Regulation of Substrate Oxidation Preferences in Muscle by the Peptide Hormone Adropin

Rigorous control of substrate oxidation by humoral factors is essential for maintaining metabolic homeostasis. During feeding and fasting cycles, carbohydrates and fatty acids are the two primary substrates in oxidative metabolism. Here, we report a novel role for the peptide hormone adropin in regulating substrate oxidation preferences. Plasma levels of adropin increase with feeding and decrease upon fasting. A comparison of whole-body substrate preference and skeletal muscle substrate oxidation in adropin knockout and transgenic mice suggests adropin promotes carbohydrate oxidation over fat oxidation. In muscle, adropin activates pyruvate dehydrogenase (PDH), which is rate limiting for glucose oxidation and suppresses carnitine palmitoyltransferase-1B (CPT-1B), a key enzyme in fatty acid oxidation. Adropin down-regulates PDH kinase-4 (PDK4) that inhibits PDH, thereby increasing PDH activity. The molecular mechanisms of adropin’s effects involve acetylation (suggesting inhibition) of the transcriptional coactivator PGC-1α, downregulating expression of Cpt1b and Pdk4. Increased PGC-1α acetylation by adropin may be mediated by inhibiting Sirtuin-1 (SIRT1), a PGC-1α deacetylase. Altered SIRT1 and PGC-1α activity appear to mediate aspects of adropin’s metabolic actions in muscle. Similar outcomes were observed in fasted mice treated with synthetic adropin. Together, these results suggest a role for adropin in regulating muscle substrate preference under various nutritional states.

Evidence suggests that the peptide hormone adropin plays a role in energy homeostasis (1,2). These studies linked adropin actions to the control of lipid and glucose metabolism, however the exact role remains unclear (1,2). Adropin is mainly expressed in liver (1) where its expression is stimulated by feeding and suppressed by fasting (1,2). Plasma adropin concentrations are also reduced by fasting (1,2). These results suggest that adropin might be involved in regulating metabolic homeostasis during the transition between fed and fasting conditions. In the fasted state, fatty acids are the major fuel in oxidative metabolism, whereas glucose oxidation is suppressed; conversely, in the fed state, glucose is a primary fuel source for oxidative metabolism (3). We therefore hypothesized that adropin regulates fuel preference in the feeding and fasting cycles.

In this study, we evaluated the role of adropin in regulating substrate oxidation preference in muscle using adropin knockout (AdrKO) and transgenic overexpression (AdrTG) mouse models. To investigate whether the release of adropin in the fed condition regulates fuel preference, we investigated fuel selection preferences in AdrKO in the
fed state and in AdrTG in the fasted state. The rationale for this approach is based on AdrKO being unable to increase adropin production with feeding, whereas in our hands, fasted AdrTG exhibit plasma adropin levels comparable to that observed in fed mice. We also investigated whether injections of synthetic adropin reverse the changes observed in fuel selection associated with fasting. These studies also focused on the roles of Sirtuin 1 (SIRT1) and peroxisome proliferator–activated receptor (PPAR)-γ coactivator-1α (PGC-1α), enzymes previously linked to metabolic adaptation in the fed/fasting cycle (4,5), in mediating the effects of adropin on fuel selection in skeletal muscle, which is quantitatively the most important organ in determining the overall levels of fatty acid and glucose oxidation (3,6).

**RESEARCH DESIGN AND METHODS**

**Animal Studies**

All mouse experiments were approved by the Institutional Animal Care and Use Committees of the Scripps Research Institute (Jupiter, FL). Whole-body AdrKO and AdrTG on a C57BL/6J (B6) background were described previously (1,2). Wild-type (WT) B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments used males aged 8 weeks that were maintained on regular laboratory chow. Indirect calorimetry was performed using a comprehensive laboratory animal monitoring system (Columbus Instruments, Columbus, OH) (1,2). Quadriceps muscles were used in the biochemical assays.

**Adropin Injections**

Adropin34–76 was provided by Ipsen (Paris, France) or purchased from ChinaPeptides (Shanghai, China). Adropin34–76 was dissolved in 0.1% BSA and administered by intraperitoneal injection at 450 nmol/kg body weight. The mice were fasted for 24 h and received three injections during fasting at 6–7-h intervals. Two hours after the last injection, mice were killed, and quadriceps muscles were collected for the subsequent assays. Previous dose-response studies indicate that this injection paradigm produces a physiological response (1).

**Substrate Oxidation Assay**

[1-14C] palmitic acid was used as the substrate in oxidation assays assessing fatty acid oxidation (FAO) in fresh whole-muscle homogenates. 14CO2 production indicates complete oxidation, whereas 14C-labeled acid-soluble metabolites (ASMs) indicate incomplete oxidation (7). Pyruvate oxidation was assessed using [1-14C] pyruvate as previously described (7). 14CO2 production from [1-14C] pyruvate also indicates pyruvate dehydrogenase (PDH) complex activity (8).

