Apelin stimulates glucose uptake but not lipolysis in human adipose tissue *ex vivo*

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Abstract

Apelin is a peptide present in different cell types and secreted by adipocytes in humans and rodents. Apelin exerts its effects through a G-protein-coupled receptor called APJ. During the past years, a role of apelin/APJ in energy metabolism has emerged. Apelin was shown to stimulate glucose uptake in skeletal muscle through an AMP-activated protein kinase (AMPK)-dependent pathway in mice. So far, no metabolic effects of apelin have been reported on human adipose tissue (AT). Thus, the effect of apelin on AMPK in AT was measured as well as AMPK-mediated effects such as inhibition of lipolysis and stimulation of glucose uptake. AMPK and acetyl-CoA carboxylase phosphorylation were measured by western blot to reflect the AMPK activity. Lipolysis and glucose uptake were measured, *ex vivo*, in response to apelin on isolated adipocytes and explants from AT of the subcutaneous region of healthy subjects (body mass index: 25.6 ± 0.8 kg/m², $n=30$ in total). *APJ* mRNA and protein are present in human AT and isolated adipocytes. Apelin stimulated AMPK phosphorylation at Thr-172 in a dose-dependent manner in human AT, which was associated with increased glucose uptake since C compound (20 μ M), an AMPK inhibitor, completely prevented apelin-induced glucose uptake. However, in isolated adipocytes or AT explants, apelin had no significant effect on basal and isoprenaline-stimulated lipolysis. Thus, these results reveal, for the first time, that apelin is able to act on human AT in order to stimulate AMPK and glucose uptake.

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Introduction

Apelin is a peptide, identified as the endogenous ligand of APJ, a ubiquitously expressed G-protein-coupled receptor (Tatemoto *et al.* 1998). Apelin is synthesized as a 77-amino-acid prepropeptide, which is cleaved in different fragments including apelin-36, apelin-17, apelin-13, and the posttranslationally [Pyr1]apelin-13 with a conversion of the N-terminal glutamate to pyroglutamate preventing enzymatic breakdown and thus preserving biological activity (Tatemoto *et al.* 1998). Prior to being revealed as an adipocyte-secreted factor (adipokine), apelin was known not only to exert several central and peripheral effects on different tissues such as the regulation of the cardiovascular, immune, and gastrointestinal functions but also on fluid homeostasis, angiogenesis, proliferation of different cell types, and embryonic development (Kleinz & Davenport 2005). Recently, we have demonstrated that i.v. injection of apelin, at physiological dose, was able to decrease glycemia in mice (Dray *et al.* 2008). Moreover, during a hyperinsulinemic-euglycemic clamp, an increased glucose uptake was observed in adipose tissue (AT) and skeletal muscle (Dray *et al.* 2008). Apelin-stimulated

glucose transport in soleus muscle was dependent on AMP-activated protein kinase (AMPK) activation (Dray *et al.* 2008). A similar stimulation of AMPK by apelin has also been described in cultured C2C12 myotubes (Yue *et al.* 2009). Taken together, these independent observations support a physiological role of apelin in glucose metabolism. Recent data have also highlighted an important role for apelin in lipid metabolism. Higuchi *et al.* (2007) showed that prolonged treatment of apelin (daily i.p. injection of apelin for 2 weeks) decreased the triglycerides content of AT and the weight of different fat depots in chow-fed and obese mice. In addition, changes in uncoupling protein-1 (UCP-1) expression in brown AT as well as changes in UCP-3 expression in skeletal muscle were observed after apelin treatment but no metabolic effects were studied (Higuchi *et al.* 2007).

