystems Oy, Helsinki, Finland) at 510nm emission with excitation wavelengths of 340nm and 380nm. Baseline fluorescence was measured for 10 min and stable fluorescence readings were obtained throughout. Calcium influx was activated after administration of membrane-permeable 1-oleoyl-2-acetyl-sn-glycerol. We used the membrane-permeable 2-aminoethoxydiphenylborane to inhibit calcium influx through TRPC channels as previously described (15,16).

**Measurements of reactive oxygen species using fluorescent dye technique:** Monocytes were incubated with the dye 2′,7′-dichlorofluorescin diacetate (DCF-DA, 50µmol/L) for 60 min and then washed and resuspended in HBSS. DCF-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of reactive oxygen species, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein which was monitored spectrophotometrically at 530nm with an excitation wavelength of 485nm.

**Statistics:** All values are reported as mean ± SEM of at least 4 independent experiments. Comparisons between groups were analyzed using non-parametric Mann-Whitney test (GraphPad Prism 5.0; LaJolla CA). Data from multiple groups were analyzed using the non-parametric Kruskal-Wallis test and Dunn's multiple comparison post hoc test. The numbers given indicate the number of separate experiments. Each sample was tested in duplicate. Two-sided p values below 0.05 were considered to indicate statistical significance. Where error bars do not appear on the figure, error was within the symbol size.

**RESULTS**

**High D-glucose increased reactive oxygen species in human monocytes:** The induction of reactive oxygen species by high glucose was investigated using the dye 2′,7′-dichlorofluorescein diacetate. Compared to control conditions (D-glucose, 5.6mmol/L) the administration of 30mmol/L D-glucose for 90 minutes significantly increased reactive oxygen species from 1.0±0.01 arbitrary units to 1.84±0.17 arbitrary units (p<0.01). Furthermore the effect of D-glucose was blocked by the concurrent administration of the superoxiddismutase mimetic tempol (100µmol/L) to 0.78±0.07 arbitrary units (p<0.01 compared to D-glucose alone).

**Detection of TRP mRNA using qRT-PCR:** To investigate whether high glucose-induced oxidative stress may affect TRP channels we first analyzed TRP mRNA in human monocytes from healthy control
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U73122, 1.95±0.03; p=n.s. compared to high D-glucose alone; each n=4). It is known that TRPC-induced calcium influx is regulated by phospholipase C. However, phospholipase C seems not to be involved in glucose-induced up-regulation of TRPC mRNA expression.

To evaluate whether increased TRPC mRNA is a feature of diabetes mellitus we compared TRPC1, TRPC3, TRPC5 and TRPC6 mRNA in monocytes from 18 patients with type 2 diabetes mellitus (10 women, 8 men; mean age 57±5years; systolic blood pressure 133±3mmHg; diastolic blood pressure 79±2mmHg; serum sodium 135±1mmol/L, serum potassium 4.4±0.1mmol/L, hemoglobin 11.2±0.5g/dL; glycated hemoglobin A1c 7.5±0.3%) and from 28 age-matched control subjects (10 women, 18 men; mean age 56±4years; systolic blood pressure 132±4mmHg; diastolic blood pressure 81±2mmHg; serum sodium 136±1mmol/L, serum potassium 4.4±0.2mmol/L, hemoglobin 12.3±0.5g/dL; glycated hemoglobin A1c 5.6±0.1%). TRPC6 mRNA was significantly higher in monocytes from patients with type 2 diabetes mellitus compared to control subjects (TRPC6 normalized ratio, 0.028±0.014; n=18; vs. 0.015±0.006; n=28; p<0.05; Figure 1E). On the other hand, TRPC1, TRPC3, or TRPC5 mRNA were not significantly different between the two groups (TRPC1 normalized ratio, 0.025±0.012 vs. 0.025±0.006; p=0.62; TRPC3, 0.010±0.005 vs. 0.003±0.001; p=0.40; TRPC5, 0.007±0.004 vs. 0.003±0.001; p=0.83). These data support the view that increased TRPC6 expression is a characteristic feature of peripheral blood cells from patients with type 2 diabetes mellitus.

High D-glucose and oxidative stress increased TRPC3 and TRPC6 protein expression in monocytes: TRPC protein expression in monocytes was quantified using a quantitative in-cell Western assay. As shown in Figure 2, compared to control conditions with 5.6mmol/L D-glucose, administration of high D-glucose (30mmol/L) significantly increased TRPC3 protein expression by 1.58-fold (p<0.01; n=10) and TRPC6 protein expression by 1.57-fold (p<0.01; n=8). The administration of high L-glucose (30mmol/L) did not significantly affect TRPC3 or TRPC6 protein expression. Concurrent administration of tempol (100µmol/L) blocked the stimulating effect high D-glucose on TRPC3 and TRPC6 protein expression.

