Introduction

Many recent studies have linked mitochondrial dysfunction with aging and aging-associated diseases [1]. The mitochondrial DNA (mtDNA) mutator mouse is a mouse model of premature aging that supports this connection. This mouse has a mutant mtDNA that harbors a proofreading-deficient mtDNA polymerase γ. These mice develop many features of premature aging including hair loss, anemia, osteoporosis, sarcopenia and decreased lifespan. They also have increased mtDNA mutations and marked mitochondrial dysfunction. We found that mutant mice treated with bezafibrate for 8-months had delayed hair loss and improved skin and spleen aging-like phenotypes. Although we observed an increase in markers of fatty acid oxidation in these tissues, we did not detect a generalized increase in mitochondrial markers. On the other hand, there were no improvements in muscle function or lifespan of the mutator mouse, which we attributed to the rodent-specific hepatomegaly associated with fibrate treatment. These results showed that despite its secondary effects in rodent’s liver, bezafibrate was able to improve some of the aging phenotypes in the mutator mouse. Because the associated hepatomegaly is not observed in primates, long-term bezafibrate treatment in humans could have beneficial effects on tissues undergoing chronic bioenergetic-related degeneration.

Abstract

Pharmacological agents, such as bezafibrate, that activate peroxisome proliferator-activated receptors (PPARs) and PPAR γ coactivator-1α (PGC-1α) pathways have been shown to improve mitochondrial function and energy metabolism. The mitochondrial DNA (mtDNA) mutator mouse is a mouse model of aging that harbors a proofreading-deficient mtDNA polymerase γ. These mice develop many features of premature aging including hair loss, anemia, osteoporosis, sarcopenia and decreased lifespan. They also have increased mtDNA mutations and marked mitochondrial dysfunction. We found that mutant mice treated with bezafibrate for 8-months had delayed hair loss and improved skin and spleen aging-like phenotypes. Although we observed an increase in markers of fatty acid oxidation in these tissues, we did not detect a generalized increase in mitochondrial markers. On the other hand, there were no improvements in muscle function or lifespan of the mutator mouse, which we attributed to the rodent-specific hepatomegaly associated with fibrate treatment. These results showed that despite its secondary effects in rodent’s liver, bezafibrate was able to improve some of the aging phenotypes in the mutator mouse. Because the associated hepatomegaly is not observed in primates, long-term bezafibrate treatment in humans could have beneficial effects on tissues undergoing chronic bioenergetic-related degeneration.
Results

Systemic Effects of Bezafibrate in the Mut Mouse

To improve mitochondrial function in all tissues, 2 month-old male Mut mice were placed on a standard mouse diet containing 0.5% bezafibrate (BD). This mouse group is heretofore referred to as MutBD. To ensure that the changes observed in MutBD mice were due to bezafibrate, we also placed 2 month-old WT mice on BD (heretofore referred to as WTBD). Mice on the BD were compared to same aged male Mut and WT mice that were fed a standard mouse diet (standard diet, SD). These mice will be referred to as MutSD and WTSD respectively. The animals were fed their respective diets for 8 months and then analyzed at 10 month-old to uncover the effects of long-term bezafibrate administration on physical phenotype, organ structure/function and mitochondrial function in a model of mitochondrial aging.

The Mut mouse was previously shown to have decreased body weight compared with WT mice [2,3]. Therefore, we monitored body weight changes in both mice on BD and SD monthly beginning at 2 months of age. Similar to previous reports, we found that beginning at 3 month-old MutSD mice had a lower body weight compared with WT mice (Fig. 1A). We observed that mice in all groups had a similar body weight at 2 month-old but only WTSD mice experienced a steady increase in body weight between 4 and 11 month-old (Fig. 1A). We also found that MutBD and WTBD mice weighed less than MutSD and WTSD mice respectively beginning at 3 months of age (Fig. 1A). This difference in body weight was not due to a decrease in the food intake of MutBD mice (not shown). These results indicate that bezafibrate reduced the body weight of both Mut and WT mice.

In addition, we monitored changes in bone mineral density and content (BMD and BMC respectively), body area and body fat and lean mass of Mut and WT mice on BD and SD by DEXA scanning. Mut were previously shown to have decreased BMD, BMC and body fat at 10 month-old [2,3]. We found that 10 month-old MutBD and WTBD mice had decreased body BMD, BMC and area compared with MutSD and WTSD mice (Fig. 1B-1D). The less dense bones may be related to lower locomotor activity or muscle function. Surprisingly, bezafibrate had no effect on fat levels in these mice (Fig. 1D).