In humans, different studies have reported changes in plasma apelin concentration and variations of apelin expression in different tissues between physiological and pathological situations (Carpéné *et al.* 2007). Insulin has been shown to be one of the main regulating factors of apelin expression and secretion (Boucher *et al.* 2005). Circulating apelin levels as well as AT expression increase in obese and hyperinsulinemic subjects (Boucher *et al.*

2005, Castan-Laurell *et al.* 2008). Plasma apelin levels are also raised in morbidly obese (Heinonen *et al.* 2005) and type 2 diabetic (Soriguer *et al.* 2009) subjects. Nonobese patients with impaired glucose tolerance or with type 2 diabetes also exhibited higher concentrations of apelin when compared with control subjects (Li *et al.* 2006). The high apelin concentrations can be modulated by weight loss. Indeed, it was shown that diet-induced weight loss reduces plasma apelin levels in women with moderate obesity (Castan-Laurell *et al.* 2008), but not significantly in patients with metabolic syndrome (Heinonen *et al.* 2009). Bariatric surgery leads to a significant decrease in plasma apelin in morbidly obese subjects with impaired fasting glucose or type 2 diabetes before surgery (Soriguer *et al.* 2009). Moreover, the combination of two antidiabetics such as metformin and rosiglitazone in patients with type 2 diabetes improves glycemic profile and allows an increase in plasma apelin concentrations (Kadoglou *et al.* 2010). Altogether these studies suggest that the increased levels of apelin might be, in early stage of metabolic disease, a compensatory mechanism delaying the onset of insulin resistance.

So far, to our knowledge, metabolic effects of apelin on human AT and the effect of apelin on AMPK activity in AT have not been published. Adipocytes are involved in lipid storage (lipogenesis) through lipid and glucose uptake but also in triglyceride breakdown (lipolysis) into fatty acids and glycerol (Lafontan 2008). Thus, we measured lipolysis and glucose uptake in AT (subcutaneous abdominal fat depot) from healthy subjects in response to increasing concentrations of apelin-13. The following results show that apelin stimulates AMPK and glucose uptake but had no effect on lipolysis in the conditions used.

Materials and methods

Subjects

The study was performed according to the Declaration of Helsinki, and human AT was collected according to the guidelines and approval of the Ethical Committee of Rangueil Hospital in Toulouse from healthy women undergoing abdominal dermolipectomy for plastic surgery (body mass index: 25.6 ± 0.8 kg/m², age: 43.2 ± 2.7 years, $n=30$ in total, each tissue was either attributed to one or several protocols). All of them were drug free and healthy. No clinical data from these patients were available.

Human AT preparation

Human subcutaneous AT was either digested by collagenase (type II, Sigma–Aldrich Co.) in order to get isolated adipocytes or minced into very small pieces (1–2 mg) to get explants. The explants were washed two times in PBS at 37 °C, centrifuged for 10 min at 850 g at

room temperature in order to remove the connective tissue, and the medium was discarded. Isolated adipocytes were obtained after mincing AT in 5 ml of DMEM (Gibco, Invitrogen) supplemented with 1 mg/ml collagenase and 1% albumin (BSA) for 30 min at 37 °C under shaking. Digestion was followed by filtration through a 150 µm screen and the floating adipocytes were separated from the medium containing the stroma vascular fraction and washed twice in DMEM.

APJ mRNA expression in human AT

Total RNAs (1 µg) were isolated from either AT using RNeasy Lipid Tissue kits (Qiagen, Courtaboeuf, France) or isolated adipocytes using RNeasy kit (Qiagen). They were then reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed as previously described (Boucher *et al.* 2005). Briefly, real-time PCR was performed on 12.5 ng cDNA with both sense (hAPJ sense: GCCCTTGCTTTCTGAAAATCA) and antisense (hAPJ reverse: GGACAGTTAAAGGATGTGCATAGGA) oligonucleotides (Eurogentec, Angers, France) in a final volume of 20 µl using SYBR Green qPCR Master Mix (Eurogentec, Seraing, Belgium). Fluorescence was monitored and analyzed in a GeneAmp 7500 detection system instrument (Applied Biosystems, Warrington, UK). In parallel, analysis of the 18S ribosomal RNA was performed using the ribosomal RNA control Taqman Assay kit (Applied Biosystems) to normalize gene expression.

