Apelin, a Newly Identified Adipokine Up-Regulated by Insulin and Obesity

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The results presented herein demonstrate that apelin is expressed and secreted by both human and mouse adipocytes. Apelin mRNA levels in isolated adipocytes are close to other cell types present in white adipose tissue or other organs known to express apelin such as kidney, heart, and to a lesser extent brown adipose tissue. Apelin expression is increased during adipocyte differentiation stage. A comparison of four different models of obesity in mice showed a large increase in both apelin expression in fat cells and apelin plasma levels in all the hyperinsulinemia-associated obesity and clearly demonstrated that obesity or high-fat feeding are not the main determinants of the rise of apelin expression. The lack of insulin in streptozotocin-treated mice is associated with a decreased expression of apelin in adipocytes. Furthermore, apelin expression in fat cells is strongly inhibited by fasting and recovered after refeeding, in a similar way to insulin. A direct regulation of apelin expression by insulin is observed in both human and mouse adipocytes and clearly associated with the stimulation of phosphatidylinositol 3-kinase, protein kinase C, and MAPK. These data provide evidence that insulin exerts a direct control on apelin gene expression in adipocytes. In obese patients, both plasma apelin and insulin levels were significantly higher, suggesting that the regulation of apelin by insulin could influence blood concentrations of apelin. The present work identifies apelin as a novel adipocyte endocrine secretion and focuses on its potential link with obesity-associated variations of insulin sensitivity status. (Endocrinology 146: 1764–1771, 2005)
sidering the total adipose tissue mass in the body, the role of adipocyte-produced apelin in numerous biological functions could be of importance. We therefore hypothesized that adipose tissue could be involved in the endocrine functions of apelin. We find that apelin is produced by the adipocyte itself in both mouse and human cells. We also show that apelin expression is markedly increased in vivo by nutritional status, being strongly reduced by fasting and rescued by refeeding. Finally, we demonstrate that insulin exerts a direct positive action on adipocyte apelin production both in vivo and in vitro and may influence plasma apelin levels in obese humans.

Materials and Methods

Plasma samples in humans

Eight drug-free obese and nine age-matched male controls were recruited for this study. All subjects gave their informed consent to participate in the study. Blood samples were drawn in the morning under fasting conditions. Blood was collected on EDTA, centrifuged at 1800 × g for 10 min and stored at −80°C.

Animals

Animals were handled in accordance with the principles and guidelines established by the National Institute of Medical Research. FVB/n, C5BL6/J, and C5BLKS/J db/db, db/+ , and +/+ female mice were obtained from Charles River Laboratory (l’Arbresle, France). Transgenic mice expressing human a2A-adrenergic receptor (AR-TG) under the mouse adipocyte lipid binding protein (aP2) promoter have been previously described (23). Mice were housed conventionally in a constant temperature (20–22°C) and humidity (50–60%) animal room and with a 12-h light, 12-h dark cycle (lights on at 0800 h). All mice had free access to food and water throughout the experiment. Streptozotocin treatment was performed in 13-week-old FVB/n mice. Streptozotocin (70 mg/kg) in citrate buffer was injected ip once a day during 4 d. Diets

All mice were assigned to chow or low- (LFD) or high-fat diet (HFD) (SAFE, Scientific Animal Food and Engineering, Augy, France). Energy contents of the specific diets were (percent kilocalories): 20% protein, 70% carbohydrate, and 15% fat for LFD and 20% protein, 35% carbohydrate, and 45% fat for HFD. The main source of fat in HFD was lard (20 g per 100 g of food). Five-week-old FVB/n, C5BL6/J, and AR-TG mice were fed a HFD or LFD for 8 wk. In 8-wk-old FVB/n mice, obesity was induced by a single ip injection (0.5 g/kg) of gold thioglucose (GTG) (Sigma, St. Louis, MO).

