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RESEARCH ARTICLE

Benfotiamine exhibits direct antioxidative capacity and prevents induction of DNA damage *in vitro*

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Abstract

Background Complications in diabetes mellitus are partially mediated by enhanced formation of reactive oxygen species. Among the factors involved in reactive oxygen species formation, advanced glycation end products play a key role. Owing to a reduced activity of the enzyme transketolase, which requires diphosphorylated thiamine (vitamin B_1) as cofactor, an accumulation of those deleterious glucose metabolites especially in diabetic patients can be observed. Benfotiamine, a lipophilic thiamine diphosphate prodrug, prevented early renal and retinal changes in animal studies, and reduced neuropathic pain in clinical studies. Several mechanisms for these activities have been described. We investigated for the first time direct antioxidant abilities of benfotiamine. Additionally, a potential DNA protective effect of benfotiamine was analysed.

Methods Oxidative stress was detected by flow cytometry, antioxidative capacity was measured with the ferric reducing ability of plasma (FRAP) assay, two endpoints for genomic damage were assessed: the comet assay and the micronucleus test, and the expression and activity of transketolase was quantified.

Results Benfotiamine prevented oxidative stress induced by the mutagen 4-nitroquinoline-1-oxide (NQO), the uremic toxin indoxyl sulfate, and the peptide hormone angiotensin II in three different kidney cell lines. Cell-free experiments showed a direct antioxidant effect of benfotiamine, which might account for the protective effect. Oxidative DNA damage, induced by angiotensin II, was completely prevented by benfotiamine. Incubation with benfotiamine increased transketolase expression and activity in the cells.

Conclusions Benfotiamine shows a direct antioxidant action. This effect of benfotiamine may be involved in the improvement of diabetic late complications, including peripheral neuropathy.

Keywords advanced glycation end products; diabetic complications; DNA damage; *in vitro*; oxidative stress; vitamin B_1

Introduction

Diabetic patients are exposed to increased oxidative stress, which plays a key role in the pathogenesis of both micro- and macrovascular complications [1]. Its existence is based on decreased antioxidant capacity, chronic exposure to increased levels of reactive oxygen species, as well as increased peroxidation and glycoxidation [2–4]. The mitochondrial

electron transport chain is one of the first targets of high glucose, resulting in enhanced oxygen radical formation, which is followed by stimulation of three pathways of hyperglycaemic damage: the activation of protein kinase C (PKC) isoforms, increased formation of glucose-derived advanced glycation end products (AGEs), and the increased glucose flux through the aldose reductase pathway [5]. Reduced activity of the enzyme transketolase, which converts glyceraldehyde-3-phosphate and fructose-6-phosphate to intermediates of the pentose phosphate pathway [6], seems to play an important role in all these cellular changes. Cofactor activity for transketolase is provided by diphosphorylated thiamine (vitamin B_1), which has been shown to be deficient in many diabetic patients because of malabsorption and enhanced urinary excretion [7–9]. In experimental studies on diabetes, benfotiamine, the lipophilic prodrug of thiamine, showed a prevention of early renal and retinal changes [10,11]. This is in line with clinical studies in diabetes, demonstrating a significant relief in neuropathic pain and a marked improvement in vibration perception thresholds in diabetic patients [7].

Benfotiamine (Figure 1(A)) is absorbed via passive diffusion through the intestinal mucosa and is rapidly converted to its biologically active form, thiamine diphosphate (Figure 1(B)) by closure of the open thiazole ring after absorption. Peak concentrations achieved by application of a lipophilic thiamine derivative turned out to be up to five times higher than those after application of a hydrophilic derivative [12].

Benfotiamine is thought to act by at least three different mechanisms. First, activation of the hexosamine pathway with subsequent decrease in the accumulation of deleterious glucose metabolites seems to be involved, second, normalization of PKC activity along with prevention of nuclear factor kappaB (NF-κB) activation has been found in retinas, and third, correction of imbalances in the polyol pathway by decreasing aldose reductase activity, sorbitol concentrations and intracellular glucose levels seems to play a role [11]. Here, we investigate for the first time a fourth possibility, i.e. whether benfotiamine possesses direct antioxidant properties. Additionally, potential DNA protective effects of benfotiamine were analysed.

