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RESEARCH ARTICLE

Thiamine and benfotiamine prevent increased apoptosis in endothelial cells and pericytes cultured in high glucose

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Abstract

Background High glucose induces pathological alterations in small and large vessels, possibly through increased formation of AGE, activation of aldose reductase and protein kinase C, and increased flux through the hexosamine pathway. We showed previously that thiamine and benfotiamine correct delayed replication and increase lactate production in endothelial cells subjected to high glucose. We now aim at verifying the effects of thiamine and benfotiamine on cell cycle, apoptosis, and expression of adhesion molecules in endothelial cells and pericytes, under high ambient glucose.

Methods Human umbilical vein endothelial cells and bovine retinal pericytes were cultured in normal (5.6 mmol/L) or high (28 mmol/L) glucose, with or without thiamine or benfotiamine, 50 or 100 μmol/L. Apoptosis was determined by two separate ELISA methods, measuring DNA fragmentation and caspase-3 activity, respectively. Cell cycle and integrin subunits $\alpha 3$, $\alpha 5$, and $\beta 1$ concentration were measured by flow cytometry.

Results Apoptosis was increased in high glucose after 3 days of culture, both in endothelium and pericytes. Thiamine and benfotiamine reversed such effects. Neither cell cycle traversal nor integrin concentrations were modified in these experimental conditions.

Conclusions Thiamine and benfotiamine correct increased apoptosis due to high glucose in cultured vascular cells. Further elucidations of the mechanisms through which they work could help set the basis for clinical use of this vitamin in the prevention and/or treatment of diabetic microangiopathy.

Keywords diabetic retinopathy; endothelium; pericytes; glucose; thiamine; benfotiamine

Introduction

Onset and severity of vascular complications in both type 1 and 2 diabetes are clearly associated with the duration and degree of hyperglycaemia [1,2]. Supra-physiological concentrations of glucose cause a number of alterations in small and large vessels, the mechanisms of which are not yet fully understood. Among possible mechanisms, increased formation of advanced glycation end products (AGE) [3], increased aldose reductase (AR) activation with consequent hyperglycaemic pseudohypoxia [4], glucose-induced activation of protein kinase C through *de novo* synthesis of the lipid second

messenger, diacylglycerol [3], and increased flux through the hexosamine biosynthetic pathway [5] seem to play major roles. It has been hypothesized that the possible common denominator ('unifying mechanism') of these apparently independent biochemical pathways is high-glucose-induced excess production of reactive oxygen species (ROS) inside the endothelium, as a result of increased flux through the Krebs' cycle [6]. ROS can partially inhibit glyceraldehyde-phosphate dehydrogenase, resulting in the accumulation of glycolytic metabolites, among which glyceraldehyde-3-phosphate (G3P) is particularly active in glycating proteins and AGE formation.

Thiamine acts as a co-enzyme for (1) transketolase, which shifts G3P from glycolisis into the pentose-phosphate shunt, (2) pyruvate-dehydrogenase, which converts pyruvate (the final product of glycolisis) into acetyl-CoA, which then enters the Krebs' cycle, and (3) α-keto-glutarate-dehydrogenase, which catalyses the oxidation of α-keto-glutaric acid to succinyl-CoA in the Krebs' cycle. As a result, thiamine could prevent cell damage induced by hyperglycaemia by removing excess G3P from the cytoplasm [7]. We reported previously that thiamine and its lipophilic analogue, benfotiamine, which has higher bioavailability after oral administration, are able to normalize cell replication, lactate production, and AGE formation in human umbilical vein and bovine retinal endothelial cells, when added to high glucose concentrations [7,8]. Thiamine was found to inhibit albumin glycation *in vitro* [9,10] and to increase transketolase activity while decreasing the triosephosphate pool and methylglyoxal formation in human red cells incubated in high glucose [11]. Recently, we observed that thiamine normalizes pericyte adhesion to extracellular matrix produced by endothelial cells cultured in high glucose, similar to aminoguanidine, an inhibitor of non-enzymatic glycation and AGE formation [12,13]. Finally, benfotiamine was shown to prevent experimental diabetic retinopathy [14], and high doses of thiamine and benfotiamine were shown to prevent incipient nephropathy in streptozotocin-diabetic rats [15]. To account for the above results, it was proposed that benfotiamine could normalize excess ROS production inside the altered endothelium [14], both in bovine aortic endothelial cells and in diabetic rats, through inhibition of the hexosamine pathway, AGE formation, and the diacylglycerol-protein kinase C pathway, thus leading to normalization of most branches of the 'unifying mechanism'.