Because the Mut has decreased lifespan compared to WT mice [2,3], we compared the lifespan of bezafibrate treated and SD mice. Our survival studies showed that bezafibrate did not increase the percent survival of MutSD mice (Fig. 1E).

Bezafibrate Delayed Hair Loss and Restored the Skin Structure of Mut Mice

As part of their premature aging phenotype, Mut mice develop grey hairs and start showing signs of hair loss at about 6 to 7 months of age [2,3]. Therefore, we compared the coat phenotype of MutBD with MutSD mice. We observed that at ~ 7 months of age, MutSD mice started showing signs of hair loss on their back and under their neck while age matched MutBD mice that had been on the BD for 3 months did not (Fig. 2A). The hair loss in MutSD mice advanced with age, whereas MutBD mice only began showing signs of hair loss on their back at 10 month-old (Fig. 2A). In addition, we observed that 10 month-old MutBD mice had fewer gray hairs than age matched MutSD mice (not shown). These findings indicate that bezafibrate is able to delay hair loss and graying of the hair, two phenotypes of the Mut mice.

To further characterize the effects of bezafibrate on hair loss and graying, dorsal and ventral skin were collected from 10 month-old WTSD, MutBD and MutSD mice. Paraflin embedded skin sections were stained with hematoxylin and cosin (H&E) to determine structural changes. We observed that dorsal skin sections from MutSD mice were thinner and lacking the distinct

Table 1. The effect of bezafibrate treatment in mouse models of disease.

| Mouse model | Bezafibrate dose | Treatment time | Results | Reference |
|-------------|-----------------|----------------|---------|-----------|
| MLC1F-Cox10/− | Standard mouse diet with 0.5% bezafibrate | ~2 or 5 months | ↑ mitochondrial protein and mtDNA; ↑ COX, SDH and CS activity; ↑ % ATP in tissue; ↑ PGC-1α and β; ↑ PPAR α, β/δ and γ; Improved muscle phenotype and survival | [8] (Wenz et al 2008) |
| Surf1−/− | Standard mouse diet with 0.5% bezafibrate | 1 month | ↑ PPAR α and β/δ; ↑ FAO related genes (CD36, ACOX and SCAD); No change in PGC-1α, mtDNA levels, CS or MRC activities; - Weight loss; - Hepatomegaly | [11] (Viscomi et al 2011) |
| Deleter | D12450B (rodent diet with 10% fat) with 0.5% bezafibrate | 5.5 months | - No change in PGC-1α and PPARs; - ↓ mtDNA copy number; - Muscle: ↓ COX negative fibers; ↓ mtDNA deletion load; ↓ OXPHOS genes/proteins; - Liver: hepatomegaly; ↑ FAO related genes, ↑ UCP2, ↑ FGF21; ↑ adipocyte area; Weight loss | [10] (Yatsuga et al 2011) |
| R6/2 | Standard mouse diet with 0.5% bezafibrate | ~2 months | ↑ PGC-1α, PPARs and OXPHOS related genes in brain, muscle, BAT; Brain: ↑ mitochondrial density; ↓ neurodegeneration; ↓ astrogliosis; ↓ oxidative stress; Muscle: ↑ type I fibers and ↓ type IIB; restores mitochondrial structure and organization; ↓ vacuolization of BAT; Improved mouse phenotype and survival | [9] (Johri et al 2012) |

doi:10.1371/journal.pone.0044335.t001

Bezafibrate Improves Phenotypes in Mutator Mice
layers that were present in WTSD mice, while MutBD mice had a skin structure more similar to WTSD mice (Fig. 2B, H&E). Specifically, the dorsal skin sections from MutSD mice were primarily composed of the dermis/connective tissue and had a non-continuous epidermis layer (Fig. 2B, H&E). In addition, the skin sections from MutSD mice had smaller hair follicles compared with WTSD and MutBD mice (not shown). No distinct structural differences were observed in ventral skin sections from these latter mice, which suggest that dorsal skin may be more susceptible to mitochondrial DNA damage. (not shown).

Because collagen and elastin fibers are the primary functional components of the dermis/connective tissue layer of the skin [12], we wanted to determine if bezafibrate had an effect on the expression of these components. To detect elastin fibers and collagen, dorsal skin sections were stained with the Verhoeff's Van Geison (EVG) and Masson's trichrome stain respectively. We found that elastin fibers (black/dark brown stain) were present in the connective tissue of both MutSD and MutBD mice at levels similar to WTSD mice (Fig. 2B, EVG). However, compared with WTSD mice, MutSD mice had essentially no collagen (blue stain) in the connective tissue of the skin (Fig. 2B, Masson's trichrome). Surprisingly, the collagen levels in the skin were restored in MutBD mice (Fig. 2B, Masson's trichrome).

