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

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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 downregulates 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

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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

Adropin$^{34–76}$ was provided by Ipsen (Paris, France) or purchased from ChinaPeptides (Shanghai, China). Adropin$^{34–76}$ 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-^{14}C] palmitic acid was used as the substrate in oxidation assays assessing fatty acid oxidation (FAO) in fresh whole-muscle homogenates. $^{14}CO_2$ production indicates complete oxidation, whereas ^{14}C-labeled acid-soluble metabolites (ASMs) indicate incomplete oxidation (7). Pyruvate oxidation was assessed using [1-^{14}C] pyruvate as previously described (7). $^{14}CO_2$ production from [1-^{14}C] 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-[^{14}C-*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 (Lys^{379}), phospho-AMPK-α (Thr^{172}), 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 WTs in relevant individual groups (AdrKO or AdrTG) were used as the control.

Real-Time PCR

Total RNA was extracted using the RNeasy 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 lysate 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. 1*A*), suggesting that adropin deficiency induces preferential oxidation of fat over carbohydrate. There was no change in VO_2 (Supplementary Fig. 2*A*) or food intake (Supplementary Fig. 2*B*) in AdrKO.

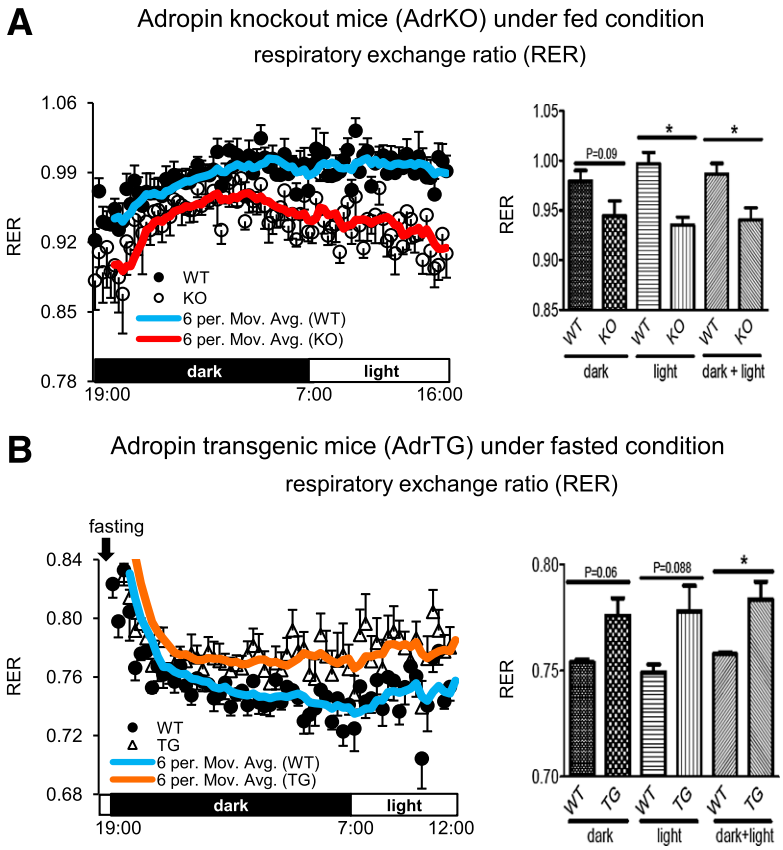

Figure 1—*A*: The AdrKO mice and WT littermates were maintained in a fed condition. RER was monitored in one dark-light cycle (n = 4–9). *B*: Food was removed from the AdrTG mice and the WT littermates before the dark onset, and RER was monitored in the following dark-light cycle (n = 6–10). The six-period 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 ± 0.53 ng/mL). In fasted animals, the RER values of AdrTG were higher compared with the WT littermates (Fig. 1*B*), indicating that adropin overexpression promotes glucose oxidation over fat oxidation. VO_2 was normal in AdrTG mice during the fast (Supplementary Fig. 2*C*).