Induction of oxidative stress in our *in vitro* experiments was achieved by the addition of three different substances. First, the known mutagen 4-nitroquinoline-1-oxide (NQO), shown to form 8-hydroxydeoxyguanosine (8-oxodG) through reactive oxygen species [13] was used. Next, indoxyl sulfate, a uremic toxin, which is known to induce oxidative stress [14], was applied, and finally, the peptide hormone angiotensin II, which is elevated in diabetes because of stimulation of the renin-angiotensin system (RAS) [15]. As previously shown, angiotensin II is able to induce oxidative stress by activating NAD(P)H oxidase probably mediated via angiotensin II receptor type 1 [16]. Benfotiamine was investigated here for its ability to prevent the induction of oxidative stress by these compounds.

To investigate the associated biological consequences, the influence of benfotiamine on the genomic damage induced by angiotensin II was analysed using two endpoints for genotoxicity. The first, the comet assay [17], detects single-strand breaks, double-strand breaks and alkali-labile sites. Secondly, the cytokinesis block micronucleus frequency test [18] was conducted. Micronuclei are chromatin-containing structures surrounded by a membrane and resulting from chromosomal fragments or whole chromosomes not correctly distributed to the daughter cells after mitosis [19].

Our findings reveal a direct antioxidant capacity of benfotiamine demonstrated with three compounds and prevention of the induction of genomic damage by angiotensin II.

Materials and methods

Materials

If not mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). Benfotiamine was donated by Wörwag Pharma GmbH (Böblingen, Germany).

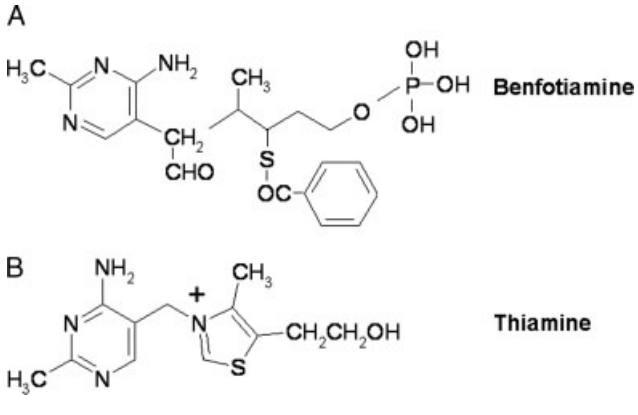

Figure 1. Chemical structures of benfotiamine (A) and thiamine (B)

Cell culture

HEK 293, a human embryonic kidney cell line with proximal tubular properties, was obtained from ATCC (Rockville, USA). Cells were grown in DMEM medium (4.5 g/L glucose) supplemented with 10% fetal calf serum, 1% glutamine and antibiotics.

NRK-52E, an epithelial rat kidney cell line with proximal tubular properties, was obtained from ECACC (Salisbury, UK) and grown in DMEM medium (4.5 g/L glucose) supplemented with 10% fetal calf serum, 1% glutamine, 1% non-essential amino acids and antibiotics.

LLC-PK1, an epithelial porcine kidney cell line with proximal tubular properties, was obtained from ATCC (Rockville, USA) and grown in DMEM medium (1.0 g/L

glucose) supplemented with 10% fetal calf serum, 1% glutamine, 25 mM HEPES buffer and antibiotics.

All cells were split routinely twice (LLC-PK1) or thrice (HEK 293, NRK) a week to ensure exponential growth and were, except for LLC-PK1 (ten passages), cultured for no more than 40 passages after thawing them from stock.