The transforming growth factor beta 1 (TGF-β1)/Smad3 signaling pathway was shown to regulate a number of collagen gene promoters in human dermal fibroblasts [13]. Also, it was shown that activated PPARδ can regulate collagen levels in skin via the TGF-β1/Smad3 pathway [14]. Since bezafibrate is...
a PPARδ agonist, we wanted to determine whether this pathway was activated in the skin of 10 month-old MutBD mice. We performed western blot for Smad3 in total homogenate from the skin of 10 month-old mice. Our results showed an accumulation of total Smad3 protein in the skin of WTBD and MutBD mice compared with WTSD and MutSD mice (Fig. 2C). The accumulation of total Smad3 protein was previously shown to be associated with increased Smad2/3 signaling in human dermal fibroblasts [15]. Therefore, our results suggest that bezafibrate activates the TGF-β1/Smad3 pathway thereby causing an increase in collagen in the skin of MutBD mice.

Bezafibrate Decreased Spleen Size and Restored the Spleen Structure of Mut Mice

Mut mice were previously shown to have enlargement of some organs, one of which was the spleen [2]. We found that bezafibrate reduced the spleen weight and size of 10 month-old MutBD mice compared with WTSD and MutSD mice (Fig. 2C). The spleen size of MutBD mice was restored to that of WTSD mice (Fig. 3A). To further investigate this effect of bezafibrate on the spleen of Mut and WT mice, we performed H&E staining of paraffin embedded spleen sections. We found that bezafibrate improved the spleen structure of MutBD mice (Fig. 3D). Our results showed that MutSD mice had an aberrant spleen structure characterized by marked atrophy and decreased white pulp (purple areas in Fig. 3D) and an increase in red pulp of the spleen (Fig. 3D). As the white pulp of the spleen synthesizes antibodies, the atrophy of this area in MutSD mice can impair their immune response [16]. Interestingly, the spleen of MutBD mice had a white and red pulp distribution and organization that were similar to WTSD mice (Fig. 3D). These findings indicate that bezafibrate protected the spleen in the Mut mouse.

Because MutSD mice had pronounced atrophy in the spleen, we performed immunohistochemistry for cleaved caspase-3 in paraffin embedded spleen sections from 10-month-old mice. After quantifying the number of positive cells, we found that compared with WT mice, MutSD mice had increased cleaved caspase-3 (Fig. 3C). However, MutBD mice had reduced number of cleaved caspase-3 positive cells (Fig. 3C), indicating that bezafibrate could...
reduce apoptosis in the spleen of Mut mice. This attenuation of apoptosis could explain the decreased atrophy and restoration of white pulp observed in the spleen of MutBD mice (Fig. 3D).

It was reported that Mut mice develop anemia, decreased red blood cells (RBC), which is a clinical feature in aging humans [2,3]. The spleen is known to recycle iron from damaged RBCs and participate in extra-medullary hematopoiesis especially during anemia [16]. Because our results showed that bezafibrate improved the spleen structure of Mut mice, we wanted to determine if bezafibrate had an effect on RBC count. We performed a complete blood cell panel (CBC) in 10 month-old mice and found that while bezafibrate had no effect on white blood cell (WBC) (not shown), MutBD and WTBD mice had a moderate but significant increase in RBC count compared with MutSD and WTSD mice (Fig. 3E). However, the RBC count in MutBD mice was not restored to that of WTSD mice. These results suggest that bezafibrate increased RBC in both WT and Mut mice and we speculate that this improvement may be associated with other beneficial effects of bezafibrate in the spleen.

