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MITOCHIP Assessment of Differential Gene Expression in the Skeletal Muscle of Ant1 Knockout Mice: Coordinate Regulation of OXPHOS, Antioxidant, and Apoptotic Genes

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Abstract

Genetic inactivation of the nuclear-encoded mitochondrial heart-muscle adenine nucleotide translocator-1 (ANT1), which exports mitochondrial ATP to the cytosol in both humans (*ANT1−/−*) and mice (*Ant1−/−*), results in lactic acidosis and mitochondrial cardiomyopathy and myopathy, the latter involving hyper-proliferation of mitochondria, induction of oxidative phosphorylation (OXPHOS) enzymes, increased reactive oxygen species (ROS), and excessive mtDNA damage. To understand these manifestations, we analyzed *Ant1−/−* mouse skeletal muscle for changes in gene expression using our custom 644 and 1087 gene MITOCHIP microarrays and for changes in the protein levels of key mitochondrial transcription factors. Thirty-four mRNAs were found to be up-regulated and 29 mRNAs down-regulated. Up-regulated mRNAs included the mitochondrial DNA (mtDNA) polypeptide and rRNA genes, selected nuclear-encoded OXPHOS genes, and stress-response genes including *Mcl-1*. Down-regulated mRNAs included glycolytic genes, pro-apoptotic genes, and *c-Myc*. The mitochondrial regulatory proteins Pgc-1α, Nrf-1, Tfam, and myogenin were up-regulated and could account for the induction of the OXPHOS and antioxidant enzymes. By contrast, c-Myc levels were reduced and might account for a reduction in apoptotic potential. Therefore, the *Ant1−/−* mouse skeletal

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muscle demonstrates that energy metabolism, antioxidant defenses, and apoptosis form an integrated metabolic network.

INTRODUCTION

Mitochondria play an important role in human health and disease as they regulate cellular energy production, reactive oxygen species (ROS) production, and apoptosis [1]. Mitochondria generate most of the cell's energy by the process of OXPHOS. During OXPHOS, electrons are passed down the electron transport chain (ETC), encompassing respiratory complexes I–IV. As electrons traverse complexes I (NADH dehydrogenase), III, and IV (cytochrome c oxidase), protons are pumped out across the mitochondrial inner membrane creating an electrochemical gradient. The resulting combination membrane potential and pH gradient, the proton motor force, is then be used by the inner membrane proton-transporting ATP synthase (complex V) as a source of potential energy to drive the phosphorylation of ADP to ATP within the mitochondrial matrix. Matrix ATP is then exported out of the mitochondrion, in exchange for cytosolic ADP, by the adenine nucleotide translocators (ANT).

Humans have four ANT isoforms that are encoded by four different genes and are distributed in different tissue specific patterns. The human *ANT1* gene is primarily expressed in the heart and in skeletal muscle [2]. Human *ANT2* is weakly expressed or absent from most tissues, while human *ANT3* is ubiquitously expressed [3]. The ANT1 and ANT3 isoforms have been proposed to export ATP from the mitochondrial matrix to the cytosol, while ANT2 has been proposed to be induced when mitochondrial energy production is impaired so that cytosolic ATP can be imported into the mitochondrion to sustain mitochondrial biogenesis and to maintain the membrane potential [4–6]. The fourth human ANT gene, *ANT4*, is expressed at highest levels in the testes and at lower levels in other organs including liver and brain [7]. Certain missense mutations in *ANT1* have been shown to cause autosomal dominant progressive external opthalmoplegia (AdPEO) [8, 9], while null mutations in *ANT1* cause autosomal recessive mitochondrial myopathy and cardiomyopathy [10].

Mice have three Ant isoforms encoded by three different genes. *Ant1* is expressed primarily in the heart and skeletal muscle, like its human homolog, while *Ant2* is expressed in virtually all tissues, but at very low levels in skeletal muscle [11, 12]. The murine equivalent to human *ANT4*, *Ant4*, has a highly restricted expression pattern, with mRNA only being detected in testes and germ cells [13, 14].

Mice in which the *Ant1* gene has been insertionally inactivated develop an autosomal recessive (*Ant1*−/−) mitochondrial myopathy and cardiomyopathy [11] that is essentially identical to that observed in humans inheriting a homozygous null *ANT1* mutation [10]. Systemic Ant1-deficient mice (*Ant1*−/−) are exercise intolerant in association with ragged red muscle fibers, massive proliferation of abnormal mitochondria, and marked elevation in muscle OXPHOS enzyme histochemistry. These mice also manifest metabolic abnormalities including elevated resting serum lactic acid and alanine and develop a progressive hypertropic cardiomyopathy [11]. The *Ant1*−/− mouse muscle and heart mitochondria also

produce elevated levels of H_2O_2, exhibit evidence of chronic oxidative stress, and have increased numbers of mitochondrial DNA (mtDNA) rearrangements [15].

In addition to being the ATP/ADP translocase, the ANTs may be important regulators of the mitochondrial permeability transition pore (mtPTP) which in turn regulates apoptosis [16, 17]. While inactivation of the *Ant1* and *Ant2* genes in mouse liver markedly decreased mtPTP calcium activation and rendered the mtPTP insensitive to modulation by adenine nucleotide levels or atractyloside, loss of Ant1 and Ant2 did not eliminate the ability of the liver mitochondria to undergo permeability transition [18]. Proteomic analysis of the mouse liver mitochondrial inner membrane revealed peptides for Ant2, Ant1, and a third Ant, presumably Ant4, in the ratio of approximately 6:3:1 [19]. However, western blot analysis did not detect *Ant4* mRNA in liver [13]. Therefore, Ant1 and/or Ant2 predominate in liver mitochondria and are important in regulating the liver mtPTP. Since over-expression of the human *ANT1* or *ANT3*, but not *ANT2*, genes have been shown to induce apoptosis in cultured human cells [20, 21], we can conclude that Ant1 (ANT1) regulates the mtPTP and thus apoptosis in both human and mouse.

The hyper-proliferation of the mitochondria in the skeletal muscle of the *Ant1*−/− mice has been correlated with an up-regulation of OXPHOS gene expression by differential display reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. This method identified 17 over-expressed genes in the mutant tissue *Ant1*−/− animals including components of the ETC as well as the muscle anti-apoptotic gene *Mcl-1* [22].

To obtain a more detailed picture of the effect of Ant1 deficiency on muscle physiology, we compared the gene expression profiles in *Ant1*−/− skeletal muscle with those in wild-type muscle using our custom cDNA microarrays. These MITOCHIPs contain either 644 or 1087 genes relevant to energy metabolism, mitochondrial function, oxidative stress, and apoptosis. We found that in *Ant1*−/− skeletal muscle 34 genes were up-regulated including a number of OXPHOS genes while 29 genes were down-regulated including genes for glycolysis. This was associated with an increase in the protein levels of a number of transcriptions factors associated with induction of muscle oxidative metabolism including peroxisome proliferator-activated receptor γ (PPARγ)-coactivator-1α (PGC-1α, human; Pgc-1α, mouse), nuclear regulatory factor-1 (Nrf-1), mitochondrial transcription factor A (Tfam), and myogenin. The cellular *Myelocytomatosis* proto-oncogene gene and protein (c-Myc) were down-regulated, which may have reduced the potential for induction of apoptosis. These results imply that in *Ant1*−/− mouse skeletal muscle, experience energy starvation, oxidative stress, and apoptotic risk, and attempt to compensate through induction of OXPHOS and antioxidant defenses and the down-regulation of apopotosis.