Comet assay

3.5×10^5 cells were treated for 2 h (NRK) or 4 h (LLC-PK1 and HEK 293) with test substances in 5 mL medium. The comet assay was carried out as described by Singh *et al*. [20] with slight modifications according to Schupp *et al*. [16] using a fluorescence microscope at a 200-fold magnification and computer aided image analysis (Komet 5, Kinetic Imaging Ltd., UK). After DNA staining with propidium iodide (20 μg/mL), 25 cells from each of two slides were measured, % tail DNA being the evaluation parameter. The comet assay detects single-strand breaks, double-strand breaks and alkali-labile sites. During electrophoresis, the DNA fragments move out of the nucleus in the direction of the anode because of their negative charge. Smaller fragments can move faster through the agarose in which the nuclei are embedded, resulting in characteristic comet-like structures.

Micronucleus frequency test

3.5×10^5 cells were incubated with test substances in 5 mL medium. After 2 h (NRK) or 4 h (LLC-PK1 and HEK 293), cytochalasin B (30 μg/mL) was added. This inhibitor of actin polymerization blocks the separation of daughter cells, but not of daughter nuclei, yielding binucleated cells. By limiting the analysis to such binucleated cells, it can be ensured that these cells have actively divided since the treatment. After 24 h, cells were harvested, applied onto glass slides by cytospin centrifugation and fixed in methanol (−20 °C) for at least 2 h. Before counting, cells were stained for 5 min with acridine orange (62.5 μL/mL in Sørensen buffer, pH 6.8), washed twice with Sørensen buffer and mounted for microscopy. From each of two slides, 1000 binucleated cells were evaluated with regard to micronucleus frequency. Evaluation criteria were according to Fenech [19]. In addition, the cytokinesis block proliferation index (CBPI); [no. of mononucleated cells + (2 × no. of binucleated cells) + (3 × no. of multinucleated cells)]/(sum of mononucleated, binucleated and multinucleated cells) was determined from 1000 cells of each sample.

Flow cytometric analysis of oxidative stress

10^6 cells were treated with test substances in 5 mL culture medium after 10 min pre-treatment with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and harvested after 2 h (NRK) and 4 h (LLC-PK1 and HEK 293). Cells were washed once with phosphate buffer (PBS)/1% bovine serum albumin (BSA), counterstained with propidium iodide (1 μg/mL) to exclude dead cells and 3×10^5 cells per sample were analysed by using a FACS LSR I (Becton-Dickinson, Mountain View, CA). Medians of the histograms were assessed using the free software WinMDI 2.8 (Scripps Research Institute Cytometry Software, http://facs.scripps.edu/software.html).

RNA isolation and semi-quantitative reverse transcriptase PCR

RNA isolation from cells treated with test substances for 2 h (NRK) and 4 h (LLC-PK1 and HEK 293) and polymerase chain reaction (PCR) was performed as described previously [20]. Primers were designed using the primer design service (MWG Biotech AG, Germany). Sequences, annealing temperatures and PCR conditions were: Transketolase NRK (For: 5′-ACC-AAC-AGC-CAT-CAT-TGC-C-3′; Rev: 5′-TGC-TCA-CCA-TGT-TCT-GCT-C-3′; 54 °C, 35 cycles); β-actin NRK (For: 5′-AGC-CAT-GTA-CGT-AGC-CAT- CC-3′; Rev: 5′-AGG-AAG-GAA-GGC-TGG-AAG-AG-3′; 54 °C, 35 cycles); Transketolase LLC-PK1 (For: 5′-CTA-TTG-CTT-GCT-GGG-AGA-CG-3′; Rev: 5′-GGA-GTG-GCC-AGG-ATC-CTC-TT-3′; 54 °C, 35 cycles); Transketolase HEK 293 (For: 5′-CCC-CAC-ATC-AAC-TGT-CTT-TTA-C-3′; Rev: 5′-TAC-CAA-ACA-TCT-TCA-GCA-GCT-C-3′; 54 °C, 35 cycles); β-actin LLC-PK1, HEK 293 (For: 5′-TCC-CTG-GAG-AAG-AGC-TAC-GA-3′; Rev: 5′-GTC-ACC-TTC-ACC-GTT-CCA-GT-3′, 54 °C, 35 cycles). Electrophoresis was conducted using a 1.5% agarose gel stained with ethidium bromide. Density of the DNA band was measured using the Gel Doc (BioRad, Hercules, CA, USA). Results are shown compared to the housekeeping gene β-actin.

