A metabolic shift induced by a PPAR panagonist markedly reduces the effects of pathogenic mitochondrial tRNA mutations

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Abstract

Mutations in mitochondrial DNA-encoded tRNA genes are associated with many human diseases. Activation of peroxisome proliferator-activated receptors (PPARs) by synthetic agonists stimulates oxidative metabolism, induces an increase in mitochondrial mass and partially compensates for oxidative phosphorylation system (OXPHOS) defects caused by single OXPHOS enzyme deficiencies *in vitro* and *in vivo*. Here, we analysed whether treatment with the PPAR panagonist bezafibrate in cybrids homoplasmic for different mitochondrial tRNA mutations could ameliorate the OXPHOS defect. We found that bezafibrate treatment increased mitochondrial mass, mitochondrial tRNA steady state levels and enhanced mitochondrial protein synthesis. This improvement resulted in increased OXPHOS activity and finally in enhanced mitochondrial ATP generating capacity. PPAR panagonists are known to increase the expression of PPAR gamma coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis. Accordingly, we found that clones of a line harbouring a mutated mitochondrial tRNA gene mutation selected for the ability to grow in a medium selective for OXPHOS function had a 3-fold increase in PGC-1α expression, an increase that was similar to the one observed after bezafibrate treatment. These findings show that increasing mitochondrial mass and thereby boosting residual OXPHOS capacity can be beneficial to an important class of mitochondrial defects reinforcing the potential therapeutic use of approaches stimulating mitochondrial proliferation for mitochondrial disorders.

Keywords: mitochondria • bezafibrate • mitochondrial disease • PPAR • PGC-1α

Introduction

Defects in the mitochondrial oxidative phosphorylation system (OXPHOS) are associated with several human diseases. The lack of ATP supply caused by the OXPHOS dysfunction affects primarily high-energy demand tissues such as brain, skeletal muscle and heart resulting in severe pathology. These defects are caused by genetic alteration of either nuclear or mitochondrial genomes [1].

Although the nuclear DNA encodes for the majority of mitochondrial proteins, the mitochondrial DNA (mtDNA) encodes for 13 subunits, 22 tRNAs and 2 rRNAs. Pathogenic mutations in most of these mitochondrial genes have been reported. Mutations in tRNA genes are particularly common in the patient population and cause a generalized defect in mitochondrial protein synthesis [1]. The mutations can cause instability of tRNA, affect interaction with the translation machinery or cause misincorporation of amino acids ultimately affecting OXPHOS function and ATP supply [2, 3].

We and others have demonstrated that OXPHOS defects with a residual ATP generating capacity can be at least partially compensated by increasing mitochondrial mass through enhanced mitochondrial biogenesis [4–7]. This mitochondrial proliferation can be achieved by activation of peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (PGC-1α), the key regulator of mitochondrial biogenesis [8]. PGC-1α interacts with PPARs, as well as with other nuclear factor including oestrogen related receptor α and the nuclear respiratory factors (NRF1 and NRF2)

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to increase transcription of mitochondrial proteins. One of the target genes is mtDNA transcription factor A (TFAM), which modulates mtDNA transcription and mtDNA replication [9]. PGC-1α expression and activity is controlled by the PPARs [9, 10] as well as by AMP-protein activated kinase (AMPK) and Sirt1 [11]. Many agonists have been identified that activate these proteins, such as bezafibrate (PPAR), resveratrol (Sirt1) and Aminoimidazol-Carboxamide-Ribonucleoside (AICAR) and metformin (AMPK). Many of them have been shown to induce mitochondrial proliferation both *in vivo* and *in vitro* and thus offer the potential of pharmacological intervention [4, 7, 12, 13]. We showed that bezafibrate can enhance OXPHOS activity (normalized to tissue mass) in a mouse model of mitochondrial myopathy caused by an isolated cytochrome *c* oxidase (COX) defect [7].

We also previously showed that adenoviral-induced PGC-1α/β expression and the associated increased mitochondrial biogenesis partially relieve single respiratory chain enzyme defects in cultured cells from patients with mitochondrial disorders [5]. Bastin *et al.* demonstrated that bezafibrate offers a pharmacological option to increase mitochondrial mass and achieve similar effects in patient cell lines harbouring mutations in nuclear genes [4].

Here we show that bezafibrate-mediated mitochondrial biogenesis is also beneficial to cell lines carrying the relatively common pathogenic mitochondrial tRNA gene mutations. Bezafibrate increased ATP synthesis capacity by increasing levels of the affected tRNA/cell resulting in increased levels of OXPHOS proteins and increased enzyme activities.