MATERIALS AND METHODS

Assembly of MITOCHIP arrays

A majority of the genes represented on the MITOCHIP arrays were selected and obtained from the NIA 15K mouse cDNA clone set [23]. The complete gene lists for MITOCHIP v3 and v4 are provided in Supplemental Table I. In addition, the arrays also contained the differential display products isolated by Murdock et al [22] as well as all 13 protein-coding

mtDNA genes and the 2 rRNA mtDNA genes that were amplified in-house. Other than the mtDNA genes, all the remaining cDNAs were amplified using the primers and PCR regimen described in NIA15K [23]. After amplification, the PCR products were purified using 96-well Qiagen PCR purification kits, and the dsDNA concentration was determined by fluorometry using the PicoGreen reagent (Invitrogen). The eluted pure PCR products were dried and reconstituted in 50% DMSO to a final DNA concentration of 75–100 ng/μl, and were then used for printing arrays. Arrays were printed using a GMS-412 arrayer, on poly-l-lysine coated glass slides. Poly-l-lysine coating, treatment and post-processing of slides were performed essentially as described by DeRisi et al [24], except that the succinic anhydride blocking step was omitted. The slides were prehybridized in a solution of 25% formamide, 5X SSC, 0.1% SDS and 1% BSA at 45°C for 45–60 min just before use, rinsed in water, dried by centrifugation at 500 rpm for 5 min and then used for hybridization.

Preparation of RNA

Total RNA was isolated from gastrocnemius muscles of wild type and mutant animals using Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. The total RNA thus isolated was then treated with RNase-free DNase I (Roche) for 30′ at room temperature to rid the sample of any contaminating DNA. After DNase I treatment, the RNA was purified using RNeasy kits (Qiagen), quantitated by spectrophotometry and used for labeling.

Hybridization of MITOCHIP arrays

25–30 μg of total RNA were labeled with either Cy3-dCTP or Cy5-dCTP by reverse transcription for 2 hrs at 42°C, in a mix containing 1X Superscript II Buffer (Invitrogen), 10mM DTT, 3 μg Oligo-dT, 200μM each of dATP, dGTP and dTTP, 100μM dCTP and 100μM Cy3-dCTP or Cy-5 dCTP (Amersham) and 400 units Superscript II (Invitrogen), with the final reaction volume being 35 μl. After reverse transcription the labeled cDNA was rid of RNA denaturation with 17.5 μl of 1.0 N NaOH at 70°C for 10′, neutralized with 17.5 μl 1.0 N HCl, and purified using PCR purification columns (Qiagen). Equal amounts (picomoles of incorporated labeled nucleotide) of wild-type and mutant labeled cDNA were mixed, dried under vacuum, resuspended in 22 μl of hybridization buffer containing 25% formamide, 5X SSC, 0.1%SDS, 1 μg polyA RNA (Sigma) and 1μg mouse Cot1 DNA (Invitrogen) and applied to the arrays. The arrays were incubated for 12–15 hrs at 55°C, and then washed three times at 55°C with 2X SSC+0.1% SDS, three times with 1X SSC+0.1% SDS at room temperature, and three times with 0.1X SSC at room temperature. The incubations and washes were carried out in a GeneMachines HybStation (Genomic Solutions). After the final wash the slides were dried by rinsing with isopropanol followed by centrifugation at 500 rpm for 5 min and scanned immediately.

Scanning and processing of data

The arrays were scanned in an Affymetrix scanner and quantitated using the Imagene 4.1 software suite (BioDiscovery). The data was then exported into the GeneSight Lite module (BioDiscovery) and normalized such that the sum of all ratios was equal to one. The normalized data were then exported into Microsoft Excel where the lists were sorted to exclude spots that were flagged and were below background intensity in each channel. The

ratios were then converted to Log_2 to provide a symmetrical distribution around one for positive and negative values and average + or − changes of greater than 1.5 obtained for both versions of the MITOCHIP or 1.7 for one version of the MITOCHIP were reported.

Western Blots

Total protein was isolated from gastrocnemius muscles of 9–12 month old wild-type and Ant1−/− mice. One gastrocnemius muscle was homogenized in 2 ml of a buffer containing 50 mM Tris pH 7.2, 100 mg/ml Sucrose, 0.1% SDS and 1 tablet/50 ml Complete Protease Inhibitor cocktail (Roche). The homogenate was spun at 1000 g for 10 min and the supernatant was carefully aspirated. Protein concentrations were determined using Bradford's reagent. μg of each protein sample were denatured in 1X (final conc) NuPage sample loading buffer (Invitrogen), run on 10% or 12% NuPage gels (Invitrogen), transferred to nitrocellulose membranes, stained with Ponceau S to ensure uniform loading and rinsed in 1X wash buffer (KPL). The blots were then blocked with 5% milk in 1X wash buffer for 1 hour, and incubated with primary antibody in 2% milk at 8–10°C overnight. The primary antibody was washed off with 5–8 washes over 30 min with 1X wash buffer, and the blots were incubated for 2 hours in 1X wash buffer containing the respective secondary antibody. The blots were washed 5–8 times over 30 min in 1X wash buffer, and developed using the ECL kit (Amersham) according to manufacturer's protocols.

Antibodies and concentrations

Horse radish peroxidase (HRP)-conjugated secondary antibodies goat anti-mouse (GAM), goat anti-rabbit (GAR) and rabbit anti-goat (RAG) were obtained from Santa Cruz Biotechnology. Primary antibodies and concentrations used to detect the various proteins were as follows: PGC-1α detected using PGC-1(P-19) or PGC-1(K-15) primary antibody (Santa Cruz Biotechnology sc-5815 and sc-5816 respectively) at 1:500 dilution, with RAG secondary at 1:5000; Nrf1 detected using anti-NRF1 serum (kindly provided by Dr. R.C. Scarpulla) at 1:5000 dilution with GAR secondary at 1:5000; Tfam detected using anti-Tfam polyclonal serum (kindly provided by Dr. Gerald Shadel) at 1:10,000 with GAR secondary at 1:5000; mt-COI detected using monoclonal antibody 1D6 (Molecular Probes) at 100 ng/ml with GAM secondary at 1:10,000; myogenin detected using M-225 primary antibody (Santa Cruz Biotechnology sc-576) at 1:500 with GAR secondary at 1:5000; Myoglobin detected using M-19 primary antibody (Santa Cruz Biotechnology sc-8081) at 1:500 with RAG secondary at 1:5000; TnI-slow detected using TnI SS C-19 primary (Santa Cruz Biotechnology sc-8119) at 1:1000 with RAG secondary at 1:5000; TnI-fast detected using TnI FS C-19 primary (Santa Cruz Biotechnology sc-8120) at 1:1000 and RAG secondary at 1:5000; Myc was detected using monoclonal anti-c-myc 9E10 (Sigma M5546) at 1:5000 with GAM secondary at 1:5000.

