Apelin Treatment Increases Complete Fatty Acid Oxidation, Mitochondrial Oxidative Capacity, and Biogenesis in Muscle of Insulin-Resistant Mice

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Both acute and chronic apelin treatment have been shown to improve insulin sensitivity in mice. However, the effects of apelin on fatty acid oxidation (FAO) during obesity-related insulin resistance have not yet been addressed. Thus, the aim of the current study was to determine the impact of chronic treatment on lipid use, especially in skeletal muscles. High-fat diet (HFD)-induced obese and insulin-resistant mice treated by an apelin injection (0.1 μmol/kg/day i.p.) during 4 weeks had decreased fat mass, glycemia, and plasma levels of triglycerides and were protected from hyperinsulinemia compared with HFD PBS-treated mice. Indirect calorimetry experiments showed that apelin-treated mice had a better use of lipids. The complete FAO, the oxidative capacity, and mitochondrial biogenesis were increased in soleus of apelin-treated mice. The action of apelin was AMP-activated protein kinase (AMPK)-dependent since all the effects studied were abrogated in HFD apelin-treated mice with muscle-specific inactive AMPK. Finally, the apelin-stimulated improvement of oxidative capacity led to decreased levels of acylcarnitines and enhanced insulin-stimulated glucose uptake in soleus. Thus, by promoting complete lipid use in muscle of insulin-resistant mice through mitochondrial biogenesis and tighter matching between FAO and the tricarboxylic acid cycle, apelin treatment could contribute to insulin sensitivity improvement.

Apelin is a circulating peptide, present in different tissues but also produced and secreted by human and mouse adipocytes (1). Apelin was identified as the endogenous ligand of the ubiquitously expressed G protein–coupled receptor named APJ (2). The apelin/APJ system exerts a large number of physiological roles, including regulation of fluid homeostasis, cardiovascular, immune, and gastrointestinal functions (3). A role for apelin/APJ in energy metabolism has also emerged recently. Acute and chronic apelin treatment has been shown to regulate glucose homeostasis (4,5). Beneficial effects of acute intravenous injection of apelin were observed in normal-chow diet (ND)-fed mice on glucose uptake, especially in skeletal muscle, through an AMP-activated protein kinase (AMPK)-dependent pathway (5). It is interesting that obese and insulin-resistant mice, exhibiting higher plasma apelin concentration than ND-fed mice (6), benefit from an acute apelin treatment since glucose tolerance was improved and muscle glucose uptake increased during a euglycemic-hyperinsulinemic clamp (5). Chronic apelin treatment also ameliorates insulin sensitivity in young db/db mice (5). Conversely, apelin KO mice develop insulin resistance especially when fed a high-fat diet (HFD) (5).

Altogether, these studies support a physiological role for apelin in the regulation of glucose homeostasis. Chronic apelin treatment also decreases lipid storage in adipose tissue since a reduction of triglycerides (TGs) in various fat depots has been observed in ND- and HFD-fed mice (7). Paradoxically, acute apelin treatment has been shown very recently to inhibit lipolysis in isolated adipocytes of non-obese mice (8) but not in human adipose tissue (9). The fate of lipids mobilized by chronic apelin treatment in obese and insulin-resistant mice is thus still unclear. More specific, the effects of apelin on fatty acid oxidation (FAO) have not yet been addressed.

To understand the impact of apelin on lipid metabolism, this study was designed on both in vivo and ex vivo approaches in obese and insulin-resistant mice chronically treated (or not) with apelin. Our data show that in apelin-treated obese and insulin-resistant mice, the main whole-body substrates oxidized in vivo were lipids. Ex vivo, in muscle of insulin-resistant mice, apelin treatment increased complete FAO, oxidative phosphorylation, and mitochondrial biogenesis but also increased insulin sensitivity by decreasing acylcarnitine levels and stimulating glucose uptake.

