Benfotiamine increases glucose oxidation and down-regulates NADPH oxidase 4 expression in cultured human myotubes exposed to both normal and high glucose concentrations

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Abstract

The aim of the present work was to study the effects of benfotiamine (S-benzoylthiamine O-monophosphate) upon glucose and lipid metabolism and gene expression in differentiated human skeletal muscle cells (myotubes) incubated for 4 days under normal (5.5 mM glucose) and hyperglycemic (20 mM glucose) conditions.

Myotubes established from lean, healthy volunteers were treated with benfotiamine for 4 days. Glucose and lipid metabolism were studied with labeled precursors. Gene expression was measured using real-time polymerase chain reaction (qPCR) and microarray technology.

Benfotiamine significantly increased glucose oxidation under normoglycemic (35% and 49% increase at 100 and 200 µM benfotiamine, respectively) as well as hyperglycemic conditions (70% increase at 200 µM benfotiamine). Benfotiamine also increased glucose uptake. In comparison, thiamine (200 µM) increased overall glucose metabolism but did not change glucose oxidation. In contrast to glucose, mitochondrial lipid oxidation and overall lipid metabolism was unchanged by benfotiamine. The expression of NADPH oxidase 4 (NOX4) was significantly downregulated by benfotiamine treatment under both normo- and hyperglycemic conditions. Gene set enrichment analysis (GSEA) showed that befotiamine increased peroxisomal lipid oxidation and organelle (mitochondrial) membrane function.

In conclusion, benfotiamine increases mitochondrial glucose oxidation in myotubes and down-regulates NOX4 expression. These findings may be of relevance to type 2 diabetes where reversal of reduced glucose oxidation and mitochondrial capacity is a desirable goal.

Keywords: benfotiamine, thiamine, myotubes, diabetes, hyperglycemia

Introduction

Chronic hyperglycemia plays a major role in the development of vascular complications associated with both type 1 and 2 diabetes (UK Prospective Diabetes Study (UKPDS) Group 1998; Brinchmann-Hansen et al. 1992; The Diabetes Control and Complications Trial Research Group 1993). These effects are believed to occur secondary to a build-up of glucose and glycolytic intermediates in the cytosol during bouts of hyperglycemia when intracellular glucose concentrations are abnormally increased (Brownlee 2001). Benfotiamine, a synthetic thiamine monophosphate analogue with improved intestinal absorption as compared to thiamine, prevents the adverse cellular changes associated with hyperglycemia and can prevent the development of diabetic retinopathy in mice (Balakumar et al. 2010; Hammes et al. 2003). These effects are believed to be mediated by increasing the activity of the thiamine-dependent and rate-limiting enzyme of the pentose phosphate pathway (PPP), transketolase (TK), which shifts excess glycolytic metabolites away from central biochemical pathways of hyperglycemic damage (Hammes et al. 2003). It has also been shown that benfotiamine functions as a direct antioxidant in rodents which may contribute to its beneficial effect (Schmid et al. 2008).

In addition to the harmful effects upon the vasculature, hyperglycaemia also contributes to insulin resistance in skeletal muscle (Richter et al. 1988). We have previously shown that myotubes exposed to chronic hyperglycemia have increased lipogenesis and intramyocellular triacylglycerol (IMTG) accumulation (Aas et al. 2004). The mechanisms by which these effects occur are complex and remain unknown, but might involve mitochondrial dysfunction (Aas et al. 2011). It has been suggested that mitochondrial dysfunction is integral in the pathophysiology of insulin resistance and that its reversal may prevent the development of a number of metabolic sequelae associated with type 2 diabetes (Pagel-Langenickel et al. 2010).

Oral benfotiamine results in a 10-40% increased incorporation of thiamine into liver and heart, whereas the incorporation is 5- to 25-fold higher into muscle and brain (Hilbig and Rahmann 1998). To improve our understanding of how benfotiamine affects glucose and lipid metabolism in cultured human skeletal muscle cells, we have therefore studied the effects of benfotiamine upon the metabolic fate of labeled glucose and fatty acids under conditions of normal glucose (5.5 mM) or chronic hyperglycemia (20 mM glucose) for 4 days. To assess whether metabolic changes were associated with changes in gene expression, we also isolated RNA and measured gene expression using real-time polymerase chain reaction (qPCR) and human gene expression microarrays.

Materials and methods

Dulbecco's Modified Eagle's Medium low glucose (DMEM), L-glutamine, penicillin/streptomycin (10,000 IE/10 mg/ml), HEPES, amphotericin B and L-carnitine were from Sigma-Aldrich, St.Louis, MO, US. Foetal calf serum (FCS), Dulbecco's Phosphate Buffered Saline (DPBS) and trypsin/EDTA (0.05%) were from Gibco/Invitrogen, Grand Island, NY, USA. Ultroser G was from Pall Corporation, St-Germain-en-Laye Cedex, France. D-[1-^{14}C]glucose (54 mCi/mmol), [^{14}C]deoxy-D-glucose (287 mCi/mmol) and [1-^{14}C]oleic acid (53 mCi/mmol) were purchased from PerkinElmer, NEN, Boston, MA, USA. D-[^{14}C(U)]glucose (5 mCi/mmol) were provided by American Radiolabeled Chemicals Inc., St. Louis, MO, USA. Insulin Actrapid® was from Novo Nordisk, Bagsvaerd, Denmark. Corning® CellBIND® microplates were from Corning B.V. Life Sciences, Schipol-Rijk, The Netherlands, 96-well UNIFILTER® microplate from Whatman, Middlesex, UK, and 96-well Isoplate, ScintiPlate®-96 TC micoplates and the scintillation liquid Optiphase Supermix was from Perkin Elmer, Waltham, Massachusetts, USA. Bio-Rad Protein Assay Dye Reagent was from Bio-Rad Laboratories, NY, USA. Agilent Total RNA isolation kit was obtained from Agilent Technologies (Santa Clara, CA, USA). Illumina Human-6 Express BeadChips version 3 arrays were from Illumina (San Diego, CA, USA).