RESULTS

Gene expression differences between skeletal muscles from *Ant1*−/− and wild type mice were studied using two versions of our mouse MITOCHIP, MITOCHIP vM3 containing 772 elements (representing 644 genes) and MITOCHIP vM4 containing 1152 elements (representing 1089 genes). Four experiments were performed with each version of the

MITOCHIP. In each experiment, a pool of skeletal muscle RNA from at least 3 *Ant1*−/− mice was compared to a pool of skeletal muscle RNA from at least 3 wild-type mice. In all, tissues from 11 different mutant animals were compared with tissues from 11 different age-matched wild-type animals over the 8 experiments.

The differential fold-expression values for each gene from the four experiments using MITOCHIPv3 (Set 1) were averaged, as were those for the four experiments using MITOCHIP v4 (Set 2). Genes showing average expression differences of at least 1.5-fold in both Set 1 and Set 2 were considered to be significant. In instances where the gene was not represented in Set 1, an average differential expression of at least 1.7-fold in the Set 2 experiments was considered significant.

Genes up-regulated in *Ant1*−/− skeletal muscle

In all, 34 different mRNAs were found to be over-expressed in *Ant1*−/− skeletal muscle relative to wild type (Table I). These genes can be classified broadly into the following functional categories: (i) Genes encoding components of the mitochondrial OXPHOS machinery; (ii) Genes for other mitochondrial components; (iii) Genes whose products are enriched in slow-twitch (oxidative) muscle fibers; (iv) Genes for stress response, and (v) Miscellaneous genes. As can be seen from Table I, a number of the genes identified as up-regulated using the MITOCHIPs were also previously shown to be up-regulated by differential and northern analysis of mRNA from *Ant1*−/− skeletal muscle (18).

(i) OXPHOS Components—Sixteen of the 34 up-regulated genes encode subunits of the mitochondrial OXPHOS. Twelve of the 13 mRNAs encoded by the mtDNA were found to be strongly up-regulated in *Ant1*−/− muscle. Of the nuclear OXPHOS subunit mRNAs that are up-regulated, 3 belonged to complex I (*Ndufs4, Ndufb5 and Ndufc2*) while one belonged to complex IV (*Cox7a2*). Interestingly, *Ndufs4* has been shown to be essential for the assembly of Complex I in humans [25], while *Cox7a2* has been shown to be essential for assembly of Complex IV in yeast [26] and *Dictyostelium* [27]. The induction of mtDNA and nDNA OXPHOS proteins is consistent with the hyper-proliferation of mitochondrial and the increased complex II and IV staining in the *Ant1*−/− skeletal muscle [11, 15, 22].

One transcript encoded by the mtDNA, *cytochrome c oxidase I* (*mt-CO1*), yielded very poor signal on our arrays, though the reason for this is unclear. Since the mitochondrial DNA H-strand is transcribed as a polycistronic unit [28], it is unlikely that transcriptional differences can account for this discrepancy. However, certain mtDNA transcripts can have short half lifes [29] and thus the *mt-CO1* mRNAs may have a differential stability relative to other mtDNA-encoded mRNAs. Indeed a previous study found that *mt-CO1* mRNA levels were disproportionately regulated relative to other mtDNA and nDNA complex IV subunits in neurons [30].

To clarify the *mt-CO1* mRNA levels, we used Real-Time PCR and found that the *mt-COI* mRNA was increased approximately 1.6-fold in *Ant1*−/− skeletal muscle (not shown). The levels of COI protein were also increased in *Ant1*−/− skeletal muscle (Fig. 1D). Hence, all of the mtDNA encoded OXPHOS genes were up-regulated in the *Ant1*−/− muscle.

(ii) Other Mitochondrial Components—mRNAs for three mitochondrial transporters were also up-regulated in *Ant1−/−* skeletal muscle. *Slc40a1* (also called ferroportin) encodes a regulated iron transporter that appears to function mainly in the export of iron. Ferroportin over-expression has been shown to cause iron depletion in cultured cells [31] and mutations in ferroportin have been shown to cause diseases of iron overload in humans [32].

Timm8a and *Timm8b* encode components of the mitochondrial protein import machinery, essential for mitochondrial biogenesis. Mutations in *Timm8a* have been shown to cause deafness-dystonia-optic atrophy (Mohr-Tranebjaerg) syndrome in humans [33].

Five other mitochondrial components were over-represented in *Ant1−/−* skeletal muscle: three components of the mitochondrial ribosome [mtDNA 12S-rRNA (*mt-rnr1*), mtDNA 16S-rRNA (*mt-rnr2*) and mitochondrial ribosomal protein L52 (*Mrpl48*)], the TCA cycle enzyme malate dehydrogenase (*Mor2*), and mitofilin. Mitofilin is a mitochondrial inner membrane protein important in cristae structure, whose reduction leads to the collapse of the cristae network, increased membrane potential, and ROS production [34]. Mitofilin is also induced in brown adipose tissue when exposed to cold [35].

(iii) Oxidative Muscle Fiber-specific genes—The strong up-regulation of mitochondrial transcripts in *Ant1−/−* skeletal muscle indicates that the mutant muscle is attempting to compensate for the energy starvation by inducing OXPHOS. This notion is supported by the fact that the products of several over-expressed genes have also been shown to be enriched in slow-twitch or oxidative (Type I) muscle fibers. The skeletal muscle LIM domain protein (Fhl1), and cytoplasmic malate dehydrogenase (Mor2) have been shown to be over-expressed in soleus (an almost-exclusively oxidative, slow-twitch, muscle type) relative to the white quadriceps muscle (an almost exclusively glycolytic, fast-twitch, muscle type) [36]. Further, the Ca++ transporting ATPase subunit (Atp2a2) as well as the Na+/K+ transporting ATPase subunit (ATP1b1) have been shown to be enriched in oxidative, slow twitch, muscle fibers [37–39] relative to glycolytic, fast-twitch, muscle fibers.

(iv) Stress Response Genes—mRNAs encoding three different stress-response proteins were found to be over-expressed in *Ant1−/−* skeletal muscle. Of these, microsomal glutathione S-transferase mu (Mgst1) is a membrane bound protein that has been shown to have gluthione transferase and glutathione peroxidase activities [40], and to protect cells from oxidative stress [41, 42]. The other two stress response genes up-regulated in *Ant1−/−* skeletal muscle are *Kin 17* and *Mcl-1*. The Kin 17 protein is induced in response to UV and ionizing radiation [43, 44] and has been shown to bind to curved DNA spanning stretches of illegitimate recombination [45]. Mcl-1 is an anti-apoptotic protein of the Bcl-2 family [46] and protects cells from apoptosis under a variety of conditions [47]. Mcl-1 has also been shown to be induced in response to oxidative stress [48].

(v) Miscellaneous—The three remaining genes that are up-regulated in *Ant1−/−* skeletal muscle (*Nrbf1, Oaz3 and Crmp2*) do not fit into any specific functional category but are interesting nevertheless. Nrbf1 was identified in a two-hybrid screen as a PPAR-α

interacting protein. Nrbf1 was also shown to be able to interact with a variety of nuclear hormone receptors such as thyroid hormone receptor β (TRβ), retinoid acid receptor α (RARα), retinoid X receptor α (RXRα), and hepatocyte nuclear factor 4 (HNF4). Nrbf1 shares homology with the yeast protein MRF-1, a putative transcription factor regulating the expression of mitochondrial respiratory proteins [49]. Crmp2 (Collapsin mediated response protein 2) belongs to a family of molecules that are involved in axonal growth cone guidance [50], while *Oaz3* encodes an ornithine decarboxylase antizyme [51].