Materials and methods

Cell lines

Previously described transmitochondrial cybrids were used (Table 1). Clones containing only mutated mtDNA and clones containing exclusively the wild-type mtDNA were selected and used in this study. Cybrids carrying the following mutations were used: A3242G tRNA$^{Leu(UUR)}$ (MELAS [mitochondrial encephalomyoptahy lactic acidosis and stroke]), A8344G tRNA^{Lys} (MERRF [myoclonus epilepsy with ragged-red fibres]), G5703A tRNA^{Asn} (mutant:W72, wild-type: W20), C3256T tRNA$^{Leu(UUR)}$ (mutant: SUB62, wild-type: SUB52) (Table 1).

Cells were grown for at least for 4 days in Dulbecco's modified Eagle's medium supplemented with 1 mM pyruvate, 50 μg/ml uridine and 10% foetal bovine serum. Bezafibrate, dissolved in dimethyl sulfoxide (DMSO), was supplemented at a concentration of 400 μM. Controls were supplemented with the equal volume of DMSO (vehicle). Cells were grown for 4 days in the supplemented media.

RT-PCR experiments

Total RNA was extracted from snap-frozen muscle by TRIZOL (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the SuperScript First Strand Kit (Invitrogen). Quantitative real-time PCR reactions were performed on the cDNAs in the presence of fluorescent dye (SYBR Green,

Table 1 Cell lines used in this study

Cell lines	tRNA	Mutation	Ref.
MELAS wt MELAS mutant	tRNA$^{Leu(UUR)}$	wild-type A3243G	[18]
MERRF wt MERRF mutant	tRNA^{Lys}	wild-type A8344G	[19]
SUB52 SUB62	tRNA$^{Leu(UUR)}$	wild-type C3256T	[21]
W20 W72	tRNA^{Asn}	wild-type G5703A	[20]
WG4 WL4	tRNA^{Asn}	G5703A, galactose-resistant clones of W72	[24]

QIAGEN, Inc., Valencia, CA, USA). All results are expressed as means $\pm$ S.E.M. The results were normalized for comparison by measuring β-actin mRNA levels in each sample.

Lactate and glucose measurements

Cells grown for 4 days in bezafibrate or DMSO-supplemented media to 70–80% confluency, when the media were exchanged for low glucose media. After 24 hrs, media were removed and glucose and lactate levels were measured with a YSI 2300 TSAT Plus Glucose & Lactate Analyzer (YSI Life Sciences, Yellow Springs, OH, USA).

ATP synthesis and levels

ATP synthesis in DMSO- or bezafibrate-treated cells was measured as described previously [14]. ATP levels were determined after extraction from cells using perchloric acid as described [14]. ATP concentrations were determined using the luciferase-based ENLITEN ATP Assay System (Promega Corporation, San Luis Obispo, CA, USA), and values were normalized to milligram of tissue.

Measurement of mitochondrial enzyme activities

Mitochondrial enzyme complex activities were measured spectrophotometrically in cells using a DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA) as described elsewhere [15]. The activities of NADH-cytochrome *c* oxidoreductase (complex I + III [CI + III]), COX (complex IV) and citrate synthase (CS) were determined by following the cytochrome *c* reduction (CI + III) or oxidation (complex IV) at 550 nm [15]. All assays were performed at 37°C (except the CS at 30°C).

Western blots analysis

Western blot analysis was performed as described [5]. Antibodies against different subunits of the oxidative phosphorylation complexes and voltage dependent anion channel 1 (VDAC1) were obtained from MitoSciences, Inc. (Eugene, OR, USA), and an antibody against tubulin was obtained from Chemicon International (Temecula, CA, USA).

Measurement of tRNA levels

RNA was isolated from cells using TRIzol reagent (Gibco-BRL, Invitrogen). RNA samples were tested by ultraviolet absorption ratio A_{260}/A_{280} for purity and concentration. Values for A_{260}/A_{280} were >1.8 for all RNA extraction. A total of 5 μg of tRNA was alkaline denatured and applied to ZETA-probe membrane *via* slot-blotting followed UV crosslinking. Hybridization was performed as described previously [16]. 45-mer oligonucleotides complementary to the tRNAs end-labelled with [γ^{32}-P]-ATP were used as hybridization probes. 4.5S RNA was used as loading control.

Mitochondrial labelling

Mitochondrial protein labelling was performed in transmitochondrial cybrids grown in the presence of bezafibrate or DMSO as described [17].

Results

We have studied the effect of bezafibrate administration in four different transmitochondrial cybrid cell lines, each of them carrying a homoplasmic mutation in mtDNA encoded tRNAs (Table 1): The first cell line harboured the A2343G tRNA$^{Leu(UUR)}$ mtDNA mutation, which causes MELAS [18]. The second cell line carried the A8344G tRNA^{Lys} mutation, which is associated with MERRF [19]. Additionally, we studied cell lines with two additional mutations: G5703A tRNA^{Asn} (mutant: W72, wild-type: W20) [20] and C3256T tRNA$^{Leu(UUR)}$ (mutant: SUB62, wild-type: SUB52) [21]. These cell lines share the common feature of a respiratory defect due to a generalized OXPHOS defect with more than one OXPHOS complex deficiency.

