Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy

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Three of the major biochemical pathways implicated in the pathogenesis of hyperglycemia induced vascular damage (the hexosamine pathway, the advanced glycation end product (AGE) formation pathway and the diacylglycerol (DAG)–protein kinase C (PKC) pathway) are activated by increased availability of the glycolytic metabolites glyceraldehyde-3-phosphate and fructose-6-phosphate. We have discovered that the lipid-soluble thiamine derivative benfotiamine can inhibit these three pathways, as well as hyperglycemia-associated NF-κB activation, by activating the pentose phosphate pathway enzyme transketolase, which converts glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphates and other sugars. In retinas of diabetic animals, benfotiamine treatment inhibited these three pathways and NF-κB activation by activating transketolase, and also prevented experimental diabetic retinopathy. The ability of benfotiamine to inhibit three major pathways simultaneously might be clinically useful in preventing the development and progression of diabetic complications.

Diabetes-specific microvascular disease is a leading cause of blindness, renal failure and nerve damage. Diabetes-accelerated atherosclerosis leads to increased risk of myocardial infarction, stroke and limb amputation. Large prospective clinical studies show a strong relationship between glycemia and diabetic microvascular complications in both type 1 and type 2 diabetes[1,2], and hyperglycemia seems to have an important role in the pathogenesis of diabetic macrovascular disease[2,3]. Four major molecular mechanisms have been implicated in glucose-mediated vascular damage: increased polyol pathway flux, increased hexosamine pathway flux, increased AGE formation, and activation of PKC isoforms through *de novo* synthesis of the lipid second messenger DAG. In aortic endothelial cells, hyperglycemia also activates the pro-inflammatory transcription factor NF-κB. All of these mechanisms reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain[4,5]. This superoxide partially inhibits the glycolytic enzyme glyceraldehyde phosphate dehydrogenase (GAPDH), thereby diverting upstream metabolites from glycolysis into the four major glucose-driven signaling pathways that cause hyperglycemic damage[5,6] (Fig. 1).

Two of these upstream metabolites, fructose-6-phosphate and glyceraldehyde-3-phosphate, are also end products of the non-oxidative branch of the pentose phosphate pathway. These metabolites are produced by the thiamine-dependent enzyme transketolase, which is the rate-limiting step of that branch of the pathway. However, because the *in vivo* concentration of transketolase metabolites is a least one order of magnitude lower than the K_m values, the net flux and direction of the transketolase reaction is determined by substrate concentration[7]. Hyperglycemia-induced increases in fructose-6-phosphate and glyceraldehyde-3-phosphate concentration could thus divert these metabolites to pentose-5-phosphates and erythrose-4-phosphate (Fig. 1) under conditions where transketolase is fully activated by its thiamine cofactor. Because it has previously been reported that diabetic patients have a subnormal erythrocyte transketolase activity[8], we hypothesized that thiamine supplementation might prevent activation of the three major pathways of hyperglycemic damage that depend on fructose-6-phosphate and glyceraldehyde-3-phosphate (the hexosamine pathway, the intracellular AGE formation pathway and the DAG-PKC pathway) and thus possibly prevent the development of diabetic complications (Fig. 1). Benfotiamine, a lipid-soluble thiamine derivative, has a greater bioavailability than does thiamine[9]. We evaluated the effect of benfotiamine on transketolase activity in both cultured endothelial cells and animal tissues. We also evaluated the effect of benfotiamine on hyperglycemia-induced activation of the hexosamine pathway, intracellular AGE formation, and the DAG-PKC pathway, as well as hyperglycemia-induced activation of NF-κB, in both cultured endothelial cells and retinas from long-term diabetic animals. Finally, we evaluated the effect of benfotiamine administration on the development of experimental diabetic retinopathy.

