The effects of apelin treatment on skeletal muscle mitochondrial content

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Frier BC, Williams DB, Wright DC. The effects of apelin treatment on skeletal muscle mitochondrial content. Am J Physiol Regul Integr Comp Physiol 297: R1761–R1768, 2009. First published September 30, 2009; doi:10.1152/ajpregu.00422.2009.—Adipose tissue is recognized as a key player in the regulation of whole body metabolism. Apelin, is a recently identified adipokine that when given to mice results in increases in skeletal muscle uncoupling protein 3 (UCP3) content. Similarly, acute apelin treatment has been shown to increase the activity of 5'-AMP-activated protein kinase (AMPK), a reputed mediator of skeletal muscle mitochondrial biogenesis. Given these findings, we sought to determine the effects of apelin on skeletal muscle mitochondrial content. Male Wistar rats were given daily intraperitoneal injections of apelin-13 (100 nmol/kg) for 2 wk. We made the novel observation that the activities of citrate synthase, cytochrome c oxidase, and β-hydroxyacyl CoA dehydrogenase (βHAD) were increased in triceps but not heart and soleus muscles from apelin-treated rats. When confirming these results we found that both nuclear and mitochondrial-encoded subunits of the respiratory chain were increased in triceps from apelin-treated rats. Similarly, apelin treatment increased the protein content of components of the mitochondrial import and assembly pathway. The increases in mitochondrial marker proteins were associated with increases in PGC-1α or Pgc-1-related co-activator (PRC) mRNA expression. Chronic and acute apelin treatment did not increase the protein content and/or phosphorylation status of AMPK and its downstream substrate acetyl-CoA carboxylase. These findings are the first to demonstrate that apelin treatment can induce skeletal muscle mitochondrial content. Given the lack of an effect of apelin on AMPK signaling and PGC-1α mRNA expression, these results suggest that apelin increases skeletal muscle mitochondrial content through a mechanism that is distinct from that of more robust physiological stressors.

PGE-1; rat; adipokine; respiratory chain; AMPK

Skeletal muscle mitochondrial biogenesis is a tightly regulated and complex process involving the coordinated regulation of nuclear and mitochondrially encoded genes. This process is controlled, at least in part, by the peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) family of transcriptional coactivators, PGC-1α, PGC-1β, and PGC-1-related coactivator (PRC) (1, 4, 14, 27, 28, 30). These molecules bind to and coactivate transcription factors that regulate the expression of nuclear genes encoding mitochondrial proteins. Similarly, PGC-1 coactivators also control the expression of mitochondrial transcription factor A, a protein involved in mitochondrial DNA transcription and replication (42).

The assembly of mitochondrial respiratory chain complexes involves the targeting and import of nuclear-encoded proteins into the mitochondria. Cytosolic chaperones target nuclear-encoded mitochondrial proteins to the transport protein complexes of the outer mitochondrial membrane. The precursor proteins are then shuttled from the outer to the inner membrane translocase complex (TIM) where mitochondrial chaperones, such as mitochondrial heat shock protein 70 (mHSP70/GRP75) and HSP60 are involved in the processing of the protein to its mature conformation (9, 20). Increases in the components of this pathway have been reported following exercise training, contractile activity, and thyroid hormone treatment (10, 36, 37).

Adipose tissue was originally viewed as a dormant storage organ, but is increasingly recognized as a multtargeting, endocrine effector capable of influencing whole body metabolic regulation (41). Investigating the vibrancy of adipose tissue has led to the relatively recent discovery of a novel adipokine, apelin (6, 7, 51). Apelin is secreted as a 77-amino acid prepropeptide that is subsequently processed into several biologically active peptides with the terminal 13 amino acid fragment conferring the highest degree of biological activity (23). Apelin acts in an endocrine fashion via its G protein-coupled receptor putative receptor protein related to AT-1 (APJ), which is expressed in a variety of peripheral tissues including skeletal muscle and heart (35). Apelin has been reported to be involved in the regulation of blood pressure (52), cardiac contractility (49), water intake (50), and angiogenesis (25). Apelin expression and secretion is increased in obese individuals and in rodents following the long-term consumption of diets high in fat, while fasting leads to marked reductions in the expression and circulating levels of apelin (6).