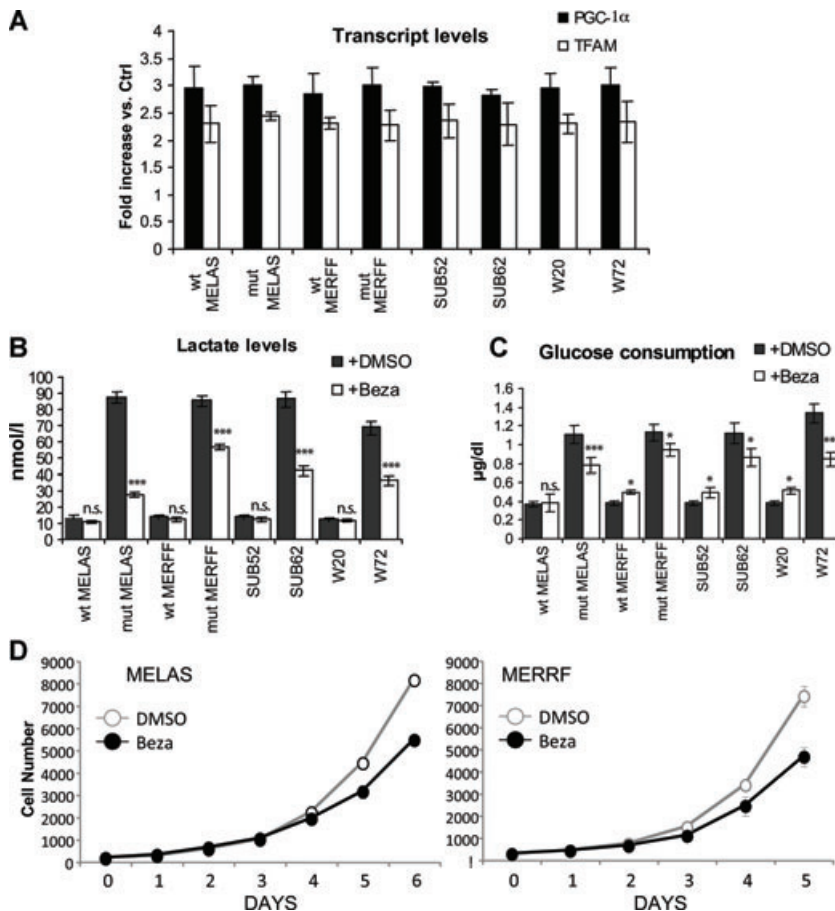### Bezafibrate induces expression of PGC-1α and TFAM in cybrids carrying tRNA mutations

Cybrid cell lines carrying mtDNA mutations and control cell lines harbouring only wild-type mtDNA were cultured in presence of 400 μM bezafibrate or DMSO (vehicle) for 4 days. PPAR agonists can be toxic to some cell lines in high concentration and can induce mitochondrial dysfunction. Bezafibrate appears to be one of the fibrates with no deleterious effects on OXPHOS function [22] or cell viability at concentrations up to 500 μM (not shown). The concentration and time period used have been shown to induce mitochondrial biogenesis in patient cell lines carrying mutations in the nuclear genome. In neither bezafibrate nor DMSO-grown cells, major differences in cell morphology or survival were observed in glucose-containing media (data not shown). We performed RT-PCR experiments to assess the levels of PGC-1α and TFAM, both key regulators of mitochondrial biogenesis. Bezafibrate-supplementation increased the expression of both PGC-1α (~3-fold *versus* DMSO treated) and TFAM (~2–2.5-fold *versus* DMSO treated) in both wild-type and mutant cell lines (Fig. 1A).

Bezafibrate supplementation decreases lactic acidosis and glucose consumption

We next studied changes in accumulation of lactic acid in the growth media of DMSO- or bezafibrate-supplemented cell. Lactate is a byproduct of glycolysis and is formed in the cytosol catalysed by enzyme lactate dehydrogenase using pyruvate and NADH as substrates. Lactic acid accumulates when OXPHOS function is impaired and the NADH : NAD ratio increases, shifting the equilibrium of the lactate dehydrogenase reaction towards lactate. In patients, this effect results in lactic acidosis, which, if untreated, can be fatal [23].

We found that mutant cells grown in the presence of the vehicle only, showed dramatically increased lactate concentration in the media after a 24 hr growth period (Fig. 1B). In all of the analysed cell lines, lactate concentration was increased 7–9-fold compared to cells carrying wild-type mtDNA. In contrast, mutant cells grown in bezafibrate-supplemented media showed decreased lactate concentration compared to vehicle-treated mutant cells: Lactic acid concentration in bezafibrate-treated MELAS cells was only ~30 nmol/l, which marks a drastic decrease to the vehicle-treated MELAS cells, where the lactate concentration was ~90 nmol/l. Bezafibrate-grown cells harbouring the MERRF mutation had ~60 nmol/l lactic acid in the media compared to a ~90 nmol/l in the vehicle-treated MERRF cells. The lactate concentration in the SUB62 cell line decreased from ~90 nmol/l to ~40 nmol/l upon bezafibrate supplementation. Likewise, the lactate concentration in the growth media from W72 cells dropped from ~70 nmol/l to ~35 nmol/l when grown in the presence of bezafibrate. These results indicate that bezafibrate treatment can attenuate lactic acid production associated with mitochondrial tRNA mutations in cybrid lines.

