PGC-1alpha Down-Regulation Affects the Antioxidant Response in Friedreich’s Ataxia

Daniele Marmolino¹, Mario Manto¹,², Fabio Acquaviva³, Paola Vergara³, Ajay Ravella¹, Antonella Monticelli⁴, Massimo Pandolfo¹*

¹ Laboratoire de Neurologie Expérimentale, Université Libre de Bruxelles (ULB), Brussels, Belgium, ² Fonds National de la Recherche Scientifique (FNRS), Brussels, Belgium, ³ Department of Cellular and Molecular Biology, University of Naples “Federico II”, Naples, Italy, ⁴ IEOS, Consiglio Nazionale delle Ricerche (CNR), Naples, Italy

Abstract

Background: Cells from individuals with Friedreich’s ataxia (FRDA) show reduced activities of antioxidant enzymes and cannot up-regulate their expression when exposed to oxidative stress. This blunted antioxidant response may play a central role in the pathogenesis. We previously reported that Peroxisome Proliferator Activated Receptor Gamma (PPARγ) Coactivator 1-alpha (PGC-1α), a transcriptional master regulator of mitochondrial biogenesis and antioxidant responses, is down-regulated in most cell types from FRDA patients and animal models.

Methodology/Principal Findings: We used primary fibroblasts from FRDA patients and the knock-in-knock out animal model for the disease (KIKO mouse) to determine basal superoxide dismutase 2 (SOD2) levels and the response to oxidative stress induced by the addition of hydrogen peroxide. We measured the same parameters after pharmacological stimulation of PGC-1α. Compared to control cells, PGC-1α and SOD2 levels were decreased in FRDA cells and did not change after addition of hydrogen peroxide. PGC-1α direct silencing with siRNA in control fibroblasts led to a similar loss of SOD2 response to oxidative stress as observed in FRDA fibroblasts. PGC-1α activation with the PPARγ agonist (Pioglitazone) or with a cAMP-dependent protein kinase (AMPK) agonist (AICAR) restored normal SOD2 induction. Treatment of the KIKO mice with Pioglitazone significantly up-regulates SOD2 in cerebellum and spinal cord.

Conclusions/Significance: PGC-1α down-regulation is likely to contribute to the blunted antioxidant response observed in cells from FRDA patients. This response can be restored by AMPK and PPARγ agonists, suggesting a potential therapeutic approach for FRDA.

Citation: Marmolino D, Manto M, Acquaviva F, Vergara P, Ravella A, et al. (2010) PGC-1alpha Down-Regulation Affects the Antioxidant Response in Friedreich’s Ataxia. PLoS ONE 5(4): e10025. doi:10.1371/journal.pone.0010025

Editor: Antoni L. Andreu, Hospital Vall d’Hebron, Spain

Received January 31, 2010; Accepted March 16, 2010; Published April 7, 2010

Copyright: © 2010 Marmolino et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the GOFAR Association (grant to M.P.), the Fonds Erasme (grant Jean Van Damme for Orphan Diseases to M.P.), the Belgian Fonds National de la Recherche Scientifique (grant n. 3.4572.08 to M.P.), M.M. is supported by FNRS-Belgium. Takeda pharmaceuticals provided the compound pioglitazone-AD4833. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: massimo.pandolfo@ulb.ac.be

Introduction

Friedreich’s ataxia (FRDA) is an autosomal recessive inherited disorder affecting approximately 1 in every 40 000 individuals [1] in Western Europe. It is characterized by progressive gait and limb ataxia, dysarthria, areflexia, loss of vibratory and position sense, and progressive weakness of central origin. Additional features include scoliosis, high risk of diabetes [2–6] and a hypertrophic cardiomyopathy that can cause premature death [3,4,7]. Age of onset is usually in childhood or adolescence, but it may vary from infancy to adulthood.

A large GAA repeat expansion in the first intron of the FXN gene is the most common mutation underlying FRDA [8]. Patients are homozygous for this mutation, or, rarely, are compound heterozygotes for the GAA repeat expansion and a different FXN loss-of-function mutation. They show severely reduced levels of the FXN-encoded mitochondrial protein frataxin, a highly conserved protein with homologs in all eukaryotes and in Gram-negative bacteria [8]. FXN down-regulation has been linked to the property of long GAA repeats to adopt a triple helical structure that directly impedes transcription in vitro [9,10]. In the nucleus of cells from human patients and mouse models, FXN silencing is associated with epigenetic marks of transcription repressive heterochromatin near expanded GAA repeats [11,12]. Whether the triplex forming ability of GAA repeats is involved in this chromatin remodeling process is unknown.

Frataxin is an essential protein in higher organisms, as first revealed by the embryonic lethality of fav gene knockout in the mouse [13]. Yeast cells can instead survive without frataxin, but they progressively lose mitochondrial function and mitochondrial DNA [14].