Ferric reducing ability of plasma (FRAP) assay

This method determines the reduction of a ferric tripyridyltriazine complex to its ferrous, coloured form, in the presence of plasma antioxidants or other antioxidative substances [22,23]. The ferric reducing ability of plasma (FRAP) assay was performed photometrically as described by Benzie and Strain [24] using dilutions of benfotiamine and thiamine instead of plasma.

Transketolase activity assay

Transketolase activity was measured using a reaction mix containing 14.8 mM ribose-phosphate, 0.25 mM NADH, 92.5 U triosephosphate isomerase (Roche), 37.5 U glycerol-3-phosphate isomerase and 0,002% thiamine pyrophosphate. The reaction was started by the addition of 100 μL cell lysate. Absorbance at 340 nM was measured for 30 min and the rate of decrease in absorbance between 5 and 20 min was used to assess the rate of oxidation of NADH.

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Statistics

If not mentioned otherwise, data from at least three independent experiments (± standard deviation) are depicted. Statistical significance among multiple groups was tested with Kruskal-Wallis over all the groups and Mann-Whitney test was used to determine significance between two groups. Results were considered significant if the p value was ≤ 0.05.

Results

Oxidative stress

To assess the potential antioxidant effect of benfotiamine, oxidative stress was induced in three kidney cell lines of human, rat and porcine origin by three compounds. The elevated oxidative stress induced by the application of 10.5 µM NQO was reduced by simultaneous, but not by the previous incubation with 300 µM benfotiamine (Figure 2(A)). This concentration of benfotiamine proved to have the best antioxidative effects and least cytotoxicity in preliminary studies and was used for all further experiments. The uremic toxin indoxyl sulfate (IS, 250–1000 µM) also induced oxidative stress in LLC-PK1 pig kidney cells which was again reduced by the addition of 300 µM benfotiamine (Figure 2(B)). Generation of oxidative stress by 200 nM angiotensin II was completely prevented by the addition of 300 µM benfotiamine (Figure 3(A)), but not by thiamine (Figure 3(B)).

These findings imply a direct antioxidant action of benfotiamine, which was confirmed in the photometric FRAP assay, which shows that under acid conditions benfotiamine, but not thiamine, is able to reduce the ferric tripyridyltriazine complex to its ferrous, coloured form (Figure 4).