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. 2*A*) and a trend toward increased incomplete oxidation in skeletal muscle (Fig. 2*A*). Carnitine palmitoyltransferase-1B (CPT-1B) is a key enzyme in muscle mitochondrial FAO (24); *Cpt1b* expression (Fig. 2*B*) and acyltransferase activity (Fig. 2*C*) were increased in AdrKO. We also observed significant increases in LC-ACs (Fig. 2*D*), 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. 2*E*), and *Cpt1b* expression (Fig. 2*F*) and CPT-1 activity were reduced (Fig. 2*G*). The levels of LC-AC were also decreased in AdrTG (Fig 2*H*), 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. 4*A* and *B*).

Stimulation of Muscle Pyruvate Oxidation by Adropin

Glucose oxidation was assessed by measuring the oxidation of [1-^{14}C] 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. 3*A*) and was increased in AdrTG muscle (Fig. 3*A*), 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 AdrTG group, 90 ± 5 nmol/mg/h; fed WT in the AdrKO group,

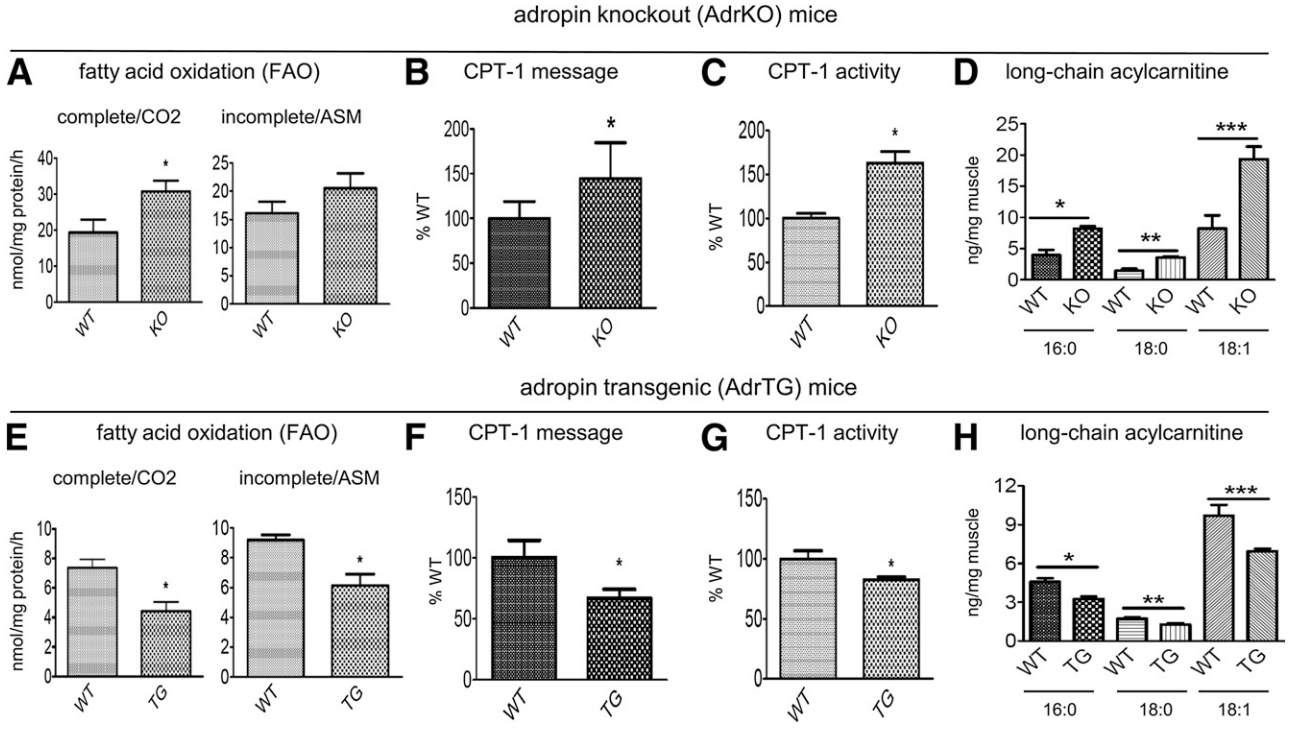

Figure 2—*A*: In the AdrKO group, complete FAO, indicated by CO_2 production, and incomplete FAO, indicated by ASM production, in muscle lysate were measured (n = 7–8). *B*: *Cpt1b* message levels were measured (n = 6–7). *C*: Muscle mitochondria were isolated and CPT-1 activity measured. The activity 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 (palmitoylcarnitine), 18:0 (stearoylcarnitine), and 18:1 (oleoylcarnitine), 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 ***$P < 0.05$.