Genes Down-regulated in *Ant1−/−* skeletal muscle

In all, mRNAs from 29 different genes were found to be down-regulated in *Ant1−/−* skeletal muscle relative to wild type (Table II). These genes encompass a variety of functions including metabolic enzymes, pro-apoptotic genes, signaling molecules, transporters, and others. Two classes of these genes (metabolic enzymes and apoptotic genes) are particularly interesting in the context of this study and will be discussed in more detail below.

(i) Metabolic enzymes—Six metabolic enzyme genes were down-regulated in *Ant1−/−* skeletal muscle relative to wild type (Table II). Two of these are key glycolytic enzymes and thus genes, 6-phosphofructo 2-kinase (*Pfkfb3*) and Aldolase B (*Aldob*). Like mitochondrial proliferation, the down-regulation of these glycolytic genes may be indicative of the shift of the skeletal muscle in *Ant1−/−* animals to a more oxidative physiology. To explore this possibility further, we tabulated the relative expression levels of all the genes involved in glucose metabolism across our eight MITOCHIP experiments (Table III).

As can be seen from Table III, genes encoding glycolytic enzymes appear generally down-regulated in *Ant1−/−* skeletal muscle. In addition to Pfkfb3 and AldoB mentioned above, the genes for Aldolase I (*Aldoa*), Triosephosphate isomerase (*Tpi1*) and Enolase I (*EnoI*) were all down-regulated at least 1.4-fold. Also, *Ldh1* (encoding lactate dehydrogenase A chain which converts pyruvate to lactate) and *Aat2* (muscle specific alanine aminotransferase which converts pyruvate to alanine) were down-regulated by 1.5 and 1.4-fold, respectively.

The MITOCHIP results also suggest that the *Ant2* gene may have been down-regulated, though the very low expression of this gene in skeletal muscle [11, 12] renders any estimate of extent of change immaterial. What is clear is that *Ant2* was NOT up-regulated in the *Ant1−/−* muscle. This has been confirmed for both *Ant2* mRNA in Northern blots and Ant2 protein in western blots (see Figure 3 c and d, respectively in [11]). The absence of induction of the *Ant2* gene in the face of Ant1 deficiency initially seemed counter-institutive, since the simplest way to resolve the Ant1 deficiency would seem to have been induction of another Ant isoform that would complement the defect. Since the *Ant2* gene is the only other systemic mouse Ant isoform, induction of this gene would have been the logical choice. One possible explanation for this anomaly could be that the mouse Ant2 protein has a similar function and properties as the human ANT2 protein. In contrast to ANT1 and ANT3, human ANT2 has been proposed to import cytosolic ATP into the mitochondria during periods of mitochondrial dysfunction, for example in hypoxia [5, 6]. If this is true for the mouse Ant2, then up-regulation of the Ant2 protein would not

complement the Ant1 deficiency, but rather make the sarcoplasmic ATP deficiency worse. This would also explain the very low expression of Ant2 in skeletal muscle, where ATP would consistently need to be transported out of the mitochondrion to support muscle contraction and plasma membrane transport. Similarly, if Ant2 functions to import ATP into the mitochondria during hypoxia, then the gene should be induced during hypoxia, not hyperoxia as would be the situation in *Ant1*−/− skeletal muscle. Therefore, the lack of induction of *Ant2* may reflect its converse function, relative to Ant1, rendering it both biochemically unable to complement Ant1 deficiency and inappropriately regulated to do so.

Table III also shows that mRNAs for several enzymes of the TCA cycle are moderately up-regulated in *Ant1*−/− skeletal muscle. This is consistent with the mitochondrial proliferation and associated induction of mitochondrial energy production in an effort to compensate for the cytosolic ATP deficiency.

Interestingly, *G6pdx* [glucose-6-phosphate dehydrogenase, which catalyzes the first and rate-limiting step of the Pentose Phosphate Pathway (PPP)] is over-expressed 1.5-fold in Ant1−/− skeletal muscle. The PPP provides much of the reducing power in the cell in the form of NADPH, and NADPH is important for maintaining reduced glutathione. Since flux through the PPP and the rates of NADPH production are directly proportional to glucose-6-phosphate dehydrogenase activity; then increased *G6pdx* expression may be is indicative of an increased demand for NADPH to manage oxidative stress.

The three remaining down-regulated genes were *Ppox*, *Cyp11a1*, *and Odc1*. The *Ppox* gene encodes the enzyme protoporphyrinogen oxidase, which catalyzes the penultimate reaction in heme biosynthesis. Mutations in the *Ppox* gene have been shown to cause variegated porphyria in humans [52]. The *Cyp11a1* gene encodes the cytochrome P450 side chain cleaving enzyme that catalyzes the first step of cholesterol biosynthesis [53]. The *Odc1* gene encodes ornithine decarboxylase, the key enzyme in the biosynthesis of polyamines which play central roles in cell proliferation and survival [54]. Down-regulation of *Odc1* should cause the down-regulation of polyamine biosynthesis in *Ant1*−/− skeletal muscle.

(ii) Genes that regulate apoptosis—Several genes that encode pro-apoptotic genes were also down-regulated in *Ant1*−/− skeletal muscle including *Myc*, Modulator of apoptosis *(Moap1)*, Bcl2-interacting killer *(Bik)*, Chk2 checkpoint homolog *(Chek2)*, and Phospholipase A2, group VI *(Pla2g6)*. This suggests that the apoptotic threshold may be higher in the *Ant1*−/− skeletal muscle. The *Myc* oncogene regulates two complex and seemingly contradictory sets of regulatory pathways. While *Myc* over-expression or activation has been shown to be a key event in many cancers, *Myc* over-expression can also activate apoptotic pathways in a normal cell under conditions of serum deprivation or DNA damage [55]. *Myc* has been shown to promote cell death by suppressing the activity of the Cdk-inhibitor p21/Cip1 [56] and thus shifting the choice of cellular DNA damage response from cytostatic to apoptotic. The down-regulation of *Myc* in *Ant1*−/− skeletal muscle was also confirmed at the protein level by Western blotting (Fig. 1F).

The *Moap1* gene was identified in a two-hybrid screen as encoding a protein that interacts with the apoptosis effector Bax. *Moap1* has been shown to cause caspase-dependent apoptosis when over-expressed in cell culture [57, 58].

The *Bik* gene also encodes a potent death agonist. Over-expression of *Bik* causes caspase-dependent apoptosis in cell culture, and its apoptotic activity can be suppressed by co-over-expression of *Bcl-2* or *Bcl-XL* [59]. The mouse *Chek2*, is required for p53 mediated DNA-damage response. Analyses of *Chek2−/−* mice have revealed that Chek2 is required for p53 mediated apoptosis in response to ionizing radiation. Thus, Chek2, like Myc, also appears to be involved in shifting the choice of the p53 pathway from cytostatic to apoptotic [60]. Finally, Pla2g6 has been shown to mediate Fas-induced cell death by causing the release of arachidonic acid. Pla2g6 may also be involved in regulating ion-channel activity and membrane phospholipids remodeling during apoptosis and inhibition of Pla2g6 has been shown to decrease Fas-induced cell death [61].

