High Glucose–Induced Oxidative Stress Increases Transient Receptor Potential Channel Expression in Human Monocytes

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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 (5.6 mmol/l D-glucose), high glucose (30 mmol/l D-glucose or L-glucose), 100 μmol/l peroxynitrite, or high glucose in the presence of the superoxide dismutase mimetic tempol (100 μmol/l). TRP mRNA and TRP protein expression was 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 peroxynitrite significantly increased the expression of TRP canonical type 1 (TRPC1), TRPC3, TRPC5, TRPC6, and TRPC6 melastatin type 6 (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 dipiperidone 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 compared with 28 control subjects (P < 0.05).


CARDIOVASCULAR COMPLICATIONS DUE TO ATHEROSCLEROSIS—Cardiovascular complications due to atherosclerosis are a frequent cause of morbidity and mortality in patients with diabetes (1). Epidemiologic 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 sites 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 show increased generation of reactive oxygen species because of chronic high glucose levels (6–8). An increased activation of monocytes from patients with diabetes 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 transient receptor potential (TRP) channel expression under diabetic high glucose conditions. One study observed TRPC type 1 (TRPC1), TRPC4, and TRPC6 regulation and impaired capacitative calcium entry in vessels of diabetic patients compared with nondiabetic 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.

RESEARCH DESIGN AND 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, CaCl2 1.0, KH2PO4 0.44, Na2HPO4 0.34, HEPES 10, pH 7.4. Monocytes were counted in Neubauer chamber and adjusted in each experiment.

To evaluate the effects of high glucose and oxidative stress on TRP channel expression, monocytes were exposed to 5.6 mmol/l D-glucose (control), 30 mmol/l D-glucose, or 100 μmol/l peroxynitrite (ONOO−) for 4 h. Additional experiments were performed using 30 mmol/l L-glucose or 30 mmol/l D-glucose in the presence of the superoxide dismutase mimetic tempol (TMP, 100 μmol/l) or diphenylene boro-
µmol/l). We also evaluated the effects of lipopolysaccharide-induced oxidative stress, tumor necrosis factor-α (TNF-α)-induced activation of NADPH oxidase, and production of superoxide radicals as well as reduction of superoxide radicals using diphenylethylene iodide. Next, the effects of selective inhibition of phosphatidylinositol-specific phospholipase C by Bayk-2388, 9-yl camptothecin (1680) or the inhibition of phospholipase C activation by t-0-0-(2-[(17β)-3-methoxyxestra-1,3,5[10]-trien-17-yl]amino[hexyl]-1H-pyrole-2,5-dione (U73122) on TRP expression in monocytes.

RNA isolation and reverse transcriptase. 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 2 µg total RNA using oligo dt (12–18) and 5 units avian myeloblastosis virus reverse transcriptase at 50°C for 60 min, followed by heating to 85°C for 5 min.

Quantitative real-time RT-PCRs. Quantitative (q) real-time RT-PCRs for TRPC1, TRPC3, TRPC5, TRPC6, TRP melastatin type 6 (TRPM6), TRP7, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using a LightCycler-Start DNA Master SYBR Green 1 Kit (Roche Diagnostics). The primers were as follows: TRPC1 (Reference Sequence [RefSeq] database accession number: NM_003304.4), forward, 5’-TGGCTTA CCAAATGCTGTTG3’; reverse, 5’-AACTGGTTTGGCCATTGACA3’; TRPC3 (NM_003305), forward, 5’-GAAATCCGACAGAAGAGC3’; reverse, 5’-TGGAGTCTCAATCGAATAAG3’; TRPC5 (NM_004621), forward, 5’-GCAATCGGAAGCTGGAAT3’; reverse, 5’-TGGA GTCAACGAAGTCTGAC3’; TRPM6 (NM_017662.4), forward, 5’-GGAGTAGTGAACACCAC3’; reverse, 5’-TTATGGATGGCTGAGTCCTTG3’; TRPM7 (NM_017672.3), forward, 5’-CTTATGAGCAGGCACTGTTG3’; reverse, 5’-CATCTTTCGTAAGAGGCTG3’; TRPs (NM_005094.2), forward, 5’-CCAGGGACCTCTCTATCACT3’; reverse, 5’-ATTGCTACAGGCTGCTA853’; GAPDH (NM_002046), forward, 5’-AATGCATGAACCCAGCTGC3’; reverse, 5’-ATGACCTTGGCCCAACAGC3’; and GAPDH 200. The expected and observed sizes of the PCR products were (in bp) TRPC1 243; TRPC2 249; TRPC3 161; TRPC5 195; TRPC6 243; TRPM6 237; TRPM7 214; TRP-α 84; and GAPDH 200.

qRT-PCR was performed using 2 µg RNA. LightCycler-Start DNA Master SYBR Green 1 Kit (Roche Diagnostics). The relative quantification method was used wherein the change in expression of the target genes (TRPC1, TRPC3, TRPC5, TRPC6, TRPM3, TRPM7, TRPM6, TRPM7, TNF-α) relative to the housekeeping gene (GAPDH) was calculated.