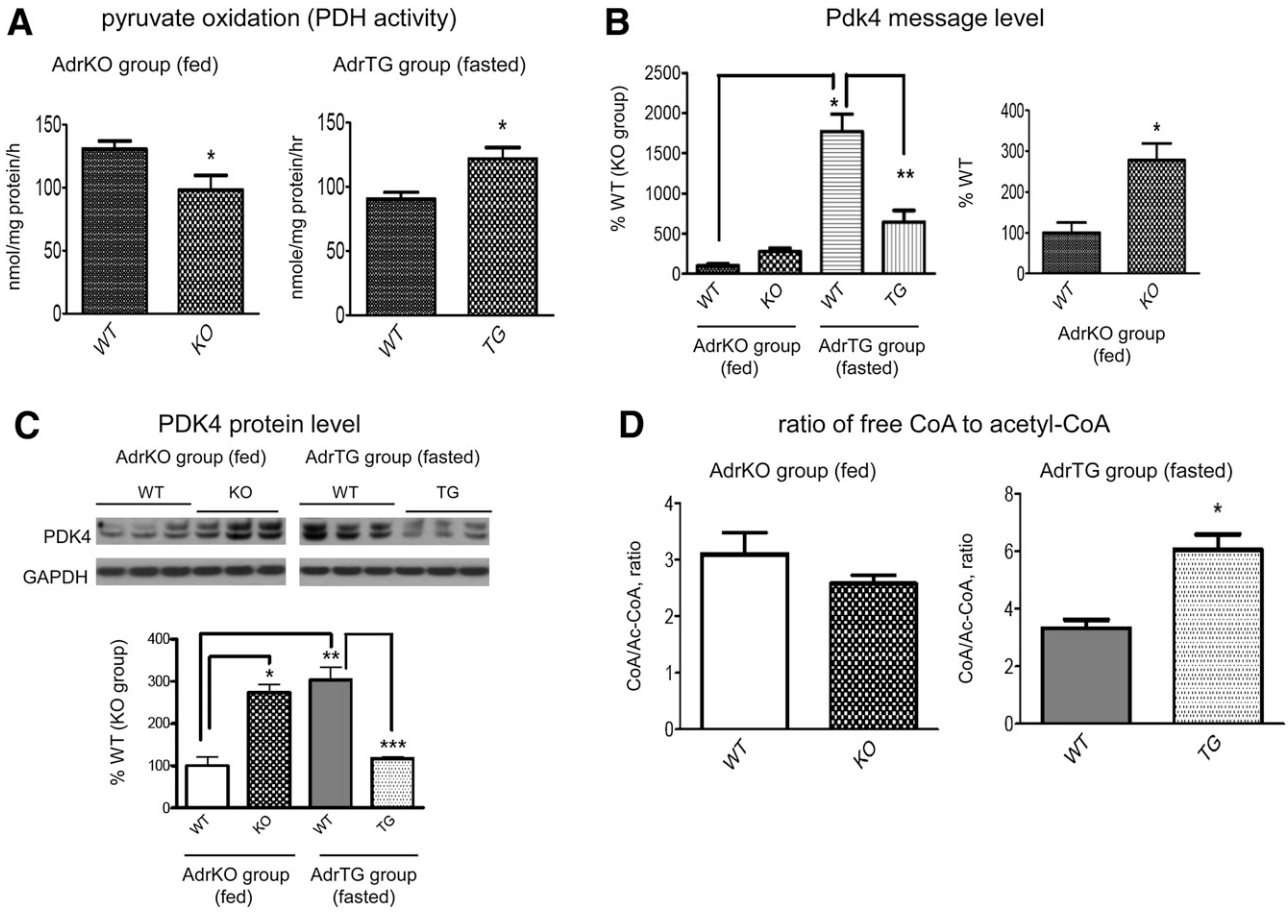

Figure 3—The AdrKO group was maintained in the fed state, whereas the AdrTG group was fasted overnight (16 h) before tissue samples were collected. *A*: Whole-muscle lysates containing intact mitochondria were prepared. Pyruvate oxidation or PDH activity was measured (AdrKO group, $n = 8$; AdrTG group, $n = 6–10$). *B*: *Pdk4* message levels were measured (AdrKO, $n = 6$; AdrTG, $n = 5–7$). *C*: PDK4 protein levels were measured (AdrKO, $n = 6$; AdrTG, $n = 6$). *D*: The ratios of the free CoA/acetyl-CoA (CoA/Ac-CoA) were calculated (AdrKO, $n = 7–8$; AdrTG, $n = 6–10$). *,**, and ***$P < 0.05$.

130 ± 6 nmol/mg/h; $P < 0.05$). 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. 3*B* and *C*). As a control, we confirmed previous findings (3,28) that mRNA and protein levels of PDK4 are upregulated with fasting (Fig. 3*B* 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. 3*D*) as a result of reduced acetyl-CoA level (Supplementary Fig. 4*B*). In AdrKO mice, no significant changes in the CoA/acetyl-CoA ratio were observed (Fig. 3*D*), despite an increase in CPT-1 activity. Similarly, we could not find changes in the CoA/acetyl-CoA ratio following fasting (Supplementary Fig. 4*C*), 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. 