To further investigate the effects of bezafibrate in the spleen, we performed quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR) to determine the expression level of PPARγ and PGC-1α. Both genes are known targets of bezafibrate, and PPARγ has been shown to be involved in regulating anti-inflammatory responses [17], which can influence spleen size. We found that PPARγ mRNA levels were elevated in the spleen of MutSD compared with WTSD mice and its levels were restored in MutBD mice (Fig. 3F). PPARγ levels were also reduced in WTBD mice indicating that this effect was due to bezafibrate (Fig. 3F). These findings suggest that bezafibrate may reduce inflammation and thus also the inflammatory response regulated by PPARγ in the spleen. We also found that PGC-1α levels were reduced in the spleen of MutSD mice compared with WTSD mice and there was a trend towards an increase in PGC-1α levels in MutBD mice (Fig. 3E). However, the RBC count in MutBD mice was restored to that of WTSD mice. To determine if this mild increase in PGC-1α levels caused an increase in mitochondrial content in the spleen of MutBD mice, we performed western blot to assess mitochondrial protein levels in total spleen homogenate. We did not observe an increase in mitochondrial protein levels in bezafibrate treated...
mice. (Fig. 3G). However, it is possible that some cell types in the spleen may have increased mitochondrial protein levels in response to bezafibrate that was undetectable in total spleen homogenate. These results indicate that beneficial effects of the bezafibrate in the spleen are not due to a robust increase in mitochondrial content but perhaps due to reduced apoptosis and anti-inflammatory responses regulated by PPARγ.

Bezafibrate Induced Fatty Acid Oxidation but did not Increase Global Mitochondrial Content in the Skeletal Muscle of Mut Mice

As part of their premature aging phenotype, Mut mice develop sarcopenia and mitochondrial dysfunction in the skeletal muscle [18]. To determine if bezafibrate was able to induce mitochondrial content/function in the skeletal muscle of Mut mice, we performed western blot to detect the levels of PGC-1α and mitochondrial proteins in total quadriceps homogenate from 10 month-old mice. We found that MutSD mice had reduced PGC-1α protein levels compared with WTSD mice and these levels were also not increased in MutBD mice (Fig. 4B). Also, bezafibrate did not increase mitochondrial protein levels or citrate synthase activity (CS) in the quadriceps of MutBD and WTBD mice (Fig. 4C and 4D respectively). We also did not detect an increase in cytochrome c oxidase (COX) activity in total quadriceps homogenate of MutBD when compared to MutSD mice (not shown). To further characterize the effects of bezafibrate on mitochondria in the skeletal muscle, we quantified mtDNA levels in the quadriceps by qPCR. Paradoxically, we found that MutBD and WTBD mice had decreased mtDNA levels compared with MutSD and WTSD (Fig. 4E). These results indicate this bezafibrate regimen did not induce mitochondrial content in the skeletal muscle of Mut and WT mice.

To determine the effect of bezafibrate on the expression of PPARs in the skeletal muscle, we performed qRT-PCR and found that bezafibrate had no effect on the mRNA levels of PPARs in the skeletal muscle of Mut mice (Fig. 4B). However, bezafibrate did not increase mitochondrial protein. We found that bezafibrate did not increase mitochondrial content/function in the quadriceps of MutBD mice (Fig. 4G). Neverthless, we went on to compare the motor function/skeletal muscle function of MutBD and WTBD mice to that MutSD and WTSD mice by assessing their performance on a treadmill test. We began testing mice at 3 months of age and monitored their performance monthly. Our results showed that between 4–6 months of age both MutBD and WTBD mice fell more than MutSD and WTSD mice (Fig. 4H). This trend continued between 7–10 months of age for MutBD (Fig. 4H). These results indicate that bezafibrate did not prevent the development of sarcopenia or improved the skeletal muscle function of Mut mice.

Bezafibrate Induced Hepatomegaly and Fatty Acid Oxidation in the Liver of Mut and WT Mice

Bezafibrate was previously shown to have hepatoproliferative effects on rodent liver [10,19]. Therefore, we monitored the liver phenotype of our mice. We found that bezafibrate doubled the liver weight of 10 month-old MutBD and WTBD mice compared to MutSD and WTSD mice (Fig. 5A). To determine whether there was a liver pathology, the liver of MutBD and WTBD and standard controls were subjected to histological analysis. H&E staining of the liver showed that MutBD and WTBD mice seemed to have enlarged hepatocytes around central veins of the hepatic lobules, consistent with the observed hepatomegaly (Fig. 5B). Furthermore, we performed a hepatic panel and found that 10 month-old MutBD and WTBD had significantly increased levels of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in their blood compared to MutSD and WTSD mice, a sign of liver damage (Fig. 5C). These results indicate that bezafibrate induced hepatomegaly and liver damage in Mut and WT mice.