**Carnitine Palmitoyltransferase-1 Activity Assay**

Mitochondria-enriched fractions were obtained from fresh muscle tissues (9). Acyltransferase activity was determined by the radiometric method using L-[14C-Me] carnitine and palmitoyl-CoA as substrates as previously described (10).

**Acylcarnitine and Acyl-CoA assay**

Long-chain acylcarnitines (LC-ACs) were extracted and subjected to high-performance liquid chromatography and mass spectrometry for quantification as described previously (11–13). The levels of short-chain acyl-CoAs were measured as reported before (14).

**Antibodies, Immunoprecipitation, and Western Blotting**

Antibodies to PDH kinase-4 (PDK4), PGC-1α (for immunoprecipitation), and histone H4 were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies to acetylated lysine, acetylated p53 (Lys379), phospho-AMPK-α (Thr172), AMPK-α, conformation-specific anti-rabbit IgG, and GAPDH were from Cell Signaling Technology (Danvers, MA). Total PGC-1α and Sirt1 were measured using antibodies from Millipore (Billerica, MA). Antibodies against p53, COXIV, and ATP5F1 were purchased from Novus Biologicals (Littleton, CO). Standard immunoprecipitation procedures (Cell Signaling Technology) and Western blotting procedures (Invitrogen, Carlsbad, CA) were performed, with GAPDH used as a loading control for whole-tissue samples; histone H4 protein was used as the loading control for nuclear extracts. Quantitation by densitometry used Scion image software (Frederick, MD), with data expressed as the ratio of band intensity of the protein of interest to loading control. When immunoblotting procedures of protein samples from the AdrKO and AdrTG groups were performed in the same run; the data were expressed as a ratio of the WT in the AdrKO group. When the blotting procedures were performed separately, the WT in relevant individual groups (AdrKO or AdrTG) were used as the control.

**Real-Time PCR**

Total RNA was extracted using the RNaseasy Mini Kit from Qiagen (Valencia, CA), and cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit from Applied Biosystems (Carlsbad, CA). PCR was performed according to the instructions from the TaqMan Gene Expression Assays on a 7900 Fast Real-time PCR System (Applied Biosystems). GAPDH was used as the loading control.

**SIRT1 Deacetylase Activity Assay**

Deacetylase activity was measured using the nuclear lyase prepared based on the nuclear extraction protocol from Pierce Biotechnology (Rockford, IL) (15). The activity was assessed using a SIRT1 fluorometric assay kit from Enzo Life Sciences (Farmingdale, NY) (16) as described by the manufacturer. Resveratrol, an activator of SIRT1 (17), stimulated SIRT1 deacetylase activity by twofold (7.2 vs. 3.4 arbitrary fluorescence units/min/μg protein with and without resveratrol, respectively), which validates the assay.

**NAD Determination**

Muscle tissue was acid extracted, neutralized, and used for NAD determination (16). NAD levels were measured based on a lactate dehydrogenase cycling reaction (BioAssay...
Systems, Hayward, CA) (18). The NAD levels in the current study are similar to those previously published (18,19).

**Plasma Adropin Level Measurement**
Plasma adropin concentrations were measured using a peptide enzyme immunosorbent assay protocol from Peninsula Laboratories (San Carlos, CA) (2,20).

**Citrate Synthase Activity Assay**
Citrate synthase (CS) activity was measured spectrophotometrically as described elsewhere (21).

**Mitochondrial Respiration**
Mitochondria were isolated from fresh muscle as previously described (9,22). Respirometry of isolated mitochondria was performed using an XF96 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) according to the manufacturer’s instructions.

**Statistics**
Data are presented as mean ± SEM and were evaluated by Student t test or one-way ANOVA (Prism; GraphPad Software, La Jolla, CA). For all tests, \( P < 0.05 \) was considered statistically significant. Between-group differences were tested using unpaired Student t test. For multiple-group comparisons, one-way ANOVA followed by Neuman-Keuls multiple comparison test was performed to evaluate the differences. Statistical significance among individual groups in the multiple-group comparisons is indicated by lines drawn between bars with single, double, or triple asterisks in the figures.

**RESULTS**

**Regulation of Whole-Body Substrate Oxidation by Adropin**
Consistent with previous results (1,2), plasma adropin concentrations decreased from 2 to 3 ng/mL as early as 2 h after fasting initiation and were undetectable after overnight fast (Supplementary Fig. 1). Plasma adropin concentrations increased rapidly with refeeding and were restored to fed levels after 24 h (Supplementary Fig. 1).