Immunohistochemical study of APJ

AT was fixed overnight in a paraformaldehyde 4% solution, then hydrated, and paraffin-embedded. AT sections were blocked with 1% BSA in Tris buffer for 1 h at room temperature. Sections were then incubated with APJ monoclonal (anti-human) antibody (R&D Systems, Lille, France) overnight at 4 °C (1:25 dilution). Control sections were stained with IgG mouse serum used at the same dilution as APJ antibody. As a secondary antibody, alkaline phosphatase (DakoCytomation, Trappes, France) was used at 1:100 dilution. Antigen visualization was achieved with an alkaline phosphate system (BCIP/NBT Substrate System) added with Levamisole (DakoCytomation) in order to suppress nonspecific staining.

Glucose uptake

AT explants were preincubated for 10 min in Krebs–Henseleit (KH) buffer, pH 7.4, containing BSA (2 mg/ml) and 20 mM Hepes. Explants were then incubated for 45 min in the presence or absence of different concentrations of [Pyr1]apelin-13 (BACHEM distribution services) or 100 nM insulin (Sigma–Aldrich

Co.). [Pyr1]apelin-13 has been chosen as a stable biological active apelin isoform (Kleinz & Davenport 2005). In order to test the involvement of AMPK, C compound was added 20 min before apelin. For glucose transport, explants were transferred to another vial containing KH medium supplemented with 0.1 mM 2-deoxyglucose (2-DG) and D-[³H]-2-DG (0.25 µCi/ml) for 10 min. All the incubations were carried out at 37 °C under a 95% O₂/5% CO₂ atmosphere. Explants were then washed two times in PBS under shaking and then lysed by using Precellys 24 automated biological sample lyser with CK-14 beads vials (Ozyme, Saint Quentin, France) in 500 µl NaOH (1 M). The infranatant was recovered for further extraction and 500 µl HCl (1 M) were added to each sample in order to restore pH neutrality. The total amount of D-[³H]-2-DG was quantified after addition of a perchloric acid (6%) solution. In an other vial, D-[³H]-2-DG 6-phosphate was precipitated by the use of zinc sulfate (0.3 M) and barium hydroxide (0.3 M). The amount of 2-DG internalized was calculated by the difference between the radioactivity found in total 2-DG and nonphosphorylated 2-DG found in the supernatants.

Glucose uptake by isolated adipocytes was measured as previously described (Iglesias-Osma *et al.* 2005). Briefly, adipocytes (1:10 dilution) were incubated with 1 µM of apelin or 100 nM insulin as a positive control for 45 min at 37 °C and 0.4 µCi 2-DG were added at a final concentration of 0.1 mM for 10 min. Assays were stopped with 100 µM cytochalasin B and aliquots of cell suspension were centrifuged in microtubes containing di-isononyl phthalate, which allowed separation of adipocytes from the buffer and counting of the radioactive intracellular 2-DG.

Lipolysis

Lipolysis was realized in Krebs–Ringer–Hepes (KRH) buffer with 2% BSA. In all, 100 µl AT microexplants or adipocytes (1:10 dilution) were incubated in 1 ml KRH for 1 h at 37 °C under gentle shaking in the presence of different concentrations of apelin or in 1 µM isoprenaline (a β-adrenergic agonist) as a positive control. The reaction was stopped once the tubes were on ice. Glycerol released in the medium was measured in a 30 µl aliquot using the Glycerol-Free Reagent kit (Sigma) while nonesterified fatty acids (NEFA) were measured in 15 µl of the medium by the WAKO NEFA kit (WAKO Chemicals, Montbonnot St Martin, France).

Western blots

AT explants were lysed as described above (Precellys) and loaded (50 µg protein per lane) on 10% SDS–PAGE gel and transferred to nitrocellulose membrane (Schleicher-Schuell). Membranes were blotted with

anti-phospho-AMPKα-Thr-172 or anti-phospho-acetyl-CoA carboxylase (ACC)-Ser79 antibodies (Cell Signaling Technology, Beverly, MA, USA) used at 1:1000 dilution in Tris-buffered saline containing 5% BSA and Tween-20 at 0.01%. As a secondary antibody, anti-rabbit-HRP was used (1:3000 dilution). Membranes were probed with β-actin for total proteins. Immunoreactive proteins were detected using the ECL Plus (GE Healthcare, Orsay, France) and quantified by Image Quant TL software (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Statistical analysis

Data are presented as means ± s.e.m. Analysis of differences between the groups was performed with one-way ANOVA followed by *post hoc* Bonferroni's or Dunnett's test, when appropriate and *P* < 0.05 was considered to be significant.