To determine the effect of bezafibrate on the RNA levels of PGC-1α and β, as well as PPARs in the liver of Mut and WT mice, we performed qRTPCR. We found that bezafibrate had no effect on the mRNA levels of PPARγ, PPAR and PGC-1β in liver of 10 month-old mice (Fig. 5D). However, bezafibrate reduced the mRNA levels of PPAR in both MutBD and WTBD mice and we found a trend towards increased mRNA for PGC-1α levels in the liver of MutBD mice (Fig. 5D). To determine if this increase in PGC-1α RNA resulted in increased mitochondrial content in the liver, we performed western blot to detect the levels of mitochondrial protein. We found that bezafibrate did not increase the levels of mitochondrial proteins in the liver of MutBD and WTBD mice (not shown). Finally, it was recently shown that bezafibrate induces fatty acid oxidation in the liver of mice [10]. We confirmed this by performing qRT-PCR. Our results showed that bezafibrate increased the mRNA levels of ACOX and CD36 in the liver of both MutBD and WTBD mice (Fig. 5E). However, there was no change in the gene expression of CPT1 and SCAD (Fig. 5E). These results indicate that bezafibrate induced fatty acid oxidation in the liver of Mut and WT mice. However, further experiments are needed to confirm this finding.

Discussion

We hypothesized that bezafibrate would induce increased mitochondrial content in the Mut mouse and the latter would ameliorate some of the premature aging phenotypes observed in these mice. Our results showed a more complex picture, where beneficial effects were observed for some tissues, but not others. Moreover, the beneficial effects were not clearly linked to increased mitochondrial content.

In the skin, the most striking finding was the restoration of collagen synthesis is a feature of skin aging [12], bezafibrate seemed to have protected the skin in Mut mice from premature aging by altering connective tissue metabolism. Both skin collagen synthesis and skin thickness were increased in MutBD mice compared to WTSD and MutSD mice (Fig. 5A). Furthermore, we performed an histological analysis and found that MutBD mice developed less inflamation than MutSD mice (Fig. 5A).

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Figure 4. The effect of bezafibrate on the skeletal muscle of Mut and WT mice. (A) Western blot of PGC-1α and loading control actin in total quadricep homogenate from 10 month-old mice and quantification of PGC-1α band intensity normalized to actin (n = 4/group). (B) Gene expression of PGC-1α and PPARs in the quadricep of 10 month-old mice normalized to actin (n = 4/group). (C) Citrate synthase activity in the total quadricep homogenate from 10 month-old mice (n = 4/group). (D) Quantification of western blot of mitochondrial proteins in the total homogenate from the quadriceps of 10 month-old mice (n = 4/group). (E) Quantification of western blot of mitochondrial proteins in the total homogenate from the quadriceps of 10 month-old mice (n = 4/group). (F) Relative ND1 copy number normalized to GAPDH. (G) Weight (g) of quadriceps and gastrocnemius. (H) Number of falls at each age group.
aging-like phenotypes. Furthermore, because collagen is an important structural component of the dermis skin layer where the hair follicles are found, restoration of collagen levels may have contributed to the preservation of hair follicles and the delay in hair loss we observed in MutBD mice.

Although we were surprised by the beneficial effects of bezafibrate in the skin; several studies have highlighted the importance of PPARs in skin biology [7]. PPARs have been shown to play a role in skin permeability, epidermal cell growth/differentiation, skin inflammatory response and wound healing [7]. In fact, activation of PPARδ was shown to be able to influence collagen expression and promote wound healing in the skin via TGF-β1/Smad3 signaling [14]. We showed that bezafibrate treated mice had an accumulation of total Smad3 protein in the skin, an indicator of increased Smad2/3 signaling [15], suggesting that this pathway may have been activated by bezafibrate to promoting an increase in collagen levels.

Figure 5. Bezafibrate induces hepatomegaly and fatty acid oxidation in Mut and WT mice. (A) Liver weight of 10 month-old mice (n = 4–6/group). (B) H&E staining of the liver of 10 month-old mice showing hepatocytes and central vein (n = 4/group). (C) The level of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood of 10 month-old mice (n = 6/group). (D) Gene expression of PGC-1 coactivators and PPARs in the liver of 10 month-old mice normalized to actin (n = 4/group). (E) The mRNA level of markers of fatty acid oxidation in the liver of 10 month-old mice normalized to actin (n = 4/group). Acyl-coenzymeA oxidase 1 (ACOX), cluster of differentiation 36 (CD36), carnitine palmitoyl transferase (CPT1) and short-chain-acyl-coenzymeA dehydrogenase (SCAD). *, P<0.05; **, P<0.01, ***P<0.001, Student’s t-test. Error bars represent the SEM. doi:10.1371/journal.pone.0044335.g005
We also showed that mice on the BD had reduced spleen weight and restoration of the red and white pulp organization in the spleen. The increased cleaved caspase-3 positive staining detected in the spleen of MutSD mice was not surprising since Mut mice were shown to have increased apoptosis in many tissues [3]. However, we found that MutBD mice had less cleaved caspase-3 positive staining; suggesting that bezafibrate was reducing apoptosis in the spleen. We also found that bezafibrate treated mice had increased red blood cell count which may have resulted from the restoration of spleen structure. In addition, PPARG mRNA levels were lower in the spleen of MutBD mice. Studies have shown that PPARG controls inflammation by inhibiting the expression of inflammatory cytokines [6,7]. Therefore, we speculate that PPARG levels were increased in MutSD mice to promote an anti-inflammatory response and this response was no longer needed in MutBD mice and so PPARG levels were reduced.