The aim of this study was to verify if thiamine and benfotiamine are able to counteract the damaging effects of high glucose on cell cycle, apoptosis, and adhesion molecule expression in endothelial cells and pericytes.

Materials and methods

Reagents

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

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical cords and cultured with a partial modification of Jaffe's method [16], as previously described [7]. Pools of cells from 3 to 5 cords were grown in Medium 199 – Hepes Modification (M199), added with 20% fetal calf serum (FCS), until confluent. In secondary cultures, HUVECs were kept in M199 + 20% FCS + 50 μg/mL endothelial cell growth supplement.

Bovine retinal pericytes (BRPs) were obtained from pools of 15 to 20 bovine retinas, with a partial modification of the method of Wong *et al*. [17] and McIntosh *et al*. [18], as previously described [19]. BRP were characterized by 3G5 (a specific membrane ganglioside) fluorescence immunostaining [20]. They were grown in Dulbecco's Modified Eagle's Medium (DMEM) 5.6 mmol/L glucose with 20% FCS in primary cultures and 10% FCS in secondary cultures.

Dose-response curves

To determine the lowest effective thiamine concentration, HUVECs and BRPs were kept in culture with either 5.6 (normal) or 28 mmol/L (high) D-glucose, with or without the addition of increasing concentrations of thiamine and benfotiamine (0, 25, 50, 75, 100, 150, 200 μmol/L). DNA synthesis was measured at day 2, by *Cell Proliferation ELISA, BrdU* assay (Roche Diagnostics, Mannheim, Germany), and cell counts were performed at day 7 in Bürker chambers, after staining with Trypan blue.

Cell cycle

After 2 days of culture in normal or high glucose, with or without 50- or 100-μmol/L thiamine or benfotiamine, cells were fixed and permeabilized with 70% ethanol and their DNA stained with propidium iodide (20 μg/mL in PBS + 0.1% BSA). Fluorescence was evaluated by flow cytometry, using FACScan equipment (Becton Dickinson Immunocytometry Systems, San Josè, CA, USA).

Apoptosis

Apoptosis was evaluated after 3 days of culture in the above-mentioned media, using the *Cell Death Detection ELISA^{PLUS}* (Roche), according to the manufacturer's instructions. This is a photometric enzyme immunoassay, which measures mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates, as an index of DNA fragmentation, which, in turn, is an early marker of apoptosis.

Since activation of the caspase family represents the first step of apoptosis in eukariotic cells, the results

obtained by measuring DNA fragmentation were checked by *Caspase-3 Colorimetric Activity Assay Kit* (Chemicon International, Temecula, CA, USA).

Adhesion molecules

Integrin subunits $\alpha 3$, $\alpha 5$, and $\beta 1$ concentrations were determined in HUVECs by cytofluorimetry, following a 6-day culture in the relevant media and indirect immunofluorescence staining, using, as primary antibodies, 10-μg/mL mouse anti-human CD49e, CD49c, and CD29 monoclonal antibodies (Cymbus Biotechnology, Chandlers Ford, UK) and, as a secondary antibody, 1 : 15 FITC-Conjugated Rabbit Anti-Mouse IgG (DAKO, Copenhagen, Denmark).

Statistical analysis

Because of the batch-to-batch variations, data are expressed as percentages (mean ± SD) of the results obtained with positive control conditions (i.e. cells grown in 5.6 mmol/L glucose alone) within each experiment, unless otherwise stated. Statistical comparisons between groups were carried out by Wilcoxon's rank sum test. Results were considered significant if the p-value was 0.05 or less.