Compared to control conditions oxidative stress induced by 100µmol/L peroxinitrite also significantly increased the TRPC3 protein expression by 1.70-fold (p<0.01; n=9) and TRPC6 protein expression by 1.8-fold (p<0.01; n=9).

High D-glucose-induced oxidative stress promoted increased TRPC-mediated calcium influx: Cytosolic calcium was measured in fura-2-loaded monocytes. 1-oleoyl-2-acetyl-sn-glycerol (OAG) increased cytosolic calcium to 2.41±0.01 (n=15), whereas OAG in the presence of high D-glucose increased cytosolic calcium to 2.79±0.03 (n=14; p<0.01). Furthermore, it should be noted that concurrent preincubation of cells with tempol (100µmol/L) and high D-glucose normalized OAG-induced calcium influx (2.49±0.02; n=13; p=n.s. compared to control). Representative traces are shown in Figure 3A. These data indicate that high D-glucose-associated oxidative stress promotes increased TRPC-mediated calcium influx. After treatment of monocytes with high D-glucose for 4 hours the administration of peroxynitrite subsequently increased intracellular calcium levels. In the presence of a membrane-permeable TRPC blocker, 2-aminoethoxydiphenylborane, the peroxynitrite induced calcium increase was significantly attenuated (3.99±0.05 vs. 4.56±0.04; each n=15; p<0.01 compared to peroxynitrite alone; Figure 3B).

DISCUSSION
Our present data show that high D-glucose-induced oxidative stress causes increased TRP expression and calcium influx in human monocytes. An increased calcium influx through TRP channels may be responsible for an increased activation of monocytes and enhanced atherosclerosis in patients with diabetes mellitus.

The presence of high glucose levels is a characteristic feature of diabetes mellitus, and considered as a major pathogenic factor for increased atherosclerosis and consecutive diseases including cardiovascular disease (19). Both an increased activation of monocytes and an increased generation of reactive oxygen species in monocytes have been described in patients with diabetes mellitus (6,20). The production of mitochondrial superoxide has been implicated as a major underlying mechanism linking high glucose and cellular dysfunction (21,22). In the present study we confirmed that high glucose increases reactive oxygen species in monocytes. The high glucose-induced increase of reactive oxygen species was blocked by the superoxiddismutase mimetic tempol.

Activated monocytes in patients with diabetes mellitus are characterized by increased calcium concentrations (9-11). According to our present results increased TRPC channel expression in monocytes may be responsible for an increased calcium influx following high glucose-induced oxidative stress. We observed that high D-glucose-induced oxidative stress increased both the expression of TRPC3 and TRPC6 mRNA and TRPC3 and TRPC6 channel proteins in monocytes. The stimulating effect of high glucose could be blocked by concurrent administration of tempol, supporting the relevance of increased oxidative stress after high D-glucose. Recently, Shanmugam et al. showed that exposure of monocytes to high glucose increased the expression of several inflammatory cytokines, including tumor necrosis factor-alpha in an oxidant stress-dependent manner (23). Our present results support these findings indicating that high-glucose-induced upregulation of TRP expression was associated with enhanced mRNA levels of the inflammatory cytokine TNF-α. We observed that high glucose elevates the expression of TNF-α even higher than peroxinitrite. The increased TNF-α-response after high glucose compared to peroxinitrite indicates that irrespective of oxidative stress other pathways, e.g. the polyol pathway, contribute to increased inflammatory response in diabetes mellitus (24). Taken together, high-glucose-induced oxidative stress activates monocytes by enhancing inflammatory cytokines and TRP channel expression promoting transmembrane calcium influx. Indeed, our experiments using the fluorescent dye fura-2 showed that high glucose-induced oxidative stress promotes increased TRP-mediated calcium influx.

Balzer et al. suggested that TRPC channels are the molecular basis of oxidant-activated cation channels in endothelial cells (25). Poteser et al. reported that redox modulation of TRPC channels is responsible for oxidative stress-induced changes in cytosolic calcium signaling (26). The present study extends their findings, showing that oxidative stress increased both the expression of TRPC3 and TRPC6 mRNA and proteins. Therefore high glucose-induced oxidative stress in patients with diabetes mellitus may activate monocytes both by interaction with TRPC channels and by enhanced TRPC expression. The present study indicates that induction of oxidative stress by several pathways may affect TRP expression. Induction of oxidative stress using lipopolysaccharide or TNF-α-induced activation of NAD(P)H oxidase oxidase significantly increased TRPC3 mRNA expression whereas the reduction of superoxide radicals using diphenylene iodonium significantly reduced TRPC3
mRNA expression. In the present study we also confirmed that high D-glucose-induced oxidative stress increases TRPM6 and TRPM7 mRNA expression in agreement with data from Yamamoto et al. who showed the regulation of TRPM expression by oxidative stress (27).