Results

Expression of APJ receptors in human AT

We have previously shown that *APJ* mRNAs were present in human AT (Castan-Laurell *et al.* 2008, Dray *et al.* 2010). In this study, we further delineated the presence of both *APJ* mRNA and protein in isolated adipocytes compared to the entire AT. As shown in Fig. 1, *APJ* mRNA levels were lower in isolated adipocytes than in AT. *APJ* proteins were also visualized in human AT since a positive immunostaining was observed in the periphery of adipocytes compared to control.

Effect of apelin on AMPK

AMPK has been revealed as a target of apelin signaling in muscle (Dray *et al.* 2008, Yue *et al.* 2009), thus the effect of apelin on AMPK activation was studied in human AT. Time-course studies in AT explants revealed that 10 nM apelin induced the phosphorylation of AMPK that was maximal at 10 min (Fig. 2A). In total, 2 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) incubated for 60 min was used as a positive control. Moreover, apelin stimulated, in parallel, the phosphorylation of AMPK and ACC, its downstream target enzyme, in a dose-dependent manner with a significant effect at 10 nM (Fig. 2B). Thus, apelin activates AMPK in human AT. Metabolic effects mediated by apelin involving AMPK were then studied.

Effect of apelin on lipolysis

Both glycerol and NEFA releases were measured as final products of triglyceride hydrolysis after 1 h incubation with different agents. Isoprenaline (a β-adrenergic

Figure 1 Expression of APJ, the apelin receptor, in human AT. (A) mRNA levels of APJ in entire adipose tissue (AT) and isolated adipocytes. Results are mean $\pm$ s.e.m. of eight independent experiments. (B) Representative immunostaining without (control) or with anti-APJ antibody (APJ) in human AT.

agonist) was used at $1\ \mu\text{M}$ as a control of maximal lipolytic activation and AICAR was used at $2\ \text{mM}$ as an activator of AMPK (Gaidhu *et al.* 2009). Isoprenaline stimulated the basal release of glycerol and NEFA from AT explants whereas AICAR decreased basal lipolysis (Fig. 3A). However, apelin, whatever the concentrations used, had no significant effect on basal and isoprenaline-stimulated lipolysis (Fig. 3A). In addition, in isolated adipocytes, apelin at $10\ \text{nM}$ and $1\ \mu\text{M}$ did not have significant effect on lipolysis. When added at the same time along with isoprenaline, apelin did not modify the response of the β -adrenergic agonist, while $100\ \text{nM}$ insulin was antilipolytic (Fig. 3B).

Effect of apelin on glucose uptake

Insulin, as a positive control, stimulated glucose uptake in human AT explants (Fig. 4A). Apelin stimulated glucose uptake in a dose-dependent manner, with a significant effect at $10\ \text{nM}$, but to a lower extent compared to insulin (1.56 -fold with $10\ \text{nM}$ apelin versus 2.0 -fold with $100\ \text{nM}$ insulin). On isolated adipocytes, apelin had a weak effect (basal: $0.16 \pm 0.04\ \text{pmol/g protein}$; apelin $1\ \mu\text{M}$: $0.23 \pm 0.06\ \text{pmol/g protein}$, $n=7-10$). The involvement of AMPK in apelin-stimulated glucose transport was underlined by the use of C compound, a selective AMPK inhibitor. C compound ($20\ \mu\text{M}$) prevented apelin-induced glucose uptake in human AT explants (Fig. 4B).