Results

Dose-response curves

Thiamine 75 μmol/L restored DNA synthesis at day 2, when added to HUVECs cultured in high glucose, whereas lower concentrations had no effect ($n = 6$). Benfotiamine had no effect at any concentration on DNA synthesis in HUVECs ($n = 6$) (Figure 1a and b).

At day 7, counts of HUVECs in high glucose showed a significant recovery with both thiamine and benfotiamine from 25 μmol/L upwards ($n = 6$) (Figure 1c and d).

As regards BRPs in high glucose, DNA synthesis measurements at day 2 showed a significant effect of thiamine in concentrations of 25 μmol/L upwards, while benfotiamine had effect only at 150 μmol/L ($n = 6$) (Figure 1e and f). Cell counts at day 7 demonstrated a significant effect of thiamine from 50 μmol/L upwards, and of benfotiamine from 25 μmol/L ($n = 6$) (Figure 1g and h).

We proceeded to use 50 and 100 μmol/L thiamine and benfotiamine for the following experiments.

Cell cycle

Cell cycle evaluation showed no effect of thiamine or benfotiamine in either cell type kept in high glucose ($n = 6$, data not shown).

Apoptosis

DNA fragmentation was significantly increased in high glucose, in both HUVECs ($111.0 \pm 6\%$, $p = 0.015$ vs normal glucose, $n = 6$) and BRPs ($114.0 \pm 10\%$, $p = 0.031$ vs normal glucose, $n = 6$). Thiamine and benfotiamine reversed the adverse effect of high glucose both in HUVECs and BRPs (Figure 2a and b).

These results were confirmed by measuring caspase-3 activity. Again, high glucose increased apoptosis ($n = 6$) in HUVECs ($120.0 \pm 8\%$, $p = 0.028$ vs normal glucose) and BRPs ($128.0 \pm 15\%$, $p = 0.028$). Thiamine and benfotiamine were able to normalize this effect (Figure 2c and d).

Thiamine and benfotiamine had no significant effect on HUVEC and BRP apoptosis, when added with physiological glucose.

Adhesion molecules

Integrin subunit $\alpha 3$, $\alpha 5$, and $\beta 1$ concentrations, as determined in HUVECs, did not show any significant difference, either between normal and high glucose or after addition of thiamine or benfotiamine ($n = 3$, data not shown).

Discussion

This paper shows that thiamine and its lipid-soluble derivative benfotiamine are able to prevent increased apoptosis due to high glucose in human endothelial cells and bovine retinal pericytes. These were chosen as representatives of human vascular cells and, respectively, more anatomically pertinent cells of retinal origin, which are not easily available from human tissues.

These protective effects of thiamine and its derivative are presumably the result of shifting excess G3P from glycolisis into the pentose-phosphate shunt. G3P, one of the most effective agents of AGE formation into the cytoplasm [21], is also an end product of the non-oxidative branch of the pentose-phosphate pathway and may be produced by transketolase, a thiamine-dependent enzyme. Since the *in vivo* concentration of transketolase metabolites is 10-fold lower than the K_M of the enzyme, the net flux and the direction of the reaction are dependent on substrate concentration [22]. Transketolase expression has been shown to be activated by high-dose thiamine and benfotiamine in the renal glomeruli of diabetic rats [15], as well as human red blood cells [11], bovine aortic endothelial cells, and retinas of diabetic rats [14].