Isolation of preadipocytes and adipocytes from human and mouse adipose tissues

Human adipose tissue was collected according to the guidelines of the Ethical Committee of Rangueil Hospital, Toulouse, France. It was obtained from healthy nonobese women (body mass index 24.8 ± 1.7 kg/m²; n = 7) undergoing abdominal dermolipectomy for plastic surgery. All of them were devoid of any identified metabolic disorder and may influence plasma apelin levels in obese humans.

Cell culture

The mouse preadipose cell line 3T3F442A (25) was grown at 37°C in 7% CO₂ in DMEM containing 25 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin (culture medium), and 10% donor calf serum. For differentiation, confluent preadipocytes were cultured in culture medium supplemented with 10% fetal calf serum and 50 mM insulin for 8–10 d, after which more than 90% of the cells had accumulated fat droplets. Total RNAs were prepared at different time points after induction of the differentiation using the RNeasy mini kit (Qiagen).

Real-time RT-PCR

Total RNAs (1 µg) were reverse transcribed using random hexamers and Superscript II reverse transcriptase (RT, Invitrogen). The same reaction was performed without Superscript II (RT−) to estimate DNA contamination. The range of cycle threshold (Ct) values for Ct gene was 23–26, whereas Ct RT− was 35, meaning that very little (less than 0.1%) of genomic DNA is present in our samples. Real-time PCR was performed starting with 12.5 ng cDNA and both sense and antisense oligonucleotides in a final volume of 25 µl by using the SYBR green TaqMan universal PCR master mix (Applied Biosystems, Warrington, UK). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems). Analysis of the 18S RNA was performed in parallel using the RNA control Taqman assay kit (Applied Biosystem) to normalize gene expression. Results are expressed as: 2(Ct18S−Ctgene)/(1−(1/2Ctgene−CtRT−)), where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. Oligonucleotide primers were designed using the Primer Express software (Applied Biosystems). All primers used were validated for PCR efficiency.

Immunohistochemistry

Immunohistochemical studies were performed as previously described (26) with antiapelin polyclonal antiserum (1:200) for apelin detection. For apelin antibody generation, the human/rodent shared 13-amino-acid region of the COOH terminus of apelin was conjugated to keyhole limpet hemocyanin and was used as an antigen to generate rabbit antiserum (Covaled, Lyon, France). Negative control was performed using preimmune serum instead of antiapelin polyclonal antiserum.

Apelin assay

Apelin was quantified with either the nonselective apelin-12 enzyme immunoassay or the specific human RIA kit (Phoenix Pharmaceuticals, Belmont, CA) following the manufacturer’s instructions.

Statistical analysis

Results are expressed as means ± SEM. Statistical differences between two groups were evaluated using Student’s t tests. The level of significance was set at P < 0.05.

Results

Apelin is expressed in both human and mouse white adipose tissues

To investigate whether apelin is expressed in human adipose tissue, sc adipose tissue obtained from patients undergoing plastic surgery was digested by collagenase, and mature adipocytes were separated from the stroma vascular fraction (SVF), which contains preadipocytes, fibroblasts, endothelial cells, and other cells, by selective flotation. Apelin mRNA levels were measured in each fraction using real-time PCR. As shown in Fig. 1A, apelin mRNA was detected in both mature adipocytes and SVF at approximately equal levels.

Immunohistochemical studies were performed using an
antibody directed against the human/rodent shared 13-amino-acid region of the COOH terminus of apelin and stained by alkaline phosphatase. Apelin peptide was present in adipose tissue as indicated by the positive staining observed in the periphery of adipocytes from human sc adipose depots (Fig. 1B). A negative control using preimmune serum is shown. The same results were obtained on mice adipose tissue (not shown).

To allow investigations of apelin production and regulation in adipocytes from animal models, apelin expression was also investigated in mouse adipose tissue. As in humans, apelin mRNA was present in both isolated adipocytes and SVF (Fig. 1C). By comparison, mRNA for adipocyte-specific genes such as aP2, leptin, and adiponectin were much more abundant in adipocytes than the SVF. Results are mean values ± SEM from five mice. D. Apelin mRNA in various mouse tissues. WAT, White adipose tissue; Kidn, kidney; Mus, muscle; BAT, brown adipose tissue.