The function of frataxin has not yet been completely elucidated, but its involvement in mitochondrial iron metabolism is supported by current literature. Frataxin has a compact globular structure with functionally important surface features, in particular a negatively charged ridge that binds ferrous iron with low affinity [15,16]. Under conditions of iron excess, frataxin has been reported to show ferroxidase activity and form high molecular...
weight complexes containing a ferric iron core [17]. This property, which has been proposed to be important for iron detoxification in the mitochondrial compartment, is most evident for the yeast frataxin homolog yhl1. The functional role of these iron-containing frataxin polymers, as well as of frataxin oligomers reported to form at lower iron concentration, is still controversial [18,19]. Multiple abnormalities of iron metabolism occur when frataxin levels are insufficient: decreased activities of iron-sulfur cluster (ISC) containing proteins [20], accumulation of iron in mitochondria and depletion in the cytosol [21], enhanced cellular iron uptake [22,23], and, in some models, reduced heme synthesis [24,25]. These abnormalities point to a defective utilization of iron for biosynthetic processes taking place in the mitochondria, in particular ISC synthesis. ISCs are prosthetic groups for several mitochondrial and extra-mitochondrial enzymes, involved in energy metabolism (aconitase and complexes I, II and III of the respiratory chain), iron metabolism (iron-responsive protein 1, IRP), and ferrochelatase), purine metabolism (xanthine oxidase) and DNA repair [26]. Accumulation of iron in the mitochondria with increased cellular uptake and cytosolic depletion occurs when ISC synthesis is defective, suggesting a role of frataxin in this process. Current evidence supports a direct interaction of frataxin with components of the mitochondrial ISC synthesis machinery, but a non-essential role in the process [27].

Evidence of oxidative stress has been found in most, though not all models of frataxin deficiency [28–35]. In FRDA patients, increased plasma levels of malonyldialdehyde (a lipid peroxidation product) [34], increased urinary 8-hydroxy-2′-deoxyguanosine (a marker of oxidative DNA damage) [35], decreased plasma free glutathione, and increased plasma glutathione S-transferase activity [29] indicate an oxidative stress condition. Oxidative stress is thought to derive from the strong reactivity of the excess mitochondrial iron with reactive oxygen species (ROS) present in that compartment, including the Fenton reaction that generates the highly toxic hydroxyl radical. Accordingly, yhl1-deficient yeast [14] and cells from FRDA patients [36] are highly sensitive to oxidants such as hydrogen peroxide H2O2. Respiratory chain dysfunction caused by decreased activity of the ISC-containing complexes I, II and III is likely to further aggravate oxidative stress by increasing leakage of electrons and formation of superoxide.

Frataxin deficient cells not only generate more free radicals, they also show a reduced ability to mobilize antioxidant defenses, in particular to induce SOD2 expression following exposure to oxidants such as H2O2 and iron [31]. The mechanism underlying this defect has not yet been understood. Its investigation has prompted the present study.

In a previous study we have shown reduced expression of the peroxisome proliferator activated receptor gamma (PPAR-γ) coactivator 1α (PGC-1α) in several tissues from frataxin-deficient mice, with the notable exception of the heart. We have observed PGC-1α down-regulation also in neural precursor cells from the subventricular zone of these animals, and in fibroblasts and lymphoblastoid cell lines from FRDA patients [37,38]. PGC-1α is a multifunctional protein found at higher levels in tissues with high metabolic requirement such as brown fat, skeletal muscle, kidney, heart, and brain [39–41], that functions as a coactivator to most nuclear receptors and to several other transcription factors [42]. It is a critical regulator that links metabolic activity to relevant environmental stimuli in multiple pathways, including those responsible for adipogenesis, gluconeogenesis, myogenesis, and mitogenesis [43]. PGC-1α has also emerged as a key factor in the induction of many antioxidant programs in response to oxidative stress, both in vivo and in vitro [44–47], in particular in neurons [46]. RNAi knockdown of Pgc1a prevents the induction by ROS of antioxidant enzymes such as superoxide dismutase 1 (Sod1), superoxide dismutase 2 (Sod2), and Gpx1, as well as the uncoupling proteins Uip1 and Uip2 [46], indicating that it mediates these protective responses [46,47].

We further observed that in C2C12 myoblasts, but not in cardiomyocytes, PGC-1α and a reporter gene under the control of the PGC-1α promoter are rapidly down-regulated when frataxin expression is inhibited by an shRNA [37], indicating that some mechanism directly links an early effect of frataxin deficiency with reduced PGC-1α transcription in this cell type, and presumably in other cells that also down-regulate PGC-1α when frataxin levels are low.

In this study we tested whether the PGC-1α down-regulation occurring in FRDA cells could be in part responsible for the blunted antioxidant response observed in frataxin-deficiency. Our results support this hypothesis, indicating a possible therapeutic target in FRDA.

