Regulation of Intracellular Glucose and Polyol Pathway by Thiamine and Benfotiamine in Vascular Cells Cultured in High Glucose*

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Hyperglycemia is a causal factor in the development of the vascular complications of diabetes. One of the biochemical mechanisms activated by excess glucose is the polyol pathway, the key enzyme of which, aldose reductase, transforms D-glucose into D-sorbitol, leading to imbalances of intracellular homeostasis. We aimed at verifying the effects of thiamine and benfotiamine on the polyol pathway, transketolase activity, and intracellular glucose in endothelial cells and pericytes under high ambient glucose. Human umbilical vein endothelial cells and bovine retinal pericytes were cultured in normal (5.6 mmol/liter) or high (28 mmol/liter) glucose, with or without thiamine or benfotiamine 50 or 100 μmol/liter. Transketolase and aldose reductase mRNA expression was determined by reverse transcription-PCR, and their activity was measured spectrophotometrically; sorbitol concentrations were quantified by gas chromatography-mass spectrometry and intracellular glucose concentrations by fluorescent enzyme-linked immunosorbent assay method. Thiamine and benfotiamine reduce aldose reductase mRNA expression, activity, sorbitol concentrations, and intracellular glucose while increasing the expression and activity of transketolase, for which it is a coenzyme, in human endothelial cells and bovine retinal pericytes cultured in high glucose. Thiamine and benfotiamine correct polyol pathway activation induced by high glucose in vascular cells. Activation of transketolase may shift excess glycolytic metabolites into the pentose phosphate cycle, accelerate the glycolytic flux, and reduce intracellular free glucose, thereby preventing its conversion to sorbitol. This effect on the polyol pathway, together with other beneficial effects reported for thiamine in high glucose, could justify testing thiamine as a potential approach to the prevention and/or treatment of diabetic complications.

Diabetic retinopathy is one of the most serious complications in diabetic patients and a leading cause of blindness. Among its earliest steps is the loss of retinal microvascular pericytes.

Hyperglycemia is a prerequisite for the development of the chronic complications of diabetes, but the precise mechanisms leading to vascular and tissue damage have not been fully elucidated. Biochemical mechanisms that have been hypothesized to account for the adverse effects of hyperglycemia include increased glucose flux through the polyol pathway (1), formation of advanced glycation end-products (AGE) (2, 3), accelerated generation of reactive oxygen species (ROS) (4, 5), and activation of the diacylglycerol-protein kinase C pathway (6, 7). It has been suggested that excess production of ROS inside the endothelium, resulting from increased glucose flux through the Krebs cycle, may represent a possible common denominator ("unifying mechanism") of these apparently independent biochemical pathways (5). In fact, ROS can partially inhibit glyceraldehyde-phosphate dehydrogenase, resulting in the accumulation of glycolytic metabolites, among which glyceraldehyde 3-phosphate (G3P) is particularly active in promoting AGE formation, protein kinase C activation, and increased flux through the hexosamine pathway.

Previous studies suggested that polyol pathway hyperactivity is implicated in the pathogenesis of diabetic retinopathy (8, 9). Aldose reductase (AR), the first and rate-limiting enzyme of the polyol pathway, catalyzes the reduction of glucose to the corresponding sugar alcohol, sorbitol, which is then converted to fructose by sorbitol dehydrogenase. The flux through this alternative route increases significantly when supranormal glucose levels saturate hexokinase, the enzyme responsible for transforming glucose into glucose 6-phosphate, the first metabolite of the long-term complications of diabetes (8, 12–17).

Potentially, thiamine could prevent cell damage induced by hyperglycemia by removing excess G3P from the cytoplasm and facilitating utilization of acetyl-CoA derived from accelerated glycolysis (18). We have shown previously that thiamine (18) and its lipophilic analogue benfotiamine (19), which has higher bioavailability after oral administration, normalize cell replication, lactate production, and AGE formation in human umbilical vein and bovine retinal endothelial cells cultured in high glucose concentrations. Thiamine was also found to inhibit albumin glycation in vitro (20, 21) and to increase transketolase (TK) activity while decreasing the triosephosphate pool and methylglyoxal formation in human red blood cells incubated in high glucose (22). In addition, benfotiamine has been shown to prevent experimental diabetic retinopathy (23), and high doses of thiamine and benfotiamine have been reported to prevent incipient nephropathy in streptozotocin-diabetic rats (24). To account for the above results, it was shown that benfotiamine normalizes excess ROS production in the endothelium, along
with the hexosamine pathway, AGE formation, and the diacylglycerol-protein kinase C pathway, thus involving three branches of the unifying mechanism (23).