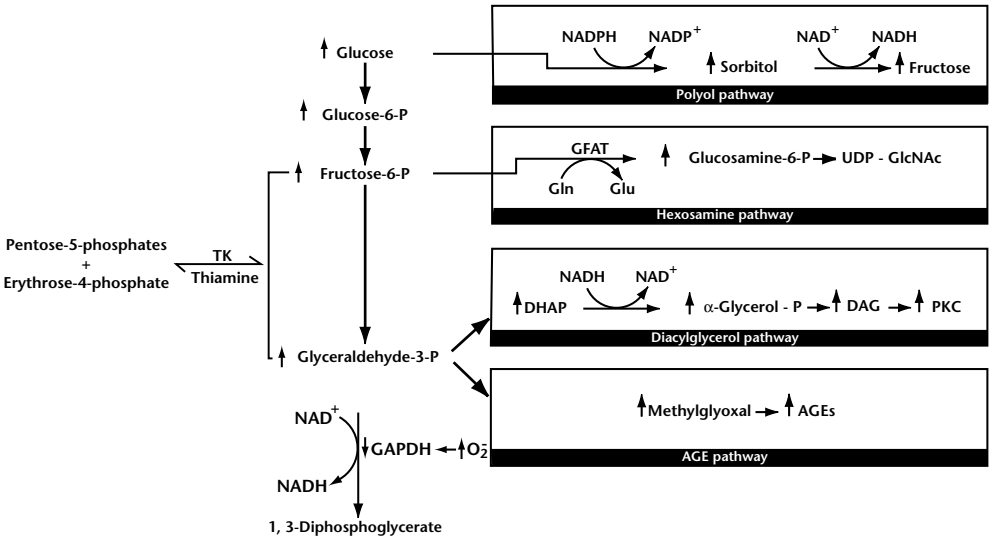


Fig. 1 Potential mechanism by which benfotiamine blocks 4 pathways of hyperglycemic damage. Hyperglycemia-induced mitochondrial superoxide overproduction partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into glucose-driven signaling pathways of glucose overuse. Benfotiamine activates the thiamine-dependent pentose phosphate pathway enzyme transketolase (TK), which converts excess fructose-6-phosphate and glyceraldehyde-3-phosphate to pentose-5-phosphates and erythrose-4-phosphate. P, phosphate; GFAT, glutamine; fructose-6-phosphate amidotransferase; DHAP, dihydroxyacetone phosphate.

Benfotiamine activates transketolase in endothelial cells

To test our hypothesis, we determined the concentration of benfotiamine required to increase transketolase activity in bovine aortic endothelial cells. Incubation in 30 mM glucose alone decreased transketolase activity, but this decrease was not statistically significant (Fig. 2). Neither 10 μM nor 25 μM benfotiamine increased transketolase activity in cells incubated in 30 mM glucose. In contrast, both 50 μM and 100 μM benfotiamine increased transketolase activity in cells by four-fold (55.9 ± 2.1 and 48.0 ± 1.6 nmol per min per mg protein for 50 μM and 100 μM benfotiamine, respectively, compared with 12.3 ± 0.3 for control; Fig. 2). We therefore selected 50 μM benfotiamine as the concentration to use in cell culture experiments, and estimated an oral dose for animal experiments based on the assumptions that distribution would be into total body water and that the estimated half-life would be 6 h[9]. There was no effect of 30 mM glucose on either intracellular thiamine pyrophosphate levels as determined by HPLC (78.8 ± 1.1 nmol/ml for 5 mM versus 82.8 ± 0.4 for 30 mM) or transketolase expression as determined by RT-PCR (0.99 ± 0.01 arbitrary units (AU) for 5 mM, 1.00 ± 0.01 for 30 mM and 0.98 ± 0.01 for 30 mM plus benfotiamine; data not shown).

