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F. Pomero • A. Molinar Min • M. La Selva • A. Allione • G.M. Molinatti • M. Porta

Benfotiamine is similar to thiamine in correcting endothelial cell defects induced by high glucose

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Abstract We investigated the hypothesis that benfotiamine, a lipophilic derivative of thiamine, affects replication delay and generation of advanced glycosylation end-products (AGE) in human umbilical vein endothelial cells cultured in the presence of high glucose. Cells were grown in physiological (5.6 mM) and high (28.0 mM) concentrations of D-glucose, with and without 150 μM thiamine or benfotiamine. Cell proliferation was measured by mitochondrial dehydrogenase activity. AGE generation after 20 days was assessed fluorimetrically. Cell replication was impaired by high glucose (72.3%±5.1% of that in physiological glucose, p=0.001). This was corrected by the addition of either thiamine (80.6%±2.4%, p=0.005) or benfotiamine (87.5%±8.9%, p=0.006), although it not was completely normalized (p=0.001 and p=0.008, respectively) to that in physiological glucose. Increased AGE production in high glucose (159.7%±38.9% of fluorescence in physiological glucose, p=0.003) was reduced by thiamine (113.2%±16.3%, p=0.008 vs. high glucose alone) or benfotiamine (135.6%±49.8%, p=0.03 vs. high glucose alone) to levels similar to those observed in physiological glucose. Benfotiamine, a derivative of thiamine with better bioavailability, corrects defective replication and increased AGE generation in endothelial cells cultured in high glucose, to a similar extent as thiamine. These effects may result from normalization of accelerated glycolysis and the consequent decrease in metabolites that are extremely active in generating nonenzymatic protein glycation. The potential role of thiamine administration in the prevention or treatment of vascular complications of diabetes deserves further investigation.

Key words Diabetic microangiopathy • Glucose • Advanced glycosylation end-products • Thiamine • Benfotiamine

F. Pomero • A. Molinar Min • M. La Selva • A. Allione
G.M. Molinatti • M. Porta (✉)
Diabetic Retinopathy Centre
WHO Collaborating Centre for Diabetes-Related Blindness
Department of Internal Medicine
University of Turin
Corso A.M. Dogliotti 14, I-10126 Turin, Italy

Introduction

Alterations of glycolysis may be involved in the mechanisms that delay replication of cultured human umbilical vein endothelial cells (HUVEC) in the presence of high glucose concentrations [1–3]. In particular, intermediate metabolites such as glyceraldehyde-3-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate are more reactive with protein NH- groups than glucose itself, and therefore more able to generate glycation products [4]. Increased cytoplasmic concentrations of these intermediate products may mediate cell damage.

In a previous report [5], we showed that thiamine (vitamin B_1), an important co-factor of some enzymes regulating glycolysis and the Krebs cycle [6–8], corrects the replication delay and increases production of advanced glycosylation end-products (AGE) in HUVEC cultured in high glucose, possibly by normalizing the glycolytic flux. The present study evaluated if similar results can be obtained in vitro with benfotiamine, a lipophilic derivative of thiamine whose pharmacokinetic properties ensure better bio-availability when the molecule is administered orally [9, 10].

Materials and methods

Cell cultures

HUVEC were cultured with a modification of Jaffe et al.'s method [11], which we have previously described [5]. In brief, cells were detached from the umbilical vein by digestion with collagenase (Boehringer Mannheim, Germany) and grown until confluent in Dulbecco's modified Eagle's medium (DMEM) with 5.6 mM glucose (Sigma Chemical, St. Louis, MO, USA), supplemented with 20% fetal calf serum (Sigma). They were then trypsinized (Sigma) and seeded, approximately 5000/cm^2, in 24-well plates for proliferation assays or in 25-cm^2 flasks for AGE determination (plasticware from Celbio, Milan, Italy). After 24 h in the new culture vessels, we added the experimental media, containing final glucose concentrations of 5.6 or 28.0 mM plus 150 μM thiamine, 150 μM benfotiamine or an equal volume of diluent (culture medium) for the control tests. Benfotiamine was kindly provided by Worwag Pharma (Böblingen, Germany).