Apelin expression increases during adipocyte differentiation

Apelin was also detected in the murine preadipose cell line 3T3F442A. Apelin mRNAs were detected in undifferentiated (preconfluent and confluent) 3T3F442A preadipocytes, and their level significantly increased during adipocyte differentiation (4.6 ± 0.4-fold after 10 d post confluence) (Fig. 2A). The kinetics of appearance of apelin during the time course of adipocyte differentiation was very similar to that of the fatty acid binding protein, aP2, and delayed about 1 d when compared with peroxisomal proliferator-activated receptor-γ2. When fully differentiated, 3T3F442A cells were incubated with DMEM alone. Apelin protein could be detected in the medium at 6, 12, and 24 h, reaching a level of 2 ng/ml (Fig. 2B).

Apelin up-regulation in obese hyperinsulinemic mice

To better understand the physiology of apelin, apelin mRNA was quantitated in adipocytes isolated from different mice strains (FVB/n, C57BL6/J, AR-TG) either fed an HFD...
or LFD and in mice rendered hyperphagic and obese by GTG treatment (see details in Material and Methods). When compared with LFD, HFD led to distinct phenotypes, depending on the mouse strain (Table 1). FVB/n mice remained lean with no alteration in plasma concentration of glucose and insulin. By contrast, both C57BL6/J and AR-TG mice exhibited a significant increase in fat mass. In C57BL6/J mice, the HFD-induced obesity was associated with a significant increase in plasma glucose and insulin concentrations, whereas no changes in these parameters were observed in obese AR-TG mice (Table 1). When compared with their control, GTG-treated mice also exhibited a significant increase in fat mass associated with a significant increase in plasma insulin and glucose concentrations. As shown in Fig. 3A, adipocyte apelin expression was significantly increased in HFD, compared with LFD, in C57BL6/J but was not changed in either FVB/n or AR-TG mice. A significant up-regulation of apelin expression was observed in GTG-treated mice when compared with controls. These results show that adipocyte apelin expression is up-regulated in obesity models exhibiting increased levels of plasma insulin and glucose. Moreover, in these two hyperglycemic, hyperinsulinemic models, a significant increase ($P < 0.05$) in plasma apelin levels was found: 0.75 ± 0.12 ng/ml in control vs. 1.50 ± 0.24 ng/ml in HFD and 3.43 ± 0.96 ng/ml in GTG-treated mice.

Covariations of apelin expression in adipocyte and plasma insulin

To further investigate whether physiological or pathological variations of insulin levels could be involved in apelin regulation, we studied the effects of a fasting/refeeding sequence in mice. When compared with fed C57BL6/J mice, 24 h of fasting led to a 5.4-fold decrease in plasma insulin concentration, and this was associated with a 6.2-fold reduction in adipocyte apelin mRNA level (Fig. 4A). Twenty-four hours after refeeding, insulin plasma levels and adipocyte apelin mRNA returned to values found in fed animals (Fig. 4A). We also found a similar regulation (e.g. decreased after fasting and recovered when refeed) in insulin sensitive tissues such as muscle and heart but no change in other tissues such as kidney (data not shown). Furthermore, plasma apelin levels significantly decreased from 3.15 ± 0.08 to 0.67 ± 0.02 ng/ml ($P < 0.05$) after 24 h of fasting and returned to normal values 24 h after refeeding (2.40 ± 0.01 ng/ml).

