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Role of adiponectin in human skeletal muscle bioenergetics

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Summary

Insulin resistance is associated with impaired skeletal muscle oxidation capacity and reduced mitochondrial number and function. Here, we report that adiponectin signaling regulates mitochondrial bioenergetics in skeletal muscle. Individuals with a family history of type 2 diabetes display skeletal muscle insulin resistance and mitochondrial dysfunction; adiponectin levels strongly correlate with mtDNA content. Knockout of the adiponectin gene in mice is associated with insulin resistance and low mitochondrial content and reduced mitochondrial enzyme activity in skeletal muscle. Adiponectin treatment of human myotubes in primary culture induces mitochondrial biogenesis, palmitate oxidation, and citrate synthase activity, and reduces the production of reactive oxygen species. The inhibition of adiponectin receptor expression by siRNA, or of AMPK by a pharmacological agent, blunts adiponectin induction of mitochondrial function. Our findings define a skeletal muscle pathway by which adiponectin increases mitochondrial number and function and exerts antidiabetic effects.

Introduction

As a consequence of the increased prevalence of obesity, type 2 diabetes (T2D) has become a global epidemic, with more than 300 million individuals worldwide projected to be afflicted with T2D by the year 2025 (Zimmet et al., 2001). Although the primary cause of T2D is unknown, it is clear that insulin resistance in skeletal muscle and liver plays a role in its pathogenesis (Lillioja et al., 1993). Insulin resistance in offspring of parents with type 2 diabetes is a strong predictor of the development of the disease later in life (Warram et al., 1990). Obese individuals and subjects with T2D are characterized by reduced adiponectin signaling (ligand and receptor; Civitarese et al., 2004; Kern et al., 2003; Rasmussen et al., 2006) and lower rates of fasting lipid utilization and impaired switch to carbohydrate oxidation in response to insulin (Kelley et al., 1999; Kelley and Mandarino, 2000). Recent evidence indicates that insulin resistance in skeletal muscle maybe be due to reduced mitochondrial oxidative capacity, fat metabolism (Kelley et al., 2002; Petersen et al., 2004), and insulin-stimulated ATP production (Petersen et al., 2005).

Bjorntorp and coworkers were among the first to demonstrate in the 1960s that mitochondria isolated from T2D subjects had decreased oxidative capacity (Bjorntorp et al., 1967). Recent studies using microarray expression analyses reported a decrease in the expression of genes encoding proteins of mitochondrial oxidative phosphorylation in the skeletal muscle of subjects with insulin resistance (Patti et al., 2003) and T2D (Mootha et al., 2003). Further studies in insulin-resistant individuals and in subjects with T2D have shown reduced mitochondrial content (Kelley et al., 2002) and lower electron transport chain (ETC) activity in total (Kelley et al., 2002; Petersen et al., 2003, 2004), intramyofibular and subsarcolemal mitochondrial fractions (Ritov et al., 2005). Taken together, these data support the hypothesis that insulin resistance in human muscle arises from defects in mitochondrial number and function.

The molecular mechanism(s) leading to mitochondrial dysfunction in obesity and T2D remain largely unknown. Bergeron et al. (2001), demonstrated that activation of 5′-AMP-activated protein kinase (AMPK) increases both mitochondrial biogenesis and oxidative capacity in skeletal muscle of rodents. Supportive of the role of AMPK in mitochondrial biogenesis, AMPK-α2 null mice display reduced PGC1α gene expression (Zong et al., 2002), a key nuclear transcription coactivator that regulates genes encoding for mitochondrial function. In animal models of T2D, the activation of AMPK by adiponectin increases muscle and hepatic fat oxidation and improves insulin sensitivity (Yamauchi et al., 2002). Collectively, these data suggest that agonists of AMPK can increase both the number and the oxidative capacity of mitochondria, thereby creating a theoretical link between adiponectin signaling and mitochondrial bioenergetics. Here, we investigate the relationship between adiponectin and mitochondrial function in three complementary settings: in muscle from humans who are predisposed to develop T2D; in muscle from insulin-resistant adiponectin KO mice, and in primary cultures of human myocytes. We define a signaling pathway in skeletal muscle by which adiponectin (ligand and receptors) exerts potent antidiabetic effects by inducing AMPKK/AMPK phosphorylation, increasing mitochondrial number and oxidative metabolism and reducing the generation of reactive oxygen species (ROS).

Results

Adiponectin pathway is associated with mitochondrial function in humans

To examine whether adiponectin signaling is associated with insulin resistance and mitochondrial dysfunction in humans, we studied young insulin-resistant offspring (n = 8) of parents with T2D (FH^+) and insulin sensitive subjects (n = 10) with no family history of T2D (FH^-) as previously described (Civitarese et al.,

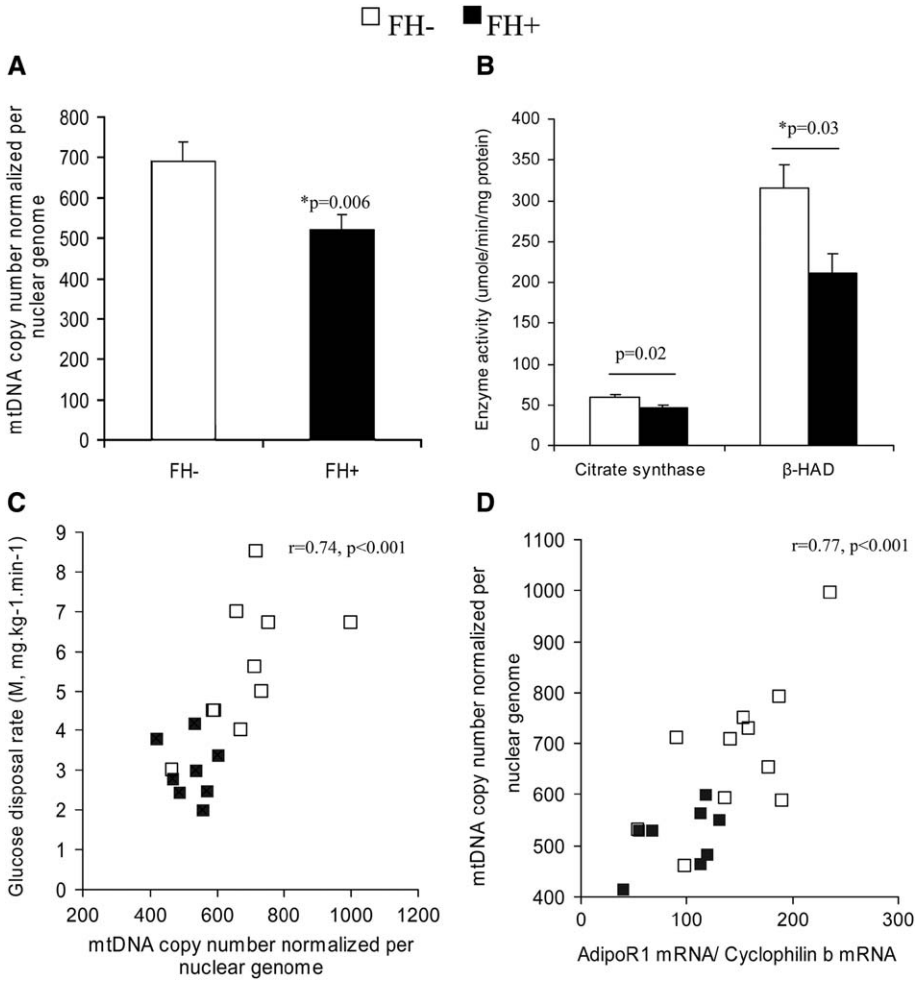

Figure 1. Comparison of skeletal muscle mitochondrial bioenergetics in insulin-resistant subjects with at least two first-degree relatives with type 2 diabetes (FH^+, ■) and insulin-sensitive subjects with no family history of type 2 diabetes (FH^-, □)

A) Mitochondrial content was assessed by mtDNA copy number.

B) Enzymatic quantification of mitochondrial oxidative capacity in the skeletal muscle of Mexican Americans revealed reduced citrate synthase (TCA cycle) and β-hydroxyacyl-CoA dehydrogenase (β-HAD; β oxidation) activity in FH^+ patients.