To explore whether adropin regulates substrate oxidation preferences, we first performed indirect calorimetry to measure the respiratory exchange ratio (RER), an indicator of substrate preference at the whole-body level (23). In the fed condition, the RER values of AdrKO mice were lower than that of WT littermates (Fig. 1A), suggesting that adropin deficiency induces preferential oxidation of fat over carbohydrate. There was no change in VO2 (Supplementary Fig. 2A) or food intake (Supplementary Fig. 2B) in AdrKO.

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**Figure 1** — A: The AdrKO mice and WT littermates were maintained in a fed condition. RER was monitored in one dark-light cycle (\( n = 4–9 \)). B: Food was removed from the AdrTG mice and the WT littermates before the dark onset, and RER was monitored in the following dark-light cycle (\( n = 6–10 \)). The six-period moving average trend line was calculated to represent the data points. *\( P < 0.05 \).
We next assessed whole-body fuel oxidation preference in AdrTG mice. Although overnight fasting reduced adropin concentrations below the detection limit in the WT controls, plasma adropin concentrations remained at fed levels in AdrTG mice (WT, not detectable; AdrTG, $3.4 \pm 0.53$ ng/mL). In fasted animals, the RER values of AdrTG were higher compared with the WT littermates (Fig. 1B), indicating that adropin overexpression promotes glucose oxidation over fat oxidation. VO$_2$ was normal in AdrTG mice during the fast (Supplementary Fig. 2C).

**Suppression of Muscle FAO by Adropin**

We next assessed the potential role of adropin in regulating FAO in muscle. AdrKO mice exhibited increased complete FAO (Fig. 2A) and a trend toward increased incomplete oxidation in skeletal muscle (Fig. 2A). Carnitine palmitoyltransferase-1B (CPT-1B) is a key enzyme in muscle mitochondrial FAO (24); Cpt1b expression (Fig. 2B) and acyltransferase activity (Fig. 2C) were increased in AdrKO. We also observed significant increases in LC-ACs (Fig. 2D), which are the end products of CPT-1 activity (13). In WT mice, fasting increases Cpt1b expression (3) and LC-AC levels (25) (Supplementary Fig. 3), indicating increased CPT-1 activity (26). Taken together, the data suggest that adropin deficiency and fasting induce similar changes in FAO in skeletal muscle.

Examination of FAO in AdrTG mice revealed opposite changes to those observed in AdrKO. Complete and incomplete FAO were reduced in AdrTG (Fig. 2E), and Cpt1b expression (Fig. 2F) and CPT-1 activity were reduced (Fig. 2G). The levels of LC-AC were also decreased in AdrTG (Fig 2H), which is consistent with the reduced CPT-1 activity. Malonyl-CoA is a physiological inhibitor of CPT-1 (27). In the present studies, no changes in the malonyl-CoA level were evident in either AdrKO or AdrTG (Supplementary Fig. 4A and B).

**Stimulation of Muscle Pyruvate Oxidation by Adropin**

Glucose oxidation was assessed by measuring the oxidation of [1-14C] pyruvate, which also indicates activity of the PDH complex, a key step in the glucose oxidative pathway (28,29). Radiolabeled glucose was not used as the substrate because data interpretation is confounded by the conversion of significant portions of glucose to glycogen or fatty acids (28,29). Pyruvate oxidation or PDH activity was decreased in AdrKO muscle (Fig. 3A) and was increased in AdrTG muscle (Fig. 3A), which is consistent with adropin-promoting glucose oxidation.

Starvation decreases muscle glucose oxidation levels in part by inhibiting PDH activity (3,29,30). We confirmed this finding in this study (fasted WT in the AdrTG group, 90 $\pm$ 5 nmol/mg/h; fed WT in the AdrKO group,

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**Figure 2**—A: In the AdrKO group, complete FAO, indicated by CO$_2$ production, and incomplete FAO, indicated by ASM production, in muscle lysate were measured ($n = 7$–8). B: Cpt1b message levels were measured ($n = 6$–7). C: Muscle mitochondria were isolated and CPT-1 activity measured. The activity assay was performed in three separate groups. The results in individual groups were converted to % WT and pooled ($n = 11$–13). D: LC-ACs, including 16:0 (palmitoyl carnitine), 18:0 (stearoyl carnitine), and 18:1 (oleoyl carnitine), in muscle were quantified ($n = 3$–5). E: In the AdrTG group, complete FAO and incomplete FAO in muscle lysate were measured ($n = 4$–11). F: Cpt1b message levels were measured ($n = 5$–8). G: Muscle CPT-1 activity assay was performed in two separate groups. The results in individual groups were converted to % WT and pooled ($n = 6$–10). H: LC-ACs in muscle were quantified ($n = 4$–7). *,**, and ***$P < 0.05$. 