**Results**

**PGC-1α reduction is associated to reduced SOD2 in FRDA fibroblasts and does not increase after H2O2 incubation**

The baseline expression levels of SOD2 and PGC-1α were reduced in fibroblasts from FRDA patients when compared to healthy controls (Fig. 1A). Treatment with 100 μM H2O2 for 48 and 72 hours increased both SOD2 and PGC-1α mRNA and protein in control cells, but not in FRDA fibroblasts (Fig. 1B–D). Incubation with H2O2 of SKNBE neuroblastoma cells was also accompanied by PGC-1α and SOD2 induction at both mRNA and protein level, showing that this response is not specific for fibroblasts (Fig. 1E–G).

**PGC-1α down-regulation by RNAi results in lack of SOD2 response to H2O2**

Incubation of fibroblasts from healthy and FRDA subjects for 72 hours with a PGC-1α specific siRNA significantly decreases mRNA and protein levels when compared to cells transfected with a control non-specific siRNA, as shown in figure 2A–C. In these conditions, SOD2 mRNA and protein reduction was significant both in control and FRDA fibroblasts. These data are in agreement with reports indicating that PGC-1α controls SOD2 expression [47,48,49]. We also confirmed our previous finding [37] that PGC-1α silencing results in frataxin down-regulation.

**Effect of PPARγ and AMPK agonists on the antioxidant response in FRDA fibroblasts**

We tested whether drugs known to up-regulate PGC-1α could restore SOD2 induction by H2O2 in FRDA cells. PPARγ and the AMP-dependent protein kinase (AMPK) are major inducers of PGC-1α activity and expression [50–54].

In a first set of experiments we evaluated the effect of the PPARγ agonist Pioglitazone. We had previously shown that a different potent PPARγ agonist, Azelaoyl-PAF, was able to increase PGC-1α and frataxin expression in FRDA and control fibroblasts [55]. Pioglitazone at a concentration of 10 μM was able to increase PGC-1α mRNA and protein levels after 72 and 96 hours of incubation of both control and FRDA fibroblasts, as shown in Fig. 3A–C. In the same conditions, SOD2 levels were also increased, with a more robust effect in FRDA cells. Frataxin expression showed a non-significant trend toward up-regulation in this set of experiments (Fig. 3A–C). Interestingly, in SKNBE neuroblastoma cells, Pioglitazone incubation for 96 hours at
10 μM increased PGC-1α and SOD2 levels as well as frataxin amount (Fig. 3D-F).

We then exposed cells to the AMPK agonist AICAR, showing that this molecule at a concentration of 2 mM, strongly upregulated PGC-1α and SOD2 in both healthy controls and FRDA fibroblasts after 48 hours of incubation (Fig. 4A-C).

Accordingly, Pioglitazone 10 μM or AICAR 2 mM, incubation for 5 hours with a following addiction of 100 μM H2O2 for 48 hours of incubation results in a significant increase of PGC-1α and SOD2 levels in both healthy controls and FRDA fibroblasts after 72 hours of incubation (Fig. 4A-C).

Figure 1. SOD2 and PGC-1α expression in FRDA fibroblasts and H2O2 treatments. A. Quantitative Real-Time PCR analysis: FXN (blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in primary fibroblasts from healthy controls and FRDA patients. B. Quantitative Real-Time PCR analysis: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in primary fibroblasts from healthy controls and FRDA patients after incubation with 100 μM H2O2 at 48 and 72 hours. C. Western Blot analysis: β-Actin (Act), PGC-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in primary fibroblasts from healthy controls and FRDA patients after incubation with 100 μM H2O2 at 48 and 72 hours. D. Densitometric scan analysis of five independent Western blots from healthy controls and FRDA patients after incubation with 100 μM H2O2 at 48 and 72 hours: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. E. Quantitative Real-Time PCR analysis: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in SKNBE neuroblastoma cells after incubation with 100 μM H2O2 at 48 and 72 hours. F. Western Blot analysis: β-Actin (Act), PGC-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in SKNBE neuroblastoma cells after incubation with 100 μM H2O2 at 48 and 72 hours. G. Densitometric scan analysis of five independent Western blots from SKNBE cells after incubation with 100 μM H2O2 at 48 and 72 hours: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. Results are expressed as a fold increase of the means (mean ± SEM) over the value of expression in the respective untreated control cells arbitrarily set as 1. (n = 5, ***p<0.001, **p<0.01, *p<0.05; Mean ±/− SEM) for all the experiments. doi:10.1371/journal.pone.0010025.g001

Figure 2. PGC-1α downregulation by a specific RNAi. A. Quantitative Real-Time PCR analysis: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in primary fibroblasts from healthy controls and FRDA patients after 72 hours transfection with a PGC-1α specific siRNA, as control a fluoresceine-conjugated siRNA was used. B. Western Blot analysis: β-Actin (Act), PGC-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in primary fibroblasts from healthy controls and FRDA patients after 72 hours transfection with a PGC-1α specific siRNA, as control a fluoresceine-conjugated siRNA was used. C. Densitometric scan analysis of five independent Western blots from healthy controls and FRDA patients after 72 hours transfection with a PGC-1α specific siRNA, as control a fluoresceine-conjugated siRNA was used: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. (n = 5, ***p<0.001, **p<0.01, *p<0.05; Mean ±/− SEM) for all the experiments. doi:10.1371/journal.pone.0010025.g002
72 hours increase SOD2 expression in FRDA fibroblasts (Fig. 4D–F). In these conditions, an increase in PGC-1α levels was also observed when compared to treatment with H2O2 alone (Fig. 4D–F).