C) Insulin sensitivity assessed by euglycemic-hyperinsulinemic clamp (M value, mg glucose · kg^{-1} · min^{-1}) versus mtDNA copy number levels in skeletal muscle.

D) mtDNA copy number versus AdipoR1 mRNA expression in skeletal muscle.

Data represented mean ± SEM; n = 8, FH^+; n = 10, FH^- subjects.

2004). Fasting plasma insulin concentrations were higher in FH^+ versus FH^- subjects (10 ± 1 versus 6 ± 1 μU/ml, p = 0.02), whereas glucose disposal rate during a hyperinsulinemic-euglycemic clamp (3.0 ± 0.3 versus 5.6 ± 0.05 mg · kg^{-1} · min^{-1}, p = 0.001) and plasma adiponectin concentration (5.4 ± 0.41 versus 8.9 ± 1.3 μg/ml, p = 0.03) were lower in FH^+ versus FH^- subjects (Behre et al., 2006; Civitarese et al., 2004). RNA and DNA were extracted from a muscle biopsy collected in the fasting condition to examine by qPCR the expression of 27 candidate genes involved in adiponectin binding, FFA transport and oxidation, de novo lipogenesis, AMPK signaling, oxidative transcription, mitochondrial biogenesis, and oxidative stress (Table S1). The expression level of AMPK-α1 (p = 0.04), PPARγ coactivator-1β (p = 0.04), and mitochondrial transcription factor A (mtTFA; p = 0.03) were lower in FH^+ subjects, suggesting lower mitochondrial content. Consistent with these results, mitochondrial DNA (mtDNA; p = 0.006; Figure 1A), a proxy marker for mitochondrial content (Wang et al., 1999), citrate synthase (CS; TCA cycle; p = 0.02), and β-hydroxy-acyl-coenzyme A dehydrogenase (β-HAD; β oxidation; p = 0.03) enzyme activity (Figure 1B) were all reduced in FH^+ subjects, indicating lower mitochondrial bioenergetic capacity. Analysis of heteroplasmy revealed no difference between groups, suggesting that mitochondrial dysfunction was not due to large mtDNA deletions (data not shown). In addition, mtDNA content alone accounted for 55% of the variance in insulin sensitivity (Figure 1C).

We have previously shown that both AdipoR1 and AdipoR2 expression are lower in FH^+ versus FH^- subjects and both correlate positively with insulin sensitivity (Civitarese et al., 2004). Now we show that mtDNA is strongly related to the expression of both adiponectin receptors (AdipoRs) with the principal correlation with AdipoR1 mRNA (r = 0.77, p < 0.001; Figure 1D). Consistently, mtDNA and AdipoR1 were positively associated with CS activity (r = 0.71, p = 0.007 and r = 0.58, p = 0.037, respectively; data not shown). In addition, plasma adiponectin concentration correlated positively with insulin sensitivity (M values; r = 0.52, p = 0.03), mtDNA content (r = 0.59, p = 0.03), β-HAD (r = 0.71, p = 0.007), and CS activity (r = 0.58, p = 0.037; data not shown). Collectively, these data reveal that individuals with a family history for T2D have lower mtDNA copy number and oxidative capacity and that adiponectin signaling (circulating plasma adiponectin and AdipoRs expression) is associated with mitochondrial number and oxidative capacity in human skeletal muscle.

Adiponectin KO mice display decreased mitochondrial content

Mice expressing dominant negative AMPK-α2 display reduced PGC1α mRNA (Zong et al., 2002), whereas transgenic mice (Combs et al., 2004) and rats (Satoh et al., 2005) overexpressing adiponectin display improved insulin sensitivity and increased markers of mitochondrial function in adipose tissue (Combs

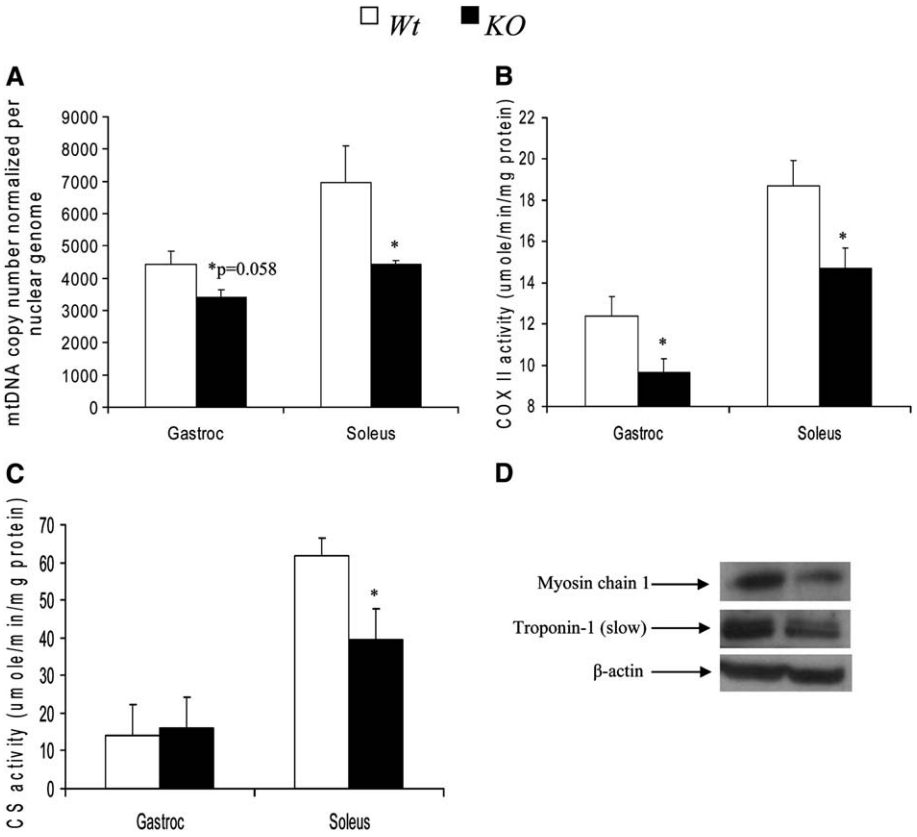

Figure 2. Molecular characterization of 12-week-old wt and adiponectin KO mice

A) Gastrocnemius (Gastroc) and Soleus muscle from mice was used in qPCR analysis of mtDNA copy number and mitochondrial enzyme activity. Analysis of mtDNA copy number (**A**), cytochrome c oxidase (**B**), and citrate synthase enzyme activity (**C**) in KO mice revealed reduced mitochondrial bioenergetics. Data represented mean ± SEM; wt, n = 5; KO, n = 5. *p < 0.05 compared to wt mice. Protein content of myosin chain 1 and troponin-1 (slow) in gastrocnemius muscle (**D**). Immunoblotting was undertaken in six mice (wt, n = 3; KO, n = 3) and data are shown as a representative blot.

et al., 2004). To investigate the relationship between adiponectin and mitochondria, we examined mitochondrial function in the muscle of adiponectin null mice. Thiazolidinediones (TZD) are effective insulin sensitizers in part by inducing the transcription of genes involved in preadipocyte differentiation (Gurnell et al., 2000) and increasing the production of circulating adiponectin (Maeda et al., 2002). To maximize differences in the concentration of adiponectin, male wt and adiponectin KO mice were maintained on a high-fat diet for 8 weeks in conjunction with rosiglitazone treatment at a dose of 10 mg/kg/day during the last 10 days before sacrifice. This treatment was reported to increase circulating adiponectin by ~4-fold in wild-type mice (Nawrocki et al., 2006). Both groups (wt and KO) had comparable body weights after TZD treatment but adiponectin deficient mice were glucose intolerant as evidenced by an oral glucose tolerance tests (2.5 mg glucose/g body weight; Nawrocki et al., 2006). These data are consistent with previous reports of insulin resistance in adiponectin null mice (Kubota et al., 2002; Maeda et al., 2002). Consistent with our hypothesis, transcription of mitochondrial markers (PGC1α and PGC1β) and of the fat oxidative transcription factor PPARα was reduced in the gastrocnemius of adiponectin KO mice compared to wt mice (data not shown). MtDNA content (4103 ± 188 versus 5261 ± 509 mtDNA copy number, $p < 0.05$), and CS activity (49 ± 7 versus 74 ± 10 μmol/min/mg protein, $p < 0.05$), were lower in KO mice relative to wt mice, whereas COX activity was not (23 ± 5 versus 35 ± 6 μmol/min/mg protein, $p = 0.12$). To exclude the potential confounding effects of TZD treatment (Bogacka et al., 2005), we also examined mitochondrial number and function in 12-week-old female mice. MtDNA content was reduced by ~22% in the gastrocnemius muscle (mixed fiber type) of KO mice relative to wt mice (Figure 2A; $p = 0.058$) and by ~37% in the oxidative soleus muscle (Figure 2A; $p = 0.025$). PGC1α mRNA was lower in both fiber types in KO animals, whereas PGC1β was only different between fiber types and not genotype (Table 1). The data is consistent with previous reports in C57/BL6 mice showing elevated PGC1α mRNA in oxidative muscle (Lin et al., 2002). Furthermore,