5*A*), confirming previous findings of the increased PGC-1α activity during fasting (19,34). AdrKO exhibited reduced PGC-1α acetylation (Fig. 4*A*), indicating activation. Conversely, AdrTG exhibited increased PGC-1α acetylation (Fig. 4*A*), indicating inhibition. There were no significant changes in PGC-1α protein expression (Fig. 4*B* and *C*) or mRNA levels (Supplementary Fig. 5*B*) 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. 6*A*). CS protein content and the levels of the OXPHOS proteins, including COXIV and ATP5F1, were also not changed (Supplementary Fig. 6*B*). Furthermore, expression of mitochondrial transcription factor A, an essential regulator of mitochondrial biogenesis (37), was not significantly altered in these mouse models (Supplementary Fig. 6*C*). Finally, mitochondrial respiration capacity indicated by FCCP-induced VO_2 was not altered by AdrKO or overexpression (Supplementary Fig. 6*D*). 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. 8*A*). SIRT1 protein levels were increased in fed AdrKO and decreased in fasted AdrTG mice compared with the relevant controls (Fig. 5*A*). 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. 5*B*). In contrast, SIRT activity was reduced in AdrTG (Fig. 5*B*). Moreover, acetylation of p53, a surrogate marker of SIRT1 activity (15,40,41), was decreased in AdrKO and increased in AdrTG (Fig. 5*C*). 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