Accumulating evidence would suggest that apelin is involved in the regulation of skeletal muscle metabolism. For instance, acute apelin treatment has been shown to increase rates of skeletal muscle glucose disposal (12), while long-term treatment has been reported to lead to reductions in weight gain and fat pad mass while increasing the expression of uncoupling protein 3 (UCP3) in murine skeletal muscle (17). Although not a universal finding (5, 43), increases in UCP3 protein content have been shown to parallel increases in skeletal muscle mitochondrial content (24, 53). Moreover, apelin has been shown to increase the activity of 5'-AMP-activated protein kinase (AMPK) (12), a reputed mediator of PGC-1α expression (21, 22) and mitochondrial biogenesis (39, 57, 60). Collectively these results suggest that apelin may be involved in the regulation of skeletal muscle mitochondrial content. Within this context, the purpose of the present study was to determine whether long-term apelin treatment would increase mitochondrial content across a range of muscles with varying oxidative capacity. It was hypothesized that 2 wk of daily apelin treatment would lead to increases in mitochondrial enzyme activity and protein content and increases in components of the mitochondrial import and assembly pathway. We further surmised that these changes would be associated with an upregulation of PGC-1α expression and the activation of AMPK.
METHODS

Materials. Reagents, molecular weight marker, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Antibodies against cytochrome c oxidase (COX) IV (cat. no. A6403), COXI (cat. no. A6403) and core 1 (cat. no. A21362) were purchased from Molecular Probes (Eugene, OR). HSP70 (cat. no. SPA-S810), HSP60 (cat. no. SPA-S829), and GRP75 (cat. no. SPS-856D) antibodies were from Stressgen (Ann Arbor, MI). Antibodies directed against Tim23 (cat. no. 611222) were purchased from BD Transduction Laboratories (Mississauga, ON). Antibodies directed against the APJ receptor (cat. no. SC338323) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-actin antibodies (cat. no. 2172) were a product of Sigma (St. Louis, MO), while tubulin antibodies (cat. no. ab7291) were from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Glucose standards were obtained from NERL Diagnostics (East Providence, RI), and glucose reagents were from Diagnostic Chemicals (PEI, Canada). Rat insulin ELISA kits and adiponectin ELISA kits were purchased from MesoScale (Gaithersburg, MD). Taqman Gene Expression Assays for β2-microglobulin (β2MG) and PRC were from Applied Biosystems (Foster City, CA). Forward and reverse primers for PGC-1α and PGC-1β, were from Integrated DNA Technologies (Corvallis, IA), while probes for these genes were from Applied Biosystems (Foster City, CA). Citrate synthase and COX assay kits were purchased from Sigma (St. Louis, MO).

Treatment of animals. All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington, MA) weighing ~200 g were housed two per cage with a 12:12-h light-dark cycle. Rats were given standard rat chow and water ad libitum. Food was weighed every other day and food intake was calculated as the average of the daily food intake during the duration of the 2-wk study. Rats were given a daily intraperitoneal injection of apelin-13 (0.1 μmol/kg body wt⁻¹·day⁻¹) for 2 wk. We chose this dose and mode of administration as it has been shown to cause an approximately threefold increase in plasma apelin levels 1 h following an acute apelin injection (17), and an identical protocol has not changed following 2 wk of apelin treatment (control 24.58 ± 0.13, mean ± SE, raw CT values). Relative differences in gene expression between saline and apelin-treated rats were determined using the 2⁻∆∆CT method (31).

Citrate synthase activity, COX activity, and β-hydroxacyl cCoA dehydrogenase activity. Samples were prepared as described above in Western blot analysis. Citrate synthase and COX activity were determined using commercially available kits from Sigma. For the determination of citrate synthase activity, the formation of 5-thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm. COX activity was determined by measuring the oxidation of cytochrome c at 550 nm. β-Hydroxacyl cCoA dehydrogenase (BHAD) activity was measured by determining the production of NAD⁺ spectrophotometrically at 412 nm as described previously (38). The coefficient of variations of these assays in our laboratory is <10%.

Determination of plasma glucose, insulin, adiponectin, and apelin. Plasma glucose, insulin, and adiponectin were analyzed as described in detail previously (48). Plasma apelin levels were measured using an enzyme immunoassay kit.

