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 obesities 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)

A PELIN IS A NOVEL bioactive peptide identified as the endogenous ligand of the orphan G protein-coupled receptor, APJ (1). Several apelin-related peptides were first isolated from bovine stomach extracts as soluble factors able to increase extracellular acidification rate and inhibit cAMP production in Chinese hamster ovary cells transfected with APJ cDNA (1). A 77-amino-acid precursor can be cleaved into a 55-amino-acid fragment and then into shorter less characterized forms. Circulating high-molecular-weight forms of apelin have also been described (2). The physiologically active form is thought to be apelin-36, although shorter C-terminal sequences also elicit biological activity, the most effective being the pyroglutamylated form of apelin-13 (1). Until the discovery of apelin, the orphan seven-transmembrane domain receptor APJ was described as the closest homolog to angiotensin-II receptor. However, angiotensin II does not bind to the APJ receptor expressed in fibroblasts (3) or Chinese hamster ovary cells (1).

In rodents, mRNA expression of both apelin and APJ has been described in several tissues (4–6), but most of the phys-

iological roles of apelin in these tissues remain to be explored. However, the cardiovascular system appears to be a primary target of apelin because blood pressure decreases (4, 7, 8) and heart rate increases (8, 9) after iv administration of apelin in rats. Apelin also exerts a positive inotropic effect in rats (10, 11). Moreover, dramatic changes of apelin and APJ expression have been found in human heart with cardiac dysfunction (2, 12). An increase of both apelin and APJ expression also occurs in endothelial cells during formation of retinal vessels (13, 14). Immunolocalization of apelin and its receptor APJ have been identified in brain, including in the hypothalamus (4, 15, 16). This is in agreement with a potential role of apelin in the control of pituitary hormone release, body fluid equilibrium, and drinking behavior (4, 17). A modest, although unclear, role in the control of food intake has been described (17–19). Finally, apelin activation of APJ receptor has been described as stimulating gastric and endothelial cell proliferation (20, 21).

During the last decade, a growing number of adipocyte-derived hormones or adipokines have been identified including leptin, adiponectin, and resistin (22). These adipokines have been described to be involved in physiological regulations of fat store and development, metabolism, and eating behavior and also play a role in obesity-associated disorders including type 2 diabetes and hypertension. Although apelin has been shown to be expressed in white adipose tissue of rats (5–7), the cells directly responsible for its secretion were not identified. Numerous cell types present in this tissue, other than the adipocyte itself, could be responsible of the detection of apelin transcripts. When con-

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 influenced *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 $1800 \times g$ 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, C57BL6/J, and C57BLKS/J *db/db, db/+*, and *+/+* female mice were obtained from Charles River Laboratory (l'Arbresle, France). Transgenic mice expressing human α2A-adrenergic receptor (AR-TG) under the mouse adipocyte lipid binding protein (aP2) promotor 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-wk-old FVB/n mice. Streptozotocin (70 mg/kg/d) 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, C57BL6/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 were drug free. Mouse intraabdominal and sc adipose tissues were dissected immediately after being killed. Fresh human or mouse adipose tissues were minced in 5 ml DMEM (Life Technologies, Inc., Invitrogen, Paisley, UK) supplemented with 1 mg/ml collagenase and 1% 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. Adipocytes were washed twice in DMEM, and stroma-vascular cells were pelleted by centrifugation at $800 \times g$ for 10 min at room temperature. Isolated adipocytes and stroma-vascular fraction were further processed for RNA extraction using the RNeasy mini kit (Qiagen, GmbH, Hilden, Germany). Human sc adipose tissue explants were prepared and cul-

tured, and total RNAs of isolated adipocytes were extracted as previously described (24).

Cell culture

The mouse preadipose cell line 3T3F442A (25) was grown at 37 C in 7% CO_2 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 nM 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 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 rRNA was performed in parallel using the rRNA control Taqman assay kit (Applied Biosystem) to normalize gene expression. Results are expressed as: $2^{(Ct18S - Ctgene)}[1 - (1/2^{(Ctgene - CtRT-)})]$, 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 (Covalab, 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.