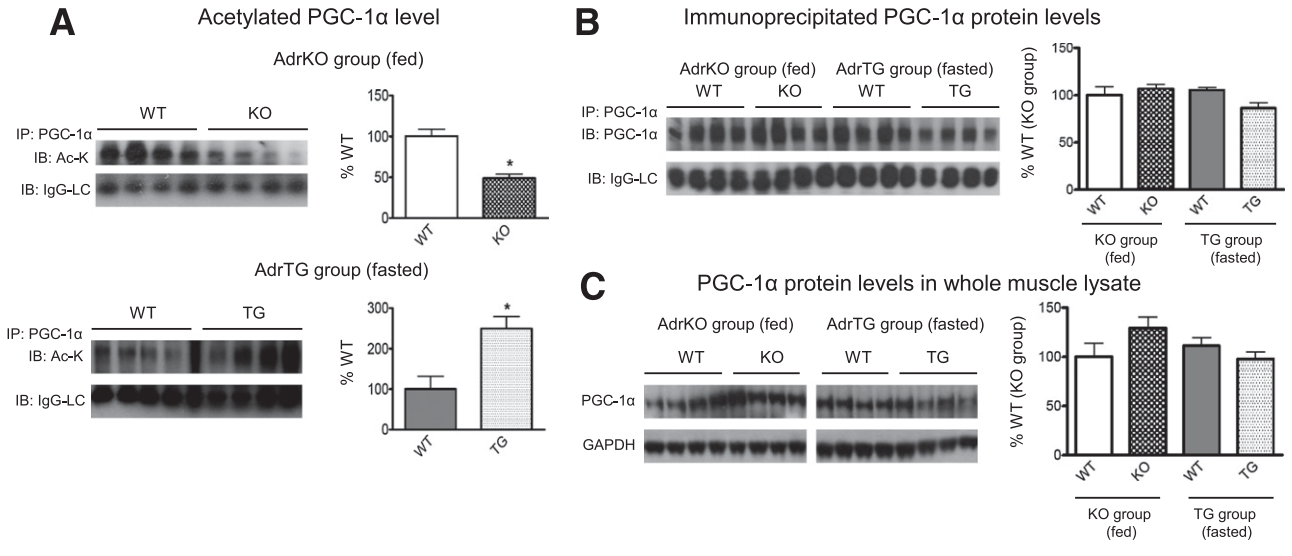

Figure 4—The AdrKO group was maintained in the fed state, and the AdrTG group was fasted overnight (16 h). *A*: PGC-1α protein was immunoprecipitated from whole-tissue lysate, and the immunoprecipitated products were immunoblotted for acetylated lysine (Ac-K) (AdrKO group, n = 5–6; AdrTG, n = 6–8). The 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. *$P < 0.05$.