The aim of this study was to verify whether thiamine and benfotiamine also modify high glucose-induced activation of the polyol pathway (i.e. the branch of the unifying mechanism for which an effect of vitamin B1 has not been explored thus far) in endothelial cells and retinal pericytes.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were purchased from Sigma-Aldrich unless otherwise stated. Benfotiamine was a kind gift of Wörwag Pharma (Böblingen, Germany).

Cell Cultures—Human endothelial cells (HUVEC) were obtained from human umbilical cords and cultured with a partial modification of Jaffe’s method (25) as described previously (18). Pooled cells from 3–5 cords were grown in medium 199-Hepes modification (M199) with 20% FCS added, until confluent. In secondary cultures, HUVEC were kept in M199 + 20% FCS + 50 μg/ml endothelial cell growth supplement.

Bovine retinal pericytes (BRP) were obtained from pools of 15–20 bovine retinas with a partial modification of the method of Wong et al. (26) and McIntosh et al. (27) as described previously (28). BRP were characterized by 3G5 (a specific membrane ganglioside) fluorescence immunostaining (29). They were grown in Dulbecco’s modified Eagle’s...
medium, 5.6 mmol/liter glucose with 20% FCS in primary cultures and 10% FCS in secondary cultures.

FIGURE 2. Effects of thiamine and benfotiamine on AR activity induced by high glucose in HUVEC (A) and in BRP (B). Results are means ± S.D. of six separate experiments expressed as milliunits/mg protein. Columns: white, 5.6 mmol/liter glucose (G5.6); black, 28 mmol/liter glucose (G28); light gray, 28 mmol/liter glucose + 50 μmol/liter thiamine (G28T50); 28 mmol/liter glucose + 100 μmol/liter thiamine (G28T100); dark gray, 28 mmol/liter glucose + 50 μmol/liter benfotiamine (G28BT50); 28 mmol/liter glucose + 100 μmol/liter benfotiamine (G28BT100). $, p < 0.028 versus 5.6 mmol/liter glucose; *, p < 0.028 versus 28 mmol/liter glucose.

medium, 5.6 mmol/liter glucose with 20% FCS in primary cultures and 10% FCS in secondary cultures.

For all experiments, cells were incubated for 7 days with one of the following: 5.6 mmol/liter glucose (G5.6), 28 mmol/liter glucose (G28), 5.6 mmol/liter glucose plus 50 or 100 μmol/liter thiamine; 5.6 mmol/liter glucose plus 50 or 100 μmol/liter benfotiamine; 28 mmol/liter glucose plus 50 or 100 μmol/liter thiamine; 28 mmol/liter glucose plus 50 or 100 μmol/liter benfotiamine.

Aldose Reductase and Transketolase mRNAs—Total RNA was isolated from HUVEC and BRP by the High Pure RNA Isolation kit (Roche Applied Science). Contaminating DNA was removed using DNase I enclosed in the kit. The yield of each RNA sample was checked by spectrophotometric measurement of absorbance at 260 nm.

RT-PCR was performed with 0.5 μg of RNA using the Qiagen OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany). The assay was designed for multiplex RT-PCR, each reaction set containing primers for AR and TK and for β-actin as an internal control, using the QuantumRNA β-actin internal standards instruction manual (Ambion, Austin, TX). Amplification was performed using the following cycling parameters: hold at 50 °C for 30 min (reverse transcriptase step), hold at 95 °C for 15 min (hot-start to PCR), 22 or 20 cycles of 95 °C (30 s)/55 °C (30 s)/72 °C (1 min) followed by a final hold at 72 °C for 10 min. The number of cycles for each of the reactions was determined by the linear range of amplification (data not shown). The RT-PCR products were visualized by electrophoresis in 2% agarose gels containing 1 μg/ml ethidium bromide and were quantified using an image analysis system (1D Image Analysis System, Kodak Co.). To determine the levels of AR and TK mRNA expression, the ratios of AR to β-actin and TK to β-actin were evaluated (according to QuantumRNA β-actin internal standards instruction manual).

Primers used for AR were as follows: 5’ primer, CCTGGAAGTCCCTCCCGAGGCGG; 3’ primer, GGTTGAATTGGAAGATGCCAAATGC (which generated a 432-bp product). Primers for TK were as follows: 5’ primer, CCCCAGCTAAAGTTGGACGACACG; 3’ primer, GGTTCATCTGGCTCTTCAGGACG (which generated a 580-bp product).