The potential link between plasma insulin levels and apelin expression in adipocyte was further investigated with both insulin-deficient (streptozotocin-treated) and a large panel of young (4–9 wk old) C57BLKS db/db, db/+ and db/+ mice. Streptozotocin treatment led to a dramatic rise in blood glucose, a fall in insulin plasma levels, and a 3.17-fold decrease in adipocyte apelin expression (Fig. 4B). On the other hand, C57BLKS db/db mice exhibited various levels of plasma glucose (from 1.8 to 5.27 g/liter) and insulin (from 0.75 to 5.66 ng/ml). Adipocyte apelin mRNA levels of C57BLKS db/db as well as their db/+ and db/+ littermates were studied, and a significant positive correlation ($r = 0.58$, $P < 0.05$) between plasma insulin and adipocyte apelin mRNA level was observed (Fig. 4C). However, no significant correlation was found between plasma glucose and adipocyte apelin mRNA levels. Taken together, these data confirm that adipocyte apelin expression is strikingly associated with plasma insulin levels.

Apelin production is regulated by insulin

To determine whether insulin could be directly involved in the regulatory mechanisms of apelin expression depicted in mice, we directly investigated the regulation of apelin expression by insulin in both in vitro and in vivo conditions.

**TABLE 1.** Parameters of mice obesity models corresponding to Fig. 3

<table>
<thead>
<tr>
<th>Model</th>
<th>Body weight (g)</th>
<th>Perigonadal fat pad weight (g)</th>
<th>Blood glucose (g/liter)</th>
<th>Plasma insulin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/n</td>
<td>22.3 ± 0.7</td>
<td>0.69 ± 0.07</td>
<td>1.52 ± 0.18</td>
<td>0.77 ± 0.25</td>
</tr>
<tr>
<td>LFD</td>
<td>22.9 ± 0.4</td>
<td>0.62 ± 0.02</td>
<td>1.41 ± 0.11</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>HFD</td>
<td>23.4 ± 0.5</td>
<td>0.18 ± 0.01</td>
<td>2.14 ± 0.25</td>
<td>0.72 ± 0.17</td>
</tr>
<tr>
<td>C57BL6/J</td>
<td>28.8 ± 1.5*</td>
<td>1.42 ± 0.32*</td>
<td>3.02 ± 0.41*</td>
<td>1.38 ± 0.23*</td>
</tr>
<tr>
<td>LFD</td>
<td>26.6 ± 1.0</td>
<td>0.78 ± 0.10</td>
<td>1.64 ± 0.18</td>
<td>0.46 ± 0.10</td>
</tr>
<tr>
<td>HFD</td>
<td>34.3 ± 0.6*</td>
<td>2.73 ± 0.17*</td>
<td>1.54 ± 0.09</td>
<td>0.54 ± 0.11</td>
</tr>
<tr>
<td>AR-TG</td>
<td>23.0 ± 0.4</td>
<td>0.32 ± 0.13</td>
<td>1.29 ± 0.07</td>
<td>0.68 ± 0.18</td>
</tr>
<tr>
<td>FVB/n</td>
<td>39.4 ± 3.1*</td>
<td>3.22 ± 0.54*</td>
<td>2.93 ± 0.45*</td>
<td>1.27 ± 0.07*</td>
</tr>
</tbody>
</table>

* $P < 0.05$ when compared with controls (LFD or Cont).
We first tested whether injection of insulin into C57BL6/J mice could modify the adipocyte mRNA level. After a 24-h fasting period, 0.04 IU insulin injection per mouse led to a 2.4- and 2.8-fold increase in apelin expression in isolated adipocytes after 3 and 6 h, respectively (Fig. 5A), whereas no variation of apelin mRNA level was observed in the other cell types of the fat pad (not shown).