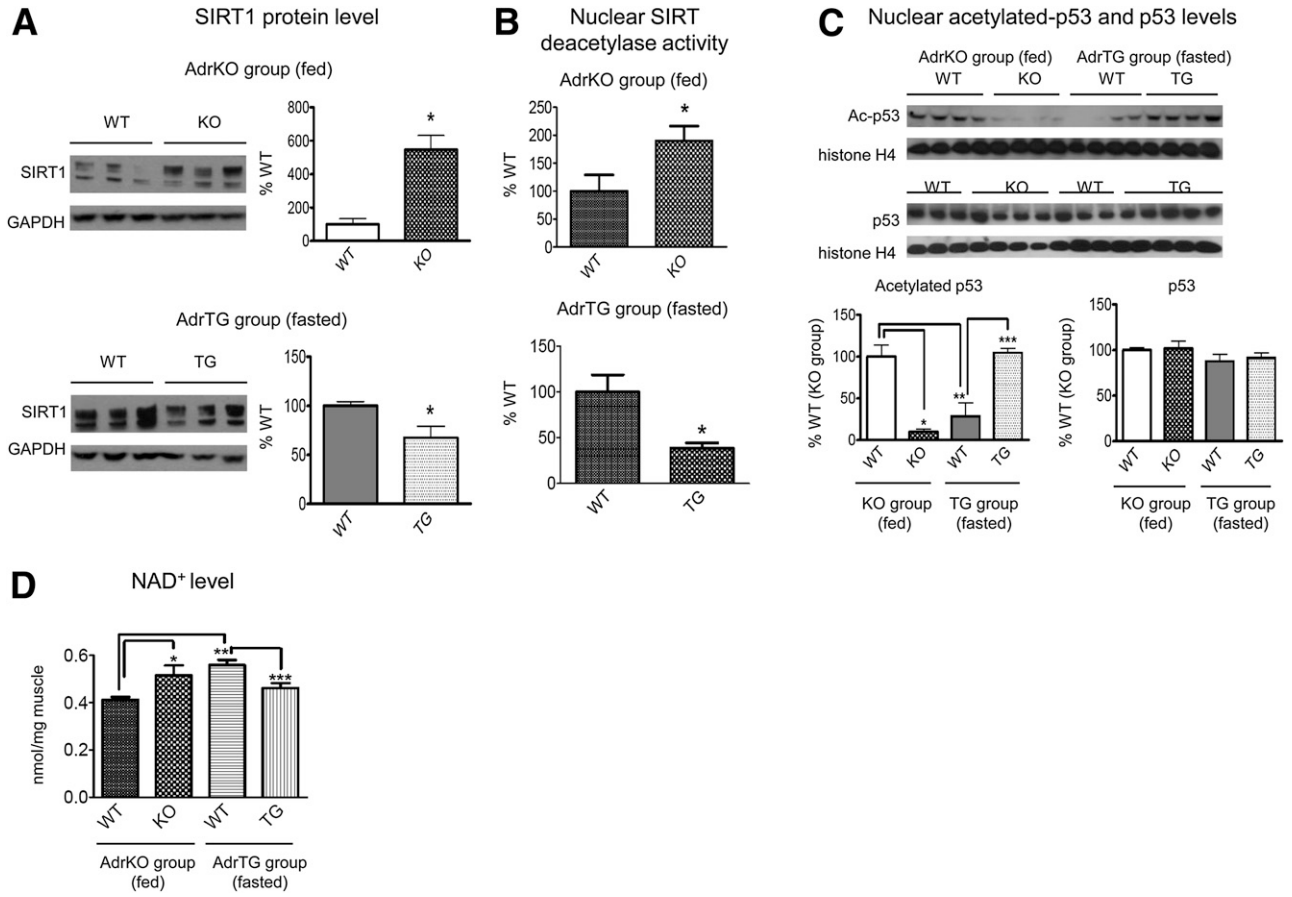

Figure 5—The AdrKO group was maintained in the fed state, and the adropin TG group was fasted overnight (16 h). *A*: SIRT1 protein levels in whole-muscle lysates were measured (AdrKO group, n = 6–7; AdrTG, n = 6–7). (Note: the blotting of the two groups was performed in separate runs.) *B*: SIRT deacetylase activities in muscle nuclear extracts were measured (AdrKO group, n = 6–7; AdrTG, n = 5–6). The activity of the fasted WT mice in the AdrTG group is significantly higher than that of the fed WT mice in the AdrKO group. *C*: Acetylated p53 (Ac-p53) levels and p53 levels in muscle nuclear extracts were measured (Ac-p53, n = 4; p53, n = 3–5). *D*: The NAD+ levels in whole-muscle extracts were measured (AdrKO group, n = 6; AdrTG, n = 6). *,**, and ***$P < 0.05$.

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. 8*B*).

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. 5*D*), which contributes to SIRT1 activation (19). Similar to fasting, adropin deletion (AdrKO) increased NAD+ levels (Fig. 5*D*), whereas adropin overexpression (AdrTG) decreased NAD+ levels (Fig. 5*D*). 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. 9*A*). Importantly, the AMPK activities were similar in AdrKO or AdrTG animals compared with the individual WT controls (Supplementary Fig. 9*B*), 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. 6*A*) without altering the mRNA level (Supplementary Fig. 8*C*).