Human skeletal muscle cell cultures

Satellite cells were isolated from the *M. obliquus internus abdominis* of 6 healthy donors, age 38.8 (± 3.8) years, body mass index 22.1 (± 1.5) kg/m^2, fasting glucose 5.0 (± 0.1) mM, insulin, plasma lipids and blood pressure within normal range and no family history of diabetes. The biopsies were obtained with informed consent and approval by the Regional Committee for Research Ethics, Oslo, Norway. The cells were cultured in DMEM (5.5 mM glucose) with 2% FCS, 2% Ultroser G, L-glutamine (4 mM), penicillin/streptomycin (P/S) and amphotericin B until 70-80% confluent. Myoblast differentiation to myotubes was then induced by changing medium to DMEM (5.5 mM glucose) with 2% FCS, 25 pM insulin, L-glutamine (4 mM), P/S and amphotericin B. Experiments were performed after 8 days of differentiation, and preincubation with benfotiamine (100-200 µM) and/or hyperglycaemia (20 mM glucose) was started after 4 days.

Substrate oxidation assay

The muscle cells were cultured on 96-well CellBIND® microplates. Substrate, [U-^{14}C]glucose (1 µCi/ml, 200 µM) or [1-^{14}C]oleic acid (1 µCi/ml, 100 µM), was given in DPBS with 10 mM HEPES (1 mM L-carnitine was also added with oleic acid as described previously (Aas et al. 2011)). A 96-well UNIFILTER® microplate was

mounted on top of the CellBIND® plate as described before (Wensaas et al. 2007), and the cells were incubated at 37°C for 4 h. The CO_2 trapped in the filter was counted by liquid scintillation (MicroBeta®, PerkinElmer). The remaining cell-associated radioactivity was also assessed by liquid scintillation, and the sum of CO_2 and cell-associated radioactivity was considered as total substrate utilization. Protein content in each well was determined (Bradford 1976), and the data are presented as CO_2/mg protein or cell-associated radioactivity/mg protein.

Scintillation proximity assay (SPA)

Radiolabeled substrates taken up and accumulated by adherent cells were measured by SPA. The cells were grown and differentiated in 96 well ScintiPlate®-96 TC SPA plates. Measurements of [^{14}C]deoxyglucose (1 µCi/ml) uptake by SPA were performed in DMEM without phenol red (Sigma, MO, cat. no. D5030) with additional 100 µM glucose. Finally, the cells were washed 3 times with PBS and harvested with 0.1 M NaOH (200 µl/well). Protein was determined according to Bradford et al. (Bradford 1976).

RNA isolation and microarray analysis

Total RNA was prepared from primary myotubes from three donors using Agilent Total RNA isolation kit according to the supplier's protocol (Agilent Technologies). RNA was used individually and RNA integrity was checked on chip analysis (Agilent 2100 bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 9.0). cRNA synthesis was performed using Illumina TotalPrep RNA Amplification (San Diego, CA, USA) according to the supplier's protocol. Hybridization, washing, and scanning of Illumina Human-6 Express BeadChips version 3 arrays (San Diego, CA, USA) were according to standard Illumina protocols. Data extraction and quality control was performed using BeadStudio version 3.1.3.0 (Illumina) and the Gene Expression module 3.2.7. Arrays were normalized using quantile normalization, and expression estimates were calculated by GC robust multiarray average background adjustment. Gene set enrichment analysis (GSEA) (Subramanian et al. 2005) was used to test the specific hypothesis that groups of genes involved in glucose metabolism were changed during benfotiamine treatment.

Collections of gene sets were obtained using a gene set browser from the Broad Institute website http://www.broad.mit.edu/ gsea/ (Molecular Signatures Database v3.0). This browser searches a number of publicly available sources (e.g., Biocarta, Kegg) where genes are grouped if they belong to the same pathway and share ontology terms or clinical phenotypes. Thus, it is possible to define specific gene sets on the basis of a particular parameter of interest. Similar use of GSEA has been published earlier (Bohn et al. 2010). Gene set collections relevant for glucose and energy metabolism were obtained using the following keywords stepwise: ”glucose”, ”insulin”, “energy”, ”lactate”, “mitochondria”, “transporter*” (*indicates truncated search), “lipid”, “DNA and Repair” and ”interleukin”. The larger, predefined gene set collections; C2, C3TFT and C5 from (www.broad.mit.edu/gsea/) were also tested. The C3 TFT (transcription factor targets) collection consists of gene sets that contain genes sharing a transcription factor-binding site defined in the TRANSFAC (version 7.4, http://www.gene-regulation.com/). The gene-sets in the C5 collection are grouped according to the gene ontology consortium. The C2 Gene sets are collected from various sources such as online pathway databases, publications in PubMed, and knowledge of domain experts. The benfotiamine treated samples were compared to untreated in paired GSEA analysis. GSEA was performed using J-express 2011 (www.molmine.com) according to the description on the J-express manual. The gene matrix was collapsed by selecting the maximum probes. Log fold change was used as the scoring method, the number of permutations was set to 1000 and gene sets with less than 10 genes or more than 500 genes were excluded from the analysis. False discovery rate (FDR) q values < 5% were used as criteria for significantly enriched gene sets. Minimum Information About a Microarray Experiment (MIAME) standards (Brazma et al. 2001) were followed in the analysis and storage of microarray data. The raw data are available at the Gene Expression Omnibus (GEO) at http: www.ncbi.nlm.nih.gov/geo/ by accession number GSE31553.