Enzyme Assays—HUVEC and BRP were harvested by trypsinization and washed with ice-cold phosphate-buffered saline, pH 7.4. Cell suspensions were lysed with 250 μl of M-PER mammalian protein extraction reagent (Pierce) and then centrifuged at 14,000 × g for 10 min at 4 °C. Protein concentrations in the supernatant fractions were determined using the Bradford reagent.

AR activity was determined in a reaction mixture containing 10 mmol/liter D,L-glyceraldehyde, 0.1 mol/liter sodium phosphate buffer, pH 6.8, 0.38 mol/liter ammonium sulfate, 10 mmol/liter NADPH, and 0.1 mmol/liter EDTA (30, 31). The enzyme reaction was started by the addition of 50 μl of supernatant fractions, and incubation was continued at 25 °C for 10 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH/min.

TK activity was determined in a reaction mixture containing 15 mmol/liter ribose 5-phosphate, 250 μmol/liter NADH, 0.1 mol/liter Tris-HCl, pH 7.8, 200 units/ml glycerol-3-phosphate dehydrogenase, and triosephosphate isomerase. The enzyme reaction was started by the addition of 100-μl supernatant fractions, and the absorbance at 340 nm was measured at 10-min intervals for 2 h. The activity was deduced from the difference in the absorbance at 10 and 80 min (23). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH/min.

Determination of Intracellular Sorbitol Levels—HUVEC and BRP were harvested by trypsinization and washed with ice-cold phosphate-buffered saline, pH 7.4. The cell suspensions were sonicated and lyophilized. The dried samples were dissolved in 0.3 mol/liter HAH-DMAP reagent (0.46 mmol/liter hydroxylamine hydrochloride and 0.33 mmol/liter DMAP, pH 6.8, 0.38 mol/liter ammonium sulfate, 10 mmol/liter NADPH, and 0.1 mmol/liter EDTA) and then centrifuged at 14,000 g for 10 min at 4 °C. The supernatant was treated with 1.5 ml of acetic anhydride, and then heated at 75 °C for 10 min. The supernatant was treated with 50 μl of 1.5 mol/liter of acetic acid, mixed, and aspirated. After additional 15 min. After the mixture was cooled at room temperature, 2 ml of 1,2-dichloroethane was added. Samples were immediately washed twice by adding 2 ml of 1.0 N hydrochloric acid, mixing, and aspirating the upper aqueous layer. The lower organic layers were placed on the heating module at 75 °C and evaporated under a stream of nitrogen.

When dry, residues were reconstituted with the mobile phase of LC/MS/MS (TSQ, Finnigan MAT, San Jose, CA).

Sorbitol levels were determined by LC/MS/MS (TSQ, Finnigan MAT) using a modification of the method of Gordon and Wayne (32). The protein concentration of the supernatant fractions was determined using the Bradford reagent.

Determination of Intracellular Glucose Levels—The intracellular glucose content in HUVEC and BRP was assessed by Amplex® Red glucose assay kit (Molecular Probes, Eugene, OR), a sensitive one-step fluorometric method for detecting glucose. Cells were harvested by trypsinization, washed twice with phosphate-buffered saline, sonicated, and centrifuged at 11000 × g at room temperature for 10 min. The glucose was measured using a modification of the method of Gordon and Wayne (32). The protein concentration of the supernatant fractions was determined using the Bradford reagent.

Determination of Intracellular Glucose Levels—The intracellular glucose content in HUVEC and BRP was assessed by Amplex® Red glucose assay kit (Molecular Probes, Eugene, OR), a sensitive one-step fluorometric method for detecting glucose. Cells were harvested by trypsinization, washed twice with phosphate-buffered saline, sonicated, and centrifuged at 11000 × g at room temperature for 10 min. The
upper clear aqueous layer was used to measure glucose concentration according to the instruction manual.

Statistical Analysis—Because of the batch-to-batch variations, AR and TK mRNA expression, intracellular glucose, and sorbitol levels are expressed as percentages (mean ± S.D.) of the results obtained with positive control conditions (i.e. cells grown in 5.6 mmol/liter glucose) within each experiment unless stated otherwise. Statistical comparisons between groups were carried out using Wilcoxon’s rank sum test. Results were considered significant if the $p$ value was 0.05 or less.