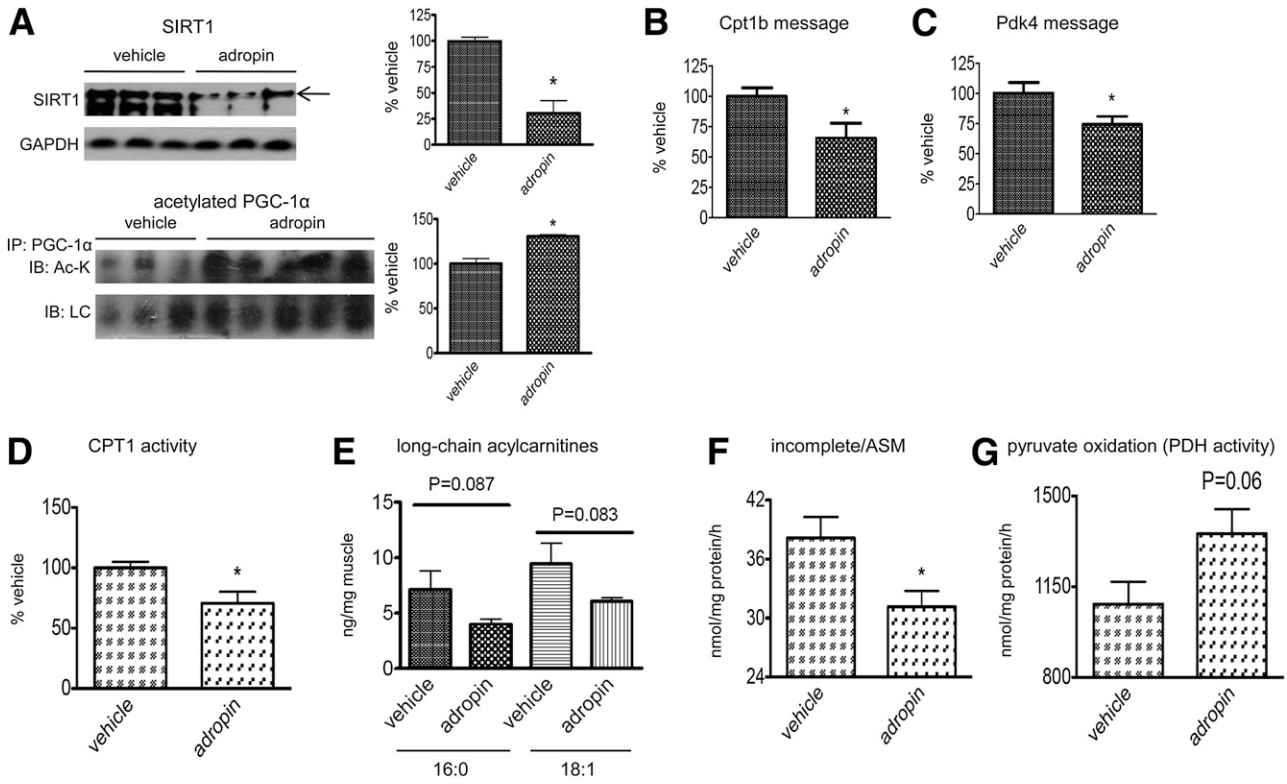

Figure 6—Food was removed in the early light cycle, and the WT lean mice were fasted for the next 24 h. Two intraperitoneal injections of adropin (or vehicle) separated by 8 h were administered during the light cycle. At the end of fasting on the next day, the third injection of adropin (or vehicle) was given. The mice were euthanized 2 h following the third injection and muscle tissues isolated. *A*: SIRT1 protein levels in whole-tissue lysates were measured (n = 3–5). PGC-1α protein was immunoprecipitated from whole lysate, and the immunoprecipitated products were immunoblotted for acetylated lysine (Ac-K) (n = 4–5). The light chain of IgG (IgG-LC) was used as the loading control. *B* and *C*: The message level of *Cpt1b* (n = 5) and *Pdk4* (n = 5) were measured. *D*: Mitochondria were isolated and CPT-1 activity measured. The activity assay was performed in two separate groups. The results in individual groups were converted to % vehicle-treated and pooled (n = 8). *E*: LC-ACs, including 16:0 (palmitoylcarnitine) and 18:1 (oleoylcarnitine), were quantified (n = 4–5). *F*: FAO levels in the lysate containing intact mitochondria were measured, and the production of ASM is shown (n = 7–8). *G*: Pyruvate oxidation or PDH activity was measured (n = 6–8). IB, immunoblotting; IP, immunoprecipitation. *$P < 0.05$.

Following adropin injection, the acetylation of PGC-1α was increased (Fig. 6*A*), and the PGC-1α target genes, including *Cpt1b* and *Pdk4*, were downregulated (Fig. 6*B* and *C*). Furthermore, CPT-1 activity and LC-AC levels were reduced (Fig. 6*D* and *E*). Accompanying the inhibition of CPT-1B activity, adropin treatment decreased incomplete FAO, indicated by the reduction of ASM production (Fig. 6*F*). Suppression of *Pdk4* expression is expected to activate PDH, and we demonstrated an increase in PDH activity and pyruvate oxidation following adropin treatment (Fig. 6*G*).

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/acetyl-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/acetyl-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. 4*A* 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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