Effect of Pioglitazone in vivo in frataxin-deficient (KIKO) mice

Based on in vivo results, we tested the effect of Pioglitazone on the levels of PGC-1α, SOD2 and frataxin in frataxin-deficient mice. For these experiments, we used the fxn(GAA)230/2(KIKO) mice that express 25–35% of wild-type frataxin levels, but have no detectable motor abnormality of pathological change. Ten KIKO mice received Pioglitazone (25 mg/Kg/day) via oral administration for one month. No change was observed in the body weight of the mice (data not shown). In the spinal cord, a primary affected tissue in the disease, no effect was observed on frataxin expression, while Pgc-1α and Sod2 levels were slightly increased (Fig. 5A–D). In the cerebellum, Pgc-1α and Sod2 levels were significantly increased (Fig. 5B–D). No effect was observed on frataxin expression. Surprisingly, in a group of 10 wt mice no effect was observed after Pioglitazone administration.

Discussion

Our results provide a link between the previous independent observations of a blunted antioxidant response in cells from FRDA patients [31,32] and the PGC-1α down-regulation occurring in most cell types with frataxin deficiency [37]. These phenomena
PGC1α and Antioxidants in FRDA

A) mRNA levels relative to B-Act

B) Western Blot of fibroblasts

C) Protein levels relative to B-Act

D) mRNA levels relative to B-Act

E) Western Blot of FRDA fibroblasts

F) Protein levels relative to B-Act
Figure 4. Effect of AICAR on the antioxidant response in FRDA fibroblasts and SKNBE neuroblastoma cells. A. Quantitative Real-Time PCR analysis: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in primary fibroblasts from healthy controls and FRDA patients after incubation with 2 mM AICAR at 48 hours. B. Western Blot analysis: β-Actin (Act), PGC-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in primary fibroblasts from healthy controls and FRDA patients after incubation with 2 mM AICAR at 48 hours. C. Densitometric scan analysis of five independent Western blots from healthy controls and FRDA patients after incubation with 2 mM AICAR at 48 hours: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. D. Quantitative Real-Time PCR analysis: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars) mRNA quantification in primary fibroblasts from FRDA patients after incubation with 100 μM H2O2 alone or in combination with 10 μM Pioglitazone or 2 mM AICAR for 72 hours. E. Western Blot analysis: β-Actin (Act), PGC-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in primary fibroblasts from FRDA patients after incubation with 100 μM H2O2 alone or in combination with 10 μM Pioglitazone or 2 mM AICAR for 72 hours. F. Densitometric scan analysis of five independent Western blots from SKNBE cells after incubation with 100 μM H2O2 alone or in combination with 10 μM Pioglitazone or 2 mM AICAR for 72 hours: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. (n = 5, ***p<0.001, **p<0.01, *p<0.05; Mean ±/− SEM) for all the experiments.

Figure 5. In vivo Pioglitazone administration in KIKO mice. A. Quantitative Real-Time PCR analysis: fxn (Blue bars), pgc-1α (Red bars) and sod2 (Green bars) mRNA quantification in the spinal cord of the KIKO mice after receiving oral administration of Pioglitazone 25 mg/Kg/day for one month. B. Quantitative Real-Time PCR analysis: fxn (Blue bars), pgc-1α (Red bars) and sod2 (Green bars) mRNA quantification in the cerebellum of the KIKO mice after receiving oral administration of Pioglitazone 25 mg/Kg/day for one month. C. Western Blot analysis: β-actin (Act), pgc-1α (PGC-1), frataxin (FXN), mitochondrial superoxide dismutase (SOD2) protein in the spinal cord and cerebellum of the KIKO mice after receiving oral administration of Pioglitazone 25 mg/Kg/day for one month. D. Densitometric scan analysis of five independent Western blots from the spinal cord and cerebellum of the KIKO mice after receiving oral administration of Pioglitazone 25 mg/Kg/day for one month: FXN (Blue bars), PGC-1α (Red bars) and SOD2 (Green bars). The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. (n = 10, **p<0.01, *p<0.05; Mean ±/− SEM) for all the experiments.

are likely to contribute to FRDA pathogenesis and constitute possible therapeutic targets.