We chose to use 50 and 100 μmol/L thiamine and benfotiamine because our dose-response curves showed little effect at lower concentrations, in agreement with other data in the literature [14]. With regard to their comparative effects, our results show an overall similar behaviour for equimolar concentrations of benfotiamine and thiamine. Previously, we observed similar effects of

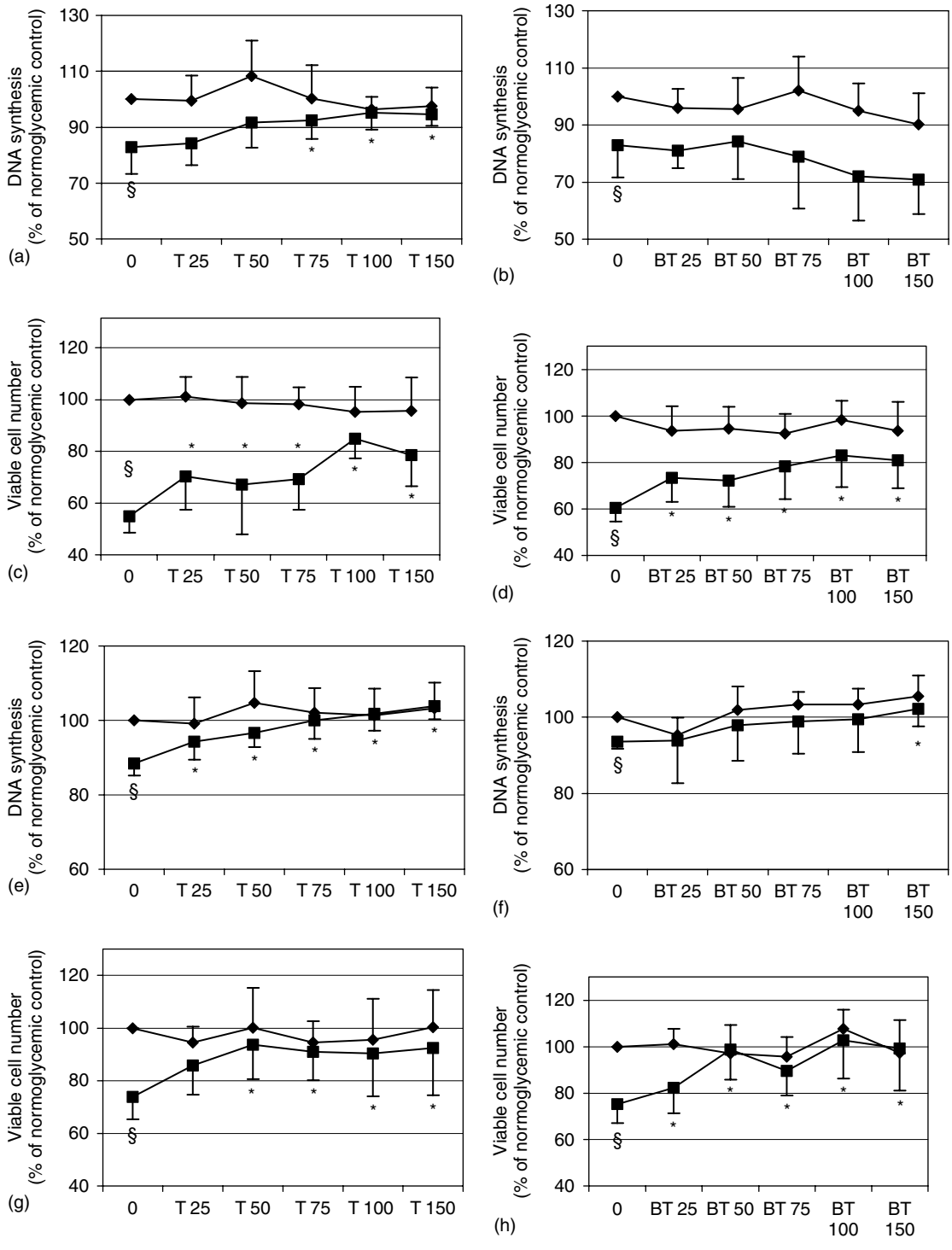

Figure 1. Dose-response curves for thiamine and benfotiamine, added to 5.6 (♦) or 28 mmol/L (■) D-glucose; (a,b) effects of thiamine and benfotiamine on DNA synthesis at day 2 in HUVECs; (c,d) effects of thiamine and benfotiamine on cell counts at day 7 in HUVECs; (e,f) effects of thiamine and benfotiamine on DNA synthesis at day 2 in BRPs; (g,h) effects of thiamine and benfotiamine on cell counts at day 7 in BRPs. § = $p < 0.005$ versus 5.6 mmol/L glucose; * = $p < 0.05$ versus 28 mmol/L glucose