Discussion

In this study, we provide evidence that acute apelin treatment of human AT from healthy subjects stimulates AMPK phosphorylation and glucose uptake but has no effect on lipolysis. Thus, this study reveals, for the first time, an activation of AMPK by apelin in AT and

Figure 2 Apelin stimulates AMPK phosphorylation. (A) Representative blot of *in vitro* time-course study of Thr-172 AMPK phosphorylation in the presence of apelin ($10\ \text{nM}$) or AICAR ($2\ \text{mM}$), used as a positive control and incubated for $60\ \text{min}$. β -Actin was used to evaluate total proteins, $n=4$. The graph shows the quantified data, $*P<0.05$, $**P<0.01$ versus control. (B) Dose-response of apelin, at the indicated concentrations (incubation time: $10\ \text{min}$ for apelin, $60\ \text{min}$ for AICAR), on AMPK and ACC phosphorylation compared to control (C). β -Actin was used to evaluate total proteins. The graph shows the quantified data, $n=4$, $*P<0.05$, $**P<0.01$ versus control.

Figure 3 Effect of apelin on lipolysis in human AT explants (A) and isolated adipocytes (B). NEFA (top) and glycerol (bottom) releases upon basal lipolysis (white columns), under stimulation with AICAR alone, apelin alone or in the presence of isoprenaline (Iso, shaded columns) and insulin 100 nM in the presence of isoprenaline. Results are the mean $\pm$ S.E.M. of six independent experiments for AT explants and four independent experiments for isolated adipocytes. * $P < 0.05$ and *** $P < 0.001$ versus basal; \$\$\$ $P < 0.001$ for insulin + isoprenaline versus isoprenaline alone.

functional metabolic effects of apelin on humans. These results are in line with our previous study performed in mice indicating that i) *ex vivo*, in soleus skeletal muscle, apelin stimulated AMPK and glucose uptake (Dray *et al.* 2008) and that ii) *in vivo*, apelin perfusion during a euglycemic-hyperinsulinemic clamp stimulated glucose uptake in AT.

Lipolysis is one of the main metabolic functions of AT. An upstream step controlling this process is the intracellular levels of cAMP. Apelin has been shown to reduce forskolin-stimulated cAMP production in different cell lines overexpressing APJ receptors through a pertussis-toxin-sensitive G-protein (Masri *et al.* 2002, Bai *et al.* 2008). Thus, an antilipolytic effect of apelin was expected but no modification of basal or isoprenaline-stimulated lipolysis was observed in the conditions used. Moreover, apelin has been shown to activate AMPK in skeletal muscle (Dray *et al.* 2008) while AMPK also regulates energy metabolism in AT (Daval *et al.* 2006). However, depending on the studies, it has been shown that, in rodents, activation of AMPK could lead either to inhibition or stimulation of lipolysis (Yin *et al.* 2003, Hutchinson *et al.* 2005, Daval *et al.* 2006, Omar *et al.* 2009). Very recently, it was shown that protein

kinase A, once activated by lipolytic agents, inactivates AMPK to promote efficient lipolysis (Djouder *et al.* 2010). AMPK activation is thus viewed as a consequence of ongoing re-esterification of fatty acids that consume energy (Gauthier *et al.* 2008, Djouder *et al.* 2010) and in fine AMPK activation restrains hydrolysis of triglycerides (lipolysis). Acute treatment (1 h) with AICAR has been shown to decrease both NEFA and glycerol release in rat adipocytes (Gaidhu *et al.* 2009). In this study, AICAR decreased both NEFA and glycerol release in agreement with the results reported by Gaidhu *et al.* (2009). Apelin incubated during the same period did not acutely inhibit lipolysis in basal conditions or during isoprenaline-stimulated lipolysis. This could be due to the less sustained effect of apelin on AMPK phosphorylation. These results are thus different from those obtained with biguanides and thiazolidinediones that activate AMPK and inhibit stimulated lipolysis in human adipocytes (Bourron *et al.* 2010). Although apelin shares with those antidiabetic drugs, a decrease in glycemia, and activation of AMPK, apelin might not act on acute lipolysis.