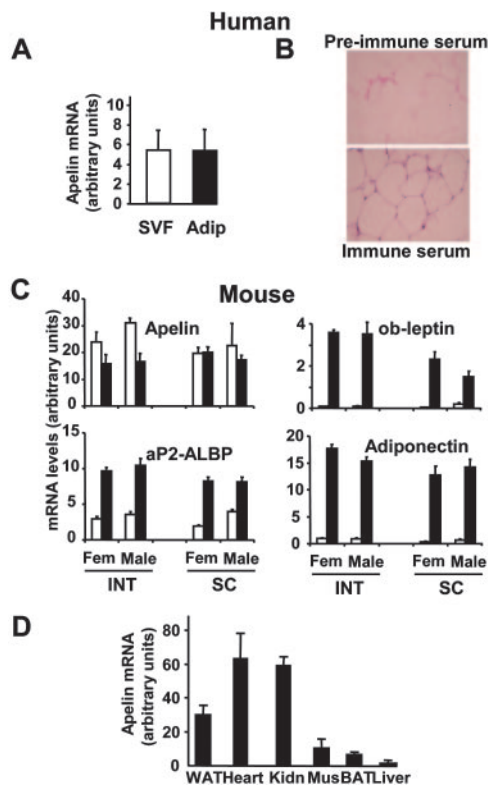Immunohistochemical studies were performed using an

FIG. 1. A, Apelin mRNA in human isolated adipocytes (adip, *black bars*) and SVF (*white bars*); results are mean values ± SEM from seven subjects. B, Immunohistochemical staining without (*upper panel*) or with (*lower panel*) an immune serum directed against the 13-COOH-terminal amino acid region of apelin (*bar*, 10 μm). C, Expression of apelin and adipocyte-specific genes in SVF (*white bars*) and adipocytes (*black bars*) from either female or male sc (SC) or intraabdominal (INT) fat pads. 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.

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. There was no difference in adipocyte apelin expression in intraabdominal and sc fat pads, whatever the gender (Fig. 1C). As previously described, apelin expression was also detected in other tissues. Among the tissues tested, expression in adipocytes was similar to that in heart and kidney, whereas lower expression was found in muscle and brown adipose tissue, and no signal was detected in liver (Fig. 1D).

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
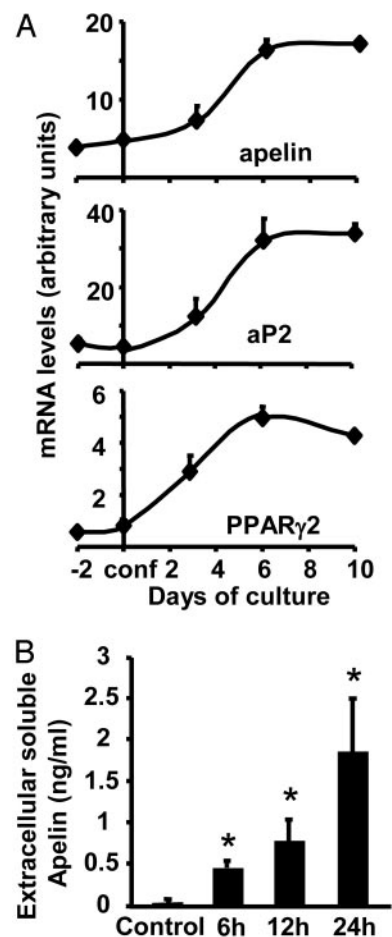

FIG. 2. A, Transcriptional regulation of apelin and adipocyte-specific genes [aP2 and peroxisomal proliferator-activated receptor (PPAR)-γ2] during 3T3F442A differentiation stages: proliferating preadipocytes (2 d before confluency), confluent cells (conf), and differentiated adipocytes (3, 6, 10 d after confluency). Results are mean values ± SEM from four separate experiments. *, $P < 0.05$ when compared with controls. B, Quantification of apelin release into the conditioned medium of 10-d differentiated 3T3F442A adipocytes 6, 12, or 24 h after medium replacement. Results are mean values ± SEM from three separate experiments.

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 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 refed) 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).