Table 1. Expression of genes involved in mitochondrial biogenesis, fiber type determination, and fat oxidation in 12-week-old wt and adiponectin-deficient mice

		Wild-Type	Adiponectin KO
Gene	Muscle	mRNA/ RPLPO mRNA (Arbitrary Units)	
PGC1α	Soleus	348 ± 13	201 ± 32[a]
	Gastrocnemius	224 ± 20[b]	158 ± 11[a,b]
PGC1β	Soleus	4.3 ± 0.4	3.4 ± 0.9
	Gastrocnemius	2.9 ± 0.7[b]	3.6 ± 0.5
NRF-1	Soleus	18 ± 1	18 ± 2
	Gastrocnemius	23 ± 2	16 ± 0.6
PPARδ	Soleus	113 ± 4	85 ± 7[a]
	Gastrocnemius	124 ± 11	98 ± 6[a]
PPARα	Soleus	94 ± 10	79 ± 12
	Gastrocnemius	32 ± 12[b]	27 ± 6[b]
UCP3	Soleus	65 ± 21	69 ± 19
	Gastrocnemius	85 ± 18	129 ± 48

PGC1α, peroxisome proliferative activated receptor gamma coactivator-1 alpha; PGC1β, peroxisome proliferative activated receptor gamma coactivator-1 beta; NRF1, nuclear respiratory factor 1; PPARδ, peroxisome proliferative activated receptor-delta; PPARα, peroxisome proliferative activated receptor-alpha; and UCP3, uncoupling protein 3. Data represented mean ± SEM, n = 5 each group.
[a] $p < 0.05$ compared to wt mice.
[b] $p < 0.05$ compared to soleus.

COX activity was reduced in both the gastrocnemius and soleus muscle (Figure 2B), whereas, CS activity was reduced in soleus muscle only (Figure 2C). Collectively, these data indicate that mitochondrial content and oxidative capacity are dependent on the presence of circulating adiponectin.

Skeletal muscle contains both oxidative (type 1) and glycolytic (types 2a and 2b) myofibers that differ in oxidative capacity mostly due to the synthesis of a distinct array of contracting proteins (i.e., troponin-1 slow), enzymes, and regulatory proteins (Berchtold et al., 2000). The fact that PGC1α is a master regulator of mitochondrial biogenesis prompted us to study the potential role of adiponectin in the regulation of proteins involved in fiber type determination in wt and adiponectin KO mice. In gastrocnemius (a muscle with a mixture of type 1 and type 2 fibers), the expression of markers of type 1 fibers such as troponin-1 (Polly et al., 2003) and slow myosin (Semper et al., 1988) was reduced in adiponectin KO mice compared to wt mice (Figure 2D) suggesting lower content of oxidative fibers. Interestingly, in primary human muscle cell culture (see below) globular adiponectin (gAD) dose-dependently induced troponin-1 mRNA (see Figure S2A in the Supplemental Data available with this article online), suggesting the reduction in troponin-1 protein in KO mice is regulated at the transcriptional level. In addition, KO mice also had reduced PPARδ mRNA (Table 1), which has been shown to induce fiber type switching in skeletal muscle (Wang et al., 2004). Taken together, the reduced protein expression of troponin-1 and slow myosin reemphasizes the role of adiponectin in muscle bioenergetics.

Adiponectin treatment increases mitochondrial bioenergetics in primary human myotubes

Adiponectin exists in human plasma as full-length protein as well as smaller globular fragments (Fruebis et al., 2001). gAD binds more avidly than full-length adiponectin to C2C12 myocytes, skeletal muscle membranes (Yamauchi et al., 2002, 2003), and mouse macrophages (Yamaguchi et al., 2005). To determine whether the adiponectin pathway was involved in the regulation of mitochondrial content, we treated primary human myotubes taken from the *vastus lateralis* muscle of five healthy male Caucasian subjects (see Experimental Procedures for clinical characteristics) with gAD. Two day treatment with 0.25 and 0.5 μg/ml of gAD increased mtDNA content, indicating an induction in mitochondrial biogenesis (Figure 3A). To rule out the possibility that the observed increases were due to increased mtDNA stability rather than to changes in true mitochondrial number, we directly measured mitochondrial mass using MitoTracker Green (Wilson-Fritch et al., 2004). gAD treatment increased mitochondrial content (Figures 3B and 3C) in parallel with increased PGC1α mRNA (Figure S1A). Consistently, citrate synthase activity, a biomarker of mitochondrial function (Figure 5B) and CO_2 production from palmitate oxidation (Figure 3D) was increased with gAD treatment. These data demonstrate that adiponectin can augment both mitochondrial mass and oxidative capacity. In addition, gAD dose-dependently increased AMPK phosphorylation (Tomas et al., 2002) and PGCIα protein (Figure 3E).

Adiponectin forms a wide range of multimers, from LMW trimer to HMW multimers in human serum (Fruebis et al., 2001; Pajvani et al., 2003). We compared the potencies of various forms of adiponectin in myotubes treated with adiponectin. Cys39Ser mutant LMW adiponectin (Pajvani et al., 2003; Figure S1B), but not HMW adiponectin, stimulated mitochondrial biogenesis (data not shown), indicating differing bioactivity of the two isoforms in skeletal muscle. Because leptin has also been shown to stimulate AMPK in skeletal muscle (Minokoshi et al., 2002), activation of AMPK and mitochondrial biogenesis may be a common mechanism by which insulin-sensitizing adipokines such as adiponectin and leptin increase insulin sensitivity. Consistent with this hypothesis, leptin treatment of primary human myotubes for 72 hr increased mitochondrial DNA content relative to control (Figure S1C).

Adiponectin treatment reduces the generation of reactive oxygen species

Cellular respiration strips electrons from fatty acids (FA), carbohydrates, and amino acids, and the electrons subsequently accumulate on the soluble electron carriers NADH and $FADH_2$. The electrons are passed down the respiratory chain to oxygen, creating a potential energy gradient of protons ($\Delta\mu_{H+}$), increased membrane potential ($\Delta\psi$) and a pH gradient (ΔpH; Luvisetto et al., 1990) to drive ATP synthesis. Increased $\Delta\psi$ is also a central bioenergetic factor that regulates the generation of reactive oxygen species (ROS); super oxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$; Maassen et al., 2002; Nicholls, 2004). In human myotubes, gAD reduced the fluorescence signals for hydrogen peroxide (Figure 4A) and superoxide production (Figures 4E and S2A). ROS production is inversely related to mitochondrial content per cell (Maassen et al., 2002). Each mitochondrion exhibits an increased workload when mitochondrial mass is reduced, and this leads to higher $\Delta\psi$ and increased ROS production. Analysis of $\Delta\psi$ using tetramethylrhodamine ethyl ester (TMRE) in cells treated with 0.25 μg/ml of gAD indicated a reduction in $\Delta\psi$ (Figure 4B). UCP3 overexpression in L6 myotubes reduces the generation of hydrogen peroxides (MacLellan et al., 2005). Analysis of UCP3 mRNA in cells treated with 0.5 μg/ml of gAD demonstrated no difference in UCP3 expression (8.6 ± 0.9 versus 10.2 ± 0.8, control versus 0.5 μg gAD, respectively; $p = 0.13$). These data suggest decreased ROS production with gAD treatment is mediated in part, by a lowering of mitochondrial $\Delta\psi$.