RNA isolation and analysis of gene expression by TaqMan® qPCR

Cells were harvested and total RNA was isolated by Agilent Total RNA isolation kit (Agilent Technologies, Santa Clara, CA, USA) according to the supplier’s total RNA isolation protocol. Total RNA was reverse-transcribed with oligo primers using a Perkin-Elmer Thermal Cycler 9600 (25°C for 10 min, 37°C for 1 h 20 min, and 85°C for 5 s) and a TaqMan reverse transcription reagents kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA). Two micrograms of total RNA were added per 20 µL of total TaqMan reaction solution. Real-time PCR was performed using an ABI PRISMT 7000 Detection System (Applied Biosystems, USA). RNA expression was determined by SYBRT Green, and primers were designed

using Primer ExpressT (Applied Biosystems, USA). Each target gene was quantified in triplicate and carried out in a 25 µL reaction volume according to the supplier's protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The housekeeping control genes GAPDH (FC -1.1, p=0.8 from microarray) and 36B4 were both measured, and transcription levels are presented as averaged change relative to levels of GAPDH and 36B4. Primer sequences: GAPDH (acc.no. NM_008084): F: CATGGCCTTCCGTGTTCCT, R: TGATGTCATCATACTTGGCAGGTT; 36B4 (acc.no. NM_007475): F: ATCTCCAGAGGCACCATTGAA, R: TCGCTGGCTCCCACCTT; NOX4 (acc.no. NM_016931): F: TGGACCTTTGTGCCTGTACTGT; R: TGAGGATGACTTATGACCGAAA.

Statistics

All data are presented as mean ± SEM. Statistical comparison between different treatments was performed by linear mixed model (LMM) and Anova repeated measures using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The parameter of interest was entered as the dependent variable, and pretreatment (with and without benfotiamine) and acute treatments were entered as fixed variables. Differences were considered statistically significant at p-values less than 0.05. All experiments were performed with at least triplicate observations, and replicate experiments were performed on cells from different donors. For microarray analysis, individual fold change (FC) for each donor was calculated by dividing the expression level after benfotiamine treatment on the expression level after control treatment. The individual fold change was then log2 transformed. Mean fold changes were calculated based on the log2-transformed individual fold changes, followed by identification of differentially expressed probe sets using Intensity-based moderated t-statistics (Sartor et al. 2006). P-values were corrected for multiple testing by using Benjamini and Hochberg's false discovery rate (FDR) method (Benjamini and Hochberg 1995). Probe sets that satisfied the criterion of FDR < 10% (q-value < 0.1) and fold-change > 1.5 or < -1.5, were considered to be significantly regulated.

Results

Effects of incubation with benfotiamine on glucose metabolism

Under normoglycemic conditions (NG, 5.5 mM glucose), basal [U-^{14}C]glucose oxidation (CO_2 formation) was significantly increased in myotubes after incubation with benfotiamine (increased by 35% [p=0.02] and 49% [p=0.003], after 100 and 200 µM benfotiamine for 4 days, respectively) (Fig. 1A). We also measured glucose oxidation after incubation with lower concentrations of benfotiamine (1 and 10 µM) without observing any effect (Fig. 1D). Under hyperglycemic conditions (HG, 20 mM glucose for 4 days), glucose oxidation was significantly increased by 70% after exposure to 200 µM benfotiamine (p=0.01) (Fig. 1A). Total metabolized glucose (sum of CO_2 formation and cell-associated glucose) was also significantly increased for 200 µM benfotiamine (p=0.001) (Fig. 1B) under NG conditions. Accordingly, we found a significant increase in fractional oxidation of [U-^{14}C]glucose (CO_2/sum of cell-associated plus CO_2) after benfotiamine treatment (100 and 200 µM) under both normoglycemic and hyperglycemic conditions (Fig. 1C). We found no significant changes in glycogen synthesis after incubation with benfotiamine (data not shown). Hyperglycemia decreased both glucose oxidation and total metabolized glucose (Fig. 1A and B) in agreement with earlier observations (Aas et al. 2011).

The effect of benfotiamine on glucose metabolism was compared with thiamine. As shown in Fig. 2 both benfotiamine and thiamine increased total metabolized glucose at the highest concentration examined (200 µM for 4 days), although the effect of benfotiamine was greater than for thiamine, 64% and 19%, respectively. There was no effect of thiamine on glucose oxidation (data not shown).

To assess whether the increase in glucose utilisation was accompanied by an increase in glucose uptake, we measured cellular [^{14}C]deoxyglucose uptake after incubation with benfotiamine. We found a small (17%) increase ($p<0.001$ overall effect) in glucose uptake after benfotiamine incubation for 4 days under NG conditions (Fig. 3).

Effects of benfotiamine on fatty acid metabolism

In contrast to the marked effects of benfotiamine upon glucose metabolism, benfotiamine had no significant effects upon [^{14}C]oleic acid (OA) oxidation (CO_2 formation) and overall lipid utilisation (CO_2 formation plus cell-associated OA) when these processes were studied separately (Fig. 4A and B). However, in contrast to glucose, benfotiamine significantly decreased fractional OA oxidation (CO_2/cell-associated OA plus CO_2) at both 100 and 200 µM benfotiamine under NG conditions as compared to NG alone (p=0.002 and 0.007

respectively, Fig. 4C), and at 200 µM benfotiamine under HG conditions as compared to HG alone ($p < 0.02$, Fig. 4C). HG treatment also decreased fractional oleic acid oxidation (Fig 4C).

