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Benfotiamine alleviates diabetes-induced cerebral oxidative damage independent of advanced glycation end-product, tissue factor and TNF-α

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Abstract

Diabetes mellitus leads to thiamine deficiency and multiple organ damage including diabetic neuropathy. This study was designed to examine the effect of benfotiamine, a lipophilic derivative of thiamine, on streptozotocin (STZ)-induced cerebral oxidative stress. Adult male FVB mice were made diabetic with a single injection of STZ (200 mg/kg, i.p.). Fourteen days later, control and diabetic (fasting blood glucose >13.9 mM) mice received benfotiamine (100 mg/kg/day, i.p.) for 14 days. Oxidative stress and protein damage were evaluated by glutathione/glutathione disulfide (GSH/GSSG) assay and protein carbonyl formation, respectively. Pro-oxidative or pro-inflammatory factors including advanced glycation end-product (AGE), tissue factor and tumor necrosis factor-α (TNF-α) were evaluated by immunoblot analysis. Four weeks STZ treatment led to hyperglycemia, enhanced cerebral oxidative stress (reduced GSH/GSSG ratio), elevated TNF-α and AGE levels without changes in protein carbonyl or tissue factor. Benfotiamine alleviated diabetes-induced cerebral oxidative stress without affecting levels of AGE, protein carbonyl, tissue factor and TNF-α. Collectively, our results indicated benfotiamine may antagonize diabetes-induced cerebral oxidative stress through a mechanism unrelated to AGE, tissue factor and TNF-α.

Keywords: Benfotiamine; Diabetes; Cerebral cortex; Oxidative stress

Diabetic neuropathy is a debilitating disorder affecting ∼50% diabetic patients. The primary phenotypes of diabetic neuropathy are manifested as sensorimotor and autonomic neuropathy due to damage of cerebral nerve fibers. Diabetic neuropathy is associated with high mortality and thus requires stringent and aggressive treatment against hyperglycemia, hyperinsulinemia, dyslipidemia and neurovascular damage [4,15]. Recent progress towards understanding the mechanisms responsible for nerve fiber degeneration and regeneration has facilitated more targeted approaches for the treatment of diabetic neuropathy including enzymatic and non-enzymatic antioxidants [15]. In addition, lifestyle changes such as smoking cessation, weight control, regular exercise and dietary restriction are beneficial to the primary care for diabetes and diabetic neuropathy [14]. Nevertheless, none of these therapeutic strategies is deemed radical for ultimate management of this devastating diabetic complication. Evidence suggests that thiamine and its lipophilic derivative benfotiamine may alleviate symptoms of diabetic retinopathy and diabetic neuropathy possibly through inhibition of advanced glycation end-product (AGE) formation [10,11,23]. AGE formation is believed to play an important role in the etiology of diabetic complications through binding to its cell-surface receptor, RAGE, thus leading to generation of intracellular reactive oxygen species and alteration of gene expression [13,22]. The therapeutic potential of thiamine against diabetic complications is in accordance with the notion of a severe thiamine deficiency in diabetes due to excessive oxidative stress [17,20]. It has been indicated that glucose autoxidation as a result of enhanced oxidative stress may oxidize thiamine into its biologically inactive products thiochrome and oxodihydrothiochrome [17]. Deficiencies in B series vitamins (such as thiamine) and folic acid, which are essential for DNA synthesis and repair, are among the key causative factors for diabetic end organ damage [17]. To further examine the impact of thiamine or its derivatives on cerebral function in diabetes, experimental diabetes was induced with streptozotocin (STZ) in adult FVB albino mice. Both control and diabetic mice received 14-day treatment of benfotiamine. Oxidative stress, protein carbonyl formation, levels of AGE, tissue factor and tumor necrosis factor-α (TNF-α) were evaluated.

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The procedures described here were approved by the Institutional Animal Care and Use Committee of University of Wyoming (Laramie, WY). In brief, eight week-old male FVB albino mice (18 ± 2 g) were injected with a single dose of streptozotocin (STZ, 200 mg/kg., i.p. in 0.01 M citrate buffer with a pH of 4.3) [9]. Weight-matched non-diabetic mice received citrate buffer only. Fasting blood glucose level was examined after 3 days of STZ injection and diabetes was confirmed by fasting blood glucose value of 13.9 mM or higher using ACCU-CHEK Advantage Glucometer (Boehringer Mannheim Diagnostics, Indianapolis, IN). On day 15 of STZ or citrate buffer injection, both diabetic and non-diabetic mice were randomly divided into two experimental groups. One diabetic and one non-diabetic group were given gavage of benfotiamine (100 mg/kg/day) for two weeks. Selection of the benfotiamine dose and duration of treatment were based on previously published data [2,3,10]. Mice were maintained on a 12 h light/12 h dark cycle and were allowed access to food and water ad libitum. The mortality rate in STZ-induced diabetic mice was ∼25% and was seemingly lesser in STZ mice with benfotiamine treatment. All mice were sacrificed four weeks after STZ or citrate injection and brain tissues (hearts also for tissue factor level) were removed, snap frozen and stored at −80°.