(iii) Other genes under-expressed in Ant1−/− muscle—As can be seen from Table II, several kinases, signaling molecules, transporters and other genes are down-regulated in *Ant1−/−* skeletal muscle. It is difficult to classify these genes further from a standpoint of metabolic pathways. However some of them do have common regulatory links. The genes for microtubule associated protein 4 (*Mtap4*) and heat shock protein 90 (*Hsp86-1*) (as well as *Odc1* and several glucose metabolism genes) are known targets of Myc [62, 63]. Thus their down-regulation may be a consequence of the down-regulation of Myc in *Ant1−/−* skeletal muscle. Also, some of the down-regulated mRNAs may also be ones that are normally enriched in glycolytic muscle fibers, and their reduced expression may also be a result of the change in the metabolic nature of the muscle fibers in *Ant1−/−* animals.

Molecular basis of the mitochondrial proliferation in *Ant1−/−* skeletal muscle

Mitochondrial proliferation and changes in muscle fiber physiology and composition can be triggered by endurance training and exercise [64]. This process has been shown to be mediated by the PPARγ associated transcriptional coactivator-1α (PGC-1α[65, 66]. PGC-1α has also been shown, in ectopic expression studies, to be a master regulator of mitochondrial proliferation and muscle fiber-type switching [67] acting through regulation of transcription factors required for mitochondrial proliferation such as Nrf1 and Tfam [66].

Since we see marked mitochondrial proliferation as well as over-expression of several slow-twitch muscle fiber markers in *Ant1−/−* muscle, we examined whether these changes were also reflected the PGC-1α transcription pathway. As can be seen in Figure 1A–C, the levels of Pgc-1α Nrf1 and Tfam proteins were all elevated in *Ant1−/−* skeletal muscle, indicating that the mitochondrial proliferation observed in *Ant1−/−* skeletal muscle is mediated by the PGC-1αpathway. Interestingly, the levels of *Pgc-1α* mRNA appear to be slightly lower in *Ant1−/−* skeletal muscle relative to wild-type, as assayed by real-time PCR (not shown). However, Pgc-1α can be regulated by either transcriptional and/or post-transcriptional mechanisms [68–75].

To investigate whether the observed changes in *Ant1−/−* skeletal muscle were due to changes in fiber-type composition of the muscle (from fast-twitch to slow-twitch), or simply

due to the metabolic nature of the muscle fibers, we examined the levels of myoglobin, Troponin I-slow (Tnni1; normally enriched in slow-twitch muscle fibers) and Troponin I-fast (Tnni2; normally enriched in fast-twitch muscle fibers) in *Ant1*−/− skeletal muscle. As can be seen from Figures 1G-I, the levels of Tnni1, Tnni2 and myoglobin are not significantly different in *Ant1*−/− skeletal muscle compared to wild-type. Immunohistochemical staining for different myosin heavy chain (MHC) isoforms also showed no significant change in the levels or distribution of the slow-twitch specific MHC isoforms in *Ant1*−/− skeletal muscle (A. Flierl and DC Wallace, unpublished results). While elevated expression of PGC-1α can induce a change in muscle-fiber type, it need not necessarily do so. Indeed, over-expression of one of the targets of PGC-1, myogenin, has been shown to promote increased oxidative metabolism in skeletal muscle fibers without effecting a complete change in muscle fiber type [76]. As can be seen in Figure 1E, levels of myogenin are significantly elevated in *Ant1*−/− skeletal muscle compared to wild-type. These results are all consistent with the idea that the skeletal muscle fibers in *Ant1*−/− animals adopt a more oxidative metabolism without changing the fiber-type composition of the muscle.

DISCUSSION

The skeletal muscle of *Ant1*−/− mice presents a fascinating regulatory conundrum from the standpoint of energy metabolism. On the one hand, the complete deficiency of Ant1 blocks virtually all transport of mitochondrial ATP into the cytosol, thus creating a severe energy deficiency within the nuclear-cytosol compartment of the muscle cells. As a result, there is little ATP for sarcoplasmic muscle contraction, ion transport, biogenesis, etc. causing severe exercise intolerance in the mice [11].

On the other hand, mitochondrial OXPHOS is fully functional and is even strongly up-regulated, as evidenced by the hyper-proliferation of skeletal muscle mitochondria and the strong induction of succinate dehydrogenase (complex II) and complex IV. Hence, the intra-mitochondrial ATP level would be expected to be near maximum. Indeed, the high resting serum lactate and alanine levels are indicative of a ‘stalled’ mitochondrial OXPHOS and TCA cycle. This would be the result if the mitochondrial matrix adenine nucleotides were maximally phosphorylated, thus depriving the ATP synthase of its ADP substrate. In the absence of ADP, the flux of protons through the ATP synthase proton channel would stop, and the mitochondrial membrane potential would remain hyper-polarized, blocking further proton pumping and stalling the ETC. Inhibition of the ETC, would also stall the TCA cycle, resulting in the accumulation of cytosolic pyruvate and NADH + H^+, which would be converted to lactate and alanine [11].

With the mitochondrial ETC stalled, in the present of unlimited calories, the electron carriers would become fully reduced. Given a hyperoxic state, the excess electrons could readily be transferred directly to O_2 to generate superoxide anion and the other ROS. The chronically elevated ROS would then damage the mitochondria and mtDNA in skeletal muscle and heart [15].

To investigate the effects of this metabolic conundrum on the molecular circuitry of energy metabolism, we employed the MITOCHIP to analyze changes in the transcript levels of over 1000 genes that are associated with energy metabolism, ROS biology, and apoptosis. These mRNA analyses were complemented by examining the protein levels of transcription factors known to regulate the expression of energy, antioxidant, and apoptotic genes. Our results revealed that the *Ant1*−/− skeletal muscle: (i) Is starved for energy and is attempting to compensate for this energy deficit by inducing oxidative metabolism while down regulating glycolysis; (ii) Is under oxidative stress and is attempting to combat this stress by up-regulating antioxidant defenses; (iii) Is inducing oxidative metabolism and antioxidant defenses through increased levels of nuclear (Pgc-1α, Nrf1, and myogenin) and mitochondrial (Tfam) DNA transcription factors; (iv) Is at increased risk for apoptosis and is compensating by up-regulating anti-apoptotic and down-regulating pro-apoptotic genes; and (v) Appears to be down-regulating the apoptotic pathways by decreased c-Myc.

The Metabolic Reorganization of Ant1−/− Mouse Skeletal Muscles

Previously, we had shown that as human myoblasts differentiate into myotubes, the expression of mtDNA and nDNA-encoded OXPHOS genes and of nDNA-encoded glycolytic genes change expression reciprocally. When myoblasts are differentiated in normoxic conditions, OXPHOS genes are up-regulated in myoblasts, down-regulated after commitment to differentiation, and up-regulated again in myotubes. *ANT1* is not expressed in myoblasts but is progressively induced as myotubes are formed. By contrast, the glycolytic genes are down-regulated in myoblasts, up-regulated after commitment, and decline as myotubes are formed. If the myoblasts are differentiated into myotubes under hypoxic condition, however, the glycolytic gene expression levels remain high in the myotubes. As a consequence, by seven days after commitment the glycolytic mRNAs have increased six fold relative to OXPHOS transcripts [77].