RESEARCH DESIGN AND METHODS

Researchers were handled in accordance with the principles and guidelines established by INSERM. C57Bl6/J wild-type (WT) mice were obtained from Harlan (Gannat, France). Mice with muscle-specific inactive AMPK (AMPK-DN mice) were provided by Birnbaum (Prof. Morris J. Birnbaum, Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA). Apelin-deficient (apelin−/−) mice were generated as described previously (10) and backcrossed to C57Bl6/J mice >10 times. Mice were housed conventionally in a constant temperature (20–22°C) and humidity (50–60%) animal room, with a 12/12 h light/dark cycle (lights on at 7:00 a.m.) and free access to food and water. The C57Bl6/J and AMPK-DN mice were fed an ND from weaning until aged 10 weeks and then either maintained on ND (control group) or fed an...
HFD containing 20% protein, 35% carbohydrate, and 45% fat (Research Diets, New Brunswick, NJ). Apelin treatment began after the onset of insulin resistance in males aged 23 weeks. Mice were injected daily with apelin-13 (Phoenix Biotech) at 0.1 μmol/kg/day i.p. as previously described (7) for 28 days. At the end of the treatment period, mice were fasted for 120 min prior to the fourth injection and 23 weeks. Standard mice were also treated with a specific APJ receptor antagonist (F13A) (Phoenix Biotech) at 0.2 μmol/kg/day during the 28 days or with the combination of apelin and F13A. All mice were killed 24 h after the last injection in a fed state. Plasma apelin concentrations, measured after a bolus of apelin (0.1 μmol/kg i.p.) in HFD mice, were increased 2.4-fold (10 ± 0.96 vs. 1.73 ± 0.24 ng/mL before the injection, n = 5), but plasma insulin concentrations were not different between PBS- and apelin-treated mice at the end of the treatment (Table 1).

Body mass composition. To determine fat and lean mass, mice were placed in a clear plastic holder, without anesthesia or sedation, and inserted into the EchoMRI-3-in-1 system (Echo Medical Systems, Houston, TX). Total body fat and lean mass were measured before (day 0) and at the end of the treatment (day 28) in apelin- and PBS-treated mice.

Plasma measures. Plasma fatty acids (FAs) and TGs measured by colorimetric technique with the Wako NEFA kit (Wako Chemicals) and the PAP 150 Kit (bioMerieux), respectively, as well as plasma leptin, adiponectin (Quantikine; R&D Systems), and apelin (Phoenix Pharmaceuticals, Inc.), were determined in the fed state at the end of the treatment. Insulinema (Merckodia, Uppsala, Sweden) and glucemia were measured with a glucometer (Accu-check; Roche Diagnostics) in the fasted state on blood from the tail vein.

Glucose and insulin tolerance tests. Glucose and insulin tolerance tests (GTT and ITT, respectively) were performed before apelin treatment and 1 week before the end of the treatment. Mice were fasted for 6 h and were then injected with glucose (1 g/kg i.p.), and blood glucose levels from the vein tail were monitored over time using a glucometer as previously described (4). For the ITT, mice were injected with insulin (0.75 units/kg) and blood glucose levels were measured as in the GTT.

Whole-body indirect calorimetry. Indirect calorimetry was performed between the 3rd and 4th week of treatment after 24 h of acclimatization. O2 consumption (VO2) and CO2 production (VCO2) were measured (Oxylab; Panlab-Bioseb, Vitrolles, France) in individual mice at 25 min intervals during a 24-h period. The respiratory exchange ratio (RER = VCO2/VO2) was measured. Energy expenditure (in kcal/day/kg0.75 = [3.815 + 1.232 log(VO2)]/1000), and lipid oxidation (in g/mmol/gm/kg0.75) = [1.672 (VO2) − 2.305 (VCO2)]/1000) were calculated. Ambulatory activities of the mice were monitored by an infrared photocell beam interruption method (Sedacem; Panlab-Bioseb).