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 subjects using qRT-PCR. The melting curve analysis showed the presence of one single peak in monocytes after 4-h treatment under control conditions, with high glucose or ONOO. The expected and observed product sizes using gel electrophoresis were (in bp) TRPC1 243; TRPC3 249; TRPC5, TRPC7, TRPM6 161; TRPC3 243; TRPC6 237; TRPM6 347; TRPM7 214; and GAPDH 200. High glucose and oxidative stress increased TRP mRNA in monocytes. We determined the effects of high glucose and oxidative stress on TRPC3 and TRPC6 mRNA in human monocytes. As shown in Fig. 1, compared with control conditions with 5.6 mmol/l glucose, administration of 30 mmol/l glucose for 10 min significantly increased TRPC3 mRNA by 1.96-fold (P < 0.01) compared with D-glucose alone. The administration of high glucose significantly increased mRNA expression of TRPC1, TRPC5, TRPM6, or TRPM7 mRNA in monocytes (Fig. 1). Control conditions with high glucose or ONOO. The expected and observed product sizes using gel electrophoresis were (in bp) TRPC1 243; TRPC3 249; TRPC5, TRPC7, TRPM6 161; TRPC3 243; TRPC6 237; TRPM6 347; TRPM7 214; and GAPDH 200.

RESULTS

High glucose increased reactive oxygen species in human monocytes. The induction of reactive oxygen species by high glucose was investigated using the dye DCFDA. Compared with control conditions (glucose, 5.6 mmol/l), the administration of 30 mmol/l glucose for 90 min significantly increased reactive oxygen species from 1.0 ± 0.1 to 1.84 ± 0.17 arbitrary units (AU) (P < 0.01). Furthermore, the effect of glucose was blocked by the concurrent administration of the superoxide dismutase mimetic TEMP (100 µmol/l) to 0.78 ± 0.07 AU (P < 0.01 compared with 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 subjects using qRT-PCR. The melting curve analysis showed the presence of one single peak in monocytes after 4-h treatment under control conditions, with high glucose or ONOO. The expected and observed product sizes using gel electrophoresis were (in bp) TRPC1 243; TRPC3 249; TRPC5, TRPC7, TRPM6 161; TRPC3 243; TRPC6 237; TRPM6 347; TRPM7 214; and GAPDH 200. High glucose and oxidative stress increased TRP mRNA in monocytes. We determined the effects of high glucose and oxidative stress on TRPC3 and TRPC6 mRNA in human monocytes. As shown in Fig. 1, compared with control conditions with 5.6 mmol/l glucose, administration of 30 mmol/l glucose significantly increased TRPC3 mRNA by 1.55-fold (P < 0.01; n = 6) and TRPC6 mRNA by 2.58-fold (P < 0.01; n = 12). The administration of high glucose mRNA did not significantly affect TRPC3 or TRPC6 mRNA. Concurrent administration of superoxide dismutase mimetic TEMP (final concentration 100 µmol/l) blocked the stimulating effect of high glucose on TRPC3 and TRPC6 mRNA expression, indicating that high glucose causes its effects by increased oxidative stress. Compared with control conditions, oxidative stress induced by 100 µmol/l ONOO also significantly increased the TRPC3 mRNA by 1.77-fold (P < 0.01; n = 15) and TRPC6 mRNA by 3.47-fold (P < 0.01; n = 11).

The administration of high glucose significantly increased mRNA from TRPC1, TRPC5, TRPC6, or TRPM7 by 1.01, 1.10, 0.91, or 1.02-fold compared with control conditions, respectively (each P < 0.05; n = 4). The administration of ONOO significantly increased mRNA from TRPC1, TRPC5, TRPC6, or TRPM7 by 4.57, 3.55, 12.91, or 3.51-fold, respectively (each P < 0.05; n = 4). We now investigated whether upregulated TRP expression was associated with elevated monocyte inflammatory gene expression. Indeed, after the administration of high glucose or ONOO and upregulation of TRP channels, mRNA of the inflammatory cytokine TNF-α was also
significantly increased by 6.11- or 2.32-fold (each \( P < 0.05 \); \( n = 4 \)), respectively.

Next, we evaluated whether induction of oxidative stress by several pathways may affect TRPC expression. Induction of oxidative stress using lipopolysaccharide significantly increased TRPC3 mRNA expression in monocytes from 1.00 ± 0.48 to 9.60 ± 0.40 (each \( n = 5 \); \( P < 0.05 \)) and TRPC6 mRNA expression from 1.00 ± 0.23 to 3.90 ± 0.11 (each \( n = 5 \); \( P < 0.05 \)). It is known that TNF-α-induced activation of NADPH oxidase increases superoxide radicals (17). TNF-α significantly increased TRPC3 mRNA expression in monocytes from 1.00 ± 0.01 to 1.58 ± 0.21 (each \( n = 4 \); \( P < 0.05 \)) and TRPC6 mRNA expression from 1.00 ± 0.00 to 1.32 ± 0.08 (each \( n = 4 \); \( P < 0.05 \)). In agreement with these findings, the reduction of superoxide radicals using diphenylene iodonium (18) significantly reduced TRPC3 mRNA expression in monocytes from 1.00 ± 0.06 to 0.52 ± 0.05 (each \( n = 5 \); \( P < 0.05 \)) and TRPC6 mRNA expression from 1.00 ± 0.12 to 0.36 ± 0.03 (each \( n = 5 \); \( P < 0.05 \)). These data indicate that TRPC3 and TRPC6 mRNA are regulated by oxidative stress, in particular by superoxide radicals.