While the ETC is stalled in the *Ant1*−/− mouse skeletal muscle, the cardiovascular system remains functional. Therefore, oxygen continues to be delivered to the muscle but can not be consumed by the mitochondria OXPHOS. Hence, the *Ant1*−/− muscle should become hyperoxic. In the hyperoxic state *Ant1*−/− muscle is attempting to compensate for the cytosolic ATP deficiency by up-regulating mitochondrial OXPHOS. However, this is futile due to the absence of Ant1, resulting in uncontrolled proliferation of the mitochondria [11]. All the mtDNA OXPHOS genes as well as four nuclear-encoded OXPHOS genes are significantly up-regulated in the Ant1 deficient muscle, with two of the nuclear encoded proteins, Ndufs4 and Cox7a2, being involved in the assembly of complex I and complex IV, respectively [25–27]. The fact that major up-regulated nuclear OXPHOS genes are involved in complex assembly suggests that most nuclear-encoded components of the ETC are not normally limiting in muscle and that the number of functional ETC complexes may be regulated at the level of complex assembly.

The up-regulation of several oxidative muscle fiber-specific marker genes including *Fhl1*, *Mor2*, *Atp2a2*, and *Atp1b1* is also consistent the *Ant1*−/− muscle's attempting to compensate for the energy deficiency by up-regulating oxidative metabolism. However, this is not associated with fiber type switching, since the myoglobin levels and troponin I and

myosin heavy chain isoforms did not change. Hence, the primary response to the reduction in the sarcoplasmic ATP appears to be the up-regulation of mitochondrial OXPHOS.

Assuming that mouse and human muscle are under similar regulation, we would expect that the induction of mitochondrial OXPHOS should be associated with the reciprocal down-regulation of glycolysis, which we observed. Both the 6-phosphofructo-2-kinase (*Pfkfb3*) and aldolase B genes were strongly down-regulated, in parallel with the down regulation of several other glycolytic genes including the lactate dehydrogenase A (*Ldh1*) and muscle-specific alanine aminotransferase (*Aat2*) genes. Therefore, the "standard" muscle response to low sarcoplasmic ATP in the presence of normoxia is induction of OXPHOS and reduction of glycolysis.

The "standard" program for energy deficient muscle under normoxia proves to be exactly the wrong response when the cytosolic ATP deficiency is caused by Ant1 deficiency. The only source of ATP for the cytosol is then glycolysis. Since carbohydrates are not limiting, the resulting flux of carbohydrates through glycolysis is high. With OXPHOS and the TCA cycle stalled, pyruvate and NADH + H^+ accumulate and are combined by lactate dehydrogenase to generate lactate. Since lactate is both the end product of glycolysis but also a substrate for OXPHOS, it is unclear if the *Ant1*−/− muscle would strive to export the excess lactate to control intra-cellular pH or import the lactate in a further attempt to bolster mitochondrial ATP production. Insight into this question might be obtained by analyzing the expression of the proton-linked monocarboxylate transporter (MCT) isoforms. Isoform MCT1 is expressed in Type I oxidative fibers and is thought to import lactate into muscle cells to be oxidized by OXPHOS, while isoform MCT4 is present in all muscles but at lower levels in oxidative muscle and is thought to export excess glycolytic lactate [78].

An alternative approach to increasing sarcoplasmic ATP would be to induce an alternative adenine nucleotide translocator, thus restoring the export of the excess mitochondrial ATP. Ant2 would be the logical choice, since it is expressed in virtually every tissue. However, Ant2 was NOT up-regulated [11]. This anomaly might be explained if Ant2 functions like the human ANT2 to import cytosolic ATP into the mitochondrion under anoxic conditions [5, 6]. If this is the case, then Ant2 should be induced under anoxic conditions and repressed under normoxic conditions, which is consistent with what we observed in the *Ant1*−/− mouse muscle.

Given the high rate of ROS production by the *Ant1*−/− muscle [15], we would expect the mitochondrial antioxidants defenses to be induced. While the steady state mRNA levels for the mitochondrial antioxidant genes were only mildly elevated, western blot analysis revealed that mitochondrial manganese superoxide dismutase (MnSOD) is induced six fold and glutathione peroxidase-1 is induced three fold in the *Ant1*−/− muscle [15]. In addition, the MITOCHIP revealed other stress-response genes were up-regulated in *Ant1*−/− skeletal muscle: *Mgst1*, *G6pdx*, and *Kin17*. *Mgst1* is involved in protecting membranes and cells from lipid peroxidation damage, consistent with high oxidative stress. Glucose-6-phosphate dehydrogenase (*G6pdx*), the rate-limiting enzyme in the pentose phosphate pathway (PPP) and the primary source of NADPH, would be important in sustaining glutathione pathways

to detoxify peroxides. The DNA damage caused by chronic oxidative stress [15], could be amelioated by the induction of the *Kin17* DNA repair gene [43, 44].

Excessive oxidative stress and DNA damage make cells more prone to apoptosis. While the importance of the apoptotic pathway in muscle is still unclear, it has been implicated skeletal muscle denervation and remodeling, muscular dystrophy, and muscle aging [79–82]. H_2O_2 promotes apoptosis via activation of the MAP kinase and JNK pathways and subsequent inactivation of *Mcl-1* [83], the muscle isoform of the Bcl-2 family [84]. Therefore, the up-regulation of *Mcl1* indicates hat the *Ant1*−/− skeletal muscle is attempting to dampen the pro-apoptotic response. The down-regulation of the pro-apoptotic *Bik*, *Moap1*, *Chek2*, and *Pla2g6* genes would further impede apoptosis. Finally, the down-regulation of the ornithine decarboxylase-1 gene (*Odc1*) indicates a down-regulation of polyamine biogenesis, which could also inhibit apoptosis by blocking the translocation of BAX into mitochondria [85].

Transcription Factor Regulation of the Energy deficiency in the Ant1−/− Muscle

Having established that the *Ant1*−/− muscle attempts to compensate for cytosolic ATP deficiency by up-regulating OXPHOS, and knowing that PGC-1α is implicated in the regulation of nDNA encoded mitochondrial genes, we examined the protein levels of Pgc-1α. Pgc-1α induces mitochondrial biogenesis in C2C12 cultured mouse muscle cells, acting through the nuclear respiratory factors 1 and 2 (NRF1 and 2) [66, 86] and Pgc-1α null mice show a reduction in MnSOD and Gpx1 [87]. Furthermore, transgenic mice over-expressing PGC-1α mRNA 16-fold exhibit a marked increase in exercise capacity and peak oxygen uptake; an increase in skeletal muscle oxidative metabolism proteins in including cytochrome c, various OXPHOS complex subunits, fatty acid oxidation enzymes; a 166% increase in mtDNA and up-regulation of Tfam transcription factor; and an induction of the antioxidant enzymes MnSOD, thioredoxin reductase-2, and catalase [75]. Since we observed a marked up-regulation of the Pgc-1α protein in the *Ant1*−/− skeletal muscle, we can infer that the up-regulation of mitochondrial OXPHOS and the increased antioxidant defenses in the *Ant1*−/− mice is a consequence of the increased levels of Pgc-1α protein. Up-regulation of Pgc-1α results in the up-regulation of the mtDNA transcription factor *Tfam*, which would increase mtDNA transcript levels. The muscle's mitochondrial oxidative capacity can also be up-regulated by myogenin [76]. Similarly, up-regulation of Nrbf1, which interacts with PPARα as well as other transcription factors known to up-regulate mitochondrial gene expression, might also be a contributing factor [49].