Palmitate oxidation and esterification. Palmitate oxidation was determined as previously described in whole soleus muscle or adipose tissues (12). The tissues were incubated in modified Krebs-Henseleit buffer containing 1.5% FA-free BSA, 5 mM/L glucose, 1 mM/L palmitate, and 0.5 μC/mL [14C]palmitate (PerkinElmer) for 60 min. At the end of the incubation, tissues were removed and homogenized in 800 μL lysis buffer. Complete oxidation was determined by acidifying the incubation buffer with 1 mL of 1 mol/L H2PO4 and the 14CO2 was trapped by benzethonium hydroxide (Sigma-Aldrich) placed in a scintillation vial in a sealed microtube. The microtube was placed and removed in a scintillation vial, and the radioactivity was counted (Cytoscan; MP Biomedicals). A total of 500 μL homogenate was placed into glass tubes to extract lipids with chloroform-methanol (2:1) and 2 mL of 10% KOH in 250 μL buffer (0.1 mol/L KH2PO4 and 0.05% BSA (pH 7.3 at 4°C), and the assay was done on 10 μL homogenate.

Determination of skeletal muscle acylcarnitines, diacylglycerol, and TG levels. Acylcarnitines. Part of muscle homogenate (20 μL) was spotted on filter membranes (Protein Saver 903 cards; Whatman). The dried spots were then treated as reported (17). In brief, acylcarnitines were derivatized by addition of butanolic HCl and treated with the reagents of the NeoGram MSMS-AAAC kit (PerkinElmer). Free carnitine and acylcarnitines were quantified by liquid chromatography–tandem mass spectrometry. Data were acquired using QTrap 5500 Q-TOF API spectrometer equipped with a 2705 high-performance liquid chromatography module and a data system controlled by Masslynx 4.1 operating system (Waters, Milford, MA).

Neutral lipids (diacylglycerol and TG). Muscles (5–10 mg) were homogenized in 2 mL methanol per 5 mmol/L EGTA (2:1 v/v) with FAST-PREP (MP Biomedicals). A total of 100 μL was evaporated, the dry pellets were dissolved in 0.1 mL NaOH (0.1 mol/L) overnight, and proteins were measured with the Bio-Rad assay. Neutral lipids corresponding to 0.9 mL of the homogenate were extracted according to Bligh and Dyer (18) in chloroform/methanol/water (2:5:2:5:1 v/v/v) in the presence of the internal standards and measured as previously described (19).

Malonyl-CoA assay. Malonyl-CoA levels were measured on frozen soleus muscle as previously described (20). In brief, muscle was homogenized (10 mg tissue in 250 μL phosphate buffer containing 1 mol/L KPO4 and 10 mmol/L EDTA, pH 7.0) on ice with a potter and then centrifuged. Supernatant (100 μL) was then incubated for 1 h at 37°C with assay buffer (phosphate buffer with 25 mmol/L diithiothreitol, 0.2 mmol/L NADPH, 0.01% free FA BSA, 13 μmol/L acetyl-CoA, and 0.63 μCi [1-14C]malonyl-CoA (PerkinElmer), and 25 μL of FA synthetase (provided by Prof. Marc Prentki, Prentki, Centre Hospitalier de l’Université de Montréal Research Centre, Montreal, Ontario, Canada). The reaction stopped with 25 μL perchloric acid, and then ethanol and petroleum acid was added. A total of 4 mL of the upper phase was transferred in a new tube containing 2 mL water, and after centrifugation, 3 mL of the upper phase was dried and radioactivity was measured after addition of 10 mL scintillation liquid.

Western blot analysis. Western blot analyses were performed as previously described (4) by loading samples (lysed muscle) on 4–12% Criterion/XT gel (Bio-Rad) and transferring to nitrocellulose membranes (Schleicher & Schuell, Bauch & Schwarm, Dassel, Germany) that had been probed with antiphospho-AMPKα (Thr172), antiphospho-acetyl-CoA carboxylase (ACC) (Ser79) (Cell Signaling Technology, Beverly, MA), or anti-OxPhos antibodies (MitoSciences, Mundolsheim, France) used at 1/1000 dilution. Membranes were probed with β-actin or AMPK α or ACC antibodies for total proteins.