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Adropin-deficient mice in the fed state thus displayed reduced pyruvate oxidation/ PDH activity similar to that observed with fasting. In muscle, the activity of PDH is inhibited by PDK2 and PDK4, which phosphorylate and inactivate PDH (30). Along with the changes in PDH activity, Pdk4 mRNA and protein levels were increased in AdrKO mice and decreased in AdrTG mice (Fig. 3B and C). As a control, we confirmed previous findings (3,28) that mRNA and protein levels of PDK4 are upregulated with fasting (Fig. 3B and C). Alteration of protein abundance is a key mechanism in the regulation of PDK4 activity (3). Thus, in AdrKO, PDK4 activity is expected to increase, contributing to the reduction of PDH activity, whereas in AdrTG, inhibition of PDK4 is expected to activate PDH. Expression of PDK2 was not affected by genotype (data presented as % WT of the KO group) (AdrKO group: WT, 100 ± 6.9; KO, 93 ± 10; AdrTG group: WT, 74 ± 14; TG, 103 ± 11), suggesting a specific role for PDK4 in mediating the metabolic response of muscle to adropin.

Both PDK4 and PDH activities are subject to the regulation of CoA and acetyl-CoA (28,31). An increase in the CoA/acetyl-CoA ratio inhibits PDK4 and activates PDH (28,31). Acetyl-CoA is the end product of mitochondrial fatty acid β-oxidation, and excessive β-oxidation beyond tricarboxylic acid cycle capacity can result in sequestration of free CoA in acetyl-CoA, reducing the ratio of free CoA/ acetyl-CoA (25). Because adropin inhibits CPT-1 activity, it is expected that the CoA/acetyl-CoA ratio would also be increased. Indeed, in AdrTG mice, we observed an increase in the CoA/acetyl-CoA ratio (Fig. 3D) as a result of reduced acetyl-CoA level (Supplementary Fig. 4B). In AdrKO mice, no significant changes in the CoA/acetyl-CoA ratio were observed (Fig. 3D), despite an increase in CPT-1 activity. Similarly, we could not find changes in the CoA/acetyl-CoA ratio following fasting (Supplementary Fig. 4C), despite finding that the in vivo CPT-1 activity is elevated in the fasted state (3,26,32).

### Effect of Adropin on PGC-1α Acetylation State

PGC-1α is a key transcriptional regulator of oxidative metabolism (4). PGC-1α coactivates various transcriptional
factors, such as PPARs, to control the expression of genes, including Cpt1b and Pdk4 (4). On the basis of the observed changes in Cpt1b and Pdk4 expression, we hypothesized a role for PGC-1α in mediating the metabolic actions of adropin. Acetylation of lysine residues is a key mechanism in regulating PGC-1α activity; increased acetylation usually inhibits transcriptional activity (33). Fasting reduced PGC-1α acetylation in muscle of WT mice (Supplementary Fig. 5A), confirming previous findings of the increased PGC-1α activity during fasting (19,34). AdrKO exhibited reduced PGC-1α acetylation (Fig. 4A), indicating activation. Conversely, AdrTG exhibited increased PGC-1α acetylation (Fig. 4A), indicating inhibition. There were no significant changes in PGC-1α protein expression (Fig. 4B and C) or mRNA levels (Supplementary Fig. 5B) in either fasted, AdrKO, or AdrTG mice. Together, these data suggest that adropin suppresses PGC-1α activity in muscle.

PGC-1α controls mitochondrial biogenesis (35). We measured CS activity, a biomarker of mitochondrial content (36). CS activity in muscle lysate was not altered by fasting, adropin deletion, or overexpression of adropin (Supplementary Fig. 6A). CS protein content and the levels of the OXPHOS proteins, including COXIV and ATP5F1, were also not changed (Supplementary Fig. 6B). Furthermore, expression of mitochondrial transcription factor A, an essential regulator of mitochondrial biogenesis (37), was not significantly altered in these mouse models (Supplementary Fig. 6C). Finally, mitochondrial respiration capacity indicated by FCCP-induced VO₂ was not altered by AdrKO or overexpression (Supplementary Fig. 6D). Thus, adropin does not appear to regulate mitochondrial content, despite affecting PGC-1α.

We next measured the expression of several key transcription factor partners of PGC-1α involved in FAO. There were no significant changes in the expression levels of these transcription factors in AdrKO or AdrTG (Supplementary Fig. 7). This suggests that PGC-1α mediates adropin’s actions of Cpt1b and Pdk4 expression by altering the activity, rather than the amount, of the transcription factor partners.