Statistical analysis. Data are presented as means ± SE. Comparisons between the means of saline and apelin-treated groups were made using an unpaired Student’s t-test. Statistical significance was set at P < 0.05.
RESULTS

Physical characteristics and blood-borne factors. Initial body weights of saline-treated animals and apelin-treated animals were not different. Similarly, final body weights were not different between saline- and apelin-treated animals. Food intake over 2 wk was similar between control and apelin groups. There was no difference in either epididymal or retroperitoneal fat pad masses between control and apelin groups. Levels of plasma glucose were not different between groups, while insulin levels were reduced in apelin-treated rats (Table 1). Chronic treatment with apelin-13 did not result in differences in fasting apelin levels. An acute injection of apelin-13 led to an ∼50% increase in total plasma apelin levels 1 h following injection compared with saline-treated controls (2.58 ± 0.17 saline, 3.65 ± 0.17 apelin ng/ml, P < 0.05, n = 4/group) with levels returning to control values 3 h postinjection. It should be noted that commercially available methods do not distinguish between various apelin isoforms such as apelin 12, 13, 36, etc. A relatively large increase in plasma apelin-13 levels could well be masked if apelin-13 constitutes a small percentage of the total circulating apelin pool. Given this, it is likely that the rise in total apelin concentration in the present study is underestimating the true increase in plasma apelin-13 values postinjection.

Apelin increases markers of mitochondrial biogenesis. Increases in mitochondrial enzyme activities are classically used as markers of increased mitochondrial content (18, 19). Two weeks of daily apelin treatment led to increases in citrate synthase, COX (∼15%), and βHAD activities in rat triceps but not soleus or heart muscles (Fig. 1). Confirming these results, apelin treatment increased the protein content of mitochondrial-respiratory chain complexes. Apelin treatment resulted in increases in the content of these proteins in rat triceps muscle. Data are presented as means ± SE for 6–13 samples/group. *P < 0.05 compared with the corresponding muscle from saline-injected rats.

Apelin increases components of the mitochondrial import and assembly pathway. Chaperone proteins such as HSP60, HSP70, and mtHSP70 are involved in the assembly of mitochondrial respiratory chain complexes. Apelin treatment resulted in increases in the content of these proteins in rat triceps muscles (Fig. 3). Similarly, the content of TIM23, a constituent of the inner mitochondrial membrane transport complex, was also increased in triceps muscles from apelin-treated rats (Fig. 3).

Apelin increases the mRNA expression of PGC-1β. The mRNA expression of PGC-1β was increased in triceps muscles from apelin-treated rats (Fig. 4). We did not see increases in the mRNA expression of the related transcriptional coactivators PGC-1α or PRC.

AMPK signaling is not increased by either acute or chronic apelin treatment. Following 2 wk of daily apelin treatment, the total content and phosphorylation of AMPK was unaltered. Additionally, the phosphorylation of ACC, a direct substrate of AMPK, remained unchanged (Fig. 5). To determine whether

Table 1. Effect of 2 wk of apelin treatment on body weight, epididymal and retroperitoneal fat pad weight, food intake, and fasting hormones and metabolites in male Wistar rats

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Apelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight, g</td>
<td>231.4±3.0</td>
<td>234.9±3.6</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>334.3±4.2</td>
<td>327.7±3.9</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>102.9±3.9</td>
<td>92.4±4.5</td>
</tr>
<tr>
<td>Epididymal fat pad, g</td>
<td>3.7±0.2</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Retroperitoneal fat pad, g</td>
<td>2.7±0.2</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Food intake, g·rat⁻¹·day⁻¹</td>
<td>31.1±0.7</td>
<td>31.8±0.7</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>127±7</td>
<td>114±7</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>0.64±0.05</td>
<td>0.51±0.04*</td>
</tr>
<tr>
<td>Plasma adiponectin, µg/ml</td>
<td>4.9±0.3</td>
<td>4.4±0.3</td>
</tr>
<tr>
<td>Plasma apelin, ng/ml</td>
<td>0.65±0.05</td>
<td>0.69±0.03</td>
</tr>
</tbody>
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Data are presented as means ± SE for 6–13 rats/group. *P < 0.05 compared with saline-treated control rats.
Apelin treatment had an acute effect on AMPK signaling, rats were given an intraperitoneal injection of apelin, and their tissues were harvested 30 min postinjection. This protocol has previously been shown to increase AMPK signaling in murine skeletal muscle (12). As seen in Fig. 5, acute apelin treatment did not increase AMPK or ACC phosphorylation in rat triceps muscle. As a positive control, rat triceps muscles taken immediately following 30 min of swim exercise were used. As expected, exercise led to approximately threefold increases in the phosphorylation of AMPK and ACC.