Similar to previous findings in deletor [10] and Surf1 KO mice [11], bezafibrate did not induce increased mitochondrial content/function in skeletal muscle of Mut mice. Interestingly, we found that PGC-1α protein levels were reduced in the skeletal muscle of Mut mice and they were not restored by bezafibrate. This raises the question of whether reduction in PGC-1α contributes to the skeletal muscle phenotype of Mut mice. Nevertheless, we found that bezafibrate increased markers of fatty acid oxidation in the skeletal muscle, similar to what was shown in Surf1 KO mice [11]. However, while bezafibrate increased the expression of PPARGs in the skeletal muscle of Surf1 KO mice [11], PPARG expression was either decreased or unaffected in all the tissues we analyzed in bezafibrate treated Mut mice. We cannot eliminate the possibility that bezafibrate increased PPARG expression in Mut mice, but this was not observed when we analyzed 10 month-old mice on BD for 8 months.

Our laboratory previously showed that bezafibrate increased PGC-1α expression and mitochondrial content in MLC1F-Cox10−/− mice thereby delaying the onset of the myopathy [8]. However, we could not demonstrate a general increase in mitochondrial content in the Mut mice, although the expression of some genes coding for mitochondrial proteins associated with β-oxidation were increased. We suspect that this difference in effect of bezafibrate in the muscle of Mut mice compared to MLC1F-Cox10−/− mice may be due to the low levels of PGC-1α in the muscle of the Mut mouse. Another possibility is that this discrepancy may be due to the difference in bezafibrate treatment time. Long-term bezafibrate treatment in mutator mice could have exacerbated the toxic effects of bezafibrate on the liver, which in turn would reduce the potential benefits of bezafibrate on the muscle. We previously showed that transgenic expression of PGC-1α in the skeletal muscle of Mut mice increased mitochondrial content and function and improved mouse treadmill performance [4]. Taken together, these findings showed that overexpression of PGC-1α confers more benefits to the skeletal muscle of Mut mice than bezafibrate administration.

In this study, we confirmed that bezafibrate has a toxic effect on mouse liver; it induced hepatomegaly in both WT and Mut mice. This is a rodent specific effect of bezafibrate that was also observed in the deletor and Surf1 KO mice [10,11]. In addition, bezafibrate induced markers of fatty acid oxidation in the liver of both WT and Mut mice, a pathway known to be regulated by PPARGs [6]. Interestingly, we observed that bezafibrate induced an increase in CD36, a marker of fatty acid oxidation, in the liver but not in the skeletal muscle. Furthermore, we found that bezafibrate treated WT and Mut mice had decreased body weight, likely because of the reduced BMD, BMC and body area. We also observed that bezafibrate caused a greater decrease in body weight in WT than Mut mice. This difference may be due to reduced weight of the Mut mice compared to the WT or a partial compensatory effect of the bezafibrate treatment in the Mut mice. In addition, we suspect that decreased locomotion observed in bezafibrate treated mice is related to the effects of bezafibrate on BMD and BMC. We speculate that these non-beneficial effects of bezafibrate on the overall well-being of the mice may have obscured its pharmacological beneficial effects. The early death of MutBD mice may be the result of the liver damage they sustained in combination with the phenotypes that were not ameliorated by bezafibrate. It is important to note that the concentrations of bezafibrate used here are approximately 80-fold higher than what is used to treat dyslipidemias in humans [20] highlighting the need to study the effect of bezafibrate on mitochondrial function in humans with mitochondrial defects.

Here we showed that bezafibrate improved some premature aging-like phenotypes of Mut mice, most clearly observed in skin and spleen. Recently, it was shown that mitochondrial dysfunction in somatic stem cells may contribute to the progeroid phenotypes present in Mut mice [21]. Therefore, our findings suggest that bezafibrate may help maintain the stem cell population in replicating tissues like skin and spleen in the Mut mice. Interestingly, the benefits observed did not correlate with generalized increased mitochondrial content or function, and may also result from the effects of PPARGs in other related regulatory pathways involved in the control of inflammation, fatty acid metabolism and apoptosis.