We next asked whether bezafibrate causes a decrease in glucose consumption. ATP production by glycolysis is more 'expensive' than by OXPHOS. Hence, if glycolysis is the major ATP supply, more glucose is consumed compared to ATP supply by OXPHOS. We found that all analysed cell lines carrying mutations in mitochondrial tRNAs had ~3–4-fold increased glucose consumption compared to control cells after a 24 hr growth period. Bezafibrate supplementation significantly decreased the glucose consumption in the mutant cell lines with W72 mutant cells having the greatest benefit (Fig. 1C). In MELAS mutant cells, glucose consumption dropped by ~25%. MERRF mutant cells showed only a small, but still significant decrease in glucose consumption when grown in bezafibrate. SUB62 cell, when supplemented with bezafibrate, had ~20% lower glucose consumption. W72 cell grown in the presence of bezafibrate consumed ~40% less glucose. These results indicate that bezafibrate treatment resulted in reduced glucose consumption, likely mediated by a metabolic shift from glycolysis to OXPHOS, which results in a lower glucose consumption and decreased lactate levels. Cells grown in bezafibrate grew slightly slower than untreated cells in high glucose media, probably due to the metabolic shift mentioned above (Fig. 1D). MERRF and MELAS cells were not able to grow in galactose medium (which depends on robust OXPHOS function) and

Fig. 1 Bezafibrate induces a metabolic shift in cultured cells. (**A**) Relative expression of PGC-1α and TFAM in mutant and control cell lines grown in bezafibrate-supplemented media. Cells grown in media with vehicle (DMSO) were used as a reference ($n = 3$ and bars represent S.D.). $P < 0.001$ for all samples. (**B**) Lactate concentration of bezafibrate and DMSO-treated mutant and control cell lines in media after 24 hrs growth ($n = 3$ and bars represent S.D.). (**C**) Glucose consumption of mutant and control cell lines after 24 hrs growth ($n = 3$ and bars represent S.D.). (**D**) Growth curves of MELAS and MERRF mutant cells treated with bezafibrate and cultured in glucose medium. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

treatment with bezafibrate was not robust enough to reverse this phenotype (not shown).

Bezafibrate stimulates oligomycin-sensitive ATP synthesis and overall ATP yield

The decreased glucose consumption in mutant cells upon bezafibrate administration indicated that OXPHOS function and hence OXPHOS-mediated ATP supply was enhanced. To verify this hypothesis, we measured the oligomycin-sensitive ATP synthesis in DMSO- and bezafibrate-treated mutant and control cells. As expected, DMSO-treated mutant cells had a drastically decreased ATP synthesis rate both with complex I and complex II substrates and showed only 10–20% of the ATP synthesis rate of the control cells (Fig. 2A and B). However, when grown in bezafibrate, all analysed mutant cell lines showed ~2-fold increased oligomycin-sensitive ATP synthesis with either complex I or complex II substrates indicating stimulation of aerobic ATP synthesis. This finding is in line with the decreased glucose consumption and lactate levels in bezafibrate-treated mutant cell lines. We also observed

that oligomycin-sensitive ATP synthesis was boosted in the control cells (Fig. 2A and B). Bezafibrate treatment also resulted in increased ATP-levels in both wild-type and mutant cell lines (Fig. 2C) in agreement with the enhanced ATP generating capacity.

Bezafibrate increases overall OXPHOS enzyme activities

We next addressed the question, how OXPHOS-mediated ATP synthesis is increased in the mutant cells by bezafibrate supplementation. Therefore, we measured the activities of OXPHOS enzymes. We found that mutant cells lines grown in DMSO had severely reduced COX and combined CI + III activities as reported previously (Fig. 3A and B). When grown in bezafibrate, both activities were significantly increased in the mutant cells: MELAS mutant and SUB62 cells regained ~25% of the wild-type COX and ~40% of the CI+III activity upon bezafibrate supplementation. MERRF mutant and W72 cells recovered ~50% of COX and CI+III activity, when grown in bezafibrate. COX and CI+III activity in the control cell lines were also increased upon bezafibrate supplementation.