Oxidative stress has been considered a major pathogenic mechanism in FRDA, even though the data are in same case controversial. Previous studies [56,57] found no evidence of oxidative stress in the target tissues of conditional knockout mouse models (heart and nervous system), which nevertheless develop FRDA-like pathology and show the typical biochemical defects of FRDA, including multiple ISC enzyme deficiencies and, in late stages, gross mitochondrial iron accumulation. One possible explanation could be that the total absence of frataxin, as found in targeted cells in conditional knock-outs, leads to an almost
complete respiratory chain shut down, so less ROS are eventually generated. Indeed, in yeast studies evidence of oxidative damage rapidly follows frataxin silencing before the loss of mitochondrial function [50]. The respiratory chain, though impaired, remains partially functional in FRDA target tissues and in animal models with reduced frataxin levels, as well as in cell models with partial frataxin deficiency or expressing a mutated frataxin protein [59,60]. Accordingly, studies in these systems [59,61,62], including tissue samples from FRDA patients, have shown evidence of chronic oxidative stress, and oxidative stress markers have been found in the blood [29,34] and urine [35] of FRDA patients. In the case of the fibroblasts utilized in the present study, we obtained further evidence of increased ROS production by revealing higher levels of superoxide than in control fibroblasts (Methods S1 and Fig. S1). Then, we confirmed [31,32] that exposure of FRDA fibroblasts to moderate oxidative stress, as induced by exogenously added H2O2 or iron, or by partial respiratory chain inhibition, fails to up-regulate antioxidant enzymes. Only strong stressors, like very high iron or H2O2 concentration in the medium have shown to possibly up-regulate SOD2 in FRDA fibroblasts [31] by triggering NFKB signaling. These data indicate the failure of a response mechanisms dealing with the control of lower, chronic levels of oxidative stress. With reference to pathogenesis, it is conceivable that, while this deficient response does not appear to be harmful to unaffected cells in FRDA like fibroblasts, unless they are exposed to additional oxidative stress, it may be deleterious for vulnerable cell types, such as neurons even in basal conditions.

The observed failure to induce antioxidant defenses contrasts with the expected homeostatic response. Several pathways are physiologically activated by ROS, leading to increased levels and activity of antioxidant enzymes and to mitochondrial biogenesis [42–44,46,47,63,64]. Key factors for antioxidant enzyme induction are nuclear factor-E2-related factor-2 (Nrf2), a transcription factor that serves as a cellular sensor for oxidative stress [64,65], and PGC-1α [66].

PGC-1α is also a key player in the ROS-induced mitochondrial biogenesis, along with the transcription factor nuclear respiratory factor-1 (NRF-1) and the mitochondrial transcription factor Tfam [48,66–71].

A recent study suggested that impaired nuclear translocation of Nrf2 may underlay the lack of oxidative stress response in FRDA cells [71]. Though this mechanism may play a role, Nrf2-regulated genes primarily include heme oxygenase-1 (HO-1), NAD(P)H-quinone oxireductase-1 (NQO1), glutathione S-transferases, and the glutathione-synthesizing enzymes glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM), while PGC-1α may be more relevant for SOD2 induction.

The importance of PGC-1α in these metabolic programs was further revealed through the generation of PGC-1α null mice. These mice display a reduced basal expression of many mitochondrial genes in liver, brain, skeletal muscle, and heart compared with wild-type (WT) animals [72–74]. Furthermore, PGC-1α knockout (KO) mice underlying PGC-1α as an important factor in brain structure and function. PGC-1α KO animals present neurodegenerative lesions in the striatum, as well as behavioral abnormalities [74]. These lesions present characteristic similar to those observed in many models with altered ROS levels. Thus, PGC-1α could play an important role in ROS control. The precise role of PGC-1α in ROS metabolism is still undiscovered. Several groups have reported that the expression of mitochondrial ROS-detoxifying enzymes increases with PGC-1α [75–77]. Conversely, muscle from PGC-1α knockout mice shows a mild reduction of SOD2 [78]. Interestingly, PGC-1α direct down-regulation by RNAi results in the downregulation of SOD2 and other antioxidant enzymes, and particularly generates a lack of their induction after exposure to stressors such as H2O2, markedly resembling the situation in frataxin-deficient cells [46]. This important finding, together with our previous observation that frataxin deficiency leads to reduced levels of PGC-1α and its target genes in most investigated cell types prompted us to study whether PGC-1α could be involved in the blunted antioxidant response in FRDA.

PGC-1α promotes the metabolic program that is very functional and can be stimulated in order to stimulate a downstream response.

Materials and Methods

Ethics statement

Patients and healthy controls were enrolled on a voluntary basis at the "Federico II" University in Naples, Italy. Written informed consent to participate in the study and provide a skin biopsy was obtained according to a protocol approved by the "Federico II" ethics committee.
Patients
The study included five FRDA patients from the department of Neurology “Federico II” Naples, and five unrelated healthy controls. Patients were homozygous for GAA repeat expansions between 500 and 1,200 repeats, with an age of onset for the disease ranging between 20 and 30 years old. All enrolled patients started treatment with Idebenone 3 mg/kg after the skin biopsies were obtained. They were also following a standard protocol of physiotherapy.

Cell cultures
Primary fibroblast primary cell cultures were obtained from skin biopsies of FRDA patients and healthy controls. Human neuroblastoma derived cells (SKNBE) are commercially available (in Europe from ATCC-LGC Standards, line number CRL-2271). Primary fibroblasts and SKNBE cells were grown in DMEM supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (P/S). All experiments with fibroblasts were conducted between fourth and eleventh passages.