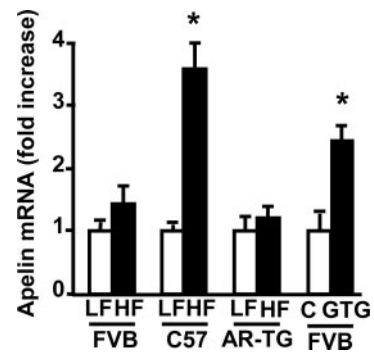The potential link between plasma insulin levels and ape-

FIG. 3. Transcriptional regulation of apelin in various obesity models. Apelin mRNA levels were quantified in isolated adipocytes from FVB/n, C57BL6/J, and AR-TG (see *Materials and Methods*) mice fed a HFD (HF, *black bars*) or LFD (LF, *white bars*) fat diet for 8 wk. Apelin mRNA levels were also quantified in isolated adipocytes from 13-wk-old, GTG-injected mice. A single ip injection with 0.5 g/kg GTG was performed in 8-wk-old FVB/n mice (GTG, *black bars*) and compared with saline-injected mice (C, *white bars*). Results are mean values ± SEM from four mice per group. *, $P < 0.05$ when compared with controls.

lin 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 +/+ 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 +/+ 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

Model	Body weight (g)	Perigonadal fat pad weight (g)	Blood glucose (g/liter)	Plasma insulin (ng/ml)
FVB/n				
LFD	22.3 ± 0.7	0.69 ± 0.07	1.52 ± 0.18	0.77 ± 0.25
HFD	22.9 ± 0.4	0.62 ± 0.02	1.41 ± 0.11	0.64 ± 0.06
C57BI6/J				
LFD	23.4 ± 0.5	0.18 ± 0.01	2.14 ± 0.25	0.72 ± 0.17
HFD	28.8 ± 1.5^a	1.42 ± 0.32^a	3.02 ± 0.41^a	1.38 ± 0.23^a
AR-TG				
LFD	26.6 ± 1.0	0.78 ± 0.10	1.64 ± 0.18	0.46 ± 0.10
HFD	34.3 ± 0.6^a	2.73 ± 0.17^a	1.54 ± 0.09	0.54 ± 0.11
FVB/n				
Cont	23.0 ± 0.4	0.32 ± 0.13	1.29 ± 0.07	0.68 ± 0.18
GTG	39.4 ± 3.1^a	3.22 ± 0.54^a	2.93 ± 0.45^a	1.27 ± 0.07^a

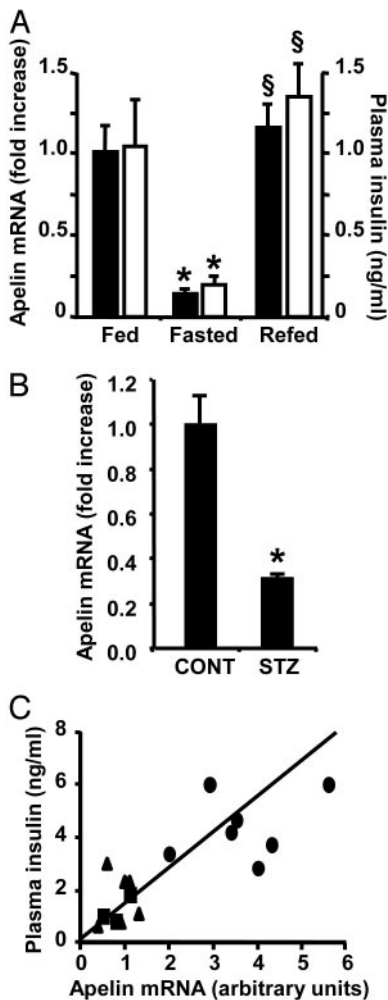[a] $P < 0.05$ when compared with controls (LFD or Cont).

FIG. 4. A, Effect of fasting/refeeding on transcriptional regulation of apelin in C57BL6/J mice adipocytes. Twelve-week-old female mice were divided into three groups. One group was allowed free access to food; the other groups were fasted for a 24-h period. At the end of the cycle, one group served as a fasting group, whereas the other was refed for 24 h. Plasma insulin levels (*white bars*) and adipocyte apelin mRNA levels (*black bars*) were quantified in the three groups. Results are mean values ± SEM from six mice per group. *, $P < 0.05$ when compared with fed group; §, $P < 0.05$ when compared with fasted group. B, Apelin mRNA levels were quantified in isolated adipocytes from streptozotocin-treated FVB/n mice (STZ) and compared with buffer citrate-treated mice (CONT). Results are mean values ± SEM from four mice per group. *, $P < 0.05$ when compared with controls. C, Plasma insulin levels and apelin mRNA from isolated adipocytes in leptin receptor-deficient mice C57BLKS homozygous db/db (*circles*), heterozygous db/+ (*squares*), and wild-type +/+ (*triangles*) littermates (n = 8, 4, and 6, respectively).