Effects of benfotiamine upon gene expression

To examine whether the effects of benfotiamine treatment could be explained by changes in gene expression, microarray analysis was performed. Under normoglycemic conditions, a total of more than 100 genes were significantly upregulated after exposure to 200 µM benfotiamine, whereas more than 200 genes were downregulated (Tables 1 and 2 for the top 20 most down- and upregulated genes; all regulated genes supplementary tables s1 and s2). NADPH oxidase 4 (NOX4) and growth factor midkine (MDK) were significantly downregulated after benfotiamine treatment (-2.6 and -3.2 FC, respectively). Under hyperglycemic conditions, the expression of more than 50 and 100 genes were significantly up- and downregulated after benfotiamine treatment, respectively (Tables 1 and 2 for the top 20 most down- and upregulated genes, respectively). The expression of NOX4 and MDK were again found to be significantly downregulated by benfotiamine (-3.1 and -3.3 FC, respectively). NOX4 expression was verified by qPCR, confirming that benfotiamine had a marked downregulatory effect under both NG and HG conditions (Figure 5, $p<0.05$ overall effect). Contrastingly, SERPINB7 (a serine proteinase inhibitor) was significantly upregulated under both normo- and hyperglycaemic conditions (+3.4 and +2.3 FC, respectively). Most of the genes regulated by benfotiamine treatment were regulated both under NG and HG conditions. We did not find any significant changes in the expression of genes encoding for proteins directly involved in lipid or glucose metabolism after benfotiamine treatment.

In addition, we have used GSEA to test the hypothesis that benfotiamine treatment of human myotubes can affect groups of genes associated with glucose metabolism when compared to control cells. A substantial number of relevant gene sets in the defined collections were significantly downregulated after benfotiamine treatment (table 3). Five gene sets defined by “energy”, one gene set defined by “lipid” and two gene sets defined by “transporter” were downregulated after exposure to benfotiamine. We found one upregulated gene set from the “glucose” collection. This gene set was associated with glucuronidation processes. Moreover, 11 gene sets from “lipid” collection were upregulated after benfotiamine treatment. Among these genes sets there were gene sets involved in peroxisomal fatty acid oxidation and organelle membrane function (including genes important for mitochondrial function). Lists of the differentially regulated gene sets and the genes that contributed to the regulation can be viewed in supplemental table s3.

Microarray data also showed that the level of muscle differentiation markers such as myogenin (-1.40 FC) and myoD (-1.07 FC) were not significantly affected by benfotiamine, indicating that this treatment does not interfere with the differentiation process of the myotubes.

Discussion

Our findings are the first to show that benfotiamine increases glucose oxidation (CO_2 formation) whilst down-regulating NOX4 expression in cultured human myotubes. Benfotiamine also increased overall glucose utilization, which was also shown for thiamine. In contrast to glucose oxidation, we found no significant change in oxidation and overall utilization of oleic acid after incubation with benfotiamine.

Hammes et al. reported that there was no change in TCA cycle activity and that activation of transketolase (TK) was responsible for the effects of benfotiamine in cultured endothelial cells (Hammes et al. 2003). Our contrasting findings suggest that the increase in glucose oxidation that we observed in myotubes does not occur in endothelial cells. Preliminary data in human umbilical vein endothelial cells (HUVEC) supports this, as although we observed a marked increase in cell-associated glucose, we did not see the same marked increase in glucose oxidation as we saw in myotubes (data not shown).

It is unclear whether an increase in mitochondrial glucose oxidation, such as we observed, would have an adverse effect upon muscle cell function *in vivo*. In endothelial cells, it has been proposed that the adverse changes observed in endothelial cells exposed to hyperglycemia results from increased glycolytic flux causing an increase in electron donors (NADH and $FADH_2$) into the electron transport chain and the subsequent generation of superoxide from the mitochondria (Brownlee 2005). However, other studies have indicated that an increase in pyruvate entering the mitochondria is beneficial as this increases the NAD/NADH ratio and thereby reduces the intracellular redox potential (Tilton et al. 1992; Van den Enden et al. 1995). GSEA analysis did not show any effect of benfotiamine on pathways directly involved in glucose metabolism (e.g. glycolysis). However gene sets involved in peroxisomal lipid oxidation and organelle membrane function (including mitochondrial OXPHOS genes) were upregulated after benfotiamine treatment.

The marked downregulation of NADPH oxidase 4 (NOX4) we observed after incubation with benfotiamine, if also present in other cell types, may be a key mechanism by which this compound exerts its beneficial effects in animal models of diabetes. NOX4 is upregulated in response to hyperglycemia (Xia et al. 2006), is a primary source of reactive oxygen species (Basuroy et al. 2009) and is highly expressed in kidney (Gorin et al. 2005) and vascular endothelial cells (Chen et al. 2008). Benfotiamine has previously been shown to prevent genomic damage to cells exposed to prooxidants *in vitro* (Schmid et al. 2008) and *ex vivo* (Schupp et al. 2008). Inhibition of NOX upregulation has been shown to prevent the development of nephropathy in STZ-induced diabetic rat model (Xu et al. 2009). Notably, we found that benfotiamine also downregulated NOX4 expression under normoglycaemic conditions, suggesting that benfotiamine may have effects upon the

constitutive expression of NOX4 unrelated to ambient plasma glucose concentrations. The significant downregulation of growth factor midkine (MDK) we observed after benfotiamine treatment may also exert a beneficial effect since it has been shown to play a key role in the development of diabetic nephropathy in a STZ-induced diabetic mouse model (Kosugi et al. 2006).

Our finding that the serine proteinase inhibitor SERPINB7 was significantly upregulated by benfotiamine under both normo- and hyperglycaemic conditions was unexpected. Recent studies have suggested that upregulation of SERPINB7 expression in response to hyperglycemia increases mesangial matrix accumulation and accelerates diabetic nephropathy in mice (Miyata et al. 2002). This may be secondary to inhibition of plasmin and matrix metalloproteinase activity (Ohtomo et al. 2008). This finding does not concord with a previous study showing that benfotiamine actually protects against diabetic nephropathy in animal models (Babaei-Jadidi et al. 2003). The reasons for this discrepancy are unclear, but may be related to differences in myotubes as compared to mesangial cells where SERPINB7 is predominantly expressed.

In addition to increasing TK activity, it has previously been found that benfotiamine increases the expression of TK in renal glomeruli (Loew 1996). However, we did not find any change in the gene expression of TK in human myotubes using microarray. In addition, we did not find any significant changes in genes encoding enzymes involved in glycolysis and/or the TCA cycle that might suggest that the increased oxidation we observed may be secondary to changes in enzyme activity rather than gene expression levels.