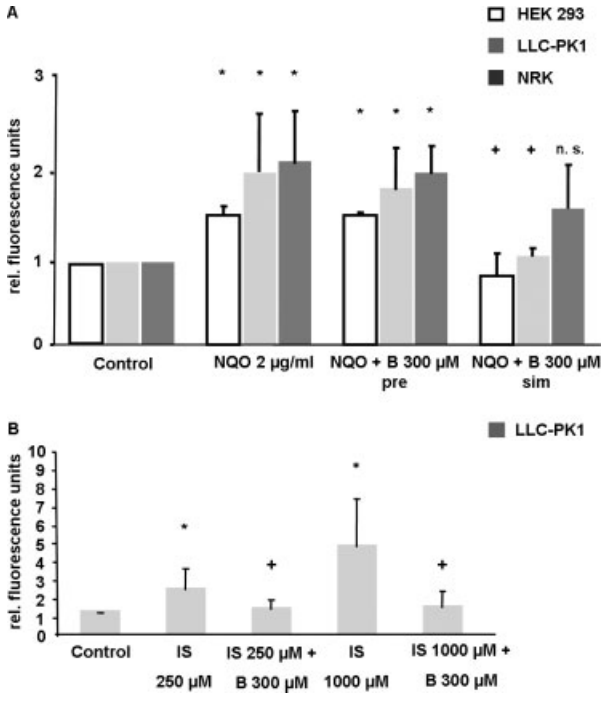

Figure 2. Oxidative stress induced by 4-nitroquinoline-1-oxide (NQO) alone, with simultaneous incubation with benfotiamine or with pre-incubation with benfotiamine in kidney cell lines. Shown are means ± standard deviation of three independent experiments, each normalized to its control. Significance ($p \leq 0.05$) compared to the control is shown by '*', significant reduction ($p \leq 0.05$) of the induced effect by '+' and not significant reduction by 'n. s.' (A). Oxidative stress is induced by indoxyl sulfate (IS) alone and in combination with benfotiamine in LLC-PK1 cells. Shown are means ± standard deviation of three independent experiments. Significance ($p \leq 0.05$) compared to the control is shown by '*', significant reduction ($p \leq 0.05$) of the induced effect by '+' (B)

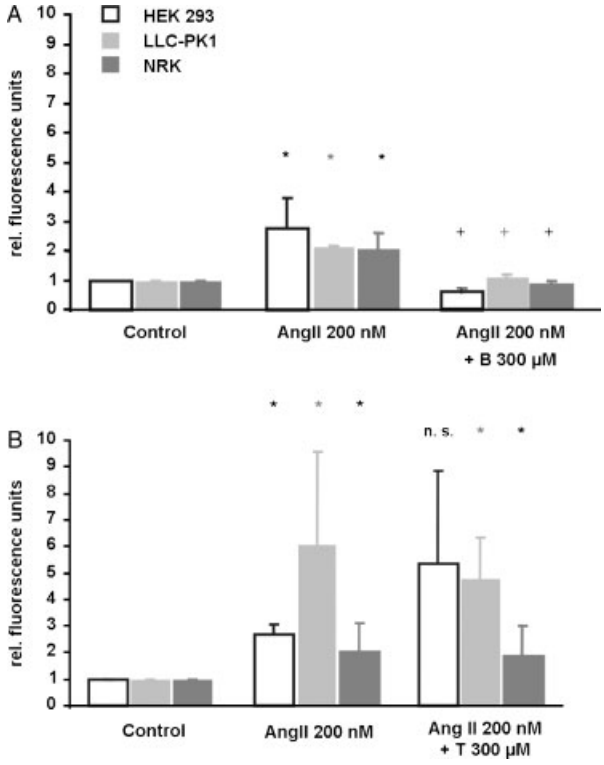

Figure 3. Oxidative stress induced by angiotensin II alone and in combination with benfotiamine (A) or thiamine (B) in kidney cell lines. Shown are means ± standard deviation of three independent experiments, each normalized to its control. Significance ($p \leq 0.05$) compared to the control is shown by '*', significant reduction ($p \leq 0.05$) of the induced effect by '+' and not significant induction or reduction by 'n. s.'

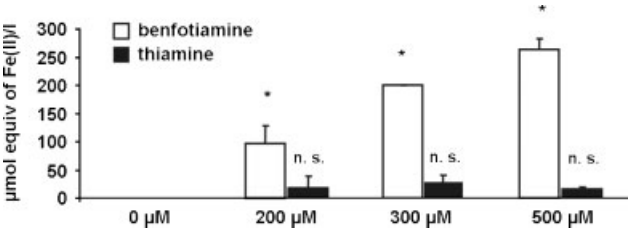

Figure 4. Ferric reducing ability of cell-free solutions of benfotiamine or thiamine in various concentrations assessed by utilization of the photometric FRAP assay. Shown are means ± standard deviation of three independent experiments, significant increase in antioxidative capacity ($p \leq 0.05$) compared to the control is shown by '*', not significant increase by 'n. s.'