We also investigated a possible role of phospholipase C on glucose-induced TRPC expression. Compared with control conditions, the inhibition of phosphatidylcholine-specific phospholipase C by D609, or the inhibition of phospholipase C activation by U73122, did not significantly affect high \( \beta \)-glucose–induced TRPC3 mRNA expression (control 1.00 ± 0.01; high \( \beta \)-glucose 1.57 ± 0.10 [\( P < 0.05 \) compared with control]; high \( \beta \)-glucose plus D609 1.47 ± 0.03 [not significant compared with high \( \beta \)-glucose alone]; high \( \beta \)-glucose plus U73122 1.95 ± 0.03 [not significant compared with high \( \beta \)-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 upregulation of TRPC mRNA expression.

To evaluate whether increased TRPC mRNA is a feature of diabetes, we compared TRPC1, TRPC3, TRPC5, and TRPC6 mRNA in monocytes from 18 patients with type 2 diabetes (10 women, 8 men; mean age 57 ± 5 years; systolic blood pressure 133 ± 3 mmHg; diastolic blood pressure 79 ± 2 mmHg; serum sodium 135 ± 1 mmol/L; serum potassium 4.4 ± 0.1 mmol/L; hemoglobin 11.2 ± 0.5 g/dL; A1C 7.5 ± 0.3%) and from 28 age-matched control subjects (10 women, 18 men; mean age 56 ± 4 years;
systolic blood pressure 132 ± 4 mmHg; diastolic blood pressure 81 ± 2 mmHg; serum sodium 136 ± 1 mmol/l; serum potassium 4.4 ± 0.2 mmol/l; hemoglobin 12.3 ± 0.5 g/dl; A1C 5.6 ± 0.1%). TRPC6 mRNA was significantly higher in monocytes from patients with type 2 diabetes compared with control subjects (TRPC6 normalized ratio 0.028 ± 0.014 [n = 18] vs. 0.015 ± 0.006 [n = 28]; P < 0.05; Fig. 1E). On the other hand, TRPC1, TRPC3, or TRPC5 mRNA was not significantly different between the two groups (TRPC1 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.

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 Fig. 2, compared with control conditions with 5.6 mmol/l D-glucose, administration of high D-glucose (30 mmol/l) did not significantly affect TRPC3 or TRPC6 protein expression in monocytes. Concurrent administration of TMP (100 μmol/l) and high D-glucose normalized OAG-induced calcium influx. These data indicate that high D-glucose–associated oxidative stress promotes increased TRP-mediated calcium influx.

After treatment of monocytes with high D-glucose for 4 h, the administration of ONOO subsequently increased intracellular calcium levels. In the presence of a membrane-permeable TRPC blocker, 2-APB, the ONOO-induced calcium increase was significantly attenuated (3.99 ± 0.03 vs. 4.56 ± 0.04; each n = 15; P < 0.01 compared with ONOO alone; Fig. 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. The presence of high glucose levels is a characteristic feature of diabetes and is considered 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 (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 superoxide dismutase mimic TMP.

Activated monocytes in patients with diabetes 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 after high-glucose–induced oxidative stress. We observed that high glucose–induced oxidative stress increased the expression of both TRPC3 and TRPC6 mRNA and proteins in monocytes. The stimulating effect of high glucose increased calcium influx after high glucose–induced oxidative stress. We observed that high-glucose–induced oxidative stress increased the expression of both TRPC3 and TRPC6 channel proteins. Therefore, high-glucose–induced oxidative stress in patients with diabetes 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 TRPC expression. Induction of oxidative stress using lipopolysaccharide- or TNF-α–induced activation of NADPH 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 glucose–induced oxidative stress increases TRPM6 and TRPM7 mRNA expression in agreement with data from Yamamoto et al. (27), who showed the regulation of TRPM expression by oxidative stress.

To evaluate whether increased TRPC mRNA is a feature of diabetes, we also compared TRPC mRNA in monocytes from patients with type 2 diabetes and control subjects. We observed increased TRPC6 mRNA in monocytes from patients with type 2 diabetes. Increased TRPC6 protein expression has already been reported in platelets from patients with diabetes compared with control subjects (28), supporting the view that increased TRPC6 expression is a characteristic feature of peripheral blood cells from patients with diabetes. Furthermore, Hu et al. (29) showed increased TRPC6 expression in the adrenal medulla from Ossabaw miniature pigs with pre-diabetic metabolic syndrome. The effects of high glucose could be cell specific. For example, Graham et al. (30) showed that high glucose downregulates TRPC6 in cultured mesangial cells. 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 phosphatidylinositol 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.

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FIG. 3. Recordings of fura-2 fluorescence in human monocytes. **P < 0.01.
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