Glutathione and glutathione disulfide levels were determined and the ratio of GSH/GSSG was used as an indicator for oxidative stress. In brief, cerebral cortex was homogenized in 4 volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16,000 × *g* (30 min) and supernatant fractions collected. Supernatant fractions were assayed for total GSH and GSSG by the standard recycling method. The procedure consisted of using one-half of each sample for GSSG determination and the other half for GSH. Samples for GSSG determination were incubated at room temperature with 2 μl of 4-vinyl pyridine (4-VP) per 100 μl sample for 1 h after vigorous vortexing. Incubation with 4-VP conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH. The GSSG (as GSHx2) was then subtracted from the total GSH to determine actual GSH level and GSH/GSSG ratio [19].

To assess oxidative protein damage, the carbonyl content of protein was extracted from cerebral cortex and was lysed to prevent proteolytic degradation. Nucleic acids were eliminated by treating samples with 1% streptomycin sulfate for 15 min followed by centrifugation (11,000 × *g* for 10 min). Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) solution. Samples were incubated at room temperature for 15–30 min. 500 μl of 20% TCA was added and samples were centrifuged for 3 min. The resultant supernatant was discarded, the pellet was washed in ethanol:ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min and the ethanol:ethyl acetate steps were repeated twice more. The precipitate was resuspended in 6 M guanidine solution, centrifuged for 3 min and insoluble debris was removed. The maximum absorbance (360–390 nm) was read against blank (2 M HCl) and carbonyl content was calculated using the molar absorption coefficient of 22,000 M^{-1} cm^{-1} [12].

Cerebral cortex was thawed and homogenized on ice in PIPA lysis buffer. Lysate protein (20 μg) was diluted with Laemmli sample buffer (1:2 w/w) and was resolved on a 10–15% SDS polyacrylamide gel under reducing condition. SeeBlue Plus2® Prestained SDS–PAGE standards were used with each gel to determine the approximate molecular weight of detected bands. Gels were transferred to polyvinylidene difluoride membranes (Millipore) using a semidry transfer apparatus (Bio-Rad) at 100 V for 100 min. The membrane was then blocked with TBS-0.05% Tween 20 (TBS-T) with 5% nonfat dry milk for 60 min and incubated with mouse anti-AGE monoclonal (1:1000, Trans Genic Inc., Japan), purified Armenian hamster anti-mouse TNF-α (1: 1000, Biolegend, San Diego), rabbit anti-mouse tissue factor (5.95 μg/ml, kindly provided by Dr. James H. Morrissey, University of Illinois at Urbana-Champaign, Urbana, IL) and anti-β-actin (1:5000) antibodies in TBS-T with 5% BSA overnight at 4 °C. The membrane was washed three times for 15 min in TBS-T, incubated with horse anti-mouse IgG, HRP-linked antibody (1:5000, Cell Signaling), horse anti- rabbit IgG, HRP-linked antibody (1:5000, Cell Signaling), goat anti-Armenian Hamster IgG (1:5000 Research Diagnostic Inc., NJ) for 1 h at room temperature, and washed three times for15 min with TBS-T. The membrane was then exposed to 2 ml of a mixture of luminol plus hydrogen peroxide under alkaline conditions (SuperSignal® West Dura Extended Duration Substrate, Pierce, Rockford, IL) for 1 min, and the resulting chemiluminescent reaction was detected by Kodak X-OMAT AR Film (Eastman Kodak, Rochester, NY) [8]. For all Western blot analysis experiments, β-actin was used as an internal loading control.

Data were presented as Mean ± S.E.M. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) using Dunnett's test as the post hoc analysis.

Four weeks of STZ treatment significantly increased fasting blood glucose levels and reduced body weight gain. Two weeks of benfotiamine treatment (100 mg/kg/day) did not elicit any significant effect on fasting blood glucose levels and body weight in either control or STZ diabetic mouse group (Table 1).