Mitochondria are normally protected against the effects of ROS by a complex system of antioxidants and scavenging enzymes including heme oxygenase (HO1), NAD(P)H dehydrogenase-quinone-1 (NQO1), 8-oxoguanine DNA glycosylase (OGG1), superoxide dismutase 2 (SOD2) and glutathione peroxidase-1 (GPx1). gAD treatment for 48 hr reduced HO1, NQO1, and OGG1 mRNA (Figures S2B–S2D), which is consistent with ROS regulating the transcription of these genes (Boiteux and Radicella, 2000; Elbekai and El-Kadi, 2005; Friesen et al., 2004; Gong et al., 2004; Guo et al., 2003). In addition, SOD2 mRNA was increased relative to controls (Figure 4C), suggesting that adiponectin can induce SOD2 activity. To clarify the mechanisms by which adiponectin reduce ROS, we measured total SOD and GPx activity in primary muscle cells treated with 0.25 μg/ml of gAD. gAD increased the activity of SOD (Figure 4D), which is consistent with the reduction in the fluorescence of MitoSOX Red. SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Luo et al., 2006). We found no change in GPx activity (data not shown), suggesting the reduction in hydrogen peroxide observed with adiponectin treatment (Figure 4A) maybe the result of lower superoxide generation. Importantly, AdipoR-specific small-interfering (si) RNA to both receptors abolished the induction in SOD activity

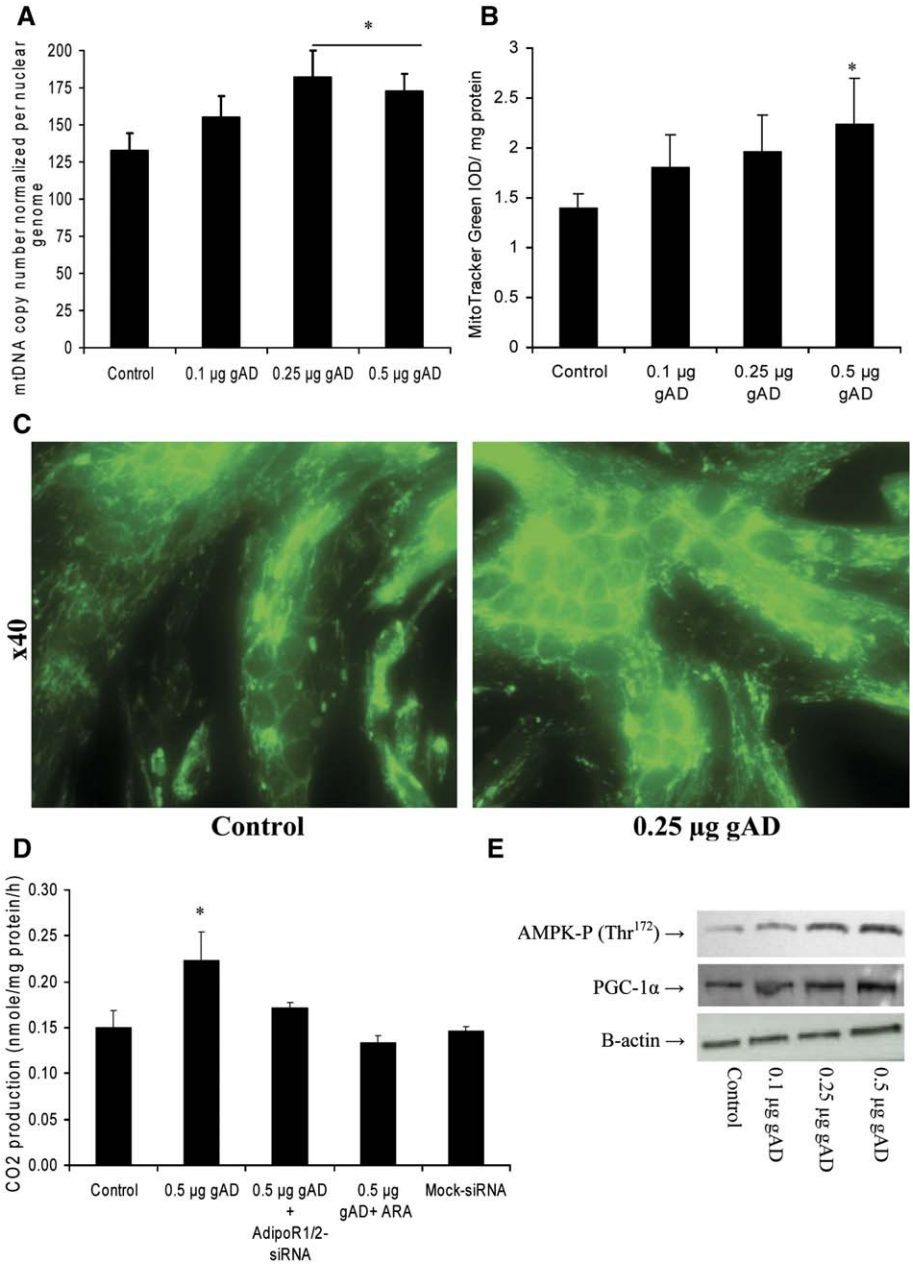

Figure 3. Effects of adiponectin treatment on mitochondrial number and oxidative capacity in primary human myotubes

A and B) Primary human myotubes were treated for 48 hr with 0.1, 0.25, and 0.5 μg/ml of gAD and mitochondrial content measured using mtDNA copy number (**A**) and MitoTracker Green (**B**).

C) Myotubes were treated with 0.25μg/ml gAD for 48 hr and cells subsequently incubated with 100 nM MitoTracker Green FM for 30 min. Mitochondrial mass is indicated by green fluorescence. Images were acquired within 5 min using a conventional wide-field microscope fitted with an ×40 Nikon plan-apo objective.

D) Effects of gAD treatment on fatty acid oxidation. Data represented as mean ± SEM; n = 5. *p < 0.05 compared to control.

E) Dose-dependent induction in AMPK-P and total PGC1α protein by 0.1, 0.25, and 0.5 μg/ml of gAD.

(Figure 4D), indicating the increase in SOD activity was specific to adiponectin signaling. Treatment of cultured human endothelial cells with 5-amino oimidazole-4-carboxamide ribonucleoside (AICAR), an AMPK activator (Kaushik et al., 2001) induced SOD2 mRNA and lowered the production ROS (Kukidome et al., 2006). In this study, treatment of primary muscle culture with 0.5 μM AICAR restored SOD activity to levels similar to those observed with gAD treatment (Figure 4D). Collectively, these data show that adiponectin lowers the production of ROS by both reducing mitochondrial membrane potential and increasing ROS scavenging vis-à-vis AMPK-SOD2.

Reduction of AdipoR reduces adiponectin-induced mitochondrial oxidative capacity

We examined the effects of AdipoR-specific siRNA on adiponectin-induced mitochondrial biogenesis in primary human myotubes. Knockdown of AdipoR1, AdipoR2, or the combination of both receptors significantly reduced gAD induction of mtDNA (data not shown), mitochondrial mass (Figure 5A), CS activity (Figure 5B), and CO_2 production (Figure 3D). These data suggest that both AdipoRs contribute to the regulation of mitochondrial mass and function in human skeletal muscle. Similar reductions in mtDNA were observed using AdipoR1-siRNA and coincubation with the Cys39Ser mutant LMW-adiponectin (Figure S1D).