Protein assay. Concentration of samples was determined using the DC protein assay kit (Bio-Rad) according to the manufacturer’s instructions.

Real-time PCR. Total RNAs (1 μg) were isolated from muscle using RNA STAT (AMS Technology, Lutterworth, U.K.) and were reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Paisley, U.K.). Real-time PCR was performed as previously described (1). Analysis of the 18S ribosomal RNA was performed using the ribosomal RNA control TaqMan Assay Kit (Applied Biosystems) to normalize gene expression.
Glucose uptake. Muscles were isolated and preincubated for 10 min in Krebs-Henseleit buffer (pH 7.4) containing 2 mg/mL BSA, 2 mmol/L sodium pyruvate, and 20 mmol/L HEPES. Muscles were then incubated for 45 min in the absence or presence of 100 nmol/L insulin as previously reported (4).

Lipolysis. Lipolysis was measured after collagenase digestion of white adipose tissue (WAT) from PBS- and apelin-treated mice as previously reported (21). In brief, isolated adipocytes were incubated in the presence (or not) of different concentrations of isoprenaline (a β-adrenergic agonist). Glycerol released in the medium was measured on 30 μL aliquot using the Glycerol Free Reagent kit (Sigma-Aldrich).

Statistical analysis. Data are presented as means ± SEM. Comparisons between groups were carried out for different parameters using Prism 5.0 software (GraphPad Software). A two-way ANOVA was applied to detect interaction between treatment and time. When appropriate, Student t test paired or nonpaired was applied. Differences at P < 0.05 were considered statistically significant.

RESULTS

Effect of chronic apelin treatment in vivo in HFD-fed mice. HFD-fed mice were obese and insulin resistant before apelin treatment. At the end of apelin treatment, HFD mice had significant lower blood glucose and were protected from hyperinsulinemia compared with HFD PBS-treated mice (Fig. 1A). HFD apelin-treated mice also had significantly reduced adiposity and plasma levels of TG, whereas FA levels and leptin and adiponectin plasma concentrations were not modified (Table 1). Moreover, apelin-treated mice were significantly more responsive to glucose and insulin at the end of the treatment (Fig. 1B and C). To determine whether this improvement of insulin sensitivity was due to a change in substrate use, indirect calorimetry was performed.

During the 24-h experiment, food intake and physical activity were not modified between PBS- and apelin-treated mice (Table 2). VO2 and energy expenditure tended to increase with apelin treatment, whereas VCO2 values were similar (Table 2). However, apelin-treated mice clearly exhibited a lower RER, especially during the feeding period (dark cycle) compared with PBS-treated mice (Fig. 2A and B), indicative of a higher use of lipids. These results were confirmed by the calculated amount of lipid oxidized, which was increased, whereas the amount of glucose oxidized was decreased in HFD apelin-treated mice (Fig. 2C).

To determine the impact of apelin per se on whole-body substrate use, indirect calorimetry was performed in apelin−/− mice. Apelin−/− mice fed an HFD for 12 weeks had increased fat mass (39.41 ± 1.71 [n = 9] vs. 35.05 ± 0.88% in WT HFD mice [n = 12]; P < 0.02) and were insulin resistant (area under the curve for ITT: 8,814.2 ± 256.7 vs. 6,200 ± 439.6 arbitrary units in WT HFD mice, n = 12; P < 0.001). The RER of HFD apelin−/− mice was similar to HFD WT mice during the dark period and did not decrease during the light period (Supplementary Fig. 1). This translated to increased glucose oxidation and decreased lipid oxidation during the light period in HFD apelin−/− mice compared with HFD WT mice.