Benfotiamine prevents hyperglycemic damage *in vitro*

We next examined the effect of benfotiamine on the three above-mentioned major molecular mechanisms implicated in glucose-mediated vascular damage. We also assessed the effect of benfotiamine on hyperglycemia-induced activation of NF-κB, which has been shown to be PKC dependent in aortic endothelial cells[4,10]. Incubation of bovine aortic endothelial cells with 30 mM glucose increased UDP–*N*-acetylglucosamine (UDP-GlcNAc) concentration, an indicator of hexosamine pathway flux, from 1.56 ± 0.12 nmol per mg protein in cells incubated with 5 mM glucose (Fig. 3*a*, bar 1) to 8.32 ± 0.56 nmol per mg protein (Fig. 3*a*, bar 2). This hyperglycemia-induced five-fold increase was completely prevented by 50 μM benfotiamine (1.79 ± 0.28 nmol per mg protein, Fig. 3*a*, bar 8). Similarly, incubation of bovine aortic endothelial cells with 30 mM glucose increased intracellular AGE formation from 793,198 ± 96,228 in cells incubated in 5 mM glucose (Fig. 3*b*, bar 1) to 1,586,978 ± 193,069 AU (Fig. 3*b*, bar 2). This hyperglycemia-induced 2.5-fold increase was also completely prevented by 50 μM benfotiamine (850,351 ± 109,263 AU; Fig. 3*b*, bar 8). Incubation of bovine aortic endothelial cells with 30 mM glucose increased the membrane fraction of intracellular PKC activity from 114.76 ± 24.3 in cells incubated in 5 mM glucose (Fig. 3*c*, bar 1) to 244.70 ± 9.92 pmol per min per mg protein (Fig. 3*c*, bar 2). Again, this hyperglycemia-induced 2.1-fold increase in PKC activity was completely prevented by 50 μM benfotiamine (91.48 ± 14.37 pmol per min per mg protein, Fig. 3*c*, bar 8). Similarly, incubation of cells in 30 mM glucose increased NF-κB activation by 2.1-fold, from 1,123.5 ± 30 AU in cells incubated in 5 mM glucose (Fig. 3*d*, bar 1) to 2,380.7 ± 38 in cells incubated in 30 mM glucose (Fig. 3*d*, bar 2); benfotiamine also completely prevented this effect of hyperglycemia (1,142.7 ± 35 AU (Fig. 3*d*, bar 8)). In each of these experiments, values for cells incubated with 5 mM glucose and 25 mM mannitol did not differ from those for cells incubated in 5 mM glucose alone, indicating that the effect of 30 mM glucose was not due to osmotic stress (Fig. 3*a–d*, bar 3).

We used an antisense strategy to test the hypothesis that benfotiamine blocks these pathways of hyperglycemic damage by

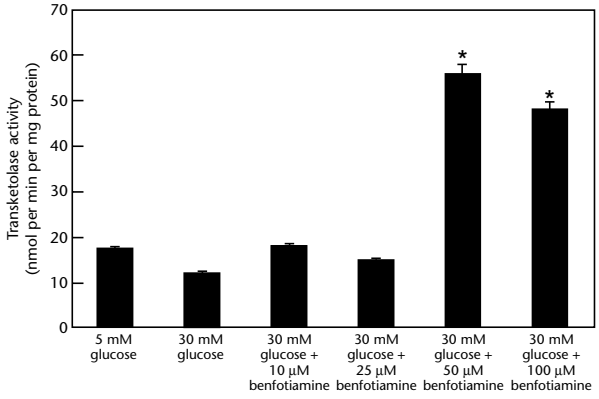


Fig. 2 Effect of glucose and benfotiamine concentration on transketolase activity in cultured bovine aortic endothelial cells. Each bar represents mean plus s.e.m. of 4 separate experiments. *, $P < 0.01$ compared with cells incubated in 5 mM glucose.

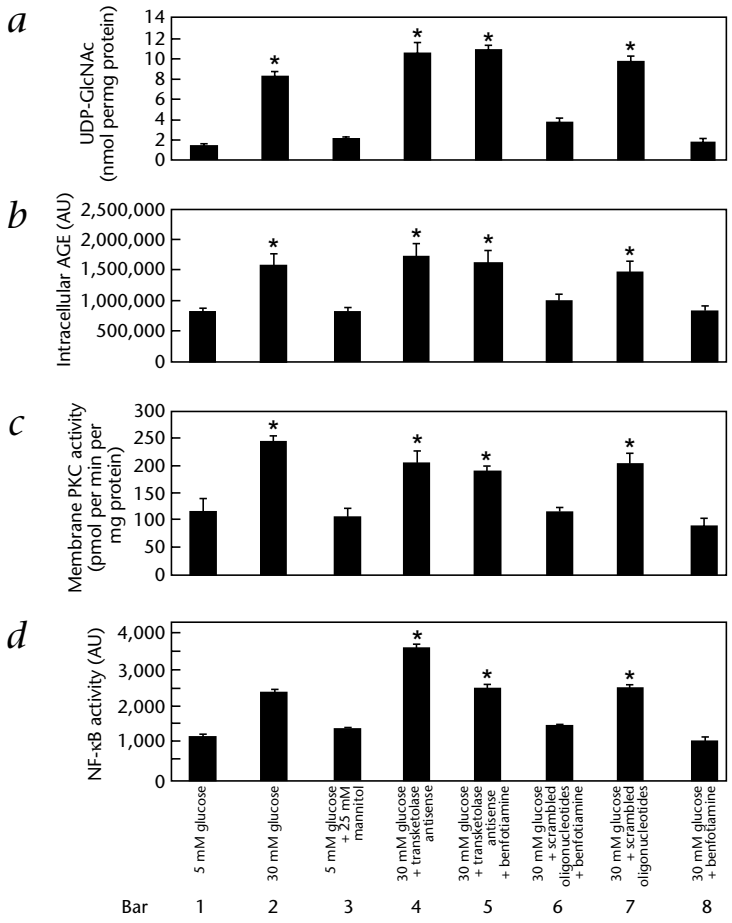
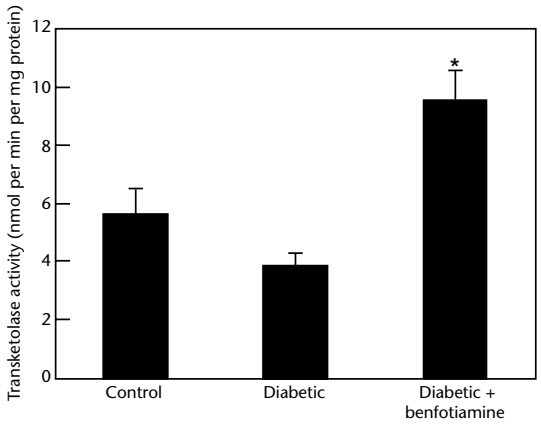