DISCUSSION

The metabolic regulation of multiple organs and tissues by adipose tissue-derived hormones, i.e., adipokines, is well established (41). In the present investigation, we have further characterized this relationship by exploring the effects of apelin on skeletal muscle mitochondrial biogenesis. Apelin is a recently identified adipokine that, when given to mice via daily intraperitoneal injections over the course of several weeks, results in increases in the expression of UCP3 in skeletal muscle, smaller fat pad mass, and reductions in weight gain (17). Given the fact that several groups have shown that the induction of UCP3 occurs in parallel to the initiation of skeletal muscle mitochondrial biogenesis (24, 53), we wanted to test the hypothesis that long-term apelin treatment would increase skeletal muscle mitochondrial content. In support of this premise we found that 2 wk of daily apelin treatment led to increases in the activities of citrate synthase, COX, and βHAD, enzymes of the citric acid cycle, respiratory chain, and β-oxidation pathways, respectively. Similarly we also found that apelin treatment had an acute effect on AMPK signaling, rats were given an intraperitoneal injection of apelin, and their tissues were harvested 30 min postinjection. This protocol has previously been shown to increase AMPK signaling in murine skeletal muscle (12). As seen in Fig. 5, acute apelin treatment did not increase AMPK or ACC phosphorylation in rat triceps muscle. As a positive control, rat triceps muscles taken immediately following 30 min of swim exercise were used. As expected, exercise led to approximately threefold increases in the phosphorylation of AMPK and ACC.
Apelin treatment increased the content of both nuclear (COXIV, core 1) and mitochondrial (COXI)-encoded proteins of the respiratory chain. In a similar manner, and consistent with what has been reported in muscle following exercise training, contractile activity, and thyroid hormone treatment (10, 36, 37), we found that constituents of the mitochondrial protein import and assembly machinery, such as TIM23, HSP60, HSP72, and mtHSP70 were increased. While markers of mitochondrial content in the soleus and heart did not change, perhaps owing to the already high levels of mitochondria in these oxidative tissues, the present results clearly demonstrate, for the first time, that long-term apelin treatment can increase markers of skeletal muscle mitochondrial content in rat triceps muscles.

The PGC-1 family of transcriptional coactivators have been identified as key players in the regulation of mitochondrial biogenesis (1, 4, 14, 27, 28, 30). For instance, PGC-1α overexpression leads to marked increases in skeletal muscle mitochondrial content (4, 30). In addition, exercise increases PGC-1α expression (3, 58) concomitantly with increases in mitochondrial biogenesis. As AMPK increases PGC-1α activity and expression in muscle (21, 22), and recent work by Dray et al. (12) has demonstrated that apelin increases the phosphorylation of AMPK and its downstream substrate ACC in mouse soleus muscle, we thought it likely that apelin treatment may increase AMPK activity and PGC-1α mRNA expression in rat triceps muscles. Surprisingly, acute apelin treatment did not increase AMPK or ACC phosphorylation, nor did chronic apelin treatment increase the protein content or phosphorylation status of AMPK. Although unexpected, the lack of an effect of apelin on AMPK is consistent with our finding that PGC-1α mRNA expression was not increased. That is, if apelin increased AMPK activity, it would be expected that PGC-1α mRNA expression would also be elevated due to the well-established stimulatory effect of AMPK on PGC-1 (21, 22). The current results point toward marked species or fiber-type-specific responses to apelin and clearly underscores the need for further studies elucidating the mechanisms by which apelin increases skeletal muscle mitochondrial content.