To test a direct effect of insulin on adipocytes, the murine 3T3F442A adipocyte cell line was used after a 10-d differentiation period. As shown in Fig. 5B, treatment of serum-starved 3T3F442A adipocytes with 50 nM insulin led to a substantial and time-dependent (4.8-fold after 3 h, 8.7-fold at 6 h) increase in apelin mRNA level. This effect of insulin on apelin expression and secretion was concentration dependent, with an EC50 ranged between 5 and 10 nM (Fig. 5C and D). To check a potential modification in apelin mRNA turnover in insulin-treated cells, we studied the disappearance of apelin mRNA after an actinomycin D treatment (5 g/ml) for 30 min and 2, 3, 6, 8, and 24 h. The kinetics of mRNA disappearance was similar when compared with insulin-treated and control cells (half-time life: 5 h 02 min vs. 4 h 57 min for control and insulin treated, respectively).
indicating that insulin increases apelin gene transcription rather than decreases mRNA degradation. Moreover, a potential cross-activation of IGF-I receptors by insulin could be excluded because insulin has no effect in undifferentiated 3T3F442A cells that express IGF-I but not insulin receptors (not shown). Finally, to test a potential regulation of apelin mRNA by glucose itself in obese mice, we treated differentiated preadipose cell line 3T3F442A with high-glucose concentration. No difference in apelin mRNA levels was observed when compared with untreated cells (not shown).

Because insulin is known to signal through various transduction pathways in adipocytes, we tested the potential intracellular targets of insulin by using specific inhibitors. We first preincubated differentiated 3T3F442A cells with the phosphatidyl inositol 3 kinase (PI3K) inhibitors, wortmannin, and LY294002 (100 nM and 10 μM, respectively, added 1 h before) significantly decreased the insulin-mediated induction of apelin mRNA. The selective inhibitor of protein kinase C (PKC), G109203X, also blocked the insulin-induced rise in apelin expression. The potential involvement of PKC was confirmed using the well-known activator of PKC, phorbol-12-myristate-13-acetate (PMA) because a 100-nM treatment induced a significant rise in apelin mRNA. A slight, but not significant, additive effect of PMA and insulin on apelin mRNA was also observed. Because insulin has been shown to signal through ERK 1 and ERK 2 in 3T3F442A adipocytes, we also checked the effects of the MAPK inhibitor PD098059. An insulin-induced increase of apelin expression was significantly inhibited by 20 μM PD098059. Taken together, these data show that the rise in apelin mRNA is clearly associated with PI3K, PKC, and MAPK activation.

**The relationship among obesity, insulin, and apelin in humans**

The influence of obesity and hyperinsulinemia in plasma apelin levels was investigated in male humans. Plasma apelin levels were quantified in moderately obese men and compared with age-matched controls (body mass index ranged from 31 to 34 and 20 to 24 kg/m², respectively). Metabolic and endocrine parameters in these obese subjects, depicted in Table 2, showed a classical increase in triglycerides and leptin but no change in glucose and cholesterol plasma levels. Plasma apelin levels were significantly higher in obese patients when compared with controls (P = 0.015). In obese patients, plasma insulin levels were also significantly increased, suggesting that the regulation of apelin by insulin could occur in humans (Fig. 6A).

To study apelin regulation by insulin in human adipocytes, no valuable human adipocyte cell line is currently available, and primary cultures are a mixture of various cell types present in the fat pad. For these reasons, we used explants prepared from human sc adipose tissue as previously described (24). Human adipose tissue explants were maintained ex vivo for 48 h with or without insulin. Then adipocytes were isolated from explants and apelin mRNA level measured. A 50-nM treatment with insulin led to a 6-fold rise in adipocyte apelin mRNA levels, compared with adipocytes from nontreated explants (Fig. 6B).

**Discussion**

An increasing number of peptides have been shown to be produced in the adipocyte. Thus far, however, few of them have been shown to possess true endocrine potencies or be regulated during metabolic perturbations and changes in nutritional status. Many studies have causally associated these adipokines with a large panel of diseases such as type 2 diabetes or obesity. The major finding of the present study is the characterization of endocrine production by adipocytes of the recently described peptide apelin and its potential link with endocrine and metabolic modifications related to obesity and/or modifications of insulin sensitivity. The presence of apelin mRNA has been previously demonstrated in several tissues, such as brain, lung, kidney, and adipose tissue (4–6), but the capacity of fat cells themselves to express and

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**TABLE 2.** Metabolic and endocrine parameters measured in control and obese subjects corresponding to Fig. 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 9)</th>
<th>Obese (n = 8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>29 ± 2</td>
<td>31 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 ± 0.6</td>
<td>32.6 ± 0.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Glucose (g/liter)</td>
<td>0.81 ± 0.03</td>
<td>0.85 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (g/liter)</td>
<td>0.7 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Cholesterol (g/liter)</td>
<td>2.1 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>0.97 ± 0.25</td>
<td>10.58 ± 2.31</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>3.27 ± 1.39</td>
<td>11.32 ± 2.83</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

NS, Not significant.