Using benfotiamine *in vitro* differs from *in vivo* since most of the benfotiamine given orally is partially dephosporylated in the gut to benzoylthiamine. Due to the enhanced membrane permeability of benzoylthiamine, oral benfotiamine leads to higher plasma and intracellular concentrations of the active metabolite, thiamine diphosphate (TPP), than thiamine (Loew 1996). Although intact benfotiamine is also delivered to cells via the reduced folate carrier-1 (RFC-1) which is then de-benzoylated to thiamine monophosphate (TMP) by cellular and plasma esterases (Thornalley 2005), it is not superior to thiamine in increasing the intracellular concentrations of thiamine *in vitro* (Volvert et al. 2008). The exposure of cells *in vivo* to intact benfotiamine is minimal (peak plasma conc. approx. 10 nM in subjects taking 250 mg benfotiamine/day) as compared to the supra-pharmacological concentrations used in this study (100-200 μM) (Ziems et al. 2000). Although doses as high as 900 mg benfotiamine per day have been used clinically (Alkhalaf et al. 2010), we must take into account the possibility that the effects we observed were associated with the high concentrations used, which might result in the generation of esterase-derived thiols at concentrations not observed *in vivo*. It is also possible that

benfotiamine, but not thiamine, induces spontaneous rearrangement of S-acylcysteine derivatives to N-acylcysteine derivatives that affects pyrimidine synthesis and related signalling (Edwards et al. 1996).

Interestingly, we also found an increase in glucose oxidation in cells exposed to normoglycemia in the presence of benfotiamine. The effects we observed are therefore not simply a result of benfotiamine abolishing the previously documented inhibitory effects of hyperglycemia upon glucose uptake in myotubes (Aas et al. 2004). However, we also found that 200 µM benfotiamine was able to reverse the inhibitory effect of hyperglycemia upon glucose oxidation. We have previously shown that chronic hyperglycemia reduces insulin-stimulated glucose uptake and increases triacylglycerol formation from labeled glucose (Aas et al. 2004). We assessed the effects of benfotiamine upon overall glucose uptake under normoglycemic conditions both with and without insulin, and only found a small (17%) but significant increase of benfotiamine. Although we cannot say whether this increased uptake preceded or was a result of the increase in glucose oxidation, this increase in uptake was relatively minor compared to the more pronounced increase in glucose oxidation (35-70%). This would suggest that the effects of benfotiamine upon glucose oxidation cannot be explained by an increased glucose uptake itself.

The decrease we noted in fractional oleic acid oxidation may reflect the inverse reciprocal relationship between glucose and lipid oxidation. This finding also makes it unlikely that the increase we observed in glucose oxidation is a result of a benfotiamine-induced change in myotube differentiation. Additionally we found no changes in muscle differentiation markers such as myogenin and myoD. However, our finding that benfotiamine decreased fractional oleic acid oxidation whilst increasing fractional glucose oxidation suggests that benfotiamine may stimulate a preferential oxidation of glucose at the expense of fatty acids. This may be secondary to increased generation of pyruvate from glycolysis. GSEA analysis also suggested that fatty acids could be channelled to peroxisomes for oxidation after treatment with benfotiamine.

Most previous research into the biochemical effects of benfotiamine have focused on cells, such as endothelial cells, susceptible to hyperglycaemic associated damage (Balakumar et al. 2010). The clinical implications of the results from the present study are that oral benfotiamine has marked metabolic effects upon cells i.e., skeletal muscle, other than those typically associated with diabetic microvascular complications. Whether our results can be extrapolated to the *in vivo* situation is not clear, as the marked effects we observed were achieved using supra-physiologic concentrations of benfotiamine. If such effects do occur *in vivo*, our results suggest that benfotiamine may not only be beneficial for microvascular complications associated with

type 1 diabetes, but could also be of relevance to type 2 diabetes and obesity where reversal of reduced mitochondrial capacity and decreased glucose oxidation is a desirable goal (Aas et al. 2011).

In conclusion, our study has shown that benfotiamine, but not thiamine, causes a marked increase in mitochondrial glucose oxidation in human skeletal muscle cells. Our finding that benfotiamine also reduces NOX4 gene expression offers a new insight into the mechanism/s by which this substance exerts its effects upon the development of diabetic complications. These findings suggest that benfotiamine has a wider range of effects in different cell types of relevance to both type 1 and type 2 diabetes than has previously been recognised.

Abbreviations: NAD: nicotinamide adenine dinucleotide, NADPH: nicotinamide adenine dinucleotide phosphate, NOX: Nicotinamide adenine dinucleotide phosphate oxidase, TK: transketolase, PPP: pentose phosphate pathway, PDH: pyruvate dehydrogenase, KDH: ketoglutarate dehydrogenase, TCA: tricarboxylic acid, IMTG: intramyocellular triacylglycerol, SPA: scintillation proximation assay, NG: normoglycemic, HG: hyperglycemic, MDK: growth factor midkine, TMP: thiamine monophosphate, TPP: thiamine diphosphate, RFC: reduced folate carrier, OXPHOS: oxidative phosphorylation.