To evaluate whether increased TRPC mRNA is a feature of diabetes mellitus we also compared TRPC mRNA in monocytes from patients with type 2 diabetes mellitus and control subjects. We observed increased TRPC6 mRNA in monocytes from patients with type 2 diabetes mellitus. Increased TRPC6 protein expression has already been reported in platelets from patients with diabetes mellitus compared to control subjects (28), supporting the view that increased TRPC6 expression is a characteristic feature of peripheral blood cells from patients with diabetes mellitus. Furthermore, Hu et al. showed increased TRPC6 expression in the adrenal medulla from Ossabaw miniature pigs with prediabetic metabolic syndrome (29). The effects of high glucose could be cell specific. For example, Graham et al. showed that high glucose downregulates TRPC6 in cultured mesangial cells (30). However, differences might be explained in part by use of native blood cells and cultured cells. In our study inhibition of phospholipase C did not affect TRPC mRNA expression. However, several pathways including phospholipase C, protein kinase C or phosphoinositide-3-kinase may modulate TRP channel protein expression and function as suggested by recent literature (31-33).

In conclusion, the present study shows that high glucose-induced oxidative stress increases TRPC3 and TRPC6 channel expression and calcium influx in human monocytes. These data point to a novel pathway for an increased activation of monocytes and hence atherosclerosis in patients with diabetes mellitus.
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High glucose affects TRPC

Figure legends

**Figure 1.** Changes of mRNA expression of (A) transient receptor potential canonical type 3 (TRPC3), (B) canonical type 6 (TRPC6), (C and D) canonical type 1 (TRPC1), canonical type 5 (TRPC5), melastatin type 6 (TRPM6), melastatin type 7 (TRPM7), and tumor necrosis factor-α (TNF-α) induced by high glucose and peroxynitrite. Exposure of monocytes to high glucose (HG; 30 mmol/L D-glucose) or peroxynitrite (ONOO; 100µmol/L) for 4h significantly increased the TRPC3 and TRPC6 mRNA expression. The effect of high glucose is attenuated by superoxide dismutase mimetic tempol (TMP; 100µmol/L). Administration of L-glucose showed no significant effect. Control (open bar) indicates control glucose (5.6 mmol/L D-glucose). The expression of each factor is normalized to GAPDH expression. Data are mean±SEM from at least four to six independent experiments. *p<0.05; **p<0.01 compared to control conditions.

**E,** Bar graph showing TRPC3 mRNA and TRPC6 mRNA expression (normalized ratio) in monocytes from 28 age-matched control subjects (open bars) and from 18 patients with type 2 diabetes mellitus (filled bars). *p<0.05 compared to control.

**Figure 2.** Quantitative in-cell Western assay of TRPC3 and TRPC6 channel protein expression in monocytes treated with high glucose (HG; 30mmol/L D-glucose) in the absence and presence of tempol (TMP; 100µmol/L), peroxynitrite (ONOO; 100 µmol/L), L-glucose (30mmol/L) or control (5.6mmol/L D-Glucose).

**A,** Representative conventional Western assay (TRPC3 protein and GAPDH protein for loading control with top values indicating densitometric analysis of blots). The TRPC3/GAPDH ratio (fold over control) is also indicated.

**B+D,** representative in-cell Western assays and

**C+E,** summary data are shown. Data are mean±SEM from at least eight independent experiments. **p<0.01 compared to control conditions.

**Figure 3.** Recordings of fura-2 fluorescence in human monocytes.

**A,** 1-oleoyl-2-acetyl-sn-glycerol (OAG)-induced calcium influx was measured under control conditions (5.6mmol/L D-glucose, open circles), in the presence of high glucose (30mmol/L D-glucose) without (filled squares) and with (filled triangle) tempol (TMP; 100µmol/L).

**B,** Summary data showing intracellular calcium levels after treatment of monocytes with high D-glucose for 4 hours (Control) stimulated with peroxynitrite (ONOO) in the absence and presence of the membrane-permeable TRPC blocker, 2-aminoethoxydiphenylborane (2-APB). **p<0.01.
High glucose affects TRPC

Figure 1

A: TRPC3 mRNA expression (fold over control)

B: TRPC6 mRNA expression (fold over control)

C: mRNA expression (fold over control)

D: mRNA expression (fold over control)

E: mRNA expression (normalized ratio)
High glucose affects TRPC

Figure 2

A

TRPC3 protein expression (fold over control)

Control HG TMP+HG ONOO L-Glucose

TRPC3/GAPDH (fold over control)

B

TRPC3

C

TRPC3 protein expression (fold over control)

Control HG TMP+HG ONOO L-Glucose

D

TRPC6

E

TRPC6 protein expression (fold over control)

Control HG TMP+HG ONOO L-Glucose
Figure 3

A

B

High glucose affects TRPC