Fig. 3 Effect of benfotiamine on pathways of hyperglycemic damage in cultured bovine aortic endothelial cells, in the absence and presence of transketolase inhibition by antisense oligonucleotides. ***a***, Hexosamine pathway activation. ***b***, Intracellular AGE formation. ***c***, PKC activation. ***d***, NF-κB activation. *, $P < 0.01$ compared with cells incubated in 5 mM glucose. *a–c*, Each bar represents mean plus s.e.m. of 4 separate experiments. *d*, Each bar represents mean plus s.e.m. of fluorescence from 40 cells measured in an *in situ* DNA-protein binding assay. AU, arbitrary units.

activating transketolase. When endothelial cells were incubated in 30 mM glucose after transfection with transketolase antisense phosphorothioate oligonucleotides, transketolase activity was inhibited by 70% (data not shown). This had no effect on the activation of the hexosamine pathway, AGE formation, PKC activation, or NF-κB activation induced by 30 mM glucose (Fig. 3*a–d*, bar 4). However, the inhibitory effect of benfotiamine on all these hyperglycemia-induced changes was completely blocked in the presence of antisense transketolase (Fig. 3*a–d*, bar 5). In contrast, benfotiamine still completely inhibited all of the changes induced by 30 mM glucose in cells transfected with scrambled phosphorothioate oligonucleotides (Fig. 3*a–d*, bar 6).

Because hyperglycemia activates all these mechanisms by a single underlying process, overproduction of superoxide by the mitochondrial electron transport chain with subsequent inhibition of GAPDH activity[5], we also evaluated the effect of benfotiamine on both hyperglycemia-induced superoxide overproduction and hyperglycemia-induced inhibition of GAPDH activity. Hyperglycemia increased superoxide production in these cells from 58.24 ± 3.40 to 161.99 ± 4.42 nmol/ml. Although *in vitro* studies have implied that thiamine could have antioxidant properties[11], benfotiamine had no effect on hyperglycemia-induced intracellular superoxide production (data not shown). Similarly, as previously reported[6], hyperglycemia decreased GAPDH activity from 528 ± 43.60 to 136 ± 48.80 nmol per sec per mg protein. Benfotiamine also had no effect on hyperglycemia-induced inhibition of GAPDH activity (data not shown). These data and those in Fig. 3 demonstrate that benfotiamine prevents activation of three major pathways of hyperglycemia-induced damage and hyperglycemia-induced activation of NF-κB by activating transketolase, the rate-limiting enzyme of the non-oxidative branch of the pentose phosphate pathway.



Benfotiamine activates transketolase in diabetic retinas

In cultured endothelial cells, benfotiamine activated transketolase and thereby prevented activation of multiple pathways of hyperglycemic damage. However, these cells were in culture for a relatively short period of time. To determine whether benfotiamine has the same effects *in vivo*, and for a much longer period of time, we evaluated the effect of benfotiamine on transketolase activity in the retinas of long-term diabetic rats (Fig. 4). Non-diabetic rats had an average body weight of 630 ± 30 g, whereas diabetic rats weighed 355 ± 63 g. Glycosylated hemoglobin was 4.75 ± 0.33% in non-diabetic rats and 16.04 ± 1.98 in diabetic rats. Benfotiamine treatment did not prevent the effects of diabetes on these two parameters (body weight was 307 ± 43 g; glycosylated hemoglobin was 19.32 ± 2.39%). After 36 weeks of hyperglycemia, transketolase activity was reduced (Fig. 4), but this reduction was not statistically significant (3.9 ± 0.5 nmol per min per mg protein for diabetics versus 5.7 ± 0.7 for non-diabetics). Benfotiamine treatment of diabetic rats for 36 weeks increased transketolase activity by 2.5-fold (9.6 ± 1.0 nmol per min per mg protein for treated diabetics versus 3.9 ± 0.5 for untreated diabetics). Thus, benfotiamine activates transketolase in long-term diabetic animal tissues as well as in short-term cell culture.