Effect of chronic apelin treatment in HFD mice on skeletal muscle lipid metabolism ex vivo. Since more lipids were used in whole-body organism of HFD apelin-treated mice, lipid metabolism in skeletal muscle was studied. As expected, muscle homogenates of HFD mice contained higher levels of diacylglycerol (DAG) (ND: 11.98 ± 2.74 nmol/mg protein, n = 5; HFD: 84.03 ± 10.97 nmol/mg protein, n = 7) and intramuscular TG (IMTG) (ND: 163.7 ± 41.4 nmol/mg protein, n = 5; HFD: 182.3 ± 23.2 nmol/mg protein,

![Graphs A, B, C showing effects of chronic apelin treatment in HFD mice.](diabetesjournals.org)
n = 7) compared with ND mice. However, apelin treatment in HFD mice was unsuccessful in reducing the amount of IMTG and DAG when compared with PBS treatment (Fig. 3A). Apelin treatment also had no effect on the rate of palmitate incorporation into TG (Fig. 3B). To further investigate the fates of lipids, both complete and incomplete oxidation of [14C]palmitate were assessed. Chronic apelin treatment significantly increased complete oxidation of [14C]palmitate to CO2 in soleus muscle when compared with PBS treatment (Fig. 3C). Of interest, incomplete oxidation was not significantly increased by chronic apelin treatment (Fig. 3C). Moreover, in soleus of HFD apelin+− mice, the complete oxidation was not increased (243.5 ± 9.6 vs. 198.4 ± 59.9 nmol CO2 released per gram protein in apelin+/− mice, n = 3–4). Altogether, these results show that apelin treatment promotes complete FAO rather than accumulation of TG or partially oxidized FA in skeletal muscle of obese and insulin-resistant mice. The effect of apelin treatment on FAO seems restricted to skeletal muscle since no significant metabolic changes were observed in brown adipose tissue and WAT (Supplementary Fig. 2).

**Effect of chronic apelin treatment in HFD mice on muscle mitochondrial activity and density.** To get further insight toward the effect of apelin, mitochondrial respiration was first assessed on freshly permeabilized muscle fibers. No difference in the glutamate/malate-driven mitochondrial respiration was found between PBS- and apelin-treated mice, suggesting that the complex I activity was not affected by the apelin treatment (data not shown). However, the succinate-driven mitochondrial respiration was significantly higher in fibers from apelin-treated mice compared with control, suggesting an increase in the oxidative capacity from complex II that uses coenzymes derived from FAO (Fig. 4A). The succinate and adenylyl-driven respiration was also significantly higher in apelin-treated mice, indicating that the capacity of the oxidative phosphorylation was increased in soleus after apelin treatment. Protein expression of complex II, III, and V also was significantly increased in apelin-treated mice (Fig. 4B). In addition, an increased citrate synthase activity, a quantitative marker of mitochondria content, was also found in muscle homogenates of apelin-treated mice compared with control (2.62 ± 0.02 vs. 2.91 ± 0.07 μmol/min/mg proteins, n = 7–9; P < 0.001). Expression of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1-α), a transcriptional coactivator mediating mitochondrial biogenesis (22), was also significantly increased in muscle of apelin-treated mice, whereas expression of PGC1-β was not modified (Fig. 4C). Moreover, expression of nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), which act in concert to increase mitochondrial oxidative phosphorylation and mitochondrial biogenesis (23), were also upregulated. Altogether, these results strongly suggest that in response to apelin treatment, mitochondrial biogenesis was increased in skeletal muscle from insulin-resistant mice. To test this hypothesis, we measured muscle mtDNA and density. The mtDNA–to–nuclear DNA ratio was significantly higher in soleus muscle of apelin-treated mice than in PBS-treated mice (Fig. 4D). Moreover, the electron microscopy demonstrated that apelin treatment significantly increased the density of intramyofibrillar (IMF) mitochondria (Fig. 4E), the largest fraction of the total mitochondria content. Fewer adverse alterations of mitochondria ultrastructure (reduced electron density of the matrix and loss of cristae) also were observed in both IMF and subsarcolemmal (SS) mitochondria of soleus muscle of apelin-treated mice (Fig. 4F), strengthening the effect of apelin on mitochondria function and biogenesis.