A Role for SIRT1 in Mediating Adropin’s Metabolic Actions in Muscle

PGC-1α is deacetylated and activated by SIRT1, a mammalian sirtuin deacetylase (33,38). SIRT1 protein levels in muscle are increased by fasting, contributing to the hypoacetylation and activation of PGC-1α (5). We confirmed the increased SIRT1 protein levels following fasting (Supplementary Fig. 8A). SIRT1 protein levels were increased in fed AdrKO and decreased in fasted AdrTG mice compared with the relevant controls (Fig. 5A). We further measured SIRT deacetylase activity in nuclei where SIRT1 is mainly localized (39). Consistent with the changes in the protein level, SIRT activity was increased by fasting and adropin deficiency (Fig. 5B). In contrast, SIRT activity was reduced in AdrTG (Fig. 5B). Moreover, acetylation of p53, a surrogate marker of SIRT1 activity (15,40,41), was decreased in AdrKO and increased in AdrTG (Fig. 5C). Collectively, these data indicate that adropin’s effect on SIRT1 activity is partly due to the changes in protein level. The fasting-induced increase in SIRT1 protein level

![Figure 4](image-url) — The AdrKO group was maintained in the fed state, and the AdrTG group was fasted overnight (16 h). A: PGC-1α protein was immunoprecipitated from whole-tissue lysate, and the immunoprecipitated products were immunoblotted for acetylated lysine (Ac-K) (AdrKO group, n = 5–6; AdrTG, n = 6–8). The light chain of IgG (IgG-LC) was used as the loading control. (Note: the blotting of the two groups was performed in separate runs.) B: PGC-1α protein was immunoprecipitated from whole-tissue lysate, and the immunoprecipitated products were immunoblotted for PGC-1α protein (AdrKO group, n = 4; AdrTG, n = 4). The IgG–LC was used as the loading control. C: The PGC-1α protein levels in the same lysate were directly measured by immunoblotting (AdrKO group, n = 4–6; AdrTG group, n = 6). IB, immunoblotting; IP, immunoprecipitation. *P < 0.05.
in muscle is mediated, in part, by increased mRNA levels (5). However, although we observed changes in SIRT1 protein levels in our mouse models, neither adropin deletion nor overexpression affected Sirt1 mRNA levels, suggesting posttranslational mechanisms (Supplementary Fig. 8B).

SIRT1 activity is also regulated by NAD+, the obligatory substrate and an activator of the deacetylase activity (42). We confirmed that fasting triggers an increase in NAD+ level in muscle (Fig. 5D), which contributes to SIRT1 activation (19). Similar to fasting, adropin deletion (AdrKO) increased NAD+ levels (Fig. 5D), whereas adropin overexpression (AdrTG) decreased NAD+ levels (Fig. 5D). By modulating cellular NAD+ levels, AMPK has been proposed to play a prominent role in regulating SIRT1 activity in muscle (43). To assess the potential role of AMPK, we first performed a fasting time-course study. Surprisingly, the levels of phosphorylated AMPK, a marker of AMPK activity, were not altered during the entire fasting period (Supplementary Fig. 9A). Importantly, the AMPK activities were similar in AdrKO or AdrTG animals compared with the individual WT controls (Supplementary Fig. 9B), indicating that adropin’s metabolic actions may not involve changes in AMPK activity.

Metabolic Adaptation of Muscle Is Inhibited by Acute Treatment With Adropin
We have presented a scenario of adropin’s metabolic actions in muscle using KO and TG models. Interpretation of data obtained from genetic models can be confounded by the compensatory mechanisms during development. We therefore wondered whether the observed changes in muscle metabolism reflect the primary effects of adropin or are secondary to the potential compensatory actions triggered by genetic manipulation. To address this concern, we used a pharmacological approach involving acute adropin administration, which less likely induces developmental compensation.

We injected fasted mice with adropin$^{34–76}$ and examined whether the metabolic adaptation of muscle to fasting would be inhibited following the treatment. Adropin treatment decreased SIRT1 protein level (Fig. 6A) without altering the mRNA level (Supplementary Fig. 8C).
Following adropin injection, the acetylation of PGC-1α was increased (Fig. 6A), and the PGC-1α target genes, including Cpt1b and Pdk4, were downregulated (Fig. 6B and C). Furthermore, CPT-1 activity and LC-AC levels were reduced (Fig. 6D and E). Accompanying the inhibition of CPT-1B activity, adropin treatment decreased incomplete FAO, indicated by the reduction of ASM production (Fig. 6F). Suppression of Pdk4 expression is expected to activate PDH, and we demonstrated an increase in PDH activity and pyruvate oxidation following adropin treatment (Fig. 6G).

**DISCUSSION**

We have observed that adropin deficiency produces changes in substrate oxidation that are similar to those observed with fasting, a state when plasma adropin concentrations are diminished. In contrast, the metabolic phenotype of fasted AdrTG where the decline in adropin is prevented recapitulates that of fed mice. Similar to the adropin overexpression, treatment of mice with synthetic adropin prevents fasting-associated changes in substrate oxidation. Taken together, the data suggest that the peptide hormone adropin plays a significant role in regulating substrate oxidation during feeding and fasting cycles.