Fig. 4 Effect of chronic benfotiamine administration on transketolase activity in retinas from long-term diabetic rats. Each bar represents mean plus s.e.m. of 4 separate retinas. *, $P < 0.01$ compared with retinas from non-diabetic rats.

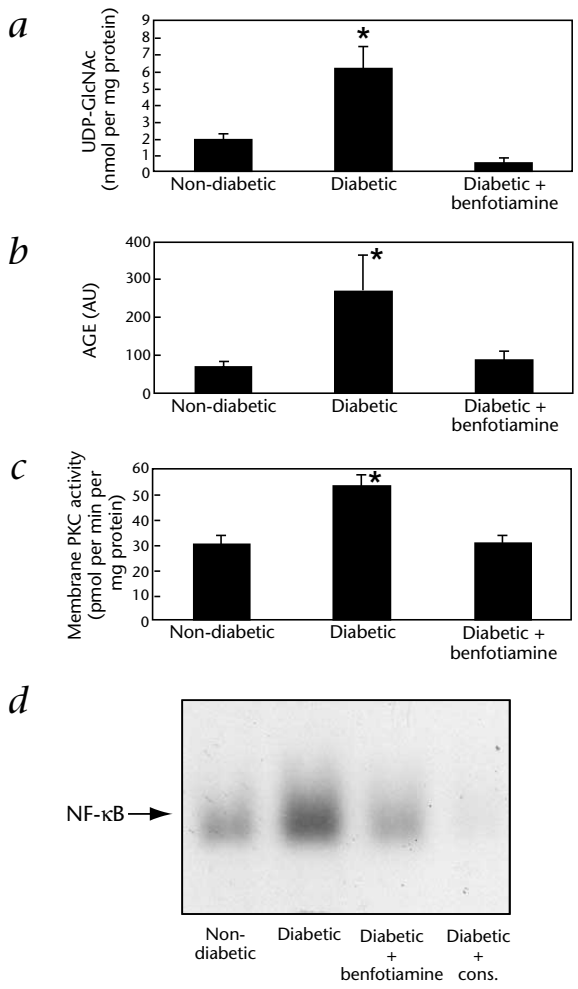


Fig. 5 Effect of chronic benfotiamine administration on pathways of hyperglycemic damage in retinas from long-term diabetic rats. ***a***, Hexosamine pathway activation. ***b***, AGE formation. ***c***, PKC activation. ***d***, NF-κB activation. *, $P < 0.01$ compared with retinas from non-diabetic animals. *a–c*, Each bar represents mean plus s.e.m. of 4 separate retinas. *d*, Representative electromobility shift assay showing NF-κB activation. Cons., excess of cold consensus NF-κB oligonucleotide.

Benfotiamine prevents pathway activation in diabetic retinas

Because benfotiamine activated transketolase *in vivo* as well as *in vitro*, we next analyzed retinas from the same groups of non-diabetic, diabetic and benfotiamine-treated diabetic rats for hexosamine pathway activity, AGE formation, PKC activity and NF-κB activation (Fig. 5). Diabetes increased hexosamine pathway activity three-fold, from 1.9 ± 0.5 to 6.3 ± 1.4 nmol GlcNAc per mg protein (Fig. 5*a*). Benfotiamine treatment of long-term diabetic rats reduced these levels to below those observed in the retinas of non-diabetic rats (0.6 ± 0.2 nmol GlcNAc per mg protein). Similarly, diabetes increased AGE accumulation from 72 ± 9 to 279 ± 43 AU. Benfotiamine treatment normalized AGE levels in the retinas of long-term diabetic rats (94 ± 11 AU; Fig. 5*b*). Diabetes increased membrane PKC activity from 30.6 ± 3.2 to 54.5 ± 3.7 pmol per min per mg protein (Fig. 5*c*). Benfotiamine treatment normalized PKC activity in the retinas of long-term diabetic rats as well (Fig. 5*c*), to 31.3 ± 2.6 pmol per min per mg protein. Diabetes also increased NF-κB activation in the retina three-fold (Fig. 5*d*). Benfotiamine treatment prevented this diabetes-induced activation of NF-κB in the retinas of long-term diabetic rats.