Materials and Methods

Mouse Model

MiDNA mutator mice (P0g1/D257A/D257A) (Mut) were previously characterized [2,3]. We placed 2 month-old male Mut and WT mice (MutBD and WTBD respectively) on a standard mouse diet containing 0.5% bezafibrate (bezafibrate diet, BD) (Bio-Serv). As a control, we placed same age male Mut and WT mice on the standard mouse diet (standard diet, SD) (MutSD and WTSD respectively). Mice were kept on their respective diets for 8 months and analyzed at 10 month-old. We choose to use only male mice to reduce the total number of animals used for our study and also to avoid experimental variability observed in female animals due to their estrous cycle.

Animal Husbandry

Mice were housed in a virus-antigen-free facility of the University Of Miami Division Of Veterinary Resources in a 12-h light/dark cycle at 22°C and fed ad libitum with irradiated standard mouse diet or bezafibrate diet.

DEXA Scan

This procedure was performed as previously described [22]. In summary, mice were anesthetized then weighed and bone mineral density and content along with fat and lean body mass was measured using a Lunar PIXImus II Densitometer (GE Medical Systems, Waukesha, WI).

Histology

The dorsal and ventral skin of euthanized mice was shaved, and skin biopsies were collected and stored in formalin for at least 24 hours before being sent to the University of Miami Lois Pope Life Centre histology core for paraffin embedding. Paraffin skin sections were stained with hematoxylin and eosin (H&E), Verhoeff’s Van Geison (EVG) and Masson’s trichrome stain at the University of Miami Dermatology Pathology Core. The
stained sections were analyzed using a light microscope. For spleen and liver analysis, deeply anesthetized mice were perfused with cold PBS and the liver and spleen were collected and stored in formalin for at least 24 hours. Fixed tissues were then paraffin embedded, sectioned, stained for H&E and analyzed for structural changes at the University of Miami Comparative Pathology Laboratory.

### Immunostaining

Paraffin embedded spleen sections were warmed then deparaffinized and rehydrated. Sections were incubated in 70% formic acid to retrieve antigen before being permeabilized with 0.2% Triton. Endogenous peroxidases were quenched then sections were blocked for 1 hour with normal goat serum (KPL). Sections were incubated overnight in 1:250 dilution of primary antibody for cleaved caspase-3 (Cell Signaling). Secondary antibody, biotinylated goat anti-rabbit (KPL), was used then sections were treated with streptavidin peroxidases (KPL) for 30 minutes before being incubated in Diaminobenzidine (DAB). Slides were dehydrated in alcohol then mounted in xylene with aqueous mounting medium and viewed with a light microscope. The number of positive (brown) staining was counted in 4 field of view from each section and averaged.

### Western Blot

Western blot was performed as previously described [23]. Primary antibodies used were Smad3 (Cell Signaling), GAPDH (GeneTex), Total OXPHOS Rodent Cocktail (Mitosciences), PGC-1α (Santa Cruz H-300), and Actin (Sigma). Primary antibodies were used at 1:1000 dilutions except PGC-1α which was used at 1:200 dilution, and incubated overnight at 4°C. Secondary antibodies were used in 0.1% Tween, and goat anti-rabbit HRP-linked (1:10,000) (Cell Signaling). Blots were either developed spectrophotometrically in total homogenate from the quadriceps of mice as previously described [4]. ASSY results were normalized to protein concentration obtained by the Bradford method.

### Spectrophotometric Assays

Citrate synthase (CS) activity was determined spectrophotometrically in total homogenate from the quadriceps of mice as previously described [4]. ASSY results were normalized to protein concentration obtained by the Bradford method.

### Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Miami (Permit Number: 12-094). All efforts were made to minimize suffering.

### Acknowledgments

We are grateful to Dr. Tongyu Cao for assistance with skin analysis, Ms. Estefania Gonzalez for help with RNA preparation, Dr. Wayne Balkan for access to the DEXA scanner, The University of Miami Comparative Pathology Laboratory for access to the DEXA scanner, The University of Miami Comparative Pathology Laboratory and Treadmill Test

**Treadmill Test**

The treadmill test was performed as previously described [4]. Mice were first acclimated to the treadmill (Columbus Instruments, Columbus, OH) then the running time was set to 10 minutes and the speed was increased every 5 minutes. Mice performance was determined by the number of times a mouse falls off the running belt onto the grid during the test.