thiamine and benfotiamine on endothelial cell cultured in high glucose [7,8]. *In vivo*, administration of the lipid-soluble derivative results in higher blood and tissue levels than the water-soluble one [23], and, in peripheral nerves of diabetic rats, benfotiamine, but not thiamine, was reported to prevent AGE formation [24]. Other authors, however, have shown both molecules to prevent incipient nephropathy in streptozotocin-diabetic rats, by inhibiting development of microalbuminuria, increasing conversion of triosephosphates to ribose-5-phosphate through activation of transketolase, and decreasing protein kinase C activation and protein glycation [15].

High glucose had been shown to delay cell cycle traversal in HUVECs after 7 to 8 days of culture, with cells

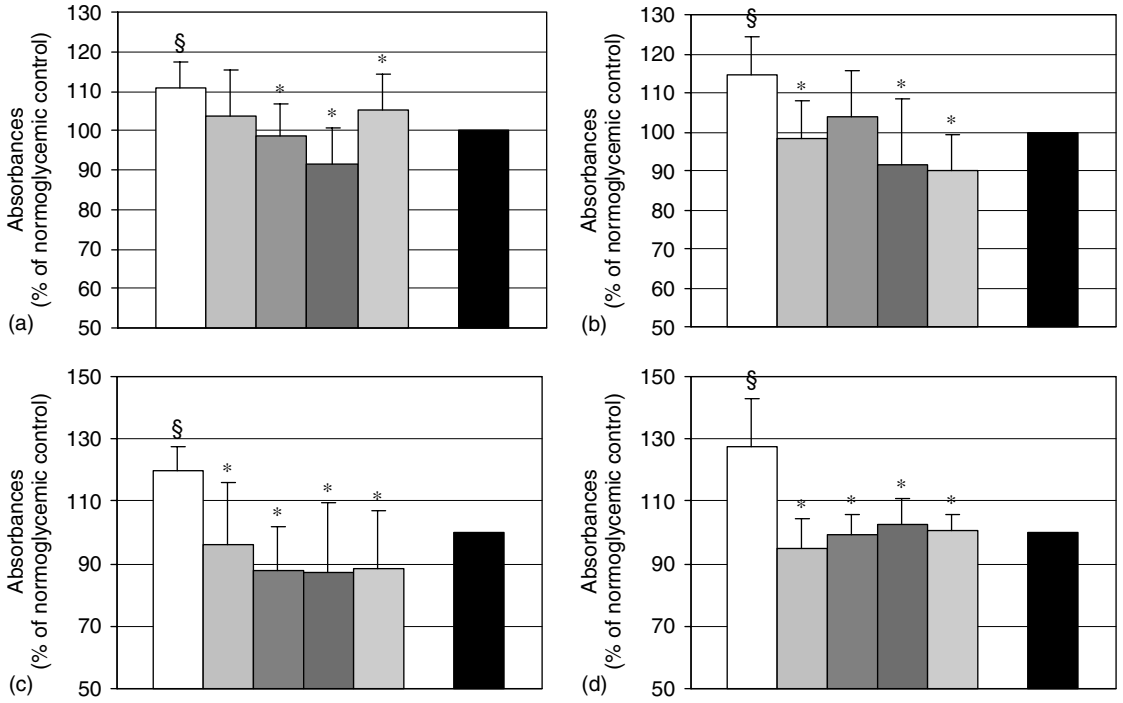

Figure 2. Effects of thiamine and benfotiamine on apoptosis induced by high glucose in HUVECs and BRPs; (a) DNA fragmentation in HUVECs; (b) DNA fragmentation in BRPs; (c) caspase-3 activity in HUVECs and (d) caspase-3 activity in BRPs. *White:* 28 mmol/L glucose; *light grey*: 28 mmol/L glucose + 50 μmol/L thiamine; *dark grey*: 28 mmol/L glucose + 100 μmol/L thiamine; *diagonals*: 28 mmol/L glucose + 50 μmol/L benfotiamine; *dots*: 28 mmol/L glucose + 100 μmol/L benfotiamine; *black*: control (5.6 mmol/L glucose). § = $p < 0.05$ versus 5.6 mmol/L glucose; * = $p < 0.05$ versus 28 mmol/L glucose