Inhibition of apoptosis may have been achieved by down-regulation of c-Myc, which was seen by both the MITOCHIP and protein analyses. Over-expression of c-Myc has been shown to promote apoptosis in cultured cells under conditions of serum deprivation and oxidative stress [88, 89], proceeding via release of mitochondrial cytochrome c and activation of pro-apoptotic caspases [90]. c-Myc has also been shown to regulate some Nrf1 target genes and promote apoptosis by de-regulating genes involved in mitochondrial function [91]. In a normal Myc expression background, Nrf1 over-expression has been shown to trigger apoptosis in serum-deprived cells [91]. In the skeletal muscle of *Ant1*−/− mice Nrf1 levels and mitochondrial proliferation are increased, but c-Myc is down-regulated. Therefore, the down-regulation of c-Myc may be counteracting the pro-apoptotic

tendency of increased Nrf1 levels. The down-regulation of c-Myc might also be important in the down-regulation of ornithine decarboxylase (*Odc1*), since *Odc1* is a target gene of c-Myc. The down-regulation of *Odc1* should diminish polyamine synthesis, inhibit the translocation of BAX into mitochondria, and repress apoptosis [85]. The inhibition of BAX translocation would also be augmented by the down-regulation of the BAX interacting Moap1 protein [57, 58].

Other c-Myc target genes that are down-regulated include *Mtap4* and *Hsp86-1*. Myc can also regulate the expression of several glycolytic genes [63] which account for the down-regulation of *Ldh1*, and *EnoI* which are known to be positively regulated by *Myc* [62, 92]. However, *Pfk1* and *Gapd* should also have been down-regulated but were not [63]. Overall, *Myc* down-regulation may have been the mechanism by which apoptosis was suppressed in the *Ant1*−/− skeletal muscle in the presence of high oxygen.

MITOCHIP Monitoring of Mitochondrial Function and for Drug Screening

Our mouse MITOCHIP is proving to be a powerful tool for analyzing the pathophysiology of mouse models of mitochondrial disease [93]. When complemented by analysis for key regulatory protein levels, the MITOCHIP and similar technologies are providing much needed readouts for identifying new mitochondrial therapeutic agents [94, 95].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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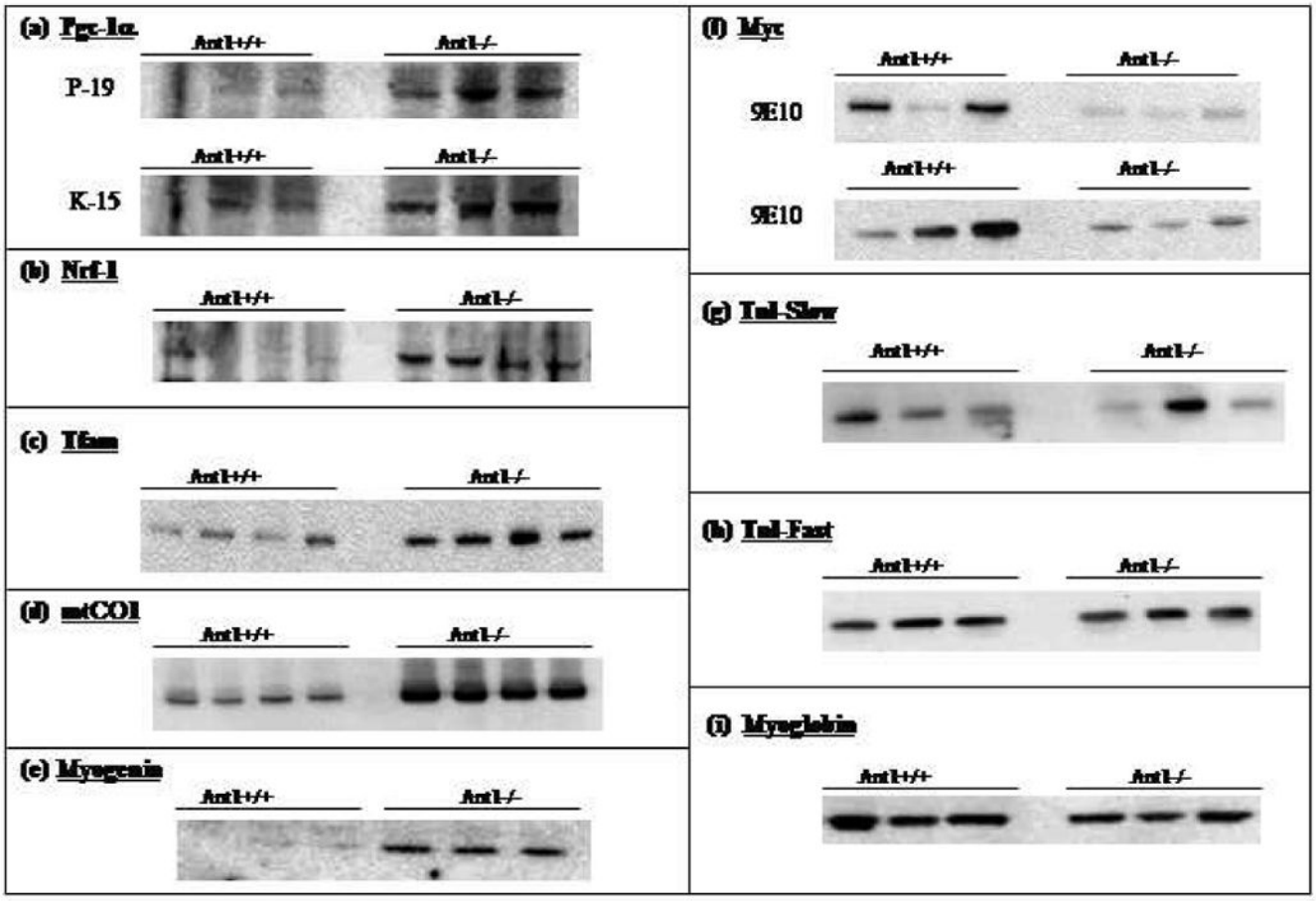

Figure 1.

Proteins that regulate mitochondrial proliferation are over-expressed in *Ant 1−/−* skeletal muscle. Western blots showing levels in Ant1+/+ and Ant1−/− skeletal muscle of the following proteins: (a) Pgc-1α detected using antibody P-19 (amino terminus-specific) and K-15 (carboxy terminus-specific), (b) Nrf-1, (c) Tfam, (d) mtCO1, (e) Myogenin, (f) Myc (two different blots, both probed with antibody 9E10, (g) Tnni1, (h) Tnni2, and (i) Myoglobin.

Table I

Genes over-expressed in *Ant1*−/− skeletal muscle. NP = Not present in that version of the MITOCHIP.