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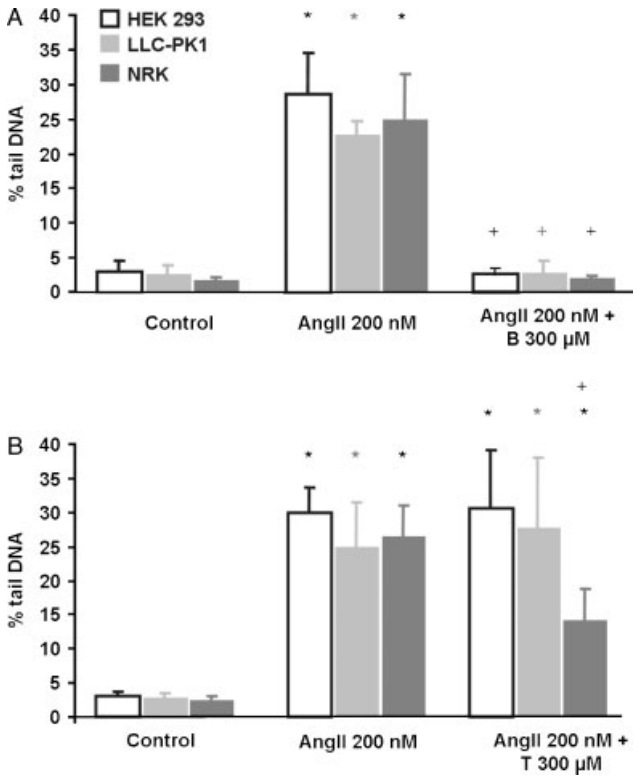

Figure 5. Comet assay analysis of angiotensin II-induced DNA damage with and without the addition of benfotiamine (A) or thiamine (B) in three kidney cell lines. Shown are means ± standard deviation of three independent experiments. Significance ($p \leq 0.05$) compared to the control is shown by '*', significant reduction ($p \leq 0.05$) of the induced effect by '+'

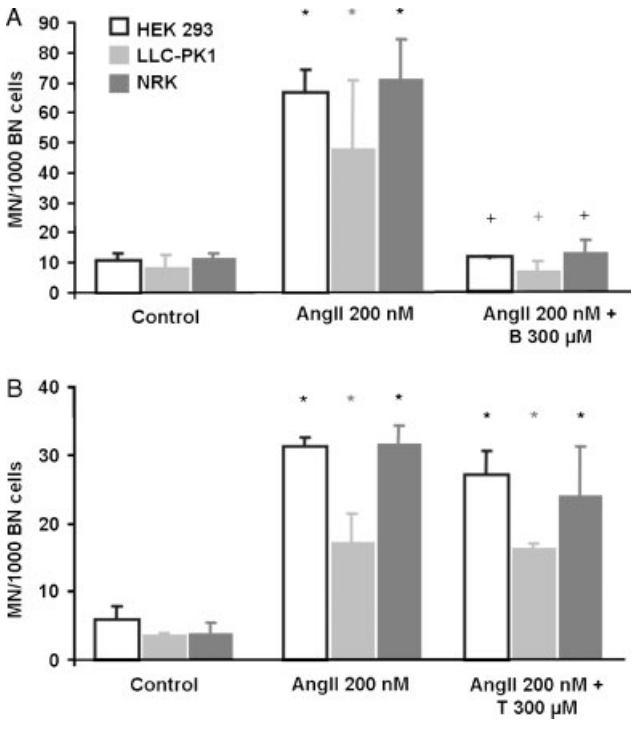

Figure 6. Micronucleus induction by angiotensin II with and without the addition of benfotiamine (A) or thiamine (B) in three kidney cell lines. Shown are means ± standard deviation of three independent experiments. Significance ($p \leq 0.05$) compared to the control is shown by '*', significant reduction ($p \leq 0.05$) of the induced effect by '+'

Genomic damage

For investigation of biological consequences of the antioxidant effect of benfotiamine, comet assay experiments were performed. DNA damage was significantly induced by 200 nM angiotensin II in all three cell lines, which was completely prevented by co-treatment with 300 µM benfotiamine but not by co-treatment with thiamine except in NRK cells (Figure 5(A), (B)). To include a second endpoint for genotoxicity, the micronucleus test was performed. After 200 nM angiotensin II, again a significant increase of micronucleated cells was found in all three cell lines and this effect was prevented by the addition of 300 µM benfotiamine, but not by co-treatment with thiamine (Figure 6(A), (B)).