To study more deeply the apelin mechanism of action, the involvement of APJ receptor in apelin effects was first determined. For this purpose, mice were treated during the same period with either apelin alone or apelin and a specific APJ receptor antagonist (F13A) (11). F13A/apelin–treated mice were glucose intolerant and had increased body weight, fat gain, and higher glycemia and insulinemia compared with apelin-treated mice (Supplementary Fig. 3). Thus, F13A antagonist behaved as a functional antagonist. In muscle of F13A/apelin–treated mice, FAO and mitochondrial biogenesis were abrogated compared with apelin-treated mice (Supplementary Fig. 3), indicating that apelin exerts its beneficial effects through APJ activation.

Next, the role of AMPK in mediating the effects of apelin was investigated since apelin is known to activate AMPK in skeletal muscle (4) and AMPK is involved in both FAO (24) and mitochondrial biogenesis (25). Apelin treatment significantly increased both AMPK and ACC phosphorylation in muscle of insulin-resistant mice (Fig. 5A). The inhibition of ACC activity (as a result of increased phosphorylation) had for consequence a significant reduction of malonyl-CoA concentrations in muscle of apelin-treated mice (Fig. 5B). In addition, the increased FAO and mitochondrial biogenesis observed in HFD WT apelin-treated mice was completely blunted in muscle of HFD AMPK-DN apelin-treated mice, and the overexpression of PGC1-α, TFAM, and NRF1 was reduced (Fig. 5C–E). Thus, AMPK is a direct target of apelin and is required for apelin effect on FAO and mitochondrial biogenesis.

**Chronic apelin treatment in HFD mice improves muscle insulin sensitivity.** Acylcarnitines represent by-products of substrate catabolism arising from incomplete FAO. Increased acylcarnitine levels have been shown to be associated with obesity and insulin resistance (26,27). Long-chain acylcarnitines were elevated in homogenates of soleus muscle from HFD insulin-resistant mice compared with ND control mice (Fig. 6A). It is interesting that in HFD apelin-treated mice, acylcarnitine levels, especially C16:1 and C18:1 species, were reduced when compared with HFD PBS-treated mice. Since chronic apelin
treatment increased complete but not incomplete FAO in soleus, we hypothesized that the resulting lower levels of acylcarnitines would correlate with improved insulin sensitivity in muscle. Indeed, insulin-stimulated glucose uptake was significantly increased in apelin-treated mice muscle compared with PBS-treated mice (Fig. 6).

DISCUSSION
Insulin resistance is a major metabolic abnormality leading to type 2 diabetes. There is considerable interest in the discovery of insulin-sensitizing agents and in the development of new therapeutic strategies. The current study shows for the first time that chronic apelin treatment increases complete FAO, mitochondrial respiratory capacity, and mitochondrial biogenesis in skeletal muscle of insulin-resistant mice. The influx of lipid in mitochondria was associated with decreased acylcarnitine levels, suggesting a tighter coupling between FAO and the tricarboxylic acid cycle. Such a tighter coupling appears important to improve insulin sensitivity since increased insulin-stimulated glucose transport in muscle of apelin-treated mice is...
observed. Increased FAO and mitochondrial biogenesis in muscle and decreased total adiposity could contribute to the overall improvement of insulin sensitivity observed with chronic apelin treatment.