Cell Treatment
Primary fibroblasts and SKNBE cells were incubated for 48 and 72 hours in presence of 100 µM H2O2 (Sigma Aldrich) or DMEM alone as control before total RNA and protein extraction. 10 µM Pioglitazone (AD-4833, Takeda pharmaceuticals) and 2 mM 5'-D-ribofuranoside (AICAR, Sigma Aldrich) were used for in vivo experiments.

Animal experiments
20 (C57BL6/j; forGAA<sup>230/-</sup>) KIKO mice eight months old were used for in vivo experiments. Precisely, 10 KIKO mice received 25 mg/Kg/day of Pioglitazone/0.1% carboxymethylcellulose sodium salt. Mice body weight was daily monitored. Mice were sacrificed by decapitation. The liver, heart, kidneys, brain and testes were extracted for the analysis. All animal procedures respected regulations and guidelines of the Belgian state and tissues were obtained. They were also following a standard protocol of physiotherapy. All animal procedures were carried out using the 2(-Delta Delta C(T)) method (2-ΔΔCt).

Western blot
For total proteins extraction, fibroblasts and SKNBE cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml of leupeptin/antipain for 30 minutes on ice. The lysate was sonicated and the concentration was determined using a Bradford assay. 100 µg of total protein were used for the analysis. The running conditions included a 10 min incubation at 10 mA (Volt and Watt at maximum) and then a step at 25 mA for 60–90 min. For blotting, a nitrocellulose membrane was used. Blotting was conducted at 5% milk, the membrane was incubated with the primary antibody (Frataxin, 1:5000; Chemicon; pgc1a, Sigma Aldrich) and a peroxidase conjugated secondary antibody was incubated lasted overnight at 4°C. After the incubation, the blot was washed and processed for chemoluminescent detection. We quantified the relative intensities of each signal using the Image J Software and normalized the values to the intensity of Actin.

Statistical analyses
Statistical analyses were performed using Sigma Stat program (SigmaStat). The Shapiro-Wilk test was used to determine whether the data were normally distributed, and then statistical significance was calculated using the one sample T-test. Differences with p values less than 0.05 (*p<0.05), less than 0.01 (**p<0.001) and less than 0.001 (***)p<0.001) were considered to be significant.
Supporting Information

Figure S1 Representative digital images of primary fibroblasts from healthy controls and two FRDA patients at basal conditions. Cells nuclei are in Blue (DAPI) and mitochondrial O2- production are in red (MitoSox). Merge is obtained by overlapping the two stain. B. Using digital image processing, the MitoSoX fluorescence intensity mean per image was calculated, averaged over three fields of view per experiment, and then averaged over three independent experiments. Data were normalized to static controls MitoSoX fluorescence. (n = 3, **p<0.01, *p<0.05; Mean +/- SEM) for all the experiments.

Found at: doi:10.1371/journal.pone.0010025.s001 (1.05 MB TIF)

Methods S1 Supplementary methods: Fluorescent detection of mitochondrial superoxide.