Apoptosis and proliferation

On the same slides that had been analysed for micronuclei, the presence of apoptotic cells as detected by morphological appearance was quantified for all treatments. Addition of 200 nM angiotensin II induced an increase in apoptosis in HEK and LLC-PK1 cells, which was not significant because of inter-experimental variation (Table 1). In HEK cells, this increase was reduced by the addition of benfotiamine. Furthermore, the cytokinesis block proliferation index (CBPI) was calculated after assessment of the numbers of cells with one, two, three and more nuclei on the same slides and was found not to be altered by any of the treatments in all the three cell lines (Table 1).

Table 1. Frequency of apoptosis and cytokinesis block proliferation index (CBPI) in the micronucleus experiments with three different renal cell lines

Cell line	Treatment (concentration)	Apoptotic cells (%)	CBPI
HEK	Control	4.2 ± 2.0	2.0 ± 0.02
	Ang II 200 nM	28.3 ± 33.9	2.2 ± 0.06
	Ang II 200 nM + B 300 µM	12.5 ± 8.8	2.1 ± 0.04
LLC-PK1	Control	7.5 ± 1.8	2.0 ± 0.01
	Ang II 200 nM	15.3 ± 11.5	2.0 ± 0.02
	Ang II 200 nM + B 300 µM	12.3 ± 5.9	2.0 ± 0.004
NRK	Control	3.3 ± 3.3	2.0 ± 0.04
	Ang II 200 nM	4.7 ± 3.0	2.1 ± 0.02
	Ang II 200 nM + B 300 µM	3.7 ± 2.5	2.0 ± 0.02

Shown are means ± standard deviation of three independent experiments.
Ang II, angiotensin II; B, benfotiamine.

Transketolase expression and activity

Since benfotiamine is thought to activate transketolase and thereby reduce concentrations of advanced glycation end products in experimental diabetes, we investigated the expression of transketolase using semi-quantitative reverse transcriptase PCR. Density measurement of the

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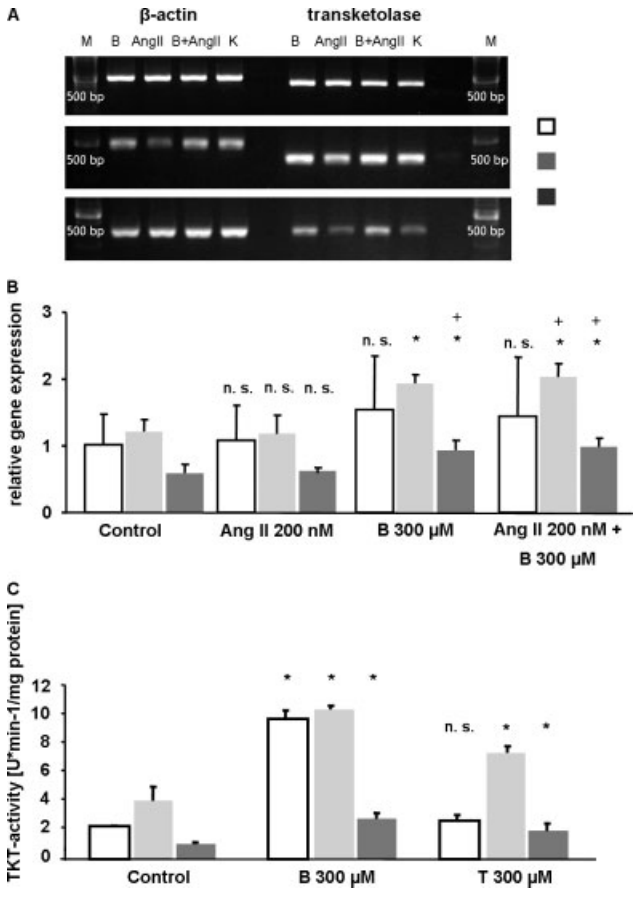