The most inoffensive fate of FAs in skeletal muscle is a matter of debate. Most studies suggest that insulin resistance develops as a consequence of diminished FAO. Instead of being directed to the mitochondria, FAs accumulate as DAG, leading to insulin resistance (28,29). More recently, it has also been suggested that insulin resistance is linked to excessive rather than to reduced FAO and that an overload of FAs in the mitochondria could exert mitochondrial stress, contributing to insulin-desensitizing effects (26). This is thought to result from high rates of incomplete FAO during which partially degraded FAs accumulate as acylcarnitines. Increased levels of acylcarnitines have already been proposed as markers of insulin resistance (26,30), and lower levels of acylcarnitines were shown to reverse HFD-induced glucose intolerance (31). In the current study, apelin treatment increased only the complete FAO and decreased acylcarnitines but not total DAG and IMTG levels in muscle. Improved insulin action in muscle has been observed after exercise intervention without modification of DAG levels (32). As previously suggested, the oxidative capacity in muscle might be a better predictor of insulin sensitivity than TG or DAG content (32). These results suggest that in muscle of HFD insulin-resistant mice treated with apelin, the lipids available to mitochondria do not exceed the capacity for their oxidation.

Impaired fuel oxidation could be attributed to mitochondrial dysfunction, a key factor contributing to insulin resistance (28,33). PGC1 proteins are transcriptional coactivators, considered as key regulators of mitochondrial biogenesis and function (22,34). PGC1-α expression in skeletal muscle is generally decreased during insulin resistance (31,35,36). Moreover, PGC1-α activation could be dependent on AMPK activation to promote mitochondrial biogenesis (25). It was previously shown that rats fed an ND and treated with apelin for 2 weeks had increased mitochondrial content and PGC1-β expression in triceps muscle (37). The current study defines the mechanisms of apelin

FIG. 3. Effect of chronic apelin treatment on palmitate partitioning in muscle of insulin-resistant mice. A: TG and DAG levels in muscle homogenates of PBS-treated (n = 7) and apelin-treated (n = 8) mice. Results are means ± SEM. B: Measure of [14C]palmitate incorporation into TG in muscle of PBS-treated (n = 11) and apelin-treated (n = 12) mice. Results are means ± SEM. C: Complete (left) and incomplete (right) FAO measured as described in RESEARCH DESIGN AND METHODS. Results are means ± SEM of PBS-treated (n = 11) and apelin-treated (n = 9) mice. **P ≤ 0.01.
FIG. 4. Chronic apelin treatment in HFD mice increased mitochondrial oxidative capacities and biogenesis in muscle. 

A: State 2 and State 3 respiration were measured on fresh permeabilized fibers prepared from soleus skeletal muscle of PBS-treated (n = 7) and apelin-treated (n = 7) mice as described in RESEARCH DESIGN AND METHODS. 

B: Representative Western blot of the different mitochondrial complexes (left) and quantification (right) in PBS-treated (n = 6) and apelin-treated (n = 7) mice. Results are means ± SEM. *P ≤ 0.05. 

C: Gene expression in soleus muscle of PBS-treated (n = 5) and apelin-treated (n = 5) mice. Results are means ± SEM. *P ≤ 0.05. 

D: mtDNA quantity calculated as the ratio of COX1 to cyclophilin A DNA levels determined by real-time PCR in soleus of PBS-treated (n = 4) and apelin-treated (n = 4) mice. **P ≤ 0.01. 

E: Transmission electron microscopy images at magnification ×6,000 and ×25,000 in SS and IMF mitochondria (left). Quantification of mitochondria number relative to the section area (analysis of three images for each mouse) from soleus of PBS-treated (n = 4) and apelin-treated (n = 5) mice (right). (A high-quality digital representation of this figure is available in the online issue.)
in skeletal muscle in a situation of insulin resistance. We propose that apelin acts through APJ activation to increase AMPK phosphorylation and activity in insulin-resistant skeletal muscle that will inhibit ACC activity and reduced malonyl-CoA levels, leading to increased FAO. Moreover, AMPK activation increased the expression of PGC1α (but not PGC1β) as well as its downstream targets NRF1 and TFAM to initiate the replication and transcription of
mtDNA. Apelin treatment not only increased the density of muscle mitochondria but also improved the ultrastructure of both IMF and SS mitochondria. Increased mitochondrial biogenesis has also been observed in mice overexpressing apelin (38). These events seem to be specific to skeletal muscle since neither FAO nor mitochondrial biogenesis was increased in response to apelin treatment in WAT and brown adipose tissue. Even if apelin has been shown to activate AMPK in WAT (8,9), it can be speculated that AMPK activation regulates substrate flux and whole-body energy distribution accordingly to the tissues as previously reported for other AMPK activators (39).