Proliferation assay

This method measures the tetrazolium salt cleavage by mitochondrial dehydrogenases of metabolically active cells [12]. HUVEC were cultured for 48 h in the different experimental media (in duplicate 16-mm wells), after which 50 μl 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Boehringer Mannheim) was added to each well. The cells were incubated at 37° C for 4 h and then solubilized with 500 μl 10% sodium dodecylsulphate in 0.01 M HCl. The plates were left overnight at 37° C and the absorbance at 580 nm was then measured using a spectrophotometer (Perkin-Elmer, Überlingen, Germany). All results were averaged readings of duplicate wells.

Fluorescent AGE determination

HUVEC were cultured for 20 days in the experimental media; the media were changed every 48 h. The cells were then detached by trypsin, washed with Dulbecco's phosphate buffered saline (Sigma) and lysed in 0.1 M NaOH. Total protein concentration was assayed by the method of Lowry and spontaneous fluorescence was measured using a fluorimeter (Jenway, mod. 6200, Dunmow, Essex, UK) at 370 nm excitation and 440 nm emission wavelengths, as described by Monnier et al. [13]. The results were calculated as fluorescence/total protein ratios. Fluorescence was taken as a non-specific marker of AGE production.

Statistic calculations

Because of the variability between different cell batches, the data observed in the different test conditions were normalized to percentages (mean±SD) of the results obtained in the presence of 5.6 mM glucose in each experiment. Differences between experimental conditions were checked by one-way analysis of variance and then confirmed by using Student's *t* test for paired data.

Results

Cell proliferation was less in 28.0 mM glucose than in 5.6 mM glucose (Table 1) (p=0.001). This reduction was partially corrected by the addition of thiamine (p=0.01 vs. 5.6 mM; p=0.005 vs. 28.0 mM glucose) or benfotiamine (p=0.008 vs. 5.6 mM glucose; p=0.006 vs. 28.0 mM glucose). The difference between 28.0 mM glucose plus thiamine and 28.0 mM glucose plus benfotiamine was not statistically significant. The addition of thiamine or benfotiamine to 5.6 mM glucose slightly reduced cell proliferation (p=0.036 and p=0.038, respectively), but the values remained significantly higher than those in 28 mM glucose (p=0.005 and p=0.002, respectively). Cell proliferation remained higher in 5.6 mM glucose plus thiamine than in 28.0 mM glucose plus thiamine (p=0.014); similarly, proliferation was greater in 5.6 mM glucose plus benfotiamine than in 28 mM glucose plus benfotiamine (p=0.037).

Fluorescence of AGE was increased by high glucose (p=0.003, Table 2). This effect was reduced by both thiamine and benfotiamine (p=0.008 and p=0.03, respectively, vs. 28 mM glucose alone) to levels that were not significantly different from those in 5.6 mM glucose. The fluorescence levels obtained with thiamine and benfotiamine added to 28 mM glucose did not differ significantly between themselves. Neither compound significantly modified the fluorescence of AGE in HUVEC kept at 5.6 mM glucose.

Table 1 Proliferation of HUVEC grown in physiological or high glucose concentrations, supplemented with 150 μM thiamine or benfotiamine. Values were normalized to that of 5.6 mM glucose and are expressed as mean (SD) of duplicate samples

	Glucose	
	5.6 mM	28.0 mM
No addition	100.0 (0)	72.3 (5.1)
Thiamine	92.0 (8.7)	80.6 (2.4)
Benfotiamine	94.0 (6.6)	87.5 (8.9)

Table 2 Fluorescence of advanced glycosylation end-products (AGE) in HUVEC cultured in physiological or high glucose concentrations, supplemented with 150 μM thiamine or benfotiamine. Values were normalized to that of 5.6 mM glucose and are expressed as mean (SD) of duplicate samples

	Glucose	
	5.6 mM	28.0 mM
No addition	100.0 (0)	159.7 (38.9)
Thiamine	116.2 (11.2)	113.2 (16.3)
Benfotiamine	115.4 (43.6)	135.6 (49.8)