References

1. Delattyki M, Williamson R, Forrest S (2000) Friedreich ataxia: an overview. J Med Genet 37: 1–6
2. Finocchiaro G, Baio G, Micossi P, Pozza G, di Donato S (1988) Glucose metabolism alterations in Friedreich’s ataxia. Neurology 38: 1292–6.
3. Filla A, De Michele G, Coppola G, Federico A, Vita G, et al. (1999) Accuracy of clinical diagnostic criteria for Friedreich’s ataxia. Mov Disord 15: 1255–60.
4. Pandolfo M (2003) Friedreich ataxia Semin Pediatr Neurol Sep 10: 163–72.
5. Pandolfo M (2006) Friedreich ataxia. In Genetic Instabilities and Neurological Diseases 2: 277–290.
6. Shapcott D, Melancon S, Butterworth R, Khoury K, Collu R, et al. (1976) Glucose and insulin metabolism in Friedreich’s ataxia. Can J Neurol Sci 3: 361–4.
7. Filla A, de Michele G, Caruso G, Marconi R, Campagnola G (1990) Genetic data and natural history of Friedreich’s disease: a study of 80 Italian patients. J Neurol 237: 345–50.
8. Campuzano V, Montemmi L, Molto MD, Pianese L, Cossee M, et al. (1996) Friedreich ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271: 1423–1427.
9. Sakamoto N, Chastain PD, Pawlikiewicz F, Ohshima K, Pandolfo M, et al. (1999) Sticky DNA: self-association properties of long GAA-ITC repeats in K-R Y triplex structures from Friedreich’s ataxia. Mol Cell 3: 465–475.
10. Grabczyk E, Mancuso M, Sammarco M (2007) A persistent RNA/DNA hybrid formed by transcription of the Friedreich ataxia triplet repeat in live bacteria, and by T7 RNAP in vitro. Nucleic Acids Res 35: 5331–9.
11. Herman D, Jenssen K, Burnett R, Sorgori E, Perlman SL, et al. (2006) Histone deacetylase inhibitors reverse gene silencing in Friedreich’s ataxia. Nat Chem Biol 2: 551–558.
12. Rai M, Soragni E, Jensen K, Burnett R, Herman D, Coppola G, et al. (2008) HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. PLoS ONE 3: 1953.
13. Cossee M, Pacico H, Gansmuller A, Kounikova H, Dierich A, et al. (2000) Isolation of the mouse frataxin homologue of frataxin-deficient cells leads to early embryonic lethality without iron accumulation. Hum Mol Genet 9: 1219–1226.
14. Babcock M, de Silva D, Oaks R, Davis-Kaplan S, Jiralerspong S, Montermini L, et al. (1998) Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism alterations in Friedreich’s ataxia cells. Hum Mol Genet 7: 2453–2468.
15. Jiralerspong S, Ge B, Hudson TJ, Pandolfo M (2001) Manganese superoxide dimutase induction by iron is impaired in Friedreich ataxia cells. FEBS Lett 509: 101–105.
16. Chaumel-Grossaud K, Gerome V, Puccio H, Koenig M, Munnich A, et al. (2001) Disabled early recruitment of antioxidant defenses in Friedreich’s ataxia. Hum Mol Genet 10: 2061–2067.
17. Fiana L, Busino L, De Base I, De Cristofaro T, Lo Casale MS, et al. (2002) Upregulation of c-Jun N-terminal kinase pathway in Friedreich’s ataxia cells. Hum Mol Genet 11: 2989–2996.
18. Emont M, Lepage G, Vanasse M, Pandolfo M (2000) Increased levels of plasma malondialdehyde in Friedreich ataxia. Neurology 55: 1732–1735.
19. Schulz JB, Schelte T, Schols H, Hard G, et al. (2000) Oxidative stress in patients with Friedreich ataxia. Neurology 55: 1719–1721.
20. Wong A, Yang J, Cavadioli P, Geller C, Lonnerdal B, et al. (1999) The Friedreich ataxia mutation confers cellular sensitivity to oxidant stress which is rescued by chelators of iron and calcium and inhibitors of apoptosis. Hum Mol Genet 8: 435–439.
21. Coppola G, Marmolino D, Lu D, Wang Q, Caporossi G, et al. (2009) Functional genomic analysis of frataxin deficiency reveals tissue-specific alterations and identifies the PPARgamma pathway as a therapeutic target in Friedreich’s ataxia. Hum Mol Genet 18: 2463–2468.
22. Marmolino D, Acquaviva F (2009) Friedreich’s Ataxia from the (GAA)n repeat mediated silencing to new promising molecules for therapy. Cerebellum 8: 245–259.
23. Emond M, Lepage G, Vanasse M, Pandolfo M (2000) Increased levels of plasma malondialdehyde in Friedreich ataxia. Neurology 55: 1732–1735.
24. Handschin C, Spiegelman BM (2006) Peroxisome proliferator-activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA sequencing, genomic organization, chromosomal localization, and tissue expression. Genomics 62: 98–105.
25. Knutti D, Kaul A, Kralli A (2000) A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. Mol Cell Biol 20: 2411–2422.
26. Liu Z, Puigserver P, Spiegelman BM (1999) Transcriptional activation of adipogenesis. Curr Opin Cell Biol 11: 609–619.
27. Puigserver P, Spiegelman BM (2003) Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) transcriptional coactivator and metabolic regulator. Endocrin Rev 24: 70–90.
28. Handschin C, Spiegelman BM (2006) Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. Endocrin Rev 27: 728–735.
29. Anderson RM, Barger JL, Edwards MG, Braun KH, O’Connor CE, et al. (2008) Dynamic regulation of PGC-1alpha localization and turnover implicates

Acknowledgments

We thank Prof. Jean-Marie Vanderwinden for helpful assistance in microscopy analysis and Prof. Sergio Coccozza for helpful discussion.