Fig. 2 Bezafibrate-treated cells have enhanced aerobic ATP synthesis and increased ATP levels. Rate of oligomycin-sensitive ATP-synthesis *via* complex I (**A**) and complex II (**B**) in digitonin-extracts from mutant and control cells grown in presence of bezafibrate and DMSO ($n = 3$ and bars represent S.D.). Activities of control cells (nmol/min/mg): CI: wt (MELAS): 33.56, wt (MERRF): 31.5, SUB52: 30.5, W20: 36.9; CII: wt (MELAS): 23.8, wt (MERRF): 22.0, SUB52: 21.0, W20: 25.8. (**C**) ATP levels in cells treated with DMSO or bezafibrate. Values were normalized by total protein content and by ATP content of DMSO-treated wild-type cells ($n = 3$ and bars represent S.D.). ATP levels of controls cells (nmol/mg protein): wt (MELAS): 21.2, wt (MERRF): 19.7, SUB52: 18.9, W20: 20.3. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
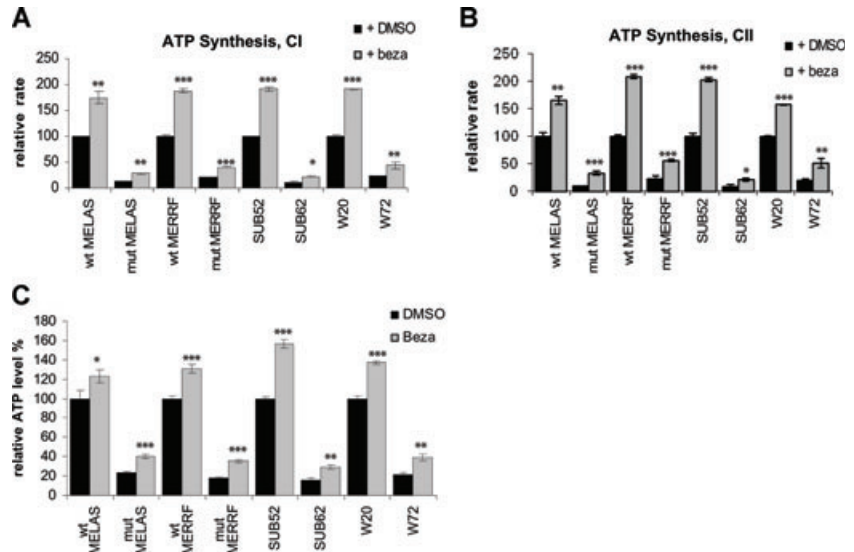
Fig. 3 Bezafibrate increases overall OXPHOS enzyme activity and steady state levels by increasing mitochondrial mass. (**A**) and (**B**) COX and combined CI + III activity in bezafibrate and DMSO-treated mutant and control cell lines ($n = 3$ and bars represent S.D.). (**C**) CS activity in bezafibrate and DMSO-treated mutant and control cell lines ($n = 3$ and bars represent S.D.). (**D**)–(**E**) Western blot analysis of bezafibrate and DMSO-treated mutant and control lines. Antibodies against ND39 (complex I), SDH (flavoprotein of complex II), COXI and COXIV (complex IV), ATPase subunit β (complex V) and VDAC1 (outer membrane protein) were used. An antibody against tubulin was used as loading control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
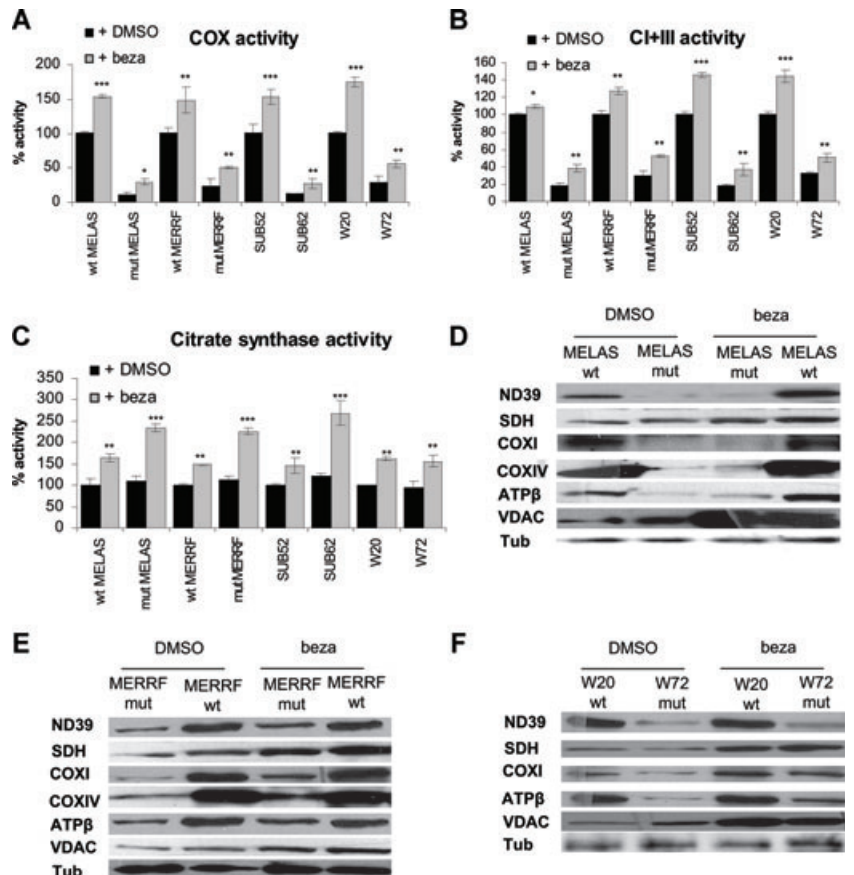
We next addressed whether the bezafibrate-induced increase of previously defective OXPHOS capacity is mediated by increased mitochondrial proliferation. Therefore, we measured the activity of the mitochondrial matrix protein citrate synthase (CS), a marker for mitochondrial mass. As expected based on the up-regulation of PGC-1α and TFAM expression (Fig. 1A), we observed increases of 1.5–2.5-fold in CS activity in both control and mutant cells lines when grown in bezafibrate (Fig. 3C). Interestingly, the mutant cell lines showed a bigger increase than the wild-type cell lines, which could be related to the metabolic shift in the mutant cell lines. The increased CS activity suggests that bezafibrate increased mitochondrial mass and hence OXPHOS capacity in the mutant cell lines resulting in enhanced ATP synthesis and decreased glucose consumption.