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Table 1. Genes downregulated by benfotiamine

Symbol	Acc	Description	FC NG	FC HG
LRRN1	NM_020873	leucine rich repeat neuronal 1	-3.97	-3.43
RARRES1	NM_206963	retinoic acid receptor responder (tazarotene induced) 1	-3.62	-2.50
COMP	NM_000095	cartilage oligomeric matrix protein	-3.39	-4.48
COL15A1	NM_001855	collagen. type XV, alpha 1	-3.29	-3.10
SPRR2G	NM_001014291	small proline-rich protein 2G	-3.28	-2.85*
MDK	NM_001012333	midkine (neurite growth-promoting factor 2)	-3.23	-3.34
COL16A1	NM_001856	collagen. type XVI, alpha 1	-3.18	-3.05
TYMP	NM_001953	thymidine phosphorylase	-2.89	-2.20*
PACSIN1	NM_020804	protein kinase C and casein kinase substrate in neurons 1	-2.89	-2.30
MYOT	NM_006790	myotilin	-2.86	-2.40*
FAM180B	XM_001716425	family with sequence similarity 180, member B	-2.81	-3.25
FRAS1	NM_025074	Fraser syndrome 1	-2.75	-2.88
MGC16121	XM_001128419	hypothetical protein MGC16121	-2.68	-1.45*
MXRA5	NM_015419	matrix-remodelling associated 5	-2.67	-2.72
SORBS2	NM_021069	sorbin and SH3 domain containing 2	-2.66	-2.96
HES4	NM_021170	hairy and enhancer of split 4 (Drosophila)	-2.57	-2.43
NOX4	NM_016931	NADPH oxidase 4	-2.56	-3.07
CTHRC1	NM_138455	collagen triple helix repeat containing 1	-2.54	-1.90
CHN2	NM_004067	chimerin (chimaerin) 2	-2.53	-1.95*
CIRBP	NM_001280	cold inducible RNA binding protein	-2.51	-1.94*

[20 genes with greatest fold-change (FC) decreased after benfotiamine treatment (200 μM, 4 days) as compared to cells not exposed to benfotiamine under NG conditions, with corresponding values under HG conditions], adjusted p-value < 0.1. *Adjusted p-value $\geq$ 0.1. N = 3 in each group.

Table 2. Genes upregulated by benfotiamine

Symbol	Acc	Description	FC NG	FC HG
SERPINB7	NM_003784	serpin peptidase inhibitor, clade B (ovalbumin), member 7	3.39	2.27
MX1	NM_002462	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	3.19	3.32*
CPA4	NM_016352	carboxypeptidase A4	3.16	1.57*
TM4SF4	NM_004617	transmembrane 4 L six family member 4	3.11	2.14*
LCE3D	NM_032563	late cornified envelope 3D	2.76	2.54
IFI44L	NM_006820	interferon-induced protein 44-like	2.62	2.54
PSG4	NM_213633	pregnancy specific beta-1-glycoprotein 4	2.43	1.98
TSGA10	NM_182911	testis specific, 10	2.38	2.55
NRXN2	NM_138732	neurexin 2	2.32	1.95
MT1G	NM_005950	metallothionein 1G	2.32	1.44
CCL26	NM_006072	chemokine (C-C motif) ligand 26	2.18	1.60*
hCG_1815504	XM_498560	hCG1815504	2.15	1.85*
BRE	NM_199193	brain and reproductive organ-expressed (TNFRSF1A modulator)	2.12	1.79
PSG11	NM_002785	pregnancy specific beta-1-glycoprotein 11	2.11	1.42*
TFPI	NM_001032281	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	2.10	1.40*
EVI2A	NM_001003927	ecotropic viral integration site 2A	2.10	1.69*
ALDH3A1	NM_000691	aldehyde dehydrogenase 3 family. memberA1	2.09	2.13
FGGY	NM_001113411	FGGY carbohydrate kinase domain containing	2.02	1.95
DEFB103B	NM_001081551	defensin, beta 103B	2.01	1.27*
IFI44	NM_006417	interferon-induced protein 44	1.98	2.07*

[20 genes with greatest fold-change (FC) increased after benfotiamine treatment (200 μM, 4 days) as compared to cells not exposed to benfotiamine under NG conditions, with corresponding values under HG conditions], adjusted p-value < 0.1. *Adjusted p-value ≥ 0.1. N = 3 in each group.

Table 3. Number of regulated gene sets comparing control and benfotiamine treatment within each group under normoglycemic (NG) conditions (paired GSEA analysis, score: log-fold change, FDR ≤ 5%).

Gene set collections	Number of gene sets in collection	Control > benfotiamine (downregulated by treatment)	Benfotiamine > control (upregulated by treatment)
C5bp	753	1	0
C3TFT	582	5	0
C2	846	2	7
Energy	63	5	1
Glucose	63	0	1
Insulin	97	0	0
Lipid	141	1	11
DNA and repair	77	0	0
Interleukin	133	1	0
Lactate	5	0	0
Mitochondria	29	0	0
Transporter*	29	2	0

A gene set browser provided by Broad institute at www.broad.mit.edu/gsea/ was used to obtain the stress-associated gene set collections. Detailed information regarding the regulated gene sets and leading edge genes are given in online supplementary table s3. *indicates that truncated search keyword was used to create the gene set collections. C2, C3TFT and C5 are pre-defined gene set collections. N = 3 in each group.

Figure legends

Figure 1. The effects of benfotiamine upon (A) [U-^{14}C]glucose oxidation (CO_2 formation), (B) total metabolized glucose (sum of CO_2 formation and cell-associated glucose) and (C) fractional oxidation of [U-^{14}C]glucose (CO_2/ sum of cell-associated plus CO_2) under NG (normoglycemic, 5.5 mM glucose) and HG (hyperglycemic, 20 mM glucose) conditions. Myotubes were treated for 4 days in the absence or presence of benfotiamine (100 and 200 µM) under NG and HG conditions. All data are normalized to normoglycemic control and presented as mean ± SEM (n = 5 for NG and n = 3 for HG, independent muscle cell donors). [a]$p<0.05$ vs. control myotubes not exposed to benfotiamine under NG condition; [b]$p<0.05$ vs. control myotubes not exposed to benfotiamine under HG condition (linear mixed model (LMM), SPSS). HG treatment significantly decreased glucose oxidation and total metabolized glucose ($p<0.001$ overall effect) (LMM). (D) Dose-response effects of benfotiamine upon [U-^{14}C]glucose oxidation (CO_2 formation) under NG conditions (mean ± SEM, n = 2).