Author Contributions

Conceived and designed the experiments: DM MUM MP. Performed the experiments: DM FA PV AR. Analyzed the data: DM MUM FA PV AM. Contributed reagents/materials/analysis tools: AR AM. Wrote the paper: DM MUM MP.
mitochondrial adaptation in calorie restriction and the stress response. Aging Cell 7: 101–111.
45. Pessayre D (2007) Role of mitochondria in non-alcoholic fatty liver disease. J Gastroenterol Hepatol 22: 820–827.
46. St-Pierre J, Drovit S, Uldry M, Sávagi JM, Rhee J, et al. (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127: 397–408.
47. Valle I, Alvarez Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1 (alpha) regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc Res 66: 562–573.
48. Spiegelman BM (2007) Transcriptional control of energy homostasis through the PGC-1 coactivators. Novartis Found Symp 286: 3–6.
49. Olmos V, Valle I, Borniquel S, Tierez A, Soria E, et al. (2009) Mutual dependence of Foxo3a and PGC-1alpha in the induction of oxidative stress genes. J Biol Chem 284: 14476–84.
50. Miglio G, Rosa AG, Rattazzi L, Collino M, Lombardi G, et al. (2009) PPARgamma stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss. Neurochem Int 55: 496–504.
51. Fujisawa K, Nishikawa T, Kukidome D, Imoto K, Yamashiro T, et al. (2009) TZDs reduce mitochondrial ROS production and enhance mitochondrial biogenesis. Biochem Biophys Res Commun 379: 45–8.
52. Wenz T, Diaz F, Spiegelman BM, Moraes CT (Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype.; PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Canto C, Anversa J, Curr Opin Lipidol. 2009 Apr;20(2):98–105. Review.
53. Thomson DM, Winder WW (2009) PPAR-activated protein kinase control of fat metabolism in skeletal muscle. Acta Physiol (Oxf) 196: 147–54.
54. Irrech I, Ljubicic V, Kirawan AF, Hood DA (2008) AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. PLoS One 3: 3614.
55. Marmolino D, Acquaviva F, Pinelli M, Monticelli A, Castaldo I, et al. (2009) PPAR-gamma agonist Azelaoyl PAF increases frataxin protein and mRNA expression: new implications for the Friedreich’s ataxia therapy. Cerebellum 8: 98–103.
56. Puccio H (2009) Multicellular models of Friedreich ataxia. J Neurol 1: 18–24.
57. Puccio H, Simon D, Cosseé M, Criqui-Filipe P, Tiziano F, et al. (2001) Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and oxidative stress leading to progressive neuronal and cardiac pathology. Cell 119: 121–135.
58. Valle I, Alvarez-Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Diabetes 55: 120–127.
59. Wenz T, Diaz F, Spiegelman BM, Moraes CT (Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype.; PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Canto C, Anversa J, Curr Opin Lipidol. 2009 Apr;20(2):98–105. Review.
60. Pi J, Zhang Q, Fu J, Woods CG, Hou Y, et al. (2009) ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function. Toxicol Appl Pharmacol, [Epub ahead of print].
61. Clark J, Simon DK (2009) Transcribe to survive: transcriptional control of antioxidant defense programs for neuroprotection in Parkinson’s disease. Antioxid Redox Signal 11: 509-20.Vitaa J, Gomez-Cabreria MC, Borras C, Froio S, Sanchis-Gomar F, Martinez-Bello VE, Pallardo FV (2009) Mitochondrial biogenesis in exercise and in ageing. Adv Drug Deliv Rev 61: 1369–74.
62. Golfrt S, Wiensser RJ (2003) Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis. Exp Physiol 88: 33–49.
63. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmann G, et al. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98: 115–24.
64. Adhikary J, Uguccioni G, Leick L, Hidalgo J, Pleigard H, et al. (2009) The role of PGC-1alpha on mitochondrial function and apoptotic susceptibility in muscle. Am J Physiol Cell Physiol 297: C217–25.
65. Mootha VK, Handschin C, Arlow D, Xie X, St Pierre J, et al. (2004) Erralpha and Galpba/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. Proc Natl Acad Sci USA 101: 6570–6575.
66. Vannett E, Dassu EP, Goncalves S, Auechre F, Loun M, et al. (2009) Impaired nuclear Nrf2 translocation undermines the oxidative stress response in Friedreich ataxia. PLoS One 4: e2525.
67. Arany Z, He H, Lin J, Hoyer K, Handschin C, et al. (2005) Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle. Cell Metab 1: 259–271.
68. Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, et al. (2004) Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha-null mice. Cell 119: 121–135.
69. St-Pierre J, Lin J, Kraus S, Tarr PT, Yang R, et al. (2003) Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators I alpha and beta (PGC-1alpha and PGC-1beta) in muscle cells. J Biol Chem 278: 26297–26303.
70. Valle I, Alvarez-Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc Res 66: 562–573.
71. Lotte L, Lyubg Sc, Wiatsewicz JF, Pleigard H (2010) PGC-1alpha is required for training-induced prevention of age-associated decline in mitochondrial enzymes in mouse skeletal muscle. Exp Gerontol Jan 18: [Epub ahead of print].
72. Wenz T, Diaz F, Spiegelman BM, Moraes CT (Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype.; Cell Metab 8: 249–56.
73. Canto C, Anversa J (2009) PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol 20: 98–105.
74. Mootha VK, Handschin C, Arlow D, Xie X, St Pierre J, et al. (2004) Erralpha and Galpba/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. Proc Natl Acad Sci USA 101: 6570–6575.
75. Valle I, Alvarez Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc Res 66: 562–573.
76. Valle I, Alvarez Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc Res 66: 562–573.
77. Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, et al. (2004) Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha-null mice. Cell 119: 121–135.
78. St-Pierre J, Lin J, Kraus S, Tarr PT, Yang R, et al. (2003) Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators I alpha and beta (PGC-1alpha and PGC-1beta) in muscle cells. J Biol Chem 278: 26297–26303.
79. Valle I, Alvarez Barrientos A, Arza E, Lamas S, Monsalve M (2005) PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc Res 66: 562–573.