We next analysed whether the bezafibrate treatment altered the steady-state levels of OXPHOS proteins. The analysed tRNA mutations cause a generalized OXHOS defect with multiple OXPHOS enzyme deficiencies resulting in largely decreased enzyme activities (Fig. 3A and B) and decreased steady-state levels of OXPHOS enzymes (Fig. 3D–F). When grown in bezafibrate, the endogenous levels of some OXPHOS subunits were increased in homogenates from MELAS (Fig. 3D), MERRF (Fig. 3E) and W72 cybrids (Fig. 3F). Surprisingly, not all peptides were increased or increased proportionally, suggesting that the functional improvement may involve not only an increase in mitochondrial biogenesis, but also other pathways. We did observe an increase in the flavoprotein of succinate dehydogenase (SDHA) and the VDAC1 in bezafibrate grown mutant cells (Fig. 3D–F). These two protein complexes are exclusively nuclear encoded and suggest an increase in mitochondrial mass. Interestingly, some mutant cells lines showed already increased SDHA and VDAC1 levels in the control treatment. This finding suggests that mitochondrial proliferation might occur even without bezafibrate treatment, but can be further boosted by treatment with bezafibrate. We also observed increased OXPHOS enzyme steady-state levels in the control cells upon bezafibrate treatment, which is in agreement with the increased ATP synthesis and OXPHOS enzyme activity observed in control cells grown in bezafibrate-supplemented media.

Steady-state levels of affected tRNAs are increased in bezafibrate-supplemented cybrids

The analysed mtDNA mutations are associated with decreased steady-state levels of the tRNA, which harbours the mutation. We also observed ~70–80% decreased levels of affected tRNAS in the MELAS (tRNA$^{Leu(UUR)}$), MERRF (tRNA^{Lys}), W72 (tRNA^{Asn}) and SUB62 (tRNA$^{Leu(UUR)}$) mutant cells (Fig. 4A, B). Mutant cells grown in bezafibrate had significantly increased levels of the affected tRNAs, up to 50% of the level of DMSO-treated control cells (Fig. 4A). We also observed that the level of the mitochondrial tRNA^{Val}, which is not affected in either of the analysed cell lines, is increased in both wild-type and mutant cell lines upon treatment with bezafibrate (not shown). Bezafibrate increased expression of TFAM (Fig. 1A), which is involved in mtDNA transcription of mito-

chondrial mRNAs, rRNAs and tRNAs. Our results imply that bezafibrate increases the synthesis of mitochondrial tRNA species, leading to an increase their steady-state levels.