Oral benfotiamine prevents experimental diabetic retinopathy

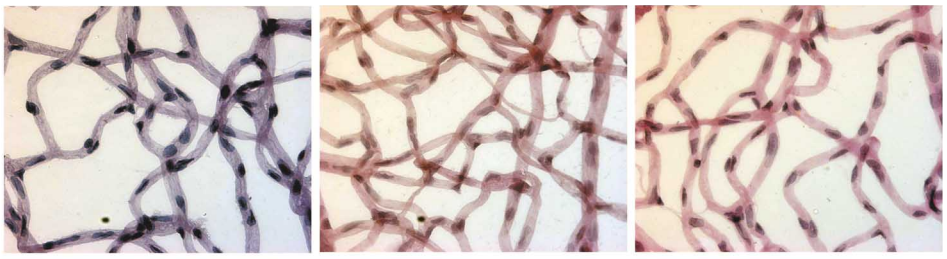
Because benfotiamine treatment prevents activation of multiple pathways of hyperglycemic damage in the retina, which are implicated in the pathogenesis of diabetic retinopathy[12,13], we also did quantitative morphological studies on retinas from the same groups of non-diabetic, diabetic, and benfotiamine-treated diabetic rats (Fig. 6*a* and *b*). After 36 weeks of diabetes, the number of acellular capillary segments in the retina increased to over 3 times that found in non-diabetics (72.50 ± 0.44 for diabetics versus 22.67 ± 3.23 for non-diabetics). In contrast, 36 weeks of the same severity of diabetes with benfotiamine treatment did not change the number of retinal acellular capillary segments (Fig. 6) from that found in non-diabetics (29.64 ± 1.29 versus 22.67 ± 3, respectively). These results show that benfotiamine treatment prevents experimental diabetic retinopathy.

Discussion

Our results in both endothelial cells cultured for several days and retinas from rats with nine months of diabetes showed that benfotiamine, a lipid-soluble thiamine derivative, prevents activation of three major pathways involved in hyperglycemia-induced vascular damage: the hexosamine pathway, the intracellular AGE formation pathway and the DAG-PKC pathway[5]. Benfotiamine also prevented hyperglycemia-induced activation of NF-κB in both cultured cells and diabetic retinas. We showed that benfotiamine blocks these pathways of hyperglycemic damage by increasing the activity of transketolase, the rate-limiting enzyme of the non-oxidative branch of the pentose phosphate pathway. Administration of benfotiamine for nine months also completely prevented the development of experimental diabetic retinopathy in rats.

We chose to use benfotiamine rather than thiamine for these studies because administration of this lipid-soluble thiamine derivative gives much higher blood and tissue levels than do equimolar doses of water-soluble thiamine derivatives[14]. Thiamine has been reported to correct hyperglycemia-induced secretion of von Willebrand factor and impaired migration in cultured aortic endothelial cells[15] and to prevent intracellular AGE formation in cultured endothelial cells[16,17]. Thiamine does not prevent AGE formation *in vivo*, in peripheral nerves of diabetic rats, whereas benfotiamine does[17]. The mechanism responsible for these effects is not known.

In our study, inhibition of the pentose phosphate pathway enzyme transketolase by antisense oligonucleotides completely blocked the effect of benfotiamine on hyperglycemia-induced activation of the hexosamine pathway, intracellular AGE formation, the DAG-PKC pathway and PKC-dependent NF-κB activation. In contrast, transfection with scrambled oligonucleotides did not alter the ability of benfotiamine to inhibit these hyperglycemia-induced activities. These data demonstrate that transketolase activity mediates the effects of benfotiamine on hyperglycemia-induced pathways of vascular damage. The observation that levels of the transketolase substrates fructose-6-phosphate and triose phosphate are elevated in erythrocytes of diabetic patients[18] is consistent with our data, although the hyperglycemia-induced reactive oxygen species that inactivate GAPDH are not produced by mitochondria in erythrocytes as they are in vascular endothelial cells. Addition of thiamine to erythrocytes incubated *in vitro* under hyperglycemic conditions increases transketolase activity, normalizes elevated triose phos-