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-
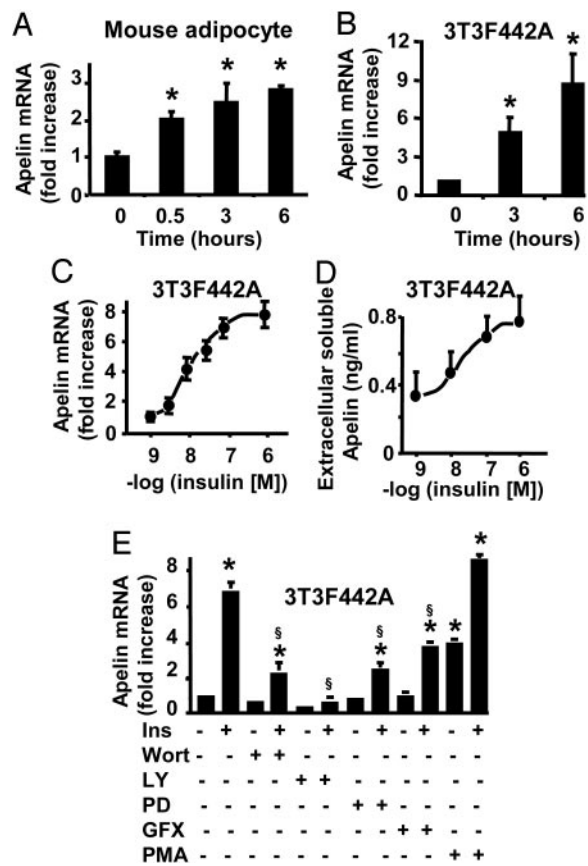

FIG. 5. Regulation of apelin expression and secretion by insulin. A, Time course of apelin mRNA levels in isolated adipocytes from insulin-treated C57BL6/J mice (single ip injection of 0.04 IU per mouse). Results are mean values ± SEM from five mice in each group, *, $P < 0.05$ when compared with sham-injected mice. B, Apelin mRNA levels in 10-d differentiated 3T3F442A adipocytes after 24 h serum deprivation and treated (3 or 6 h) or not with 50 nM insulin. Results are expressed as fold changes relative to the serum-free culture conditions (DMEM alone). Results are mean values ± SEM from five separate experiments, *, $P < 0.05$ when compared with deprived cells. C and D, Dose-response effect of insulin on apelin mRNA levels (C) or accumulation in the conditioned medium (D) in 10-d differentiated 3T3F442A adipocytes. Results are mean values ± SEM from three separate experiments. E, Apelin mRNA levels in 10-d differentiated 3T3F442A adipocytes after 24 h serum deprivation and incubated 6 h or not with 50 nM insulin (Ins) in the absence (−) or presence (+; added 1 h before insulin treatment) of wortmannin (Wort, 100 nM), LY294002 (LY, 10 μM), PD098059 (PD, 20 μM), PMA (100 nM), or GF109203X (GFX, 10 μM). Results are mean values ± SEM from four separate experiments. *, $P < 0.05$ when compared with controls; §, $P < 0.05$ when compared with insulin-stimulated.

starved 3T3F442A adipocytes with 50 nM insulin led to a substantial and time-dependent (4.8-fold after 3 h, 8.7-fold at after 6 h) increase in apelin mRNA level. This effect of insulin on apelin expression and secretion was concentration dependent, with an EC_{50} ranged between 5 and 10 nM (Fig. 5, C 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 μg/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 before administration of insulin. Figure 5E shows that 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), GF109203X, 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/m^2, 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 in-

creased, 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

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

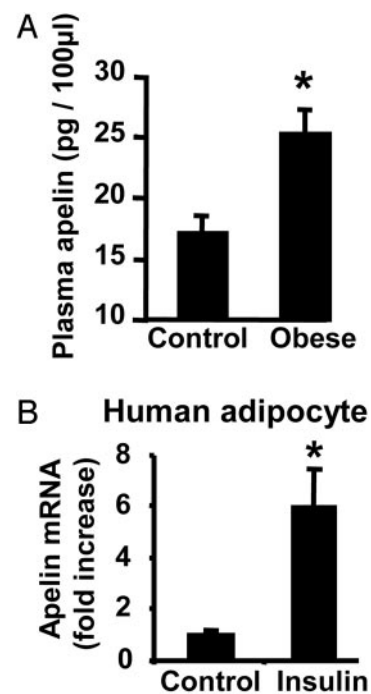|	Control (n = 9)	Obese (n = 8)	
Age (yr)	29 ± 2	31 ± 2	NS
BMI (kg/m^2)	23.4 ± 0.6	32.6 ± 0.4	$P < 0.01$
Glucose (g/liter)	0.81 ± 0.03	0.85 ± 0.03	NS
Triglycerides (g/liter)	0.7 ± 0.1	1.6 ± 0.2	$P < 0.01$
Cholesterol (g/liter)	2.1 ± 0.1	2.2 ± 0.2	NS
Leptin (ng/ml)	0.97 ± 0.25	10.58 ± 2.31	$P < 0.01$
Insulin (μU/ml)	3.27 ± 1.39	11.32 ± 2.83	$P < 0.01$