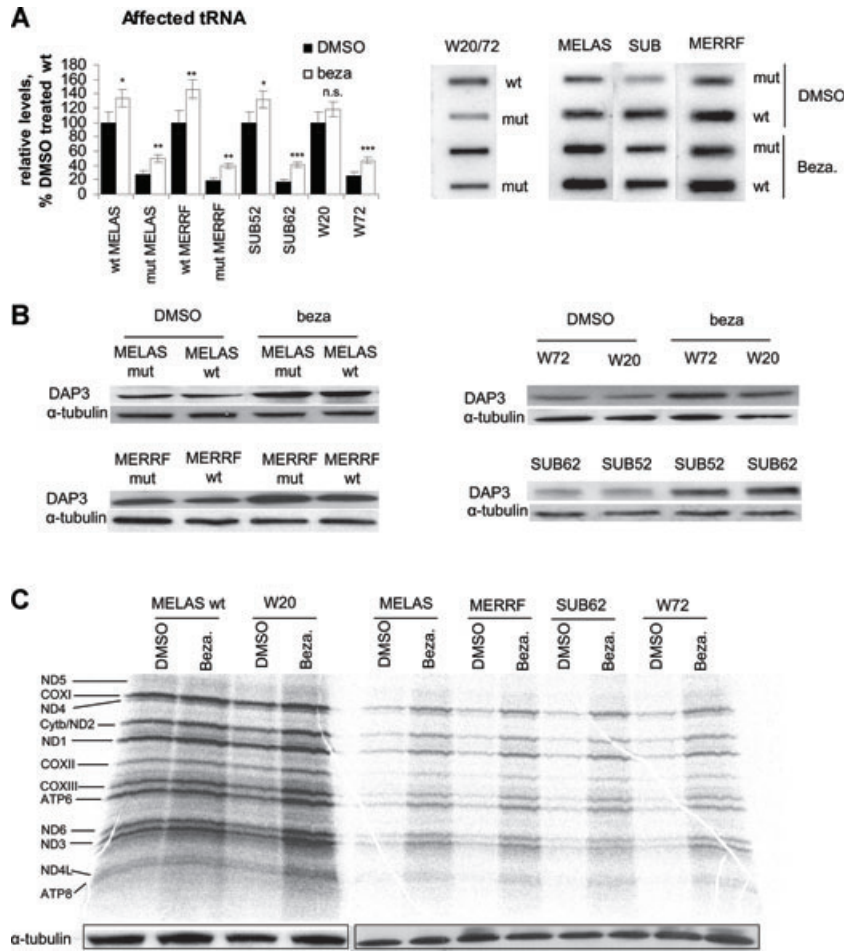## Bezafibrate improves mitochondrial protein synthesis

The decreased mitochondrial tRNA levels in the analysed mutant cell lines impair mitochondrial protein synthesis, resulting in the observed OXPHOS defects. We therefore tested if the bezafibrate-mediated increase in mitochondrial tRNA levels affects mitochondrial protein synthesis. We saw that bezafibrate-treated cells, wild-type and mutant alike, had increased levels of the mitoribosome marker Death-Associate-Protein 3 (DAP3), a protein component of the small ribosomal subunit (Fig. 4B). More importantly, we observed that upon bezafibrate treatment, mitochondrial protein synthesis is greatly enhanced in the cell lines carrying mutations in mitochondrial tRNAs as analysed by ^{35}S-methionine labelling of mitochondrial proteins (Fig. 4C).

## Suppressors of tRNA mutation G5703A have increased PGC-1α levels

Our group reported several years ago a suppression of mitochondrial tRNA mutation associated with changes in the nuclear background [24]. These suppressor cell lines, named WG9 and WL4, were obtained as galactose-resistant clones of the W72 clone. These cell lines had restored OXPHOS function and increased tRNA^{Asn} levels compared to the parental mutant clone. Although the origin of the suppressing factor was found to be nuclear, the factor could not be identified. Potentially, the nuclear factor that suppresses the tRNA mutation in WG9 and WL4 clones, might be associated with the PGC-1α/PPAR pathway. Therefore we performed RT-PCR experiments to determine changes in the PGC-1α and TFAM expression level in WG9 and WL4. We found that both gene transcripts were significantly increased in both WG9 and WL4 compared to the parental W72 clone (Fig. 5). Interestingly, the level of up-regulation in the WG9 clone was comparable to W72 cells treated with bezafibrate. In the WL4 cells, we observed an even further increased expression of PGC-1α and TFAM. However, additional adaptations must have helped optimize OXPHOS function in WG9 and WL4, as the mutant W72 treated with bezafibrate did not show the same robust growth in galactose medium (not shown).

Discussion

Diagnosis of OXPHOS disorders has advanced in the last decade. However, therapeutic options are limited or non-existent for mitochondrial disease. Work from our lab and others' has shown that activation of the PGC-1α/PPAR pathway increases mitochondrial

Fig. 4 Bezafibrate increases affected tRNA levels and enhances mitochondrial protein synthesis. (**A**) Levels of affected tRNA in bezafibrate and DMSO-treated mutant and control cell lines ($n = 3$ and bars represent S.D.). The right panel shows the slot blots used to quantitate the respective affected tRNA levels. The graph on the left shows the relative levels of these tRNAs. (**B**) Western blot analysis of bezafibrate and DMSO-treated mutant and control lines. An antibody against the small mitoribosomal subunit DAP3 was used. (**C**) *In vivo* labelling of mitochondrial translation products in bezafibrate and DMSO-treated mutant cell lines. W20 and MELAS cell lines were used as controls. Western blot against tubulin was used as a loading control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
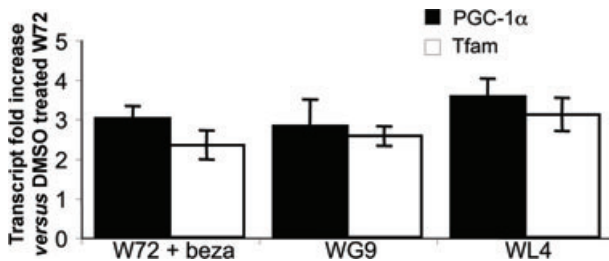
Fig. 5 PGC-1α expression is increased in suppressor cell lines of mtDNA G5703A mutation. Relative expression of PGC-1α and TFAM in suppressor lines WG4 and WL4 compared to parental W72 treated with bezafibrate. W72 cells grown in media with vehicle (DMSO) were used as a reference ($n = 3$ and bars represent S.D.). $P < 0.001$ for all samples.

