Pre- and post-translational negative effect of β -adrenoceptor agonists on adiponectin secretion: *in vitro* and *in vivo* studies

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The adipose-derived hormone, adiponectin (ApN), has a role in fuel homeostasis, insulin action and atherosclerosis. Regulation of ApN by catecholamines has scarcely been investigated. We examined the effects of β -adrenergic agonists (and their second messenger, cAMP) on ApN gene expression, production and secretion in mouse *in vitro* and *in vivo*; their effects in human fat were also briefly studied *in vitro*. β -Adrenergic agonists and cAMP inhibited ApN gene expression in human visceral adipose tissue. Likewise, cAMP down-regulated ApN mRNAs in cultured mouse explants from visceral and subcutaneous regions. The amount of ApN released into the medium decreased concomitantly. cAMP also caused qualitative changes in ApN secretion. Under basal conditions, ApN was secreted as a single 32 kDa species. In the presence of cAMP, an additional and probably immature (not modified post-translationally) 30 kDa species was also sorted. This altered secretion resulted from cAMP-induced quantitative and qualitative changes of ApN within the adipocyte. Under basal conditions, the 32 kDa form of ApN was mainly associated with high-density microsomes

(HDMs), while the 30 kDa species was confined to a pool recovered with the cytosol fraction. cAMP depleted intracellular ApN at the expense of both HDM and cytosol fractions, and abnormally targeted ApN species to the different subcellular compartments as a result of impaired maturation. β -Adrenergic agonists mimicked the inhibitory effects of cAMP on ApN mRNA and secretion, the β_3 -agonist BRL37344 being the most potent. Administration of BRL37344 to mice reduced ApN mRNAs in both adipose regions, and ApN levels in plasma. In conclusion, β -agonists inhibited ApN production and maturation, and thus exerted a dual (pre- and post-translational) negative effect on ApN secretion by cultured mouse adipose explants. ApN inhibition by β -agonists was reproduced in mouse *in vivo* and in humans *in vitro*. ApN down-regulation may have an important role in fuel homeostasis, insulin resistance and stress-induced atherosclerosis.

Key words: adipocytes, atherosclerosis, insulin resistance, leptin, obesity.

INTRODUCTION

Adipose tissue secretes a large number of physiologically active peptides [1] that often share structural properties of cytokines, and are therefore referred to collectively as ‘adipocytokines’. One of these, adiponectin (ApN), is expressed exclusively in differentiated adipocytes. This hormone is composed of an N-terminal collagenous domain and a C-terminal globular domain [2], and is secreted into the blood where its concentration is high. ApN increases muscle fatty acid oxidation and causes weight loss in mice [3]. It is also a potent insulin enhancer in mouse models of obesity, lipodystrophy and/or diabetes [4,5]. Eventually, ApN attenuates endothelial inflammatory responses and macrophage-into-foam cell transformation *in vitro*, thereby potentially preventing the development of atherosclerosis [6, 7]. Further support for the metabolic effects of ApN comes from clinical and genetic studies. Thus plasma ApN levels are decreased in human subjects with obesity [8], type 2 diabetes [9] or cardiovascular disease [6]. Recent genome-wide scans have mapped a susceptibility locus for type 2 diabetes and metabolic syndrome to the chromosome 3q27, where the ApN gene is located [10,11].

The mechanisms involved in the regulation of ApN have not been fully elucidated. Although catecholamines have a major role in fuel homeostasis, in counteracting insulin action and in promoting stress-induced atherosclerosis, their influence on ApN

production has scarcely been investigated. A single study has reported a reduction in ApN mRNA levels by isoproterenol in murine clonal 3T3-L1 pre-adipocytes differentiated *in vitro* [12]. However, ApN may be regulated at the post-translational level and these modifications may be a determinant for protein activity [2,13]. Moreover, pre-adipocyte differentiation in an *in vivo* context has been found to be a prerequisite for optimal adipocytokine expression and hormonal responsiveness [14]. Furthermore, the effects of catecholamines on circulating ApN levels have not been explored; their influence on human ApN is also currently unknown.

In the present study, we examined the effects of β -adrenergic agonists (and cAMP, their second messenger) on ApN gene expression, tissue content and distribution, and secretion in cultured mouse adipose tissue or mature adipocytes. We extended this work to mice treated with β -agonists *in vivo*. Lastly, some data on the influence of catecholamines on human fat have also been presented.

MATERIALS AND METHODS

Subjects

Visceral (omental) adipose tissue was obtained from nine subjects (three men and six women; age 48.1 ± 5.1 years; body mass

Abbreviations used: ApN, adiponectin; Cyt, cytosol; FCS, foetal calf serum; HDM, high-density microsome; LDM, low-density microsome; MEM, minimum essential medium; PM, plasma membrane; rApN, recombinant adiponectin; s.c. subcutaneous(ly); TM, total membrane; TNF α , tumour necrosis factor- α .

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index, 31.6 ± 2.3 kg/m³) undergoing elective abdominal surgery after an overnight fast. No patient had a history of diabetes or coronary heart disease, and none had undergone any significant weight change. Patients receiving medication known to influence adipose tissue mass or metabolism were excluded. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and had the approval of the local ethical committee.