### Blood Analysis

Mice were fasted overnight and the following day anesthetized and blood was collected by cardiac puncture. Blood was sent to the University of Miami Comparative Pathology Laboratory for analysis of complete blood cell count (CBC) and for a hepatic blood panel to measure markers of liver damage/inflammation.

### References

1. Dillon LM, Belcho AP, Moraes CT (2012) The role of PGC-1 coactivators in aging skeletal muscle and heart. JUBMB Life 64: 231–241.
2. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429: 417–423.
3. Kojoh GC, Hiona A, Pugh TD, Someya S, Panzer K, et al. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science 309: 401–404.
4. Dillon LM, Williams SL, Hida A, Peacock JD, Prella TA, et al. (2012) Increased mitochondrial biogenesis in muscle improves aging phenotypes in the mtDNA mutator mouse. Hum Mol Genet 21: 2289–2297.
5. Tenenbaum A, Motto M, Fissman EZ (2005) Dnnd and pan-peroxisome proliferator-activated receptors (PPAR) co-agonist: the bezafibrate lessons. Cardiaco Diabetes 4: 14.
6. Wang YX (2010) PPARs: diverse regulators in energy metabolism and metabolic diseases. Cell Res 20: 124–137.
7. Kuenzli S, Saurat JH (2003) Peroxisome proliferator-activated receptors in cutaneous biology. Br J Dermatol 149: 229–236.
8. Wenz T, Díaz F, Spiegelman BM, Moraes CT (2008) Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype. Cell Metab 8: 249–256.
9. Johri A, Calingasan NY, Hennessey TM, Sharma A, Yang L, et al. (2012) Pharmacologic activation of mitochondrial biogenesis exerts widespread beneficial effects in a transgenic mouse model of Huntington’s disease. Hum Mol Genet 21: 1129–1137.
10. Yatsuga S, Nuomalainen A (2011) Effect of bezafibrate treatment on late-onset mitochondrial myopathy in mice. Hum Mol Genet.
11. Viscomi C, Bottani E, Ciallella G, Cerutti R, Moggio M, et al. (2011) In vivo correction of COX deficiency by activation of the AMPK/PGC-1alpha axis. Cell Metab 14: 80–90.
12. Oikarinen A (1994) Aging of the skin connective tissue: how to measure the biochemical and mechanical properties of aging dermis. Photodermatol Photoimmunol Photomed 10: 47–52.
13. Ham SA, Kim HJ, Kang ES, Eun SY, Kim GH, et al. (2010) PPARdelta promotes wound healing by up-regulating TGF-beta-dependent or -independent expression of extracellular matrix proteins. J Cell Mol Med 14: 1747–1759.
14. Smaldone S, Giovino GL, Morecini G, Gabrielli A, et al. (2011) Ha-Ras stabilization mediates pro-fibrotic signals in dermal fibroblasts. Fibrogenesis Tissue Repair 4: 8.
15. Harries MJ, Paas R (2009) Scarring alopecia and the PPAR-gamma connection. J Invest Dermatol 129: 1066–1070.
16. Cesta MF (2006) Normal structure, function, and histology of the spleen. Toxicol Pathol 34: 453–463.
17. Hiona A, Sanz A, Kujoth GC, Pamplona R, Seo AY, et al. (2010) Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice. PLoS One 5: e11468.
18. De Souza AT, Cornwell PD, Dai X, Cagayong MJ, Ulrich RG (2006) Agonists of the peroxisome proliferator-activated receptor alpha induce a fiber-type-selective transcriptional response in rat skeletal muscle. Toxicol Sci 92: 378–386.
19. Djonadi F, Bastin J (2011) Species differences in the effects of bezafibrate as a potential treatment of mitochondrial disorders. Cell Metab 14: 715–716; author reply 717.
20. Ahlqvist KJ, Hamalainen RH, Yatsuga S, Uutela M, Terzioglu M, et al. (2012) Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice. Cell Metab 15: 100–109.
21. Cheung MC, Spalding PB, Gutierrez JC, Balkan W, Namias N, et al. (2009) Body surface area prediction in normal, hypermuscular, and obese mice. J Surg Res 153: 326–331.
22. Pickrell AM, Wakin M, Wang X, Moraes CT (2011) The striatum is highly susceptible to mitochondrial oxidative phosphorylation dysfunctions. J Neurosci 31: 9893–9904.