Our data indicate that skeletal muscle is a key organ in mediating adropin’s effects on whole-body substrate oxidation. In muscle, the signaling pathways underlying adropin’s metabolic actions appear to involve SIRT1 and PGC-1α. The data show that adropin inhibits SIRT1 deacetylase activity, thereby inducing hyperacetylation of PGC-1α that would be expected to inhibit activity. This would lead to downregulation of PGC-1α target genes, including Cpt1b and Pdk4. CPT-1B and PDK4 through PDH play gatekeeping roles in FAO and glucose oxidation. In muscle, the inhibition of CPT-1B and downregulation of PDK4 appear to play important roles in adropin’s inhibition of FAO and activation of pyruvate oxidation.

Regulation of PDH activity is complex and in muscle, involves another kinase belonging to the same family (PDK2); both are kinases regulated by other variables related to energy flux within the cell. However, fasting induces changes in PDK4 expression but not PDK2 (44).
We also observed no evidence for regulation of PDK2 expression by adropin, which is consistent with changes in plasma adropin being a major factor in the regulation of muscle PDH activity in fed-fasting cycles.

FAO and glucose oxidation interact to regulate the activity of each other. FAO exerts control over glucose oxidation through the CoA/acyetyl-CoA ratio that affects PDK4 and PDH activities (Randle cycle) (3). The Randle cycle seems to be operational in mediating adropin’s metabolic actions in muscle. In AdrTG, the CoA/acyetyl-CoA ratio is increased as a result of the inhibition of FAO, and this is expected to contribute to the suppression of PDK4 and activation of PDH. Regarding the effect of glucose oxidation on FAO, elevated glucose oxidation increases mitochondrial acetyl-CoA. If this exceeds the tricarboxylic acid cycle capacity, excessive acetyl-CoA is shuttled to the cytoplasm and converted by acetyl-CoA carboxylase to malonyl-CoA, an inhibitor of CPT-1 (3,45). In the current studies, adropin overexpression activates pyruvate oxidation; however, we could not find the anticipated increase in malonyl-CoA levels. These results are consistent with the hypothesis (46) that sufficient mitochondrial oxidative capacity exists in accommodating elevated substrate flux. In line with this argument, we also could not find changes in the acetyl-CoA level in either AdrKO or fasted WT mice, despite the FAO levels being elevated in both conditions. AMPK affects malonyl-CoA level by phosphorylating and inhibiting acetyl-CoA carboxylase (3). Consistent with the lack of changes in malonyl-CoA level (Supplementary Fig. 4A and B), we could not detect changes in the activities of AMPK in our mouse models.

The present results suggest that PGC-1α acts as a converging point in adropin’s actions on FAO and glucose oxidation. PGC-1α is a well-established substrate of SIRT1, and we observed changes in SIRT1 activity that correlate with changes in the PGC-1α acetylation. These data suggest that adropin’s effects on PGC-1α acetylation may be secondary to altered SIRT1 activity. Changes in PGC-1α activity can alter mitochondrial respiration capacity, and mitochondrial capacity responds to the cellular level of energy expenditure that is in turn driven by energy demand (47). However, there are no changes in muscle energy demand (and thus energy expenditure) in either AdrKO or AdrTG, and it follows that adropin’s signaling actions do not affect mitochondrial capacity. At the molecular level, PGC-1α may selectively activate the transcriptional partners involved in substrate oxidation but not those controlling mitochondrial biogenesis. Thus, in the context of the regulation of substrate oxidation, adropin appears to control fuel preference rather than the total level of energy production.

The results suggest that adropin regulates SIRT1 activity in part by modulating protein abundance. SIRT1 protein levels change in the absence of altered message levels, indicating that adropin’s effects on SIRT1 involve posttranscriptional mechanisms. Evidence suggests that SIRT1 protein stability is regulated by c-Jun N-terminal kinase (JNK). Notably, the JNK2 isoform has been shown to protect SIRT1 from ubiquitination-mediated proteasome degradation, increasing the half-life of the protein (48). Indeed, we have observed changes in JNK2 activity in AdrKO and AdrTG mice (data not shown), leaving open the possibility that adropin affects JNK2 activity to control SIRT1 protein stability. The potential roles of JNK in adropin’s signaling pathways are currently under investigation.

The adropin-mediated change in NAD+ level provides an additional mechanism underlying the alteration of SIRT1 activity. However, the mechanisms underlying the change in NAD+ level are elusive. It is known that AMPK plays a role in modulating NAD+ level by affecting the protein level of nicotinamide phosphoribosyltransferase, which is the rate-limiting enzyme in the salvage pathway in NAD+ biosynthesis. The present results do not support a role of AMPK in adropin’s metabolic actions because no changes in AMPK activity were observed. Evidence indicates that increased FAO may be a mechanistic step in upregulating NAD+ levels because treatment with the CPT-1 inhibitor etomoxir abrogates the response (19). Accordingly, we hypothesize that the observed changes in NAD+ level in our mouse models are an outcome of adropin’s actions on CPT-1B activity and FAO. The results also suggest a model where adropin triggers the inhibition of SIRT1 activity through an NAD+-independent mechanism but maintains the activity state by modulating NAD+ level. Alternatively, adropin’s effect on NAD+ level could act in a feed-forward way, amplifying the downstream actions resulting from the altered SIRT1 activity.