Table 1
General features of control or STZ-induced diabetic mice with or without benfotiamine treatment (100 mg/kg/day for 14 days)

Mouse group	Control (11)	Control-benfotiamine (12)	Diabetic (12)	Diabetic-benfotiamine (13)
Body weight (g)	24.2 ± 0.6	24.4 ± 0.5	21.3 ± 0.9[a]	21.3 ± 1.1[a]
Glucose (mM)	4.88 ± 0.43	5.42 ± 0.38	24.95 ± 1.07[a]	24.31 ± 1.51[a]

Mean ± S.E.M.
[a] $p < 0.05$ vs. corresponding control groups, (*n*): number of animals.

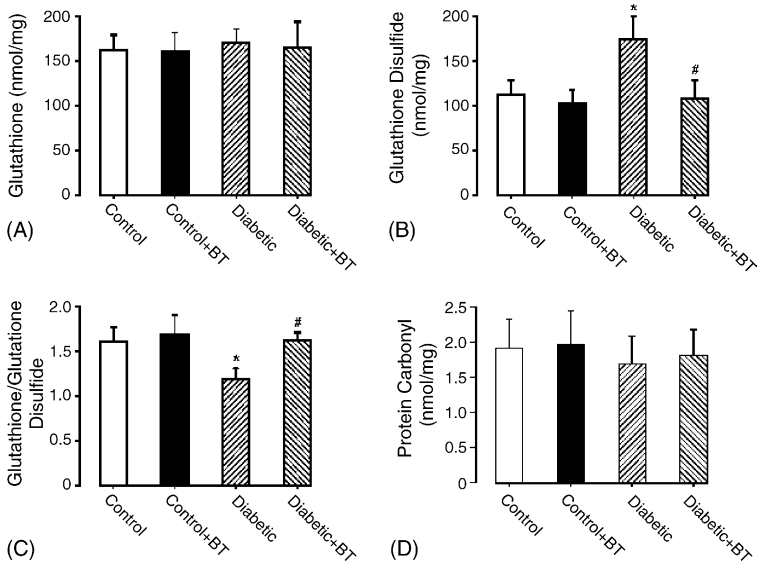

Fig. 1. GSH (panel A), GSSG (panel B), GSH/GSSG ratio (panel C) and protein carbonyl formation (panel D) in cerebral cortex from control or STZ-induced diabetic mice with or without benfotiamine (BT, 100 mg/kg/day for 2 weeks) treatment. Mean ± S.E.M., $n = 6$–12 samples per group, $^{*}p < 0.05$ vs. corresponding control group.

Diabetes is associated with enhanced oxidative stress leading to irreversible damage of membrane proteins [24]. GSH and GSSG levels are commonly used markers for oxidative stress. A low GSH/GSSG ratio suggests increased oxidative stress. Result in Fig. 1 indicates that 4 weeks STZ treatment significantly enhanced GSSG levels without affecting that of GSH. As a result, the GSH/GSSG ratio was significantly reduced in STZ-induced diabetic cerebral cortex, indicative of enhanced cerebral oxidative stress. Protein carbonyl formation, an indicative of protein damage, was not altered by 4 weeks STZ-induced diabetes. Interesting, benfotiamine treatment ablated diabetes-induced increase in GSSG levels and reduced GSH/GSSG ratio. Neither protein carbonyl formation nor GSH levels was affected by benfotiamine. Since AGE accumulation is often considered as a main source for oxidative stress [13,22], the levels of AGE was evaluated by immunoblot along with pro-inflammatory biomarkers TNF-α and tissue factor. In agreement with elevated oxidative stress in STZ-induced diabetic group, the levels of AGE and TNF-α were significantly increased in diabetic cerebral cortex following 4 weeks of STZ treatment. However, benfotiamine treatment failed to alter diabetes-induced elevation of AGE and TNF-α. We also examined the levels of circulating tissue factor in both cerebral cortex and heart ventricles. Levels of tissue factor were not affected by either STZ treatment or benfotiamine in either organ (Fig. 2).