In this study, accentuated FAO was observed not only in skeletal muscle but also in vivo. Indirect calorimetry experiments showed that HFD apelin-treated mice retained low RER during the dark period (corresponding to feeding period) compared with PBS-treated mice and, thus, relied on lipid oxidation when needed. Apelin−/− mice displayed a lipid oxidation rate during the dark cycle similar to control mice but lacked the metabolic flexibility to increase lipid oxidation during the light cycle (fasted period). This could contribute to the increased adiposity observed in apelin−/− mice. By contrast, a better lipid oxidation in vivo in HFD apelin-treated mice during the dark period could be in line with the observed decreased adiposity and the expression profile of leptin and adiponectin in adipose tissue (Supplementary Fig. 2).

Several events could thus contribute to the improved insulin sensitivity seen in HFD apelin-treated mice. It is already known that apelin−/− mice are insulin resistant, a condition that can be reversed by a 2-week apelin treatment (5). Our results are in agreement with those findings since chronic apelin treatment not only improved overall insulin sensitivity but also increased insulin-stimulated glucose uptake in soleus muscle. Moreover, chronic apelin treatment prevented hyperinsulinemia and reduced hyperglycemia in HFD mice, although plasma leptin and adiponectin were not modified. Thus, the improvement of insulin sensitivity triggered by apelin might be secondary to decreased adiposity but also due to direct action on skeletal muscle. It is likely that chronic apelin treatment improves insulin-stimulated glucose uptake in muscle as a result of increased FAO and limited accumulation of FA intermediates.

In conclusion, chronic apelin treatment triggers an amelioration of both lipid and glucose metabolism. Even
though obese and insulin-resistant mice have enhanced plasma apelin compared with ND mice, adding a bolus of exogenous apelin is beneficial. Chronic apelin treatment optimizes muscle mitochondrial performance through increased mitochondrial biogenesis and a tighter matching between FAO and the tricarboxylic acid cycle. Since mitochondrial dysfunction is now considered a central event in whole-body metabolic dysregulation with regard to type 2 diabetes, apelin treatment appears as an attractive therapeutic strategy.

ACKNOWLEDGMENTS

This work was supported by a grant from Association de Langue Française pour l’Étude du Diabète et des Maladies Métaboliques/Société Françophone du Diabète 2009.

No potential conflicts of interest relevant to this article were reported.

C.A., A.B., and C.D. researched data and contributed to discussion. C.F., S.L.G., D.D., R.G.-R., and C.R. researched data. V.B. researched data, contributed to discussion, and reviewed and edited the manuscript. K.K. and J.P. contributed to discussion. M.R.-G., T.L., R.B., L.P., and P.V. contributed to discussion and reviewed and edited the manuscript. I.C.-L. contributed to discussion and wrote the manuscript. P.V. and I.C.-L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Prof. Moris Birnbaum (Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA) and Prof. Marc Pretinki Pretinki (Centre Hospitalier de l’Université de Montréal Research Centre [CRCHUM], Montreal, Ontario, Canada) for providing DN-AMPK mice and FA synthetase enzyme, respectively. The authors also acknowledge for their expertise the Anexpol platform (pheno-otyping and lipidomic and electronic microscopy) and the staff of animal facilities; Claudiane Guay (CRCHUM) for excellent technical assistance; Chantal Bertrand (INSERM U1048), PhD student, for her help with experiments for the reviewing; and François Crampes and Jean-Christophe Audo (INSERM U1048) for fruitful discussion and advice.

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