Figure 2. The effects of thiamine and benfotiamine upon total metabolized glucose (sum of CO_2 formation and cell-associated glucose). Myotubes were treated for 4 days with or without thiamine (200 µM) or benfotiamine (200 µM) under NG (5.5 mM glucose) condition. All data are normalized to control and presented as mean ± SEM (n = 3 independent muscle cell donors). [a]$p<0.05$ vs. control myotubes not exposed to the compounds; [b]$p<0.05$ vs. thiamine (LMM).

Figure 3. The effects of benfotiamine upon [^{14}C]deoxyglucose uptake in cells under NG (5.5 mM glucose) condition. Myotubes were treated for 4 days in the absence or presence of benfotiamine (200 µM) before the addition of colorless DMEM with 100 µM glucose and [^{14}C]deoxy-D-glucose (1 µCi/ml). Data represent mean ± SEM from cells isolated from 3 individual donors. Benfotiamine significantly increased overall glucose uptake ($p<0.001$, Anova repeated measures).

Figure 4. The effects of benfotiamine upon (A) [1-^{14}C]oleic acid (OA) oxidation, (B) overall OA utilization (sum of CO_2 formation and cell-associated OA) and (C) fractional oxidation of OA (CO_2/(sum of cell-associated plus CO_2)) of OA under NG (5.5 mM glucose) and HG (20 mM glucose) conditions. Myotubes were treated for 4 days with or without benfotiamine (100 and 200 µM) under NG and HG conditions. All data are normalized to normoglycemic control cells and presented as mean ± SEM (n = 5 for NG and n = 6 for HG, independent muscle cell donors). [a]$p<0.05$ vs. control myotubes not exposed to benfotiamine under NG condition; [b]$p<0.05$ vs. control

myotubes not exposed to benfotiamine under HG condition (LMM). HG treatment significantly decreased fractional oleic acid oxidation ($p<0.01$ overall effect) (LMM).

Figure 5. Effects of benfotiamine upon expression of NADPH oxidase 4 (NOX4) mRNA measured by qPCR. Myotubes were treated for 4 days with or without benfotiamine (200 µM) under NG and HG conditions. All data presented as mean ± SEM (n = 3 independent muscle cell donors). $p<0.05$ overall effect of benfotiamine vs. control myotubes (LMM).