RESULTS

Effects of Thiamine and Benfotiamine on Aldose Reductase Expression and Activity—AR expression ($n = 6$) was increased in the presence of high glucose concentrations both in HUVEC (183.5 ± 36.3% versus G5.6, $p = 0.028$) and BRP (218.9 ± 41.6%, $p = 0.031$). This effect of high glucose was reversed by the addition of thiamine and benfotiamine, both in HUVEC (Fig. 1, $A$ and $B$) and in BRP (Fig. 1, $C$ and $D$). AR activity was also increased in high glucose ($n = 6$) in HUVEC (6.5 ± 0.9 versus 1.8 ± 0.6 nmol/min/mg protein in G5.6, $p = 0.027$), as well as BRP (4.5 ± 0.3 versus 1.1 ± 0.2 nmol/min/mg protein in G5.6, $p = 0.027$). Again, this high glucose-induced effect was reversed by thiamine and benfotiamine (Fig. 2). Thiamine and benfotiamine on their own (i.e. when added to physiological glucose) had no significant effect on AR mRNA expression and activity.

Effects of Thiamine and Benfotiamine on Transketolase Expression and Activity—TK mRNA expression ($n = 6$) was not increased by high glucose alone in HUVEC or BRP. Thiamine and benfotiamine increased TK expression only when added to high glucose (Fig. 3). Similar results

FIGURE 3. Effects of thiamine and benfotiamine on TK mRNA expression induced by high glucose in HUVEC and BRP. $A$ and $C$, quantitation of TK mRNA expression by an image analysis system in HUVEC ($A$) and in BRP ($C$). Data were normalized to β-actin mRNA expression, and results are means ± S.D. of six separate experiments expressed as percentages of the values in 5.6 mmol/liter glucose. $B$ and $D$, electrophoresis of TK and β-actin in HUVEC ($B$) and in BRP ($D$). Columns: white, 5.6 mmol/liter glucose (G5.6); black, 28 mmol/liter glucose (G28); light gray, 28 mmol/liter glucose + 50 μmol/liter thiamine (G28T50); 28 mmol/liter glucose + 100 μmol/liter thiamine (G28T100); dark gray, 28 mmol/liter glucose + 50 μmol/liter benfotiamine (G28BT50); 28 mmol/liter glucose + 100 μmol/liter benfotiamine (G28BT100). *, $p < 0.028$ versus 28 mmol/liter glucose.
Increased erythrocyte sorbitol/blood glucose ratio (33) and AR protein levels (34) were reported in diabetic patients with complications. Moreover, a recent study on rat and human retinas demonstrates that there is a role for the polyol pathway in human diabetic retinopathy and that aldose reduction inhibition prevents vascular damage (23, 24). Activation of TK may shift excess G3P from glycolysis into the non-oxidative pentose phosphate shunt, its activation by thiamine might reduce superoxide overproduction. However, the possible effects of thiamine and benfotiamine on the polyol pathway, the fourth biochemical mechanism involved in the unifying hypothesis, were never explored. Our results confirm that TK mRNA and activity increased after the addition of thiamine and benfotiamine to high glucose, although not in the presence of high glucose alone. As suggested above, this might result in shifting of free glucose into glycolysis and away from the polyol pathway, thus accounting for reduced AR mRNA and activity along with sorbitol concentrations.

Thiamine and benfotiamine were shown to prevent incipient nephropathy and retinopathy in streptozotocin-diabetic rats, in which they increased the conversion of triosephosphates to ribose 5-phosphate through activation of TK, decreased protein kinase C activation, and reduced protein glycation (23, 24). Activation of TK by high dose thiamine and benfotiamine was reported also in human red blood cells (22), bovine aortic endothelial cells, and the retinas of diabetic rats (23).

One of the goals of research in AR and the pathophysiology of diabetic complications is the development of drugs that are effective in inhibiting excess flux through the polyol pathway. We propose that thiamine and benfotiamine may exert such effects. Together with recent reports (22, 23, 36) suggesting that they may counteract several mechanisms implicated in the pathogenesis of damage induced by high glucose, our results further support the notion that vitamin B1 and its derivatives are inexpensive and easily available compounds that may prove useful in the prevention and/or treatment of diabetic complications.
FIGURE 5. Effects of thiamine and benfotiamine on sorbitol concentration in HUVEC (A) and BRP (B) and intracellular glucose also in HUVEC (C) and BRP (D) cultured in high glucose. Results are means ± S.D. of six separate experiments expressed as percentages of the values in 5.6 mmol/liter glucose. Columns: white, 5.6 mmol/liter glucose (G5.6); black, 28 mmol/liter glucose (G28); light gray, 28 mmol/liter glucose + 50 μmol/liter thiamine (G28T50); 28 mmol/liter glucose + 100 μmol/liter thiamine (G28T100); dark gray, 28 mmol/liter glucose + 50 μmol/liter benfotiamine (G28BT50); 28 mmol/liter glucose + 100 μmol/liter benfotiamine (G28BT100). §, p < 0.05 versus 5.6 mmol/liter glucose; *, p < 0.05 versus 28 mmol/liter glucose.
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