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**FIG. 6.** A, Plasma apelin levels in humans of nine controls and eight age-matched, drug-free obese. Blood samples were drawn in the morning under fasting conditions and collected on EDTA. *, P < 0.05 when compared with control subjects. B, Effect of 50 nM insulin in apelin mRNA expression on isolated adipocytes from human adipose tissue explants. Results are mean values ± SEM from eight separate experiments. *, P < 0.05 when compared with controls.
produce apelin has never been clearly shown. The results presented herein clearly demonstrate that apelin is expressed by each of the main adipose tissue depots in mice (i.e. both intraabdominal and sc anatomical location).

We also demonstrate that within adipose tissue, white adipocytes express apelin mRNA in comparable amounts when compared with the other cell types present in this tissue or with organs known to express apelin such as kidney and heart, whereas muscle and brown adipose tissue have somewhat lower levels. Moreover, by immunohistochemistry, apelin peptides can be detected in white adipose tissue, and apelin is secreted into the medium of cultured adipocytes in vitro. Apelin mRNA is detectable in nondifferentiated preadipocytes, but its production is increased 4-fold upon differentiation of the fat cells as previously found for the well-characterized adipokines, leptin and adiponectin. In vitro, in the mouse, apelin level is markedly influenced by the nutritional status. It is strongly inhibited by fasting and restored by refeeding, suggesting that insulin may regulate apelin gene expression and secretion. The potential link between plasma insulin levels and apelin expression in adipocytes has also been shown in streptozotocin-treated mice characterized by a dramatic fall in insulin production, which exhibit a large decrease in apelin expression. Moreover, in models of experimental obesity, apelin is significantly elevated but only in those states associated with hyperinsulinemia. Finally, a direct effect of insulin on apelin expression and secretion can be clearly demonstrated in vivo in mice as well as in vitro in both human and murine adipocytes. Regulation of production of apelin by insulin involves both the PI3K and PKC pathways. Such kinases have already been involved in the pleiotropic regulation by insulin of gene expression such as Foxc2 (27).

The role of insulin in regulation of apelin expression is supported by the in vitro and in vivo data. In vitro, in 3T3F442A adipocytes, the half-maximal effective concentration of insulin is in about 5 nM, in good agreement with the other well-characterized responses to insulin in these cells. Moreover a similar concentration-response to insulin between apelin mRNA and apelin peptide secretion is observed. The direct regulation of apelin by insulin identified in the differentiated murine 3T3F442A preadipocytes was confirmed in isolated mature murine adipocytes ex vivo from explants prepared from human sc adipose tissue.

Defining the relative roles of hyperinsulinemia and obesity in regulation of apelin is difficult because obesity is often, although not always, associated with hyperinsulinemia. To address this question, we compared four different models of obesity in mice: diet enrichment in fat, chemical alteration of hypothalamus with GTG, leptin receptor deficiency, and genetically engineered mice for a human-like adipocyte-hypothalamic loop could be hypothesized. Moreover, because apelin has recently been described as a mitogenic agent in epithelial
endothelial cells (21), apelin could take part in the development of the fat pad by acting on preadipocyte proliferation and/or angiogenesis. Finally, when considering the importance of total adipose tissue mass in the body, the possible impact of adipocyte-secreted apelin in various other organs also needs to be taken into consideration. Thus, apelin overproduction by fat tissues could be involved in the numerous obesity-associated disorders.

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