GENE	SET 1	SET 2
mtDNA OXPHOS subunits		
mtDNA-ATPase 6	**2.2**	**2.9**
mtDNA-ATPase 8	**3.3**	**3.1**
mtDNA-COX 2*	**1.5**	**1.6**
mtDNA-COX 3*	**2.9**	**2.9**
mtDNA-Cyt b	**3.1**	**3.9**
mtDNA-NADH 1*	**1.8**	**2.6**
mtDNA-NADH 2*	**2.6**	**2.3**
mtDNA-NADH 3	**2.5**	**1.6**
mtDNA-NADH 4*	**2.4**	**2.2**
mtDNA-NADH 4L*	**2.1**	**3.3**
mtDNA-NADH 5*	**1.9**	**2.9**
mtDNA-NADH 6*	**2.2**	**3.0**
DD69 (mtDNA_ND5)*	**2.2**	**1.7**
Nuclear OXPHOS subunits		
NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase) *(Ndufs4)**	**1.9**	**1.9**
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 (16kD) *(Ndufb5)*	**1.6**	**1.7**
NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2 (14.5kD) *(Ndufc2)*	**1.6**	**1.5**
Cytochrome c oxidase, subunit VIIa 2 *(Cox7a2)*	**1.5**	**1.8**
Other Mitochondrial Components		
Solute carrier family 40 (iron-regulated transporter), member 1 *(Slc40a1)*	**2.1**	**1.5**
Translocase of inner mitochondrial membrane 8 homolog b *(Timm8b)*	**1.6**	**1.7**
Mitochondrial Import Inner Membrane Translocase subunit TIM8 A (Deafness Dystonia Protein Homolog)	**NP**	**1.6**
mtDNA-12s rRNA	**3.6**	**3.5**
mtDNA-16s rRNA	**2.5**	**2.5**
Mitochondrial ribosomal protein L48 *(Mrpl48)**	**2.7**	**1.7**
Inner membrane protein, mitochondrial (Immt, mitofilin)	**2.4**	**1.6**
Malate dehydrogenase, mitochondrial	**1.8**	**1.6**
Gene products enriched in Oxidative Muscle Fibers		
Skeletal Muscle LIM protein (*Fhl1*)	**1.8**	**1.6**
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 *(Atp2a2)*	**1.5**	**1.5**
Mus musculus ATPase, Na+/K+ transporting, beta 1 polypeptide *(Atp1b1)*	**1.5**	**1.6**
Mus musculus malate dehydrogenase, soluble *(Mor2)**	**1.5**	**1.9**
Stress response		
Microsomal glutathione S-transferase 1 *(Mgst1)*	**1.6**	**1.5**
Antigenic determinant of RecA protein *(Kin 17)**	**NP**	**2.8**
Myeloid cell leukemia sequence 1 *(Mcl1)**	**1.5**	**1.5**

GENE	SET 1	SET 2
Miscellaneous		
Nrbf1	**NP**	**2.7**
Ornithine decarboxylase antizyme 3 (Oaz3)	**1.7**	**2.7**
Homolog of Unc33/Collaspin reponse mediated protein 2 (Crmp2)	**3.1**	**3.5**

* Also identified as up-regulated by Murdock *et al* [22]

Table II

Genes under-expressed in *Ant1*−/− skeletal muscle. NP = Not present on that version of the MITOCHIP.

GENE	SET 1	SET 2
Metabolic enzymes		
Inducible 6-phosphofructo-2-kinase (Pfkfb3)	**NP**	**−3.0**
Aldolase B	**−8.2**	**−2.7**
Protoporphyrinogen oxidase (Ppox)	**−2.0**	**−1.5**
Cytochrome P450 side chain cleavage enzyme 11a1 (Cyp11a1)	**NP**	**−1.9**
Ornithine decarboxylase antizyme 2 (Oaz2)	**−1.6**	**−1.6**
Apoptosis effectors		
Modulator of Apoptosis (Moap1)	**NP**	**−1.8**
Bcl2-interacting killer-like (Biklk), mRNA	**NP**	**−1.8**
Myelocytomatosis oncogene (Myc)	**NP**	**−1.9**
RAD53 homolog (Chek2)	**NP**	**−1.8**
Phospholipase A2, group VI (Pla2g6)	**NP**	**−2.4**
Myc target genes		
N-myc downstream regulated 1 (Ndr1)	**NP**	**−2.2**
Ornithine decarboxylase, structural (Odc)	**−3.2**	**−2.3**
Transporters		
Bumetanide-sensitive Sodium-(Potassium)-Chloride Cotransporter 1 (Slc12a2)	**−1.6**	**−1.5**
Solute carrier family 30 (zinc transporter), member 3 (Slc30a3)	**−1.6**	**−1.6**
Adenine nucleotide translocase-2 (Ant2)	**−3.4**	**−3.4**
Kinases		
LIM motif-containing protein kinase 2 (Limk2)	**NP**	**−1.7**
Casein kinase I alpha isoform (CSNK1A1)	**NP**	**−1.9**
Casein kinase 1, epsilon (Csnk1e)	**NP**	**−1.9**
Homeodomain interacting protein kinase 3 (Hipk3)	**−2.0**	**−1.7**
Mitogen activated protein kinase kinase kinase 12 (Map3k12)	**NP**	**−1.9**
Rho-associated coiled-coil forming kinase 1 (Rock1)	**NP**	**−1.7**
Signalling		
Protein tyrosine phosphatase, non-receptor type 21 (Ptpn21)	**NP**	**−2.0**
Mothers against Dpp homolog 1 (Madh1)	**NP**	**−1.7**
Signal transducer and activator of transcription interacting protein 1 (Statip1)	**NP**	**−1.7**
Activin receptor IIB (Acvr2b)	**NP**	**−1.7**
Heat Shock Proteins		
Crystallin, alpha C (Hsp 20)	**NP**	**−1.8**
Heat shock protein, 86 kDa 1 (Hsp86-1)	**NP**	**−1.7**
Other		
Ubiquinol-cytochrome c reductase core protein 1 (Uqcrc1)	**−1.6**	**−1.6**
Microtubule-associated protein 4 (Mtap4)	**NP**	**−1.9**

NP: Not present on the array.

Table III

Expression of glucose metabolism genes in *Ant1*−/− skeletal muscle.

GENE	Mean Fold Expression
Glycolysis and Pyruvate Metabolism	
Mus musculus phosphofructokinase, muscle (Pfkm)	−1.1
Mus musculus inducible 6-phosphofructo-2-kinase (Pfkfb3)	**−2.9**
Mus musculus aldolase 1, A isoform (Aldoa)	**−1.6**
Aldolase B (Aldob)	**−3.4**
Mus musculus triosephosphate isomerase (Tpi1)	**−1.5**
GAPDH	−1.3
Mus musculus phosphoglycerate mutase 1 (Pgam1)	1.0
3-Phosphoglycerate dehydrogenase	−1.3
Mus musculus enolase 1, alpha non-neuron (Eno1)	**−1.4**
Mus musculus pyruvate kinase 3 (Pk3)	−1.3
Mus musculus lactate dehydrogenase 1, A chain (Ldh1)	**−1.5**
Alanine aminotransferase	1.2
Alanine aminotransferase 2 (muscle specific)	**−1.4**
Pentose Phosphate Pathway	
Mus musculus glucose-6-phosphate dehydrogenase X-linked (G6pdx)	**1.5**
Tricarboxylic acid cycle	
Isocitrate dehydrogenase 2 (NADP+), mitochondrial (Idh2)	1.2
Isocitrate dehydrogenase 3 (NAD+) alpha (Idh3a)	1.2
Aconitase 2, mitochondrial (Aco2)	1.1
ATP-specific Succinyl-CoA synthetase beta subunit	1.2
Succinate dehydrogenase (Ubiquinone) Flavoprotein subunit, Mitochondrial	1.4
SDHD gene_Small subunit of Cytochrome b of Succinate dehydrogenase	1.5
Fumarate hydratase (FH)	1.4
Malate dehydrogenase, mitochondrial (DD67)	1.6