NS, Not significant.

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 vivo*, 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 adipocyte 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 adrenergic receptivity. The situation investigated herein clearly demonstrate that obesity is not the main determinant of the rise in apelin expression because, when fed an HFD, the genetically modified mice that lack β3-AR and express human α2-AR became obese but did not exhibit any change in apelin levels. High-fat feeding is not involved in apelin control either because the high-fat-fed FVB/n mice that did not become obese exhibited normal levels of apelin. However, there is a large increase in apelin expression in all the

hyperinsulinemia-associated obesities, *i.e.* high-fat-fed C57BL6/J, GTG-treated FVB/n, and C57BLKS db/db mice. Thus, insulin is likely a potent regulator of apelin expression. It can be noticed that fat cell size could be another parameter affecting apelin mRNA expression in mouse adipocyte. In fact, whatever the mouse model studied exhibiting a variation in apelin mRNA, a similar variation in adipocyte size could occur: obese hyperinsulinic mice exhibit hypertrophied adipocytes and increased apelin; streptozotocin-treated mice exhibit smaller fat cells and lower amounts of apelin. The only model of obese mouse (AR-TG) associated with a lack of adipocyte hypertrophy has normal plasma insulin levels and no change in apelin mRNA (23). However, when insulin is injected into mice, the up-regulation of apelin mRNA cannot be attributed to variations in fat cell size. Thus, to our point of view, the size of the adipocyte could influence apelin expression, but the direct effect of insulin is assured.

Among the numerous so-called adipokines, some have been described to be regulated by insulin both *in vivo* and *in vitro*, including leptin, plasminogen activator inhibitor type 1, resistin, and SPARC. However, a direct action of insulin is still a matter of debate for resistin (28–30) and is not uniformly found for leptin (31–34), whereas for plasminogen activator inhibitor type 1, multiple situations leading to high insulin levels *in vivo* have been shown to be effective: insulin injection in fasting animals from different species (35–37), obesity-associated hyperinsulinemia (35), and *in vitro* treatment in different fat cell models [3T3-L1 or 3T3F442A cell lines (38, 39) or human adipose tissue explants (38)]. The results depicted in the present study clearly show similar converging data on the regulation of apelin expression by insulin.

Finally, the potential relevance of apelin as an endocrine bioactive peptide is quite clear because we detected apelin in plasma from both mouse and man. The physiological variation in insulin obtained by fasting/refeeding in mouse induced a decrease/recovery of apelin mRNA expression in adipocytes and similar variations of plasma apelin levels. Moreover, significantly higher plasma apelin levels were found in both obese hyperinsulinemic mice and humans. Our values for plasma apelin are similar to those described by Foldes *et al.* (2) and ranged between 100 and 330 pg per 100 μl but lower than the levels described by Chen *et al.* (12). The differences in estimated apelin levels may be explained by the presence of shorter forms than apelin 36. Our assay and the assay used by Foldes (2) detected only the 36-amino-acid and larger forms of apelin and thus may underestimate the level of total apelin peptides present in blood. Moreover, we show that, in the obese patients, both plasma apelin and insulin levels are significantly higher, indicating that apelin homeostasis is impaired in the obese state and suggesting that the rise in plasma insulin could promote an increase in blood concentrations of apelin. The exact origin of this overproduction of apelin still remains to be elucidated, but the striking up-regulation of apelin promoted in adipocytes by insulin suggests an adipocyte origin of the increased apelin in obesity. Although somewhat controversial results (17–19), apelin and its receptor could also be involved in the regulation of food intake, and a leptin-like adipocyte-hypothalamic loop could be hypothesized. Moreover, because apelin has recently been described as a mitogenic agent in epithelial

(20) and 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.

Acknowledgments

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