Activation of AMPK in rodents (Bergeron et al., 2001) induces mitochondrial biogenesis and increases mitochondrial oxidative output. We tested whether inhibiting AMPK activity would impair adiponectin-induced activation of mitochondrial function. Adenine-9-β-D-arabinofuranoside hydrate (ara-A), an AMP analog and competitive AMPK inhibitor (Musi et al., 2001), decreased gAD-stimulated AMPK phosphorylation (see below),

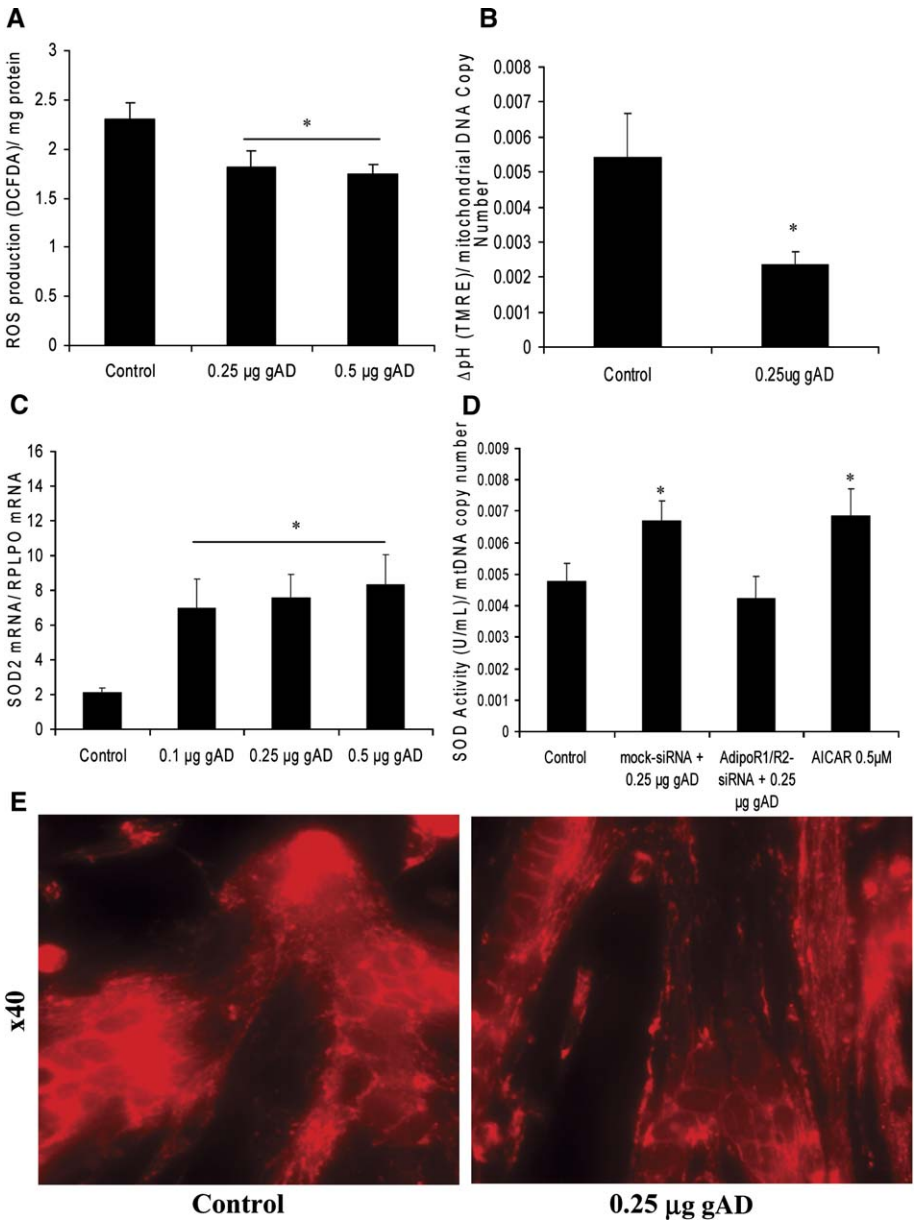

Figure 4. Effects of adiponectin treatment on mitochondrial ROS production in primary human myotubes

To analyze ROS levels in myotubes, cells were incubated with DCFDA (**A**) (indicator of hydrogen peroxide) and (**B**) Tetramethylrhodamine ethyl ester (TMRE) for estimation of membrane potential.

C) mRNA expression of SOD2 in myotubes treated with 0.1, 0.25, and 0.5 µg/ml of gAD for 48 hr.

D) Total SOD activity (Cu/Zn-, Mn-, and Fe-SOD) in cells treated with 0.25 µg/ ml of gAD, mock-siRNA, AdipoR1/R2-specific siRNA, and AICAR. Data represented as mean ± SEM; n = 5. *$p < 0.05$ compared to control.

E) The presence of ROS in myotubes treated with 0.25 µg/ml of gAD for 48 hr. Red fluorescence indicates the presence of ROS.

mitochondrial biogenesis, and CS activity (Figures 5A and 5B), indicating that signaling through AMPK is a necessary postadiponectin receptor event.

Adiponectin-mediated signaling

AMPK is allosterically activated by alterations in the AMP:ATP ratio and covalently by phosphorylation at Thr172 by upstream kinase AMPK kinase (Hawley et al., 2003; Woods et al., 2003). gAD treatment of myotubes for 48 hr increased the phosphorylation of AMPKK, AMPK (Chen et al., 2005; Yamauchi et al., 2002), and total PGC1α and COX protein content (Figure 5C). Consistently, recent work demonstrated that overexpression of PGC1α in L6 myocytes also induces COX protein (Koves et al., 2005). Suppression of AdipoR1, AdipoR2, or the combination of both receptors was associated with reduced AMPKK and AMPK phosphorylation (Fujioka et al., 2006) and lower PGC1α and COX protein content. AdipoR1-specific siRNA caused a greater reduction in AMPK phosphorylation and PGC1α protein relative to AdipoR2-siRNA. However, knockdown of both AdipoRs yielded an additive effect (Figure 5C). Collectively, these data provide evidence that AdipoR can mediate the gAD-stimulated induction in AMPK phosphorylation and PGC1α protein in human skeletal muscle.

In vitro, the binding of AMP to AMPK enhances ~3-fold the rate at which AMPK can be phosphorylated and activated by AMPKK-STRAD-MO25 complex (Hawley et al., 2003; Woods et al., 2003). Consistent with these observations, ara-A treatment decreased the phosphorylation of AMPKK and AMPK and PGC1α and COX protein content (Figure 5C). This data are in accord with mice overexpressing dominant-negative AMPK-α2, which have reduced PGC1α mRNA and mitochondrial content (Zong et al., 2002). Thus, stimulation of mitochondrial biogenesis by gAD occurs through the activation of AMPKK-AMPK in primary human myotubes.