In contrast to PGC-1α, we found that PGC-1β was increased following 2 wk of apelin treatment. Although gain and loss of function studies demonstrate that PGC-1α (27) and -1β (28, 46) control the expression of many of the same genes, these transcriptional coactivators appear to be regulated by different physiological stimuli. For instance, acute exercise, exercise training, and cold exposure robustly increase PGC-1α in skeletal muscle, whereas PGC-1β does not appear to respond to these same physiological stressors (32–34). The findings that skeletal muscle PGC-1β expression does not increase following exercise, yet the ablation of PGC-1β leads to reductions in skeletal muscle mitochondrial content has led to the hypothesis that PGC-1β may set the “tone” for basal and stress-mediated mitochondrial biogenesis, whereas increases in PGC-1α may be necessary for the muscle to respond to increases in energy demand (29). Given the increase in PGC-1β in the absence of changes in PGC-1α, it would appear that apelin-associated increases in skeletal muscle mitochondrial content may occur through a pathway that is distinct from that of more robust physiological stressors, such as exercise.

Long-term apelin treatment in mice has been shown to increase plasma adiponectin concentrations (17). Interestingly, it has been suggested that adiponectin plays a role in the maintenance of skeletal muscle mitochondrial content. For example, skeletal muscle citrate synthase and COX activities are lower in adiponectin knockout mice compared with wild-type controls (8). Similarly, adiponectin treatment has been shown to increase mitochondrial content and oxidative capacity in cultured muscle cells through an AMPK-dependent pathway (8). Within this framework, we thought it was possible that increases in mitochondrial content in our model could be secondary to changes in adiponectin. In contrast to what has previously been reported in mice (17), we found that adiponectin levels were not different between apelin and saline-treated rats. While Higuchi et al. (17) reported an approximately threefold increase in apelin levels 1 h following apelin injections (although it was not specified what apelin isoform was used), we found a much smaller increase at this time point. Given this it is a distinct possibility that differences in apelin metabolism, handling, clearance, etc., could explain, in part, the discrepancies between studies regarding adiponectin levels, weight gain, and fat pad mass. While the lack of an effect of apelin on adiponectin is surprising, it is consistent with our finding that basal levels of AMPK signaling were not increased following apelin treatment. If adiponectin was increased, it would be expected that AMPK signaling would be enhanced due to the documented effects of adiponectin on this pathway. Collectively, our results imply that elevations in adiponectin levels, at least in our model, do not mediate the effects of apelin on skeletal muscle mitochondrial content.

While we currently do not have a mechanistic explanation for the apelin-induced increases in skeletal muscle mitochondrial content, it is plausible these changes were secondary to increases in myogenesis. In support of this premise, myogenesis in murine myoblasts, a cell line that is devoid of PGC-1α, is associated with the induction of mitochondrial biogenesis and increases in the expression of PGC-1β (26). Since PGC-1β but not -1α was increased in the present study, and owing to the putative role of apelin in the regulation of myocardial progenitor cell differentiation (40, 44, 59), it is tempting to speculate that a similar effect could be occurring in skeletal muscle. Given the fact that the rats in our study were still growing, it will be necessary to determine whether apelin has
a similar effect in mature skeletal muscle. Clearly these are areas that require further investigation.

**Perspectives and Significance**

In the present study, we have made the novel observation that treating rats with apelin increases mitochondrial enzyme activities, the protein content of respiratory chain subunits, and components of the mitochondrial import and assembly pathway. In contrast to exercise and adiponectin, these changes were not paralleled by increases in the activation of AMPK or the expression of PGC-1α. Rather, we found that apelin-induced mitochondrial biogenesis was associated with increases in the expression of PGC-1β. It is interesting to note that plasma apelin concentrations are increased in obese, insulin-resistant rodents (6), a condition that is associated with increases in skeletal muscle mitochondrial content and oxidative capacity (15, 16, 18, 54). Apelin mRNA expression and secretion is increased by TNF-α (11), an inflammatory cytokine that is a reputed mediator of insulin resistance and whose expression is increased in adipose tissue from insulin-resistant

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**Fig. 5.** Chronic (A) or acute (B and C) apelin treatment does not increase the protein content and/or phosphorylation of 5′-AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). Triceps muscles were taken from rats immediately following 30 min of swimming and served as positive controls. Data are presented as means ± SE for 7–13 samples/group. Representative Western blots for the proteins of interest are shown beside (A) or above (B and C) the quantified data. *P < 0.05 compared with the corresponding muscle from control rats.
Apelin and Skeletal Muscle Mitochondria

**Disclosure**

No conflicts of interest are declared by the authors.

**References**