The major finding of our study is that the lipophilic derivative of thiamine benfotiamine elicits beneficial effect on diabetic cerebral cortex damage through a mechanism associated with reduction in oxidative stress but unlikely related to protein carbonyl formation nor accumulation of AGE, TNF-α and tissue factor. AGE accumulation is usually considered the main source of oxidative in diabetes mellitus [6]. The apparent discrepancy in benfotiamine-elicited effects on AGE formation and oxidative stress (GSH/GSSG ratio) in diabetic cerebral cortex indicates that: (1) an AGE-independent mechanism(s) may be predominantly responsible for diabetes-induced cerebral cortex oxidative stress and/or (2) benfotiamine antagonizes AGE-RAGE interaction-induced oxidative stress may occur at or downstream of RAGE receptor. Other possible explanation cannot be excluded at this time including alteration in glucose metabolism and protein kinase C activation [6,18]. Benfotiamine has been shown to participate in glucose metabolism via activation of the pentose phosphate pathway enzyme transketolase, thus facilitating conversion of glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphates [10,17]. Elevated TNF-α production has been demonstrated in diabetes to promote microvascular permeability, hypercoagulability and nerve damage [21]. Clinical agents antagonizing TNF-α may inhibit development and exacerbation of chronic diabetic complications [21]. Our observation that benfotiamine treatment failed to produce any overt effect on elevated TNF-α levels in diabetes indicates that inhibition of this pro-inflammatory mediator is unlikely a main reason for benfotiamine-elicited cerebral protection. Not surprisingly, no evidence is available suggesting any effect of benfotiamine on TNF-α production or breakdown. It is noteworthy that we failed to observe any change in protein carbonyl formation or tissue factor (both cerebral cortex and heart tissue) following 4 weeks of STZ treatment. Although diabetes has been associated with protein oxidative damage, elevation in tissue factor and hypercoagulability [16], the period of diabetes employed in our present study (4 weeks) may not be long enough to induce such changes in cerebral protein damage and tissue factor elevation. The relatively short period of diabetes is also believed to be responsible for the negative finding of diabetes or benfotiamine on AGE formation compared with those published elsewhere [7]. Nevertheless, our data provided evidence that benfotiamine may alleviate diabetic cerebral cortex oxidative stress through a mechanism(s) unrelated to AGE. Last but not the least, our data revealed that benfotiamine failed to reconcile STZ-induced body weight loss, suggesting

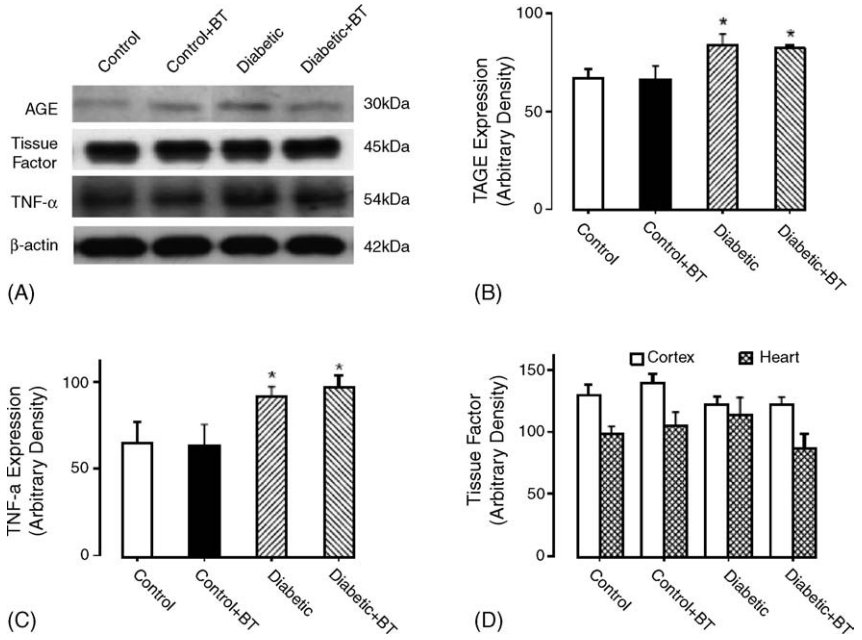

Fig. 2. Western blot analysis of AGE (panel B), TNF-α (panel C) and tissue factor (panel D) in cerebral cortex from control or STZ-induced diabetic mice with or without benfotiamine (BT, 100 mg/kg/day for 2 weeks) treatment. Panel A displays actual gel blotting using anti-AGE, anti-TNF-α and anti-tissue factor antibodies. Tissue factor expression was also examined in heart tissue for comparison. Mean ± S.E.M., $n = 5$–12 samples per group, $^{*}p < 0.05$ vs. control group.

the thiamine derivative does not likely interfere with diabetes-induced anorexic effect although potential direct toxicity from STZ should not be ruled out at this point.

In summary, our findings revealed that benfotiamine antagonizes cerebral cortex oxidative stress in STZ-induced diabetic mouse brain through a mechanism(s) unlikely dependent upon AGE formation, TNF-α or tissue factor. Given what we know about the ability of benfotiamine, thiamine and other B series vitamins to promote cell survival and alleviate diabetic complications [1–3,5,17], the in-depth mechanism of action and clinical value of employing benfotiamine in the therapeutics of diabetic neurological diseases warrants further investigation.

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