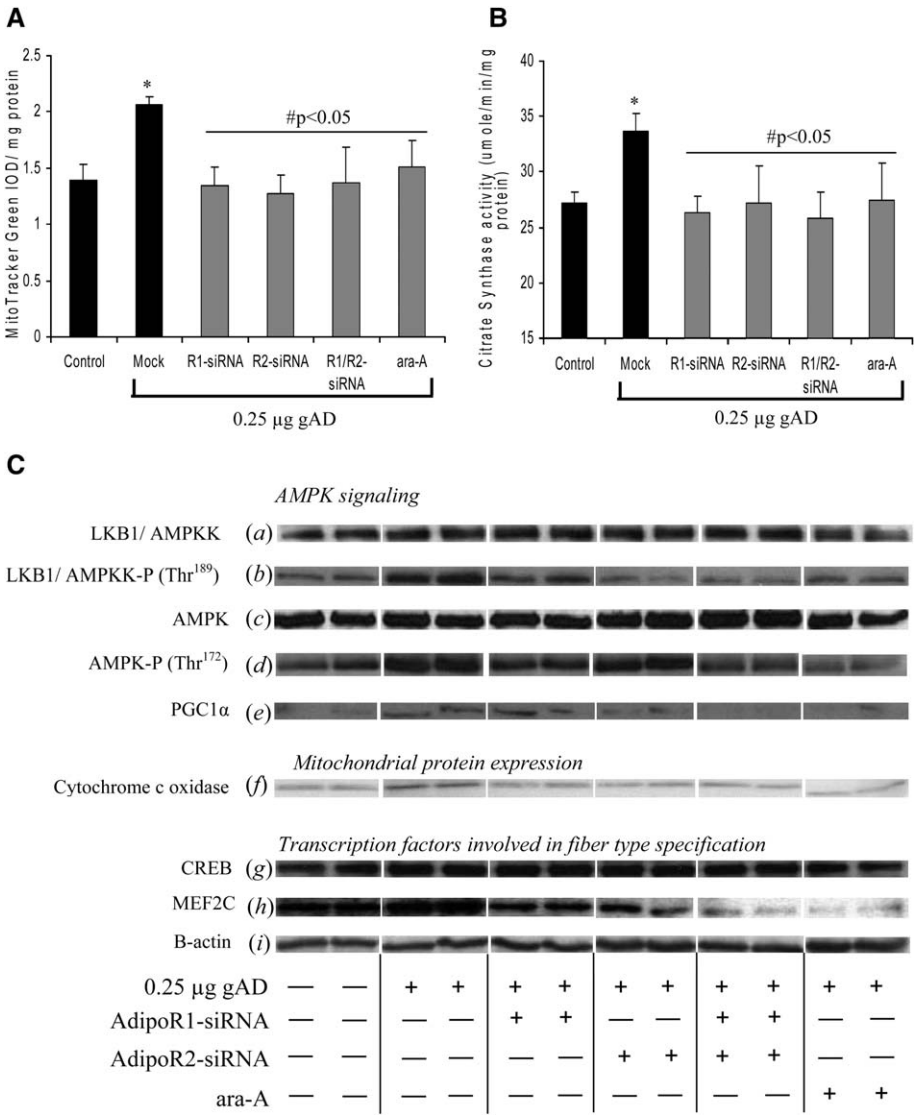

Figure 5. Effects of suppression of AdipoR1 or AdipoR2 expression by siRNA and pharmacological inhibition of AMPK activity using ara-A in primary human myotubes

A and B) mitochondrial content (**A**) and citrate synthase activity (**B**) in myotubes treated with siRNA duplex and ara-A compound. Data represented mean ± SEM; n = 5. *p < 0.05 compared to control. #p < 0.05 compared to mock-siRNA.

C) Phosphorylation and total amount of AMP-activated protein kinase kinase (AMPKK) (*a* and *b*), AMP-activated protein kinase (AMPK) (*c* and *d*), and total amount of peroxisome proliferative activated receptor γ coactivator 1-α (PGC1α) (*e*), cytochrome c oxidase (*f*), cAMP response element binding protein (CREB) (*g*), Myocyte enhancer factor 2C (MEF2C) (*h*), and β-actin protein (*i*) in primary human myotubes transfected with siRNA duplex or ara-A and incubated with 0.25 μg/ml gAD for 48 hr in duplicate cell incubations.

Myocyte enhancer factor 2C (MEF2C) and cAMP response element binding protein (CREB; Handschin et al., 2003) bind to the PGC1α promoter increasing PGC1 content, promoting mitochondrial biogenesis and increasing oxidative fiber types. MEF2C activity is regulated in part through AMPK signaling (Al-Khalili et al., 2004), while both CREB and MEF2C are activated by calcium signaling kinase calcium/ calmodulin-dependent protein kinase (CaMKIV) and calcineurin A (can; Corcoran and Means, 2001; Handschin et al., 2003). gAD increased MEF2C protein content without a change in CREB protein (Figure 5C). Conversely AdipoR-specific siRNA or chemical inactivation of AMPK with ara-A completely blocked the induction of MEF2C protein but had no effect on CREB protein. Collectively, these data suggest that the activation of mitochondrial biogenesis by gAD occurs through the induction of AMPK, MEF2C, and PGC1α.

Discussion

Adiponectin signaling is an important regulator of skeletal muscle mitochondrial oxidative capacity based on the following findings: (1) In FH^+ subjects, insulin resistance and mitochondrial dysfunction in skeletal muscle were associated with lower levels of circulating adiponectin and AdipoR mRNA expression; (2) in vivo, plasma adiponectin and AdipoR1 mRNA is a correlate of mtDNA; (3) in vitro, adiponectin induced mitochondrial biogenesis and oxidative output and reduced the production of ROS; (4) the induction of mitochondrial biogenesis and increased oxidative capacity by gAD was inhibited by targeted suppression of the AdipoRs; (5) adiponectin KO mice have reduced mitochondrial content, TCA cycle, and ETC activity and reduced markers of type-1 oxidative fibers in skeletal muscle. Collectively, these data define a pathway in skeletal muscle by which adiponectin signaling contributes to energy homeostasis by modulating mitochondrial number and function.

It has become increasingly clear that lower mitochondrial number (Kelley et al., 2002; Ritov et al., 2005) and oxidative capacity (Bjorntorp et al., 1967; Kelley et al., 2002; Petersen et al., 2003; Ritov et al., 2005) are central defects in the development of insulin resistance in skeletal muscle. Results from the current study and Petersen et al. (2004) demonstrate that mitochondrial dysfunction is present in the prediabetic state, suggesting that

mitochondrial abnormalities may be primary to the development of T2D. The functional consequence of deficient β-oxidation and impaired ETC activity might be the accumulation of intramyocellular lipids (acylcarnitines, fatty acyl CoAs, diacylglycerols, and ceramides) that can activate serine kinases that inhibit insulin signaling and glucose transport (Adams et al., 2004; Chavez and Summers, 2003; Yu et al., 2002). Elegant work by Ritov et al. (2005), showed the ETC activity of subsarcolemmal mitochondria are reduced in subjects with T2D. This latter observation is important, "since subsarcolemmal mitochondria provide energy for membrane-related processes, including signal transduction, ion exchange, and substrate transport—processes involved in insulin action, glucose uptake, and storage" (Ritov et al., 2005). The key question is what is the physiological importance of adiponectin signaling in the regulation of mitochondrial bioenergetics?

Studies in obese and diabetic rhesus monkey demonstrate that plasma adiponectin level declines in the early phases of obesity and in parallel to the progressive development of insulin resistance (Hotta et al., 2001). Furthermore, circulating plasma adiponectin levels and the expression of both AdipoRs are reduced in subjects with a family history of T2D (Civitarese et al., 2004), while prospective studies in Pima Indians demonstrate that high concentrations of adiponectin is protective against the development of T2D (Lindsay et al., 2002). Collectively, these data suggest that early defects in the secretion of adiponectin or adiponectin signaling may be a contributing factor to lower mitochondrial content and/or function in the prediabetic state. However, not all studies report lower adiponectin concentrations in FH^+ subjects (Kriketos et al., 2004; Petersen et al., 2004; Tschritter et al., 2003). Potential confounding factors may include classification of FH status (i.e., one or both parents with type 2 diabetes in FH^+ groups) (Behre et al., 2006), gender (Kern et al., 2003), and adiposity (Behre et al., 2006; Kern et al., 2003).

In our study, adiponectin treatment induced mitochondrial oxidative capacity and increased PGC1α protein in human myotubes. Recent work in both animal and cell culture models demonstrate the inability of mitochondria to oxidize lipid in response to fatty acid exposure (fasting and a high-fat diet) is dependent on PGC1α mRNA expression (Koves et al., 2005). Conversely, overexpression of PGC1α in L6 myocytes resulted in the complete oxidation of lipid and the elevated transcription of PGC1α target genes (β-oxidation and TCA cycle; Koves et al., 2005). Taken together, these data imply that the ability of skeletal muscle to effectively switch from glucose to fatty acid oxidation during increased fatty acid supply (metabolic adaptability) relies upon on mitochondrial content and the expression level of PGC1α, both of which are targets of the adiponectin signaling pathway (Figure 6).