accumulating either in the S [25] or G0/G1 phases [26]. More recently, streptozotocin diabetes was reported to promote transition of endothelial cells from the G0 to the G1 phase in rat thoracic aorta [27]. In our model, neither high glucose nor thiamine or benfotiamine showed any relevant effect on cell cycle traversal. Shorter exposure (2 days) of the cells to high glucose, and the impossibility to discriminate between G0 and G1 phases, when the cells are stained by propidium iodide, may account for these discrepancies. However, when we tried longer culture times, these increasingly resulted in cell accumulation in the G0/G1 phase (data not shown), possibly because of contact inhibition, which is characteristic of confluent monolayers. Indeed, the percentages of cells we observed in the G0/G1 phase were consistent with those reported by Kimura [27].

Apoptosis is a well-described phenomenon in microvascular cells. Diabetic retinopathy is characterized by early loss of pericytes [28], along with basement membrane thickening, whereas loss of endothelial cells is possibly a later event, usually associated with capillary nonperfusion [29]. Bovine retinal pericytes are particularly vulnerable to the effects of high glucose, as their replication is impaired at lower concentrations than those able to affect bovine retinal endothelial cells [30]. Increased expression of the pro-apoptotic Bax proteins *in vivo* and *in vitro* [31] and activation of the Nuclear Factor-κB (NF-κB) *in vitro* [32] have been associated with pericyte apoptosis. We demonstrated early apoptosis for both HUVECs and BRPs, following 2-day incubation in high glucose. Superimposable results were found measuring both DNA fragmentation and caspase-3-activity. Thiamine and benfotiamine managed to reverse such effects of high glucose, possibly by protecting against the intracytoplasmic accumulation of damaging metabolites and, consequently, AGE. The latter have been shown to exert apoptotic effects on BRPs and bovine aortic endothelial cells [33,34]. In addition, benfotiamine was shown to inhibit hyperglycaemia-associated pro-apoptotic NF-κB activation by stimulating transketolase in bovine aortic endothelial cells, as well as in diabetic rats [14].

With reference to cell–extracellular matrix (ECM) interactions, high glucose was shown to increase the levels of mRNAs encoding for the fibronectin-specific integrin receptor $\alpha5\beta1$, as well as that of the integrin subunit $\alpha3$, a component of the $\alpha3\beta1$ polyspecific receptor for fibronectin, laminin, and collagen in HUVECs, and increased expression of $\beta1$-integrin in post-mortem retinal capillaries of diabetic patients compared to that of non-diabetic controls [35]. Impaired replication of endothelium on ECM obtained from other endothelial cells grown in high glucose concentrations was described previously [35,36], and a role for excess exogenous fibronectin, altering cell–ECM interactions through increased adhesion and cytoskeletal rearrangements, was hypothesized [37]. We described previously how pericyte adhesion, rather than replication, is impaired on extracellular matrix produced by endothelium in high hexose concentrations [12,13]. Our finding that no differences could be found in integrin subunits $\beta1$, $\alpha3$, and $\alpha5$ expression in endothelial

cells cultured in high glucose and, consequently, no effect of thiamine or benfotiamine is consistent with the hypothesis that reduced adhesion to ECM in high glucose is due to excess protein glycation, corrected by aminoguanidine and thiamine, rather than altered glycoprotein composition [12,13].

There is growing evidence that thiamine and some of its derivatives may counteract the damage induced by high glucose on vascular cells in experimental culture and animal models. Further elucidation of the mechanisms through which they work could help set the basis for clinical trials to test this vitamin, already available in drug or pro-drug form, as a potential and inexpensive approach to the prevention and/or treatment of diabetic microvascular complications.

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