Discussion

Endothelial cells cultured in the presence of high glucose concentrations decrease their replication rate [1–3]. A sustained increase in the availability of glucose amplifies the processes of nonenzymatic glycosylation of proteins, which at first generate labile aldiminic bonds (Schiff bases), followed by rearrangement into stabile compounds and, finally, advanced glycosylation end-products (AGE) [4]. High levels of the hexose in the culture medium lead to increased free glucose inside cells, where it is carried by the insulin-independent transporters GLUT-1 and GLUT-3 [14] and where it may be metabolized through, among other pathways, acceleration of the glycolytic flux. Hexokinase, the initial enzyme in glycolysis, is inhibited by its product, glucose-6-phosphate and its low K_m makes it work at nearly maximal activity even when the substrate levels are physiological [15]. Nevertheless, increased hexokinase activity in the presence of raised glucose has been reported in the renal cortex of diabetic rats [16] and in the vascular cells of the *rete mirabilis* of the eel swimbladder [17]. It is not known whether this is associated with increased expression of mRNA or post-translational modifications of the enzyme.

Accelerated glycolysis may lead to higher availability of intermediate metabolites in the cytoplasm, some of which (e.g. glyceraldehyde-3-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate) are highly active as nonenzymatic glycosylators at the lysine NH_2 ε-terminals of proteins [18]. We previously showed that thiamine (vitamin B_1) corrects the replication defect and increased AGE generation, while normalizing increased lactate production, induced by high glucose in human and bovine endothelial cells [5]. These results led us to hypothesize that thiamine reduces the damaging effects of accelerated glycolysis by shifting the excess glyceraldehyde-3-phosphate (which, although short-lived in the cell, is about 200-times as reactive as glucose in glycosylating proteins) [18] into the pentose-phosphate shunt through its co-factor activity on transketolase [6]. In addition, glycolytic metabolites may be cleared away efficiently because thiamine also acts as a co-factor for two other enzymes, pyruvate dehydrogenase [7] and α-ketoglutarate dehydrogenase [8], which permit increased disposal of acetyl-CoA through the Krebs cycle.

An additional possibility is that thiamine protects endothelial cells from high glucose-induced damage by increasing the availability of reduced glutathione [19], which in turn depends on the recycling of oxidized glutathione through an NADPH-requiring reaction [20]. Because the pentose phosphate shunt is an important source of NADPH, and since it is potentianted by thiamine [21], vitamin B_1 has been suggested to act indirectly as an antioxidant [19].

Benfotiamine is a liposoluble derivative of vitamin B_1, belonging to the allithiamines family [9]. Allithiamines differ from hydrophilic molecules for the presence of an open thiazole ring which, when the compound becomes biologically active, is closed by an intracellular reducing reaction [10]. Benfotiamine was developed in the Far East in the 1950s as an alternative to thiamine for the treatment of vitamin B_1 deficiency, due to its higher bioavailability when administered orally [22]. Benfotiamine, though not thiamine, reduced the generation of AGE and *N*-carboxymethyl-lysine in Wistar diabetic rats [23]. On the other hand, we observed similar effects on cell replication and AGE generation in HUVEC for the same molar concentrations of thiamine and benfotiamine. Different methods for AGE assay may account for this discrepancy, even though the effects reported in this paper occur in vitro at relatively high concentrations of thiamine and benfotiamine. Although we were unable to obtain precise information on the circulating levels of vitamin B_1 in humans, either during steady state or after acute oral administration, a concentration of 150 μM is likely to be about one order of magnitude higher than that reached in the blood but may occur locally at interface sites, such as the vascular endothelium. Thus, a different behavior in vivo may be linked to higher concentrations in tissues resulting from the more favorable bioavailability of benfotiamine.

This paper, while confirming our previous report on thiamine [5], shows that similar effects are produced by its lipophilic derivative in an in vitro system. Whether these results can translate into useful effects of benfotiamine in the pharmacological prevention or treatment of the vascular complications of diabetes remains to be established.

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