The current data do not support the popular notion that AMPK activation is a component in the adaptive response during fasting (3). This notion is almost exclusively based on the studies of in vitro cell models. In cultured cells, glucose withdrawal is frequently used to mimic food deprivation. This challenge can easily induce cellular energy stress (i.e., reduction or even depletion of ATP), which rapidly activates AMPK (19). However, muscle cells in vivo may not experience drastic reductions in substrate availability during fasting, at least initially. Fatty acids mobilized from adipose tissue during a fast provide muscle with a fuel source in replacement of glucose (3).

In summary, we have identified a novel role of the peptide hormone adropin in regulating muscle substrate oxidation during the feeding and fasting cycle. Mitochondrial fatty acid overload in skeletal muscle may contribute to insulin resistance (25). Because adropin inhibits CPT-1B activity in muscle, pharmacological treatment with adropin has the potential to enhance glucose utilization. Adropin may therefore be a promising candidate for developing treatments to combat insulin resistance and type 2 diabetes.

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References
1. Kumar KG, Trevaskis JL, Lam DD, et al. Identification of adipron as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism. Cell Metab 2008;8:468–481
2. Ganesh Kumar K, Zhang J, Gao S, et al. Adipron deficiency is associated with increased adiposity and insulin resistance. Obesity (Silver Spring) 2012;20:1394–1402
3. de Lange P, Moreno M, Silvestri E, Lombardi A, Goglia F, Lanni A. Fuel economy in food-deprived skeletal muscle: signaling pathways and regulatory mechanisms. FASEB J 2007;21:3431–3441
4. Klein S, Nguyen-Tran V, Baré O, Huang X, Spiegelman B, Wu Z. PPARα agonist activates fatty acid oxidation via PGC-1α but does not increase mitochondrial gene expression and function. J Biol Chem 2009;284:18624–18633
5. Noriega LG, Feige JN, Canto C, et al. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. EMBO Rep 2011;12:1069–1076
6. McFarlan JT, Yoshida Y, Jain SS, et al. In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation. J Biol Chem 2012;287:23502–23516
7. Frisard MI, McMillan RP, Marchand J, et al. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. Am J Physiol Endocrinol Metab 2010;298:E988–E998
8. Abdel-aleem S, Sayed-Ahmed M, Nada MA, Hendrickson SC, St Louis J, Lowe JE. Stimulation of non-oxidative glucose utilization by L-carnitine in isolated myocytes. J Mol Cell Cardiol 1995;27:2465–2472
9. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2007;2:287–295
10. Morillas M, Gómez-Puertas P, Roca R, et al. Structural model of the catalytic core of carnitine palmitoyltransferase I and carnitine octanoyltransferase (COT): mutation of CPT I histidine 473 and alanine 381 and COT alanine 238 impairs the catalytic activity. J Biol Chem 2001;276:45001–45008
11. Gao S, Keung W, Serra D, et al. Malonyl-CoA mediates leptin hypothalamic control of feeding independent of inhibition of CPT-1a. Am J Physiol Regul Integr Comp Physiol 2011;301:R209–R217
12. Wende AR, Huss JM, Schaeffer PJ, Giguère V, Kelly DP. PGC-1α/CoT coactivates PDK4 gene expression via the orphan nuclear receptor ERRα/β: a mechanism for transcriptional control of muscle glucose metabolism. Mol Cell Biol 2005;25:10684–10694
13. Jauregui O, Sierra AY, Carrasco P, Gratacós E, Hegardt FG, Casals N. A new LC-ESI-MS/MS method to measure long-chain acylcarnitine levels in cultured cells. Anal Chim Acta 2007;599:1–6
14. Keung W, Uscher JR, Jaswal JS, et al. Inhibition of carnitine palmitoyltransferase-1 activity alleviates insulin resistance in diet-induced obese mice. Diabetes 2013;62:711–720
15. White AT, McCurdy CE, Philip A, Hamilton DL, Johnson CD, Schenk S. Skeletal muscle-specific overexpression of SIRT1 does not enhance whole-body energy expenditure or insulin sensitivity in young mice. Diabetologia 2013;56:1629–1637
16. Chabi B, Adhihetty PJ, O’Leary MFS, Menzies KJ, Hood DA. Relationship between Sirt1 expression and mitochondrial proteins during conditions of chronic muscle use and disuse. J Appl Physiol (1985) 2009;107:1730–1735
17. Lagoue M, Armgann C, Gerhart-Hines Z, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. Cell 2006;127:1109–1122