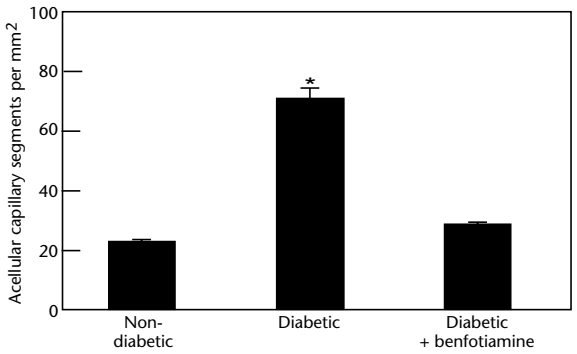


Fig. 6 Effect of benfotiamine on experimental diabetic retinopathy. ***a***, Photomicrographs of retinal vessels prepared from non-diabetic (left), diabetic (middle) and benfotiamine-treated diabetic (right) rats. ***b***, Quantitation of acellular capillary segments in retinal vessels from non-diabetic, diabetic and benfotiamine-treated diabetic rats. *, $P < 0.01$ compared with non-diabetics. Twelve retinas were analyzed morphometrically from each group. For each assessment, 10 fields per retina were randomly selected.

phate levels and increases the concentration of transketolase reaction products, consistent with our data from vascular endothelial cells[19]. Although thiamine is also a cofactor for the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex, addition of benfotiamine to aortic endothelial cells incubated in 30 mM glucose had no effect on tricarboxylic acid cycle activity (data not shown). Thus we conclude that augmentation of transketolase activity is solely responsible for the observed effects of benfotiamine.

The mechanism by which hyperglycemia may induce functional thiamine deficiency in vascular endothelial cells is not yet known. However, because diabetic patients clearly do not exhibit the classic clinical manifestations of thiamine deficiency, it seems most likely that a vascular cell–specific mechanism is involved. Vascular endothelial cells and other cell types damaged by hyperglycemia are uniquely unable to downregulate glucose transport when exposed to extracellular hyperglycemia[20]. As a result, these cells develop intracellular hyperglycemia that causes overproduction of superoxide by the mitochondrial electron transport chain[4,5]. Superoxide and superoxide-derived reactive oxygen species can oxidize thiamine to the biologically nonfunctional products thiochrome and oxodihydrothiochrome[21]. Intracellular oxidative inactivation of thiamine by hyperglycemia-induced reactive oxygen species would be consistent with all the available clinical and biochemical data.

The data reported here indicate that treatment of diabetic patients with benfotiamine or other lipid-soluble thiamine derivatives might prevent or delay the development of diabetic complications. Inhibition of hyperglycemia-induced PKC activation prevents functional and structural abnormalities in the retina and renal glomerulus of diabetic animals[13,22]. Similarly, AGE inhibitors prevent various functional and structural manifestations of diabetic vascular disease in retina, kidney, nerve and artery[12,23–25]. The ability of benfotiamine to inhibit both pathways might be clinically useful in preventing the development and progression of diabetic complications.

Methods

Materials. Eagle's MEM, nonessential amino acids and antibiotics were from Gibco (Grand Island, New York). FBS was from HyClone (Logan, Utah). Benfotiamine was obtained from Sigma (St. Louis, Missouri). Fluorescent oligonucleotides used in the NF-κB assay were obtained from Operon Technologies (Alameda, California).

Cell culture conditions. Confluent bovine aortic endothelial cells (passages 4–10) were maintained in Eagle's MEM containing 0.4% FBS, essential and nonessential amino acids and antibiotics. For the benfotiamine dose-response experiment, cells were incubated for 6 h with either 5 mM glucose, 30 mM glucose, or 30 mM glucose plus varying concentrations of benfotiamine. For all other cell culture experiments, cells were incubated with either 5 mM glucose, 30 mM glucose, 5 mM glucose plus 25 mM mannitol, 30 mM glucose plus 50 μM benfotiamine, 30 mM glucose plus transketolase antisense plus benfotiamine (see below), 30 mM glucose plus transketolase antisense, 30 mM glucose plus scrambled oligonucleotides, or 30 mM glucose plus scrambled oligonucleotides plus benfotiamine. Cells were incubated for 48 h prior to determination of hexosamine pathway activity, for 5 d prior to determination of AGE formation, 3 d prior to determination of PKC activity, and 6 h prior to determination of NF-κB activation.