mass and thereby increases overall OXPHOS capacity preventing a bioenergetic crisis both *in vivo* and *in vitro* [4, 5, 7]. Importantly, these results showed that bezafibrate, a PPAR panagonist, can induce mitochondrial proliferation and thereby compensate for

single OXPHOS defects caused by genetic alteration in the nuclear DNA [4, 7]. Here we show that activation of the PGC-1α/PPAR pathway is also beneficial for a generalized OXPHOS defect associated with mutation in mitochondrial tRNAs.

Fibrates activate PPARs, which alter the transcription rate of target genes that play key roles in the development of atherosclerosis, dyslipidemia, cardiovascular disease and metabolic syndrome [25]. Additionally, PPARs target genes coding for mitochondrial proteins [4, 26]. Thus PPARs are central in regulation of energy homeostasis. Recent clinical trials with bezafibrate showed that it reduced the risk of cardiac infarction by 17% (8 year follow-up). It also reduced the risk of myocardial infarction in patients with severe metabolic syndrome by 56% (8 year follow-up) and the development of type 2 diabetes by almost half (2 and 5 year follow-up) [26]. Given the board spectrum of target genes it is likely that these effects are not related solely to triglycerides and HDL levels, but rather a more global metabolic re-programming. Metabolic remodelling in response to fasting or exercise in liver and muscle is known to involve PGC-1α and PPAR [27]. Metabolic reprogramming from a carbohydrate to an oxidative carbon flux is

associated with positively influencing lifespan and preventing pathology in the aging heart [27, 28].

We found that treatment of cell lines harbouring homoplasmic pathological mutations in mitochondrial tRNAs with bezafibrate induced a metabolic shift towards aerobic ATP synthesis. Bezafibrate supplementation improved OXPHOS capacity as indicated by increased ATP synthesis and OXPHOS enzyme activity and the steady state level of some OXPHOS subunits. Our results imply that bezafibrate increases the steady-state levels of mitochondrial tRNAs, which results in increased mitochondrial protein synthesis and thereby enhanced OXPHOS function. However, the increase in the levels of OXPHOS proteins was not always detected. We have observed before that individual OXPHOS enzymes or subunits are increased in a non-stochiometrical manner [5]. Therefore, we believe the suppression mechanism likely involves additional PPAR-linked metabolic pathways.

A few suppressors of mitochondrial tRNA mutations are known [24, 29]. Our group found that the tRNA mutation in the W72 cell lines was suppressed in galactose-resistant clones, but could not identify the mechanisms of suppression. Interestingly, we found that the level of PGC-1α and TFAM expression in the WG9 clone was comparable to W72 cells treated with bezafibrate. Although there might be more nuclear modification that affects OXPHOS function in WG9 and WL4, our results suggest that the up-regulation of PGC-1α and TFAM and hence mitochondrial biogenesis are likely to be involved in the suppression mechanism.

In conclusion we showed that bezafibrate administration ameliorates OXPHOS defects caused by multiple enzyme deficiencies due to homoplasmic mitochondrial tRNA mutations by metabolic remodelling. Clearly, the levels of ATP did not reach wild-type ones after bezafibrate treatment, and accordingly, could not support galactose growth. However, mtDNA mutations are usually heteroplasmic in patients' tissues. It is possible that bezafibrate-mediated mitochondrial proliferation could be more beneficial in this scenario, leading to more functional OXPHOS units and further increased overall ATP generating capacity.

The fact that the bezafibrate-mediated protection can be achieved with cell lines carrying different mtDNA mutations and nuclear DNA mutations suggests that this approach is beneficial for OXPHOS defects caused by either genome. Bezafibrate administration to cell lines from patients with mitochondrial disease caused by nuclear gene mutations or mutations of unknown origin led to improved respiration [4]. Our work demonstrated that this beneficial effect of increased mitochondrial proliferation, either by bezafibrate treatment or increased PGC-1α expression, on mitochondrial ATP synthesis in the context of a mitochondrial dysfunction can also be observed in patient cell lines and *in vivo* [5, 7]. All these findings in cell lines and murine models with different mitochondrial defects imply that increasing mitochondrial mass and thereby boosting overall residual OXPHOS capacity can benefit different classes of mitochondrial defects. Induced mitochondrial proliferation might hence be a novel therapeutic strategy for mitochondrial disorders in general.

Acknowledgements

Conflict of interest

The authors declare no conflict of interests.

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