Overexpression of PGC1α in muscle results in a dramatic increase in insulin sensitive type 1 oxidative fibers (Lin et al., 2002). The increase in PGC1α activity occurs mainly through calcium signaling and activation of PGC1α promoter by CREB and MEF2 binding sites (Wu et al., 2002). In the present study, gAD treatment increased PGC1α and MEF2C protein expression in parallel to increased transcription in troponin-1. MEF2C directly binds and activates transcription of troponin-1 in C2C12 myotubes (Polly et al., 2003) and transgenic overexpression of PGC1α increase troponin-1 protein in oxidative fibers (Lin et al., 2002). Importantly, analysis of the gastrocnemius muscle

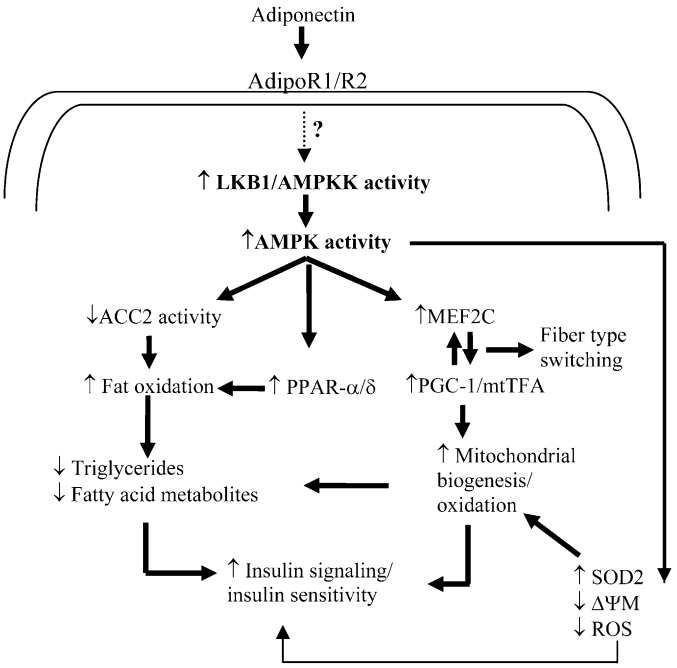

Figure 6. Adiponectin signaling and the regulation of mitochondrial function in skeletal muscle

Adiponectin binding to its AdipoRs activates AMP-activated protein kinase (AMPK) and stimulates the phosphorylation of ACC2 and fatty-acid oxidation (Yamauchi et al., 2002, 2003). Adiponectin also activates peroxisome proliferative activated receptor-α (PPARα) stimulating transcription of genes in the fatty-acid oxidation pathway and decreasing triglyceride content in muscle (Yamauchi et al., 2001, 2002) and improving insulin sensitivity. Independent of changes in transcription and mitochondrial mass, the improvements in lipid oxidation occur in less than 6 hr (Yamauchi et al., 2002). Adiponectin activation of AMPK by upstream kinase AMPK kinase (AMPKK) activates transcription of myocyte enhancer factor 2C (MEF2C) and phosphorylation of peroxisome proliferative activated receptor γ coactivator 1-α (PGC1α), which in turn increases mitochondrial content, oxidative capacity, and oxidative-fiber type composition. Central to the development of mitochondrial dysfunction is reactive oxygen species (ROS) production, which reacts with DNA, protein, and lipids leading to oxidative damage. ROS production is inversely related to mitochondrial content. Activation of the adiponectin pathway reduces the generation of ROS by two processes: (1) Increased mitochondrial content, which in turn decreases the workload for each mitochondrion leading to reduced membrane potential ($\downarrow\Delta\psi$) and lower ROS production; and (2) adiponectin increases PGC1α activity which increases the transcription/activity of the antioxidant enzyme SOD2 that decreases super oxide radical ($O_2^{\bullet-}$) (Maassen et al., 2002).

from adiponectin KO mice revealed lower PGC1α mRNA and mitochondrial content, reduced tropinin-1 protein expression. These data suggest that circulating adiponectin contributes to protein expression of genes involved in myofiber determination by a signaling mechanism involving PGC1α, MEF2C, and troponin-1.

We have also shown the induction of mitochondrial function by gAD occurs in parallel with increased AMPKK phosphorylation. Recent data in primary human myotubes demonstrate that AMPKK is not activated in response to acute gAD treatment (<6 hr), despite significant increases in AMPK Thr^{172} phosphorylation (Chen et al., 2005), suggesting that longer exposure (48 hr) to gAD is required to induce AMPKK activity and mitochondrial biogenesis. Chen et al. (2005) found impaired gAD induction of AMPK activity in primary myotubes from obese T2D subjects that was not associated with changes in the gene expression of the adiponectin receptors. This does not exclude

the possibility that the expression of both AdipoRs may be regulated by factors that are altered within the hormonal milieu of obese and obese type 2 diabetic subjects. In support of this, we (A.E.C. and B.U., unpublished data) and others (Fang et al., 2005; Tsuchida et al., 2004) have found that AdipoR1 mRNA is lowered by high concentrations of insulin (100–200 nM) and palmitate (60–150 μM; A.E.C. and B.U., unpublished data) in muscle cell cultures. Intriguingly, we observe no difference in the expression of AdipoRs in the muscle of adiponectin KO mice relative to wt mice (A.E.C. and A.R. Nawrocki, unpublished data) or in primary muscle cell culture after treatment with globular or full-length adiponectin (A.E.C. and B.U., unpublished data), consistent with data in transgenic rats overexpressing adiponectin with no difference in the skeletal muscle expression of both AdipoRs (Satoh et al., 2005). Taken together, these data do not support the common feedback regulation of hormone-ligand receptor interaction of which leptin is an example. Not all G-coupled receptors undergo immediate downregulation in response to receptor agonist. Classical work examining the regulation of the β_2-adrenergic receptor demonstrates that in the short-term, agonist-induced changes in receptor binding properties can account for much of the decrease in receptor-mediated signaling and is unrelated to any physical translocation or degradation of the receptor molecule (Hausdorff et al., 1991; Mahan et al., 1985; Wang et al., 1989). This suggests that receptors for adiponectin may also exhibit similar properties. Clearly, further studies are required investigating the regulation of the AdipoRs in skeletal muscle.

We show that adiponectin increases mitochondrial content, SOD2 activity and reduces the fluorescence of TMRE ($\downarrow\Delta\psi$) resulting in lower ROS production as measured by DCFDA (Kukidome et al., 2006; MacLellan et al., 2005). This effect is important as mitochondria from subjects with T2D have damaged inner-membrane structure (Kelley et al., 2002) as well as reduced electron transport chain activity (Petersen et al., 2004; Ritov et al., 2005). The inner mitochondrial membrane (cristae) are particularly prone to oxidative stress as a large portion of protein complexes associated with oxidative phosphorylation are embedded in this area (Gilkerson et al., 2003). In addition, in primary muscle cell culture, gAD and AICAR increased the activity of SOD. This is in agreement with Kukidome et al. (2006), who demonstrated that AICAR and overexpression of PGC1α increases SOD2 mRNA and lower ROS production. This suggests that under normal physiological conditions, AMPK and PGC1α activation by adiponectin (or other stimuli) may be a normal physiological response to reduce the production of ROS. We speculate that lower circulating adiponectin and/or receptor expression contributes to lower mitochondrial content, decreased SOD2 activity, and increased mitochondrial oxidative damage.

The lowering of mitochondrial $\Delta\psi$ with gAD treatment was unexpected and did not relate to any changes in UCP3 mRNA. It is unclear whether mitochondrial proliferation can reduce ROS production. Old mitochondria produce less ATP and have increased ROS production (Miyoshi et al., 2006). In addition, under well-coupled conditions, phosphorylating mitochondria have slightly lower $\Delta\psi$ and generate less ROS (Nicholls, 2004). It is possible that newly formed mitochondria have "more efficient" coupling and ATP production. Further, studies are required to clarify the link between adiponectin, mitochondrial biogenesis, $\Delta\psi$, and ROS regulation.

In summary, our results demonstrate that adiponectin and the AdipoRs play an important role in the regulation of mitochondrial number and function. Subjects with T2D have reduced adiponectin and decreased adiponectin receptor expression prior to the onset of hyperglycemia. Activation of the adiponectin pathway is a promising target for the treatment of insulin resistance and other components of the metabolic syndrome.

Experimental procedures

Human subjects

Subject recruitment was conducted in the General Clinical Research Center of the South Texas Veterans Health Care System, Audie Murphy Division, in San Antonio as previously described (Civitarese et al., 2004). The Institutional Review Board of the University of Texas Health Science Center at San Antonio approved the research, and all subjects gave written informed consent.