Figures

Figure 1

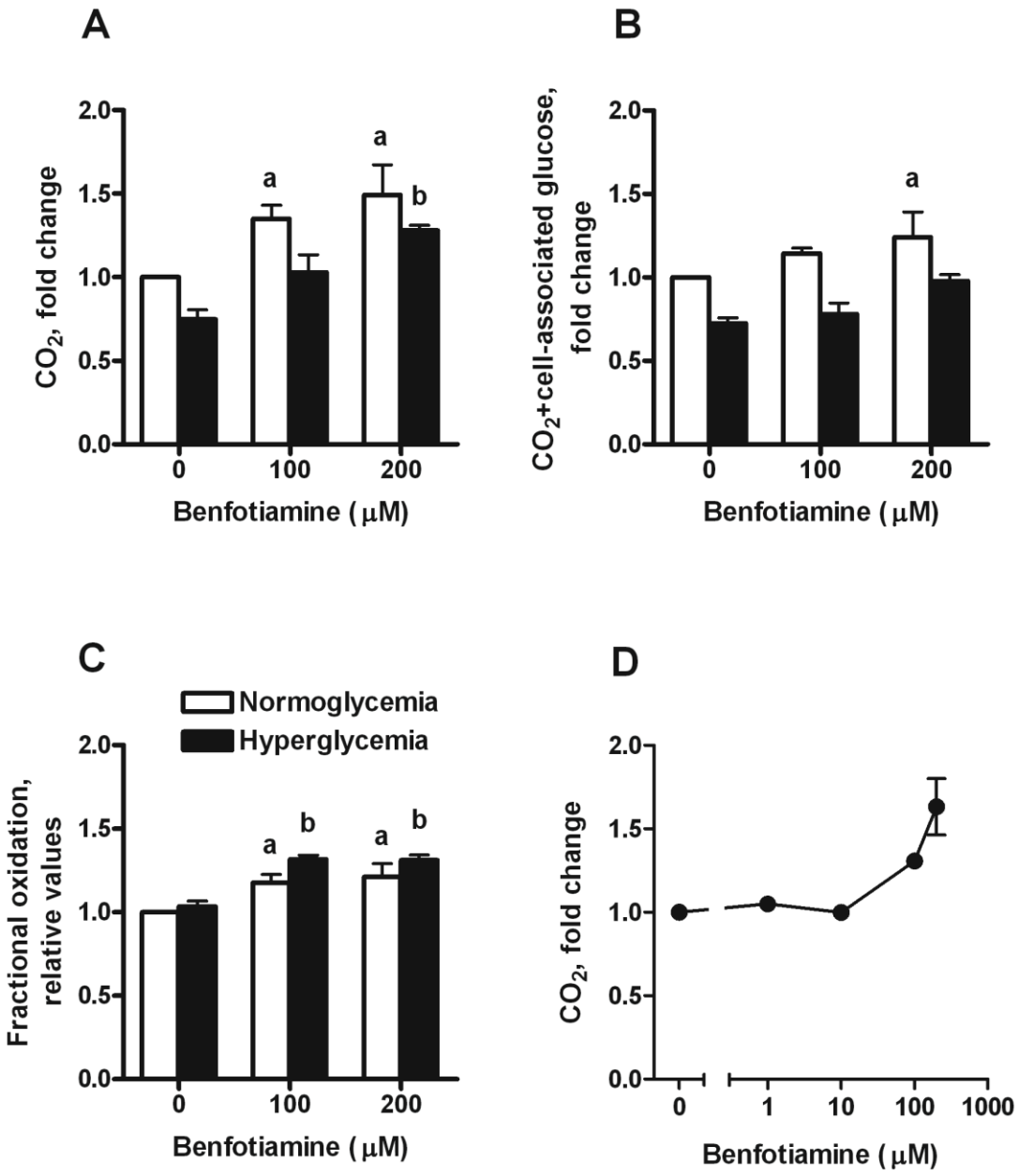


Figure 2

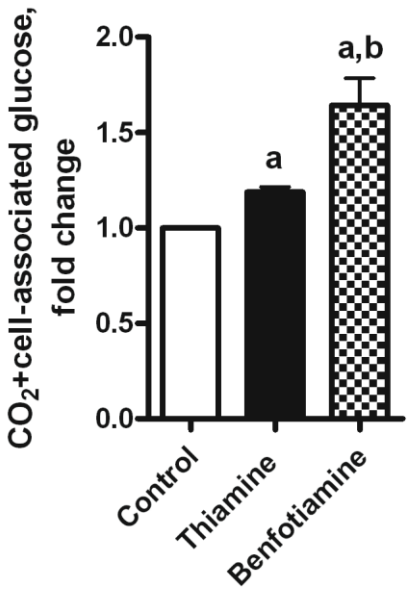

CO_2+cell-associated glucose, fold change
2.0
1.5
1.0
0.5
0.0
a
a,b
Control
Thiamine
Benfotiamine

Figure 3

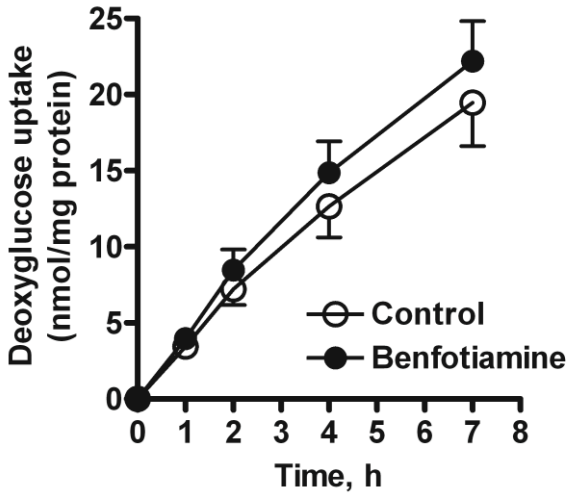

Deoxyglucose uptake
(nmol/mg protein)
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
Time, h
Control
Benfotiamine

Figure 4

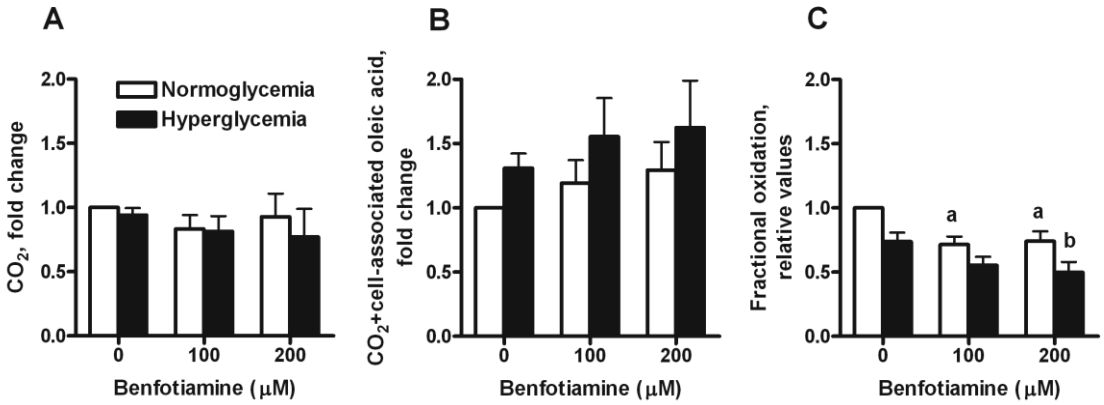
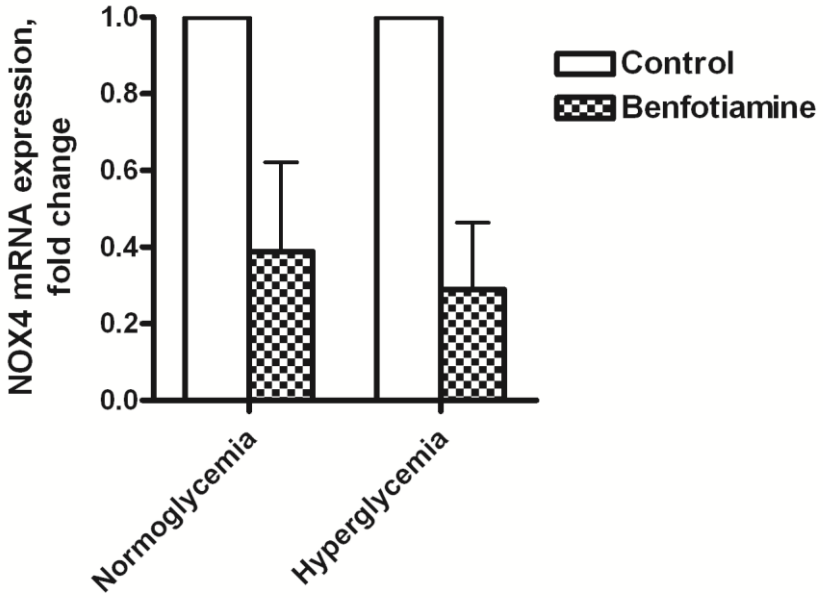

A
B
C
Normoglycemia
Hyperglycemia
CO_2, fold change
CO_2+cell-associated oleic acid, fold change
Fractional oxidation, relative values
2.0
1.5
1.0
0.5
0.0
0
100
200
Benfotiamine (μM)
a
a
b

Figure 5


NOX4 mRNA expression, fold change
1.0
0.8
0.6
0.4
0.2
0.0
Control
Benfotiamine
Normoglycemia
Hyperglycemia