18. Bai P, Canto C, Oudart H, et al. PPAR-1 inhibition increases mitochondrial metabolism through SIRT1 activation. Cell Metab 2011;13:461–468
19. Canto C, Jiang LQ, Deshmukh AS, et al. Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. Cell Metab 2010;11:213–219
20. Butler AA, Tam CS, Stanohe KL, et al. Low circulating adipron concentrations with obesity and aging correlate with risk factors for metabolic disease and increase after gastric bypass surgery in humans. J Clin Endocrinol Metab 2012;97:3783–3791
21. Cali JA, Voelker KA, Wolff AV, et al. Endurance capacity in maturing mdx mice is markedly enhanced by combined voluntary wheel running and green tea extract. J Appl Physiol (1985) 2008;105:923–932
22. Liu N, Bezprowansy S, Shilton JM, et al. Mice lacking microRNA 133a develop dynamin 2-dependent centronuclear myopathy. J Clin Invest 2011;121:3258–3268
23. Ferrannini E. The theoretical bases of indirect calorimetry: a review. Metabolism 1988;37:287–301
24. Cross DT 3rd, Moron CJ, Brown AP, et al. Endovascular treatment of epitaxias in a patient with tuberculosis and a giant petrous carotid pseudoaneurysm. AJNR Am J Neuroradiol 1995;16:1084–1086
25. Koves TR, Ussher JR, Noland RG, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab 2008;7:45–56
26. Rasmussen BB, Holmåcck UB, Volpi E, Morio-Liondore B, Padddon-Jones D, Wolfe RR. Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. J Clin Invest 2002;110:1687–1693
27. McGarry JD, Mills SE, Long CS, Foster DW. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. Biochem J 1983;214:21–28
28. Sugden MC, Holness MJ. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. Am J Physiol Endocrinol Metab 2003;284:E855–E862
29. Berger M, Hagg SA, Goodman MN, Ruderman NB. Glucose metabolism in perfused skeletal muscle. Effects of starvation, diabetes, fatty acids, acetoacetate, insulin and exercise on glucose uptake and disposition. Biochem J 1976;158:191–202
30. Holness MJ, Sugden MC. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem Soc Trans 2003;31:1143–1151
31. Pettit FH, Pelley JW, Reed LJ. Regulation of pyruvate dehydrogenase kinase and phosphatase by acetyl-CoACoA and NADH/NAD ratios. Biochem Biophys Res Commun 1975;65:575–582
32. Shuldiner AR, McLenithan JC. Genes and pathophysiology of type 2 diabetes: more than just the Randle cycle all over again. J Clin Invest 2004;114:1414–1417
33. Jenina EH, Schoonjans K, Auwerx J. Reversible acetylation of PGC-1: connecting energy sensors and effectors to guarantee metabolic flexibility. Oncogene 2010;29:4617–4624
34. Gerhart-Hines Z, Rodgers JT, Bare O, et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. EMBO J 2007;26:1913–1923
35. Wu Z, Puigserver P, Andersson U, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115–124
36. Larsen S, Nielsen J, Hansen CN, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 2012;590:3349–3360
37. Larsson NG, Wang J, Wilhelmsson H, et al. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat Genet 1998;18:231–236
38. Nemoto S, Fergusson MM, Finkel T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1alpha. J Biol Chem 2005;280:16456–16460
39. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nat Rev Mol Cell Biol 2012;13:225–238
40. Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2(SIRT1) functions as an NAD-dependent p33 deacetylase. Cell 2001;107:149–159
41. Schenk S, McCurdy CE, Philp A, et al. Sirt1 enhances skeletal muscle insulin sensitivity in mice during caloric restriction. J Clin Invest 2011;121:4281–4288
42. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000;403:795–800
43. Cantó C, Gerhart-Hines Z, Feige JN, et al. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 2009;458:1056–1060
44. Wu P, Inskeep K, Bowker-Kinley MM, Popov KM, Harris RA. Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. Diabetes 1999;48:1593–1599
45. Majerus PW, Lastra R. Fatty acid biosynthesis in human leukocytes. J Clin Invest 1967;46:1596–1602
46. Dawson KD, Baker DJ, Greenhaff PL, Gibala MJ. An acute decrease in TCA cycle intermediates does not affect aerobic energy delivery in contracting rat skeletal muscle. J Physiol 2005;565:637–643
47. Muoio DM, Newgard CB. Fatty acid oxidation and insulin action: when less is more. Diabetes 2008;57:1455–1456
48. Ford J, Ahmed S, Allison S, Jiang M, Milner J. JNK2-dependent regulation of SIRT1 protein stability. Cell Cycle 2008;7:3091–3097