Generation of adiponectin KO mice

Adiponectin KO mice were generated as previously described (Nawrocki et al., 2006). Briefly, a mouse 129/SvEv genomic library was screened and a positive adiponectin clone was isolated and inserted into a targeting vector containing a 2.74 kb BamHI fragment and a 4.3 kb SalI to ClaI fragment into a neomycin cassette (Stratagene). The correct clone was isolated by screening 750 clones by transforming ES cells in the presence of G418 and analyzed by Southern blotting. The targeted ES cells were injected into C57BL/6J blastocysts and transferred into foster females. Germline transmission was obtained from seven chimeric males, with no phenotypic differences were observed between the lines.

Immunoblotting and mRNA analysis

Immunoblotting for protein and mRNA analysis using qRT-PCR were performed as previously described (Heilbronn et al., 2005). Gene-specific primer and probe sets are provided in Table S2.

Skeletal muscle cell culture

Vastus lateralis muscle biopsy (~100 mg) were obtained from five healthy young sedentary subjects (BMI = 24.1 ± 0.5; body fat = 15 ± 1%; fasting glucose concentration = 88 ± 2 mg/dl; fasting plasma insulin = 4.4 ± 0.9 μU/ml) using the Bergstrom technique (Bergstrom, 1975). Satellite cells were isolated as previously described (Ukropcova et al., 2005). Cells were seeded into 12- (RNA/DNA) and 6-well plates (protein) at a density of 20×10^3 cells per cm^2 and grown at 37°C in a humidified atmosphere of 5% CO_2. In selected cells, two pairs of *Stealth*-siRNAs were chemically synthesized (Invitrogen), annealed, and transfected (200 pmol/ml) into 50%–70% confluent primary human myocytes using GeneSilencer siRNA transfection reagents (Gene Therapy Systems). The sequences of the sense siRNA are as follows: AdipoR1, AAACAGCACGAAACCAAGCAGAUGG; AdipoR2, AUGUCCCA CUGGGAGAC UAUAAUGG. Cells transfected with nonfunctional-jumbled siRNA (Mock) (Ambion) were used as negative controls. Differentiation of myoblasts into myotubes was initiated as previously described (Ukropcova et al., 2005). Seventy-two hours after the induction of differentiation, the medium was changed and treated with globular (R&D Systems) or LMW/HMW adiponectin for 48 hr.

Mitochondrial content and enzyme activity assay

For quantification of mitochondrial content, we used mtDNA as previously described (Heilbronn et al., 2005) and MitoTracker Green probe (Molecular Probes). Specific human and mouse primer and probe sets are provided in Table S2. MitoTracker Green probe preferentially accumulates in mitochondria regardless of the mitochondrial membrane potential and provides an accurate assessment of mitochondrial mass. Cells were washed with PBS and incubated at 37°C for 30 min with 100 nM MitoTracker Green FM (Molecular Probes). Cells were harvested using trypsin/EDTA and resuspended in PBS. Fluorescence intensity was detected with excitation and emission wavelengths of 490 and 516 nm, respectively, and values corrected for total protein (mg/ml). Mitochondrial heteroplasmy were undertaken as described previously (Heilbronn et al., 2005). Citrate synthase, cytochrome c oxidase,

and β-HAD activities were determined spectrophotometrically from cell homogenates using previously described methods (Heilbronn et al., 2005).

Antioxidant enzyme activity

Cell lysates were prepared by sonication in cold 20 mM HEPES buffer (pH 7.2), containing 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose and centrifuged at 1500 × g for 5 min at 4ºC for SOD samples and in cold 5 mM Tris-HCL (pH 7.5), containing 5 mM EDTA and 1 mM DTT and centrifuged at 10,000 × g for 15 min at 4ºC for GPx samples. Total SOD activity (Cu/Zn-, Mn-, and Fe-SOD) was measured by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. GPx activity was measured by the GPx1-mediated oxidation of glutathione with the recycled reduction of GSSG to GSH by glutathione reductase using NADPH as a reductant. Both enzyme activities were determined by an indirect assay according to the commercially available Cayman Chemicals assay kits.

Mitochondrial measurements of ROS production and mitochondrial membrane potential

Hydrogen peroxide

Myotubes were incubated in PBS containing 1 μmol/l of the H_2O_2-sensitive fluorescent probe 1 carboxy-H_2 [5-(and-6)-carboxy-2′, 7′-dichlorohydofluorescein diacetate] (DCFDA; Molecular Probes) for 15 min in the dark at 37ºC in 5% CO_2. Myotubes were washed twice with PBS and fluorescence emission measured at 490 nm and 516 nm, respectively.

Superoxide

Superoxide production was measured in myotubes grown in 96-well opaque plates and stained using MitoSOX red. Briefly, cells were incubated in Hanks balanced salt solution (HBSS) containing 2 μM of MitoSOX red for 10 min in the dark at 37ºC in 5% CO_2. Cells were washed three times in warm HBSS and fluorescence intensity detected with excitation and emission wavelengths of 510 and 580 nm, respectively. Fluorescent values for both the DCFDA and MitoSOX assay were corrected for cell density using mg of protein.

Membrane potential

Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) is a cationic, lipophilic dye that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential. For estimation of membrane potential, cells were incubated with differentiation media containing 100 nM TMRE for 20 min at 37ºC in a humidified atmosphere of 5% CO_2. At the end of 20 min, cells were washed three times with PBS, and a micro plate reader was used to measure TMRE fluorescence (excitation 549 nm, emission 574 nm). Fluorescent values were corrected for mtDNA copy number as a marker of mitochondrial mass.

Determination of fatty acid metabolism

Myocytes maintained in differentiation medium for 5 days with either gAD (0.25 and 0.5 μg/ml), mock-siRNA, AdipoR1/R2-specific siRNA, or ara-A were incubated at 37ºC in sealed 24-well plates containing 1000 μl serum-free DFM plus 12.5 mmol/l HEPES, 0.2% BSA, 1.0 mmol/l carnitine, 100 μm Palmitic acid, 50 μg/ml gentamicin, and 1.0 μCi/ml [1-14C]-palmitate (NEN). After 3 hr, the incubation media was transferred to new dishes and assayed for labeled oxidation products (CO_2 and acid-soluble metabolites [ASMs]; Hulver et al., 2005). The cells were placed on ice, washed twice with PBS, scraped into a 1.5-ml eppendorf tube in two additions of 0.30 ml 0.05% SDS lysis buffer, and then stored at −80ºC. Cell lysates were later assayed for protein.

Confocal microscopy

Mitochondrial content

In 12-well collagen-treated plates, myotubes were washed two times in PBS and cells were incubated at 37ºC for 30 min with 100 nM MitoTracker Green FM (Molecular Probes). Myotubes were washed twice with PBS, and a single in-focus optical section was acquired.

Fluorescent imaging of ROS

To analyze ROS activity in myotubes, muscle cells were incubated with the reduced form of MitoTracker Red (MitoTracker Red CM-H_2XROS; Molecular Probes), which associates with mitochondria and becomes fluorescent after exposure to ROS (Kukidome et al., 2006). The probe was removed by washing cells twice in PBS and cells were imaged using a confocal microscope (Nikon Eclipse TE2000-U) with an ×40 air objective. MitoTracker Red was imaged using the HeNe 543 line and a LP 560 filter.

Statistical analysis

The significance of difference was determined by analysis of variance. In 18 Mexican subjects, age, sex, BMI, percent body fat, insulin sensitivity (M value), fasting plasma insulin and glucose, plasma fatty acids, AMPKα1/α2 mRNA PGC1α/β mRNA, TCAD mRNA, mtTFA mRNA, citrate synthase, and β-HAD enzyme activity were entered in stepwise regression models to assess their contribution to the interindividual variability of mtDNA copy number, AdipoR1, and AdipoR2 mRNA. Pearson correlation coefficients were calculated to determine the relationship between selected variables within each group. All data are presented as means ± SEM. p values < 0.05 were considered statistically significant.

Supplemental data

Supplemental data include two figures and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/1/75/DC1/.

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