Among the other metabolic functions of AT involving AMPK, we focused on glucose uptake. In this study,

Figure 4 Apelin stimulates glucose uptake through AMPK activation. (A) AT explants were stimulated either with 100 nM insulin or with the indicated concentrations of apelin as indicated in the section 'Materials and methods'. Results are the mean $\pm$ S.E.M. of 7–12 independent experiments. * $P < 0.05$, *** $P < 0.001$ versus basal lipolysis (Basal). (B) Effect of 20 μ M C compound on basal and apelin-stimulated glucose uptake in AT explants. Insulin was used as a positive control. Values are the mean $\pm$ S.E.M., $n = 7$. * $P < 0.05$, *** $P < 0.001$ versus basal. § $P < 0.05$ for apelin 10 nM versus apelin 10 nM + C compound.

apelin had a modest effect on glucose uptake in human AT explants and isolated adipocytes. Insulin, at physiological concentrations, stimulated glucose transport in human AT explants twofold above basal, which is in agreement with previous studies performed on human isolated adipocytes (Iglesias-Osma *et al.* 2005, Wanecq *et al.* 2009) or differentiated cultured adipocytes (Hauner *et al.* 1998). It should be noticed that conversion of glucose into fatty acids (*de novo* lipogenesis) especially in AT is much lower in humans compared to rodents (Zelewski & Swierczyński 1990). We choose to work first on AT explants with the aim to reproduce the conditions used for glucose transport *ex vivo* in muscle (Dray *et al.* 2008). It has been shown that functional activities are maintained in human AT explants (Moustaid *et al.* 1996, Viguerie *et al.* 2002). Moreover, the effects of apelin were similar in AT explants and isolated adipocytes.

Only a few studies have demonstrated the role of AMPK in glucose uptake in adipose cells, and the mechanisms leading to AMPK activation in adipocytes

remain poorly understood. The use of AICAR, as a pharmacological tool to activate this kinase, has not given conclusive results regarding its physiological role (Salt *et al.* 2000, Sakoda *et al.* 2002). Indeed, over-expression of a dominant negative form of AMPK in cultured adipocytes was shown to abolish AMPK activation without affecting AICAR-induced glucose transport (Sakoda *et al.* 2002). However, adiponectin, an insulin-sensitizing adipokine, was shown to increase glucose uptake in primary rat adipocytes through AMPK activation and this effect was abrogated in the presence of AMPK inhibitors (Wu *et al.* 2003). Similar results were obtained in this study with apelin. The effect of C compound, in blocking apelin-stimulated glucose uptake as well as the phosphorylation of ACC, strongly suggests a role of AMPK and thus an insulin-independent pathway for the effects of apelin. Yet, it is not known whether glucose uptake by adiponectin or apelin is dependent on glucose transporter-4 (GLUT4) translocation. GLUT4 is essential for insulin-stimulated glucose uptake in skeletal muscle and AT. Moreover, GLUT4 mRNA levels in human AT are detected mostly in mature adipocytes compared to the stroma vascular fraction or nonadipocyte cells (Vitkova *et al.* 2007). Consequently, the GLUT4-dependent effects are mainly due to adipocyte response. Activation of AMPK in different cell types could lead to translocation of GLUT4 transporters (Mu *et al.* 2001, Li *et al.* 2004), but not in all the reports (Lemieux *et al.* 2003, Breen *et al.* 2008). Moreover, studies performed in cultured 3T3-L1 preadipocytes or in C2C12 myoblasts demonstrated that AMPK is also implicated in GLUT1-mediated glucose uptake (Abbud *et al.* 2000). Hence, apelin could activate AMPK and glucose uptake through different GLUTs depending on the cell type (preadipocytes or adipocytes) involved.

In conclusion, these results show that apelin stimulates glucose uptake in human AT but to a less extent than insulin. Given that apelin is produced and secreted by AT, a local action of apelin could affect differently AT metabolic functions compared to insulin action. It will be of interest to depict these effects in obese subjects. Moreover, we also described AMPK activation by apelin in AT. Since the activation of AMPK is viewed as a therapeutic approach in obesity-associated disorders (type 2 diabetes) and since only few compounds have been described to activate AMPK in this tissue, the apelin/APJ system could be a promising target with potential beneficial effects in humans.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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