Figure 7. Transketolase expression in three kidney cell lines after treatment with angiotensin II or benfotiamine alone and angiotensin II in combination with benfotiamine. Quantification of band densities derived from three independent experiments (with standard deviations, normalized to the housekeeping enzyme β-actin) is shown in (A,B). Transketolase activity corresponding to protein concentration in kidney cell lines after treatment with benfotiamine or thiamine is shown in (C). Shown are means ± standard deviation of three independent experiments. Significance ($p \leq 0.05$) compared to the control is shown by '*', significance compared to angiotensin II ($p \leq 0.05$) by '+' and not significant induction compared to the control by 'n. s.'. M, marker; K, control

DNA bands showed an increase of transketolase gene expression in cells treated with 300 µM benfotiamine and 200 nM angiotensin II plus 300 µM benfotiamine compared to the untreated control and angiotensin II treatment alone (Figure 7(A), (B)).

Measurements of transketolase activity showed a significant increase in all three cell lines when treated with 300 µM benfotiamine and in LLC-PK1 and NRK cells when treated with 300 µM thiamine. Furthermore, a significant increase could be observed in LLC-PK1 cells treated with 200 nM angiotensin II and 300 µM benfotiamine simultaneously (Figure 7(C)).

Discussion

Potential antioxidative effects of benfotiamine were explored by inducing oxidative stress and DNA damage in human, rat and porcine renal cells, with and without the addition of benfotiamine. Oxidative stress was induced by three different agents, the mutagenic model compound NQO [13], the uremic toxin indoxyl sulfate [14] and the peptide hormone angiotensin II, which is enhanced in diabetes because of renin-angiotensin system stimulation [15]. Genomic damage, evaluated by comet- and micronucleus-formation, was also investigated after treatment of cells with angiotensin II. Benfotiamine was able to reduce the oxidative stress and genomic damage exerted by these agents. Since the vitamin was only active upon simultaneous treatment with NQO and not if limited to a pre-treatment, a direct antioxidant activity could be inferred. This was confirmed in the FRAP assay, which is a cell-free assay. The feasible oxidation of thiamine to thiochromes by iron (III) is of little importance in this case because all *in vitro* experiments were conducted under neutral conditions and the cell-free FRAP under acid conditions, meaning that the thiochromes are not likely to be formed. To our knowledge, no comparable data concerning a direct antioxidant capacity of benfotiamine has been published to date.

In comparison to the antioxidant vitamin C, benfotiamine shows a ten-fold lower antioxidative capacity in the FRAP assay (2391 ± 67 *vs* 200 ± 1 µmol equiv of Fe(II)/L). So it is assumable that its antioxidative capacity, because of being low, is not the only means by which benfotiamine exerts direct antioxidant effects. Angiotensin II induces oxidative stress and genomic damage via the angiotensin II type 1 receptor [16], subsequently, activating phospholipase C (PLC), PKC and NAD(P)H oxidase. In an *in vivo* study with male Sprague-Dawley rats with streptozotocin-induced diabetes mellitus, activation of PKC and its suppression by high-dose benfotiamine treatment was observed [25]. This ability of benfotiamine to suppress increased PKC activation may contribute to our findings of the direct antioxidative effect.

Furthermore, an increased transketolase activity could be observed in samples incubated with benfotiamine or thiamine. This is in line with previously published findings [26]. In addition, the elevated transketolase expression after co-incubation with angiotensin II and benfotiamine corresponds to the published effects in experimental diabetes [27].

Thiamine, in contrast to benfotiamine, showed antioxidant potential neither in the cellular assays nor in the cell-free assay, while it did increase transketolase expression and activity. These observations also show that the up-regulation of transketolase, while for sure important in the organism in antagonising advanced glycation end products, is not responsible for the protective effects of benfotiamine against oxidative stress, because thiamine is ineffective in this regard.

It is conceivable that benfotiamine supplementation of diabetic patients reduces the clinical complications, including peripheral neuropathy [7,28], at least in part because of its antioxidant effect. Possibly, a reduction of genomic damage could also be achieved *in vivo*, offering a form of cancer-prevention to diabetic patients.

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Conflict of interest

None declared.

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