Oligonucleotide synthesis and treatment of cells. Phosphorothioate oligonucleotides were synthesized by Operon Technologies. The *S*-antisense transketolase had the sequence 5′-C*TCCAGCAAGCAATAGA*C-3′. Scrambled oligonucleotides (5′-A*ATACCGCTCACGCAGAA*C-3′) were used as controls. A solution of 36.3 μl oligonucleotide, 16.3 μl polyethylenimine and 945 μl medium was added to the cells for 2 h. Medium was changed to either 5 mM glucose, 30 mM glucose or 30 mM glucose + 50 μM benfotiamine 48 hrs after the addition of transketolase antisense oligonucleotide or scrambled oligonucleotide.

Transketolase activity. Transketolase activity was measured as described[19]. Enzyme activity was measured by adding 20 μl cytosolic fraction to 200 μl reaction mixture containing 14.8 mM ribose-5-phosphate, 253 μM NADH, 185 U/ml triosephosphate isomerase and 21.5 U/ml α-GAPDH (pH = 8) in Tris buffer. The optical density was measured at 340 nm immediately and then every 10 min for 2 h. The activity was calculated from the difference in the optical density readings at 10 and 80 min using the extinction coefficient for NAD. Results are expressed in nmol per min per mg protein.

Hexosamine pathway activity. Hexosamine pathway activity was assessed by measuring UDP-GlcNAc concentration. Cells were homogenized in 3 600-μl volumes of cold 0.6 M perchloric acid and kept at 0 °C for 10 min. The precipitated proteins were removed by centrifugation for 5 min at 13,500*g*. UDP-GlcNAc in the supernatant was determined by HPLC[26].

Advanced glycation end products. Equal amounts of cell extract protein were used for quantitative immunoblotting[27]. Methylglyoxal-derived imidazole AGE was detected using a 1:10,000 dilution of monoclonal antibody 1H7G5. Immunocomplexes were visualized using an enzyme-catalyzed fluorescence kit according to the manufacturer's instructions (Amersham, Piscataway, New Jersey) and quantified using a Molecular Dynamics (Sunnyvale, California) FluorImager and its ImageQuant 4.0 analytical soft-

ware. For retinal extracts, enzyme-catalyzed fluorescence was quantified using the CUE-2 Image analysis system (Olympus Opticals, Hamburg, Germany).

Protein kinase C activity. The assay was performed according to the manufacturer's instructions using the Protein Kinase C Assay System (InVitroGen, Carlsbad, California).

NF-κB activation. A fluorescence *in situ* DNA-protein binding assay was performed in cultured cells[28]. Fluorescence per cell was determined using IP Lab Spectrum (Scanalytics, Fairfax, Virginia). An electrophoretic mobility shift assay was used in the retina[29].

Animals. Diabetes was induced by intravenous injection of streptozotocin (Sigma) in 6-week-old outbred male Wistar rats (Charles River, Sulzfeld, Germany) weighing 220–250 g. After confirmation of stable hyperglycemia (blood glucose >15 mM 15 d after injection), hyperglycemic rats were randomly assigned to receive either standard rat chow containing benfotiamine (80 mg per kg body weight per day) or no treatment. Non-diabetic animals served as controls. Body weight and blood glucose values were monitored at regular intervals. Glycosylated hemoglobin was measured at the end of the study using an affinity chromatography method (Glyc Affin, Isolab, Akron, Ohio). Experiments performed in this study adhered to the Association for Research in Vision and Opthalmology statement for the "Use of Animals in Ophthalmic and Vision Research." The study was approved by the Regional Commission in Giessen, Germany (File Reference GI 20/11–3/98).

Retinal preparations. After 36 weeks of hyperglycemia, eyes were obtained by enucleation from animals under deep anesthesia. Retinas from the left eyes were microdissected at the time of sacrifice under a dissecting microscope, snap frozen in liquid nitrogen and stored at –80 °C until analysis for transketolase activity, hexosamine pathway activity, AGE formation, PKC activity and NF-κB activation. The right eyes were fixed in 4% buffered formalin. Retinas from the right eyes were subjected to an established enzymatic digestion method[30]. Acellular capillary segments were quantified as described[31]. Twelve retinas from each group were analyzed morphometrically, and 10 fields per retina were randomly selected.

Statistics. Data were analyzed using 1-factor analysis of variance to compare the means of all the groups. The Tukey–Kramer multiple comparisons procedure was used to determine which pairs of means were different.

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Competing interests statement

The authors declare that they have no competing financial interests.

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