Adipose biopsies were placed in PBS and transported to the laboratory within 5–10 min of sampling, and then were prepared for culture of explants. For each culture, adipose tissue from only one subject was used.

Animals

Female mice aged 11 weeks (strain C57BL/6J or NMRI) were purchased from IFFA Credo (Brussels, Belgium). The genetic background of mice did not influence the results, and data from both strains of mice were pooled for presentation. The animals received *ad libitum* a common laboratory chow (A04; Usine d'Alimentation Rationnelle, Villemoisson-Sur-Orge, France) and were housed at a constant temperature (22 °C) with a fixed 12 h light:12 h dark cycle.

For *in vitro* studies (adipose tissue culture), mice were killed in the morning. Visceral (intraperitoneal-retrovesical) and subcutaneous (inguinal) fat pads were quickly removed under sterile conditions. It was necessary to pool tissues from several mice to obtain enough material of a given depot to allow direct comparisons to be made between different experimental conditions.

For the *in vivo* study, treatment with the β -adrenergic agonist began at 06:00 h. BRL37344 (Sigma-Aldrich, Bornem, Belgium) was dissolved in saline and used at a concentration of 0.11 mg/ml. This compound was injected subcutaneously (s.c.) to mice at a dose of 2 mg/kg of body weight, twice after 8 hourly intervals (i.e. at 06:00 h and 14:00 h). Control mice received the vehicle only. At the end of the experiment (i.e. 16 h after the first injection), visceral and inguinal fat pads were immediately removed, frozen in liquid nitrogen and stored at -70 °C; blood samples were also saved, and plasma was stored at -20 °C.

The University Animal Care Committee has approved all procedures.

Adipose tissue culture

Small fragments of human or mouse adipose tissue (2–3 mm³; explants) were prepared and cultured for up to 12 h (human) or 10 h (mouse) in 100 mm Petri dishes containing 10 ml of minimal essential medium (MEM) with Earle's salts supplemented with 10 % (v/v) foetal calf serum (human) or 0.5 % BSA (mouse) and antibiotics as described previously [15–17]; 600 mg (human) or 200 mg (mouse) of adipose tissue was cultured per dish. In some preliminary experiments with mouse explants, reducing the amount of tissue per dish (to 40 mg) did not affect ApN mRNA levels; likewise, renewing culture medium was without effect. Cell viability, as assessed by low release of lactate dehydrogenase and triacylglycerols into the medium under basal conditions [16], did not change over the course of the culture. Glucose concentrations in the medium also remained stable ([18]; results not shown).

Different agents [all from Sigma-Aldrich, except for dobutamine (Dobutrex®, Eli Lilly, Brussels, Belgium), fenoterol (Berotec®, Boehringer Ingelheim, Brussels, Belgium) and a polyclonal antibody directed against murine tumour necrosis factor- α (TNF α ; R&D Systems Europe, Abingdon, U.K.)] were added to the medium in accordance with the experimental protocols. At the end of the culture, aliquots of medium were

saved, and explants were rinsed in PBS, frozen in liquid nitrogen and stored at -70 °C.

RNA extraction and Northern blot analysis

Total RNA was extracted and subjected to Northern blot analysis [16]. The cDNA probe for mouse ApN was obtained after reverse transcription-PCR on total RNA from mouse adipose tissue (sense primer: 5'-TGGAGAGAAGGGAGAGAGAAA-3' and antisense primer: 5'-AGAAAGCCAGTAAATGTAGAG-3'). The identity of the 528 bp product was confirmed by mapping with restriction endonucleases. The probes for human ApN and mouse cyclophilin have been described elsewhere [18,19]. After hybridization with the radiolabelled probes [18], the filters were exposed to autoradiographic films. Absorbances of the mRNA bands on the blots and of 18 S rRNA on the membranes were quantified by scanning densitometry (Image Master TotalLab, Amersham Biosciences). Levels of specific mRNA were expressed relative to those of 18 S rRNA. Internal standards (pooled RNA from 2–3 patients or from several mice) were always loaded on each gel to allow direct comparisons between different blots.

Preparation of subcellular fractions from adipose tissue or cells

Mouse explants were fractionated into total membranes (TMs, i.e. plasma and microsomal membranes) and cytosol (Cyt), as described by Le Marchand-Brustel et al. [20]. With this simple method, recovery of ApN in each fraction was complete (compare ApN levels in control homogenates with the sum of control levels in TM and Cyt; see Figure 3B). ApN concentrations in each fraction were not influenced by the use of fresh or previously frozen tissue that was homogenized in the original buffer [20], or in another, i.e. HES buffer (see below), with a motor-driven metal crusher (10 s) or a gentler Dounce homogenizer (10 strokes; results not shown).

In some experiments, we studied further ApN distribution within different membrane compartments in isolated mouse adipocytes. For each experimental condition, fat cells were prepared from 6 g of tissue (pooled dishes of cultured explants) as described previously [21]. Cells were rinsed twice in HES buffer [20 mM Hepes (pH 7.4)/250 mM sucrose/1 mM EDTA/1 mM PMSF/1 μ M aprotinin], Dounce-homogenized and then fractionated into high-density microsomes (HDMs), low-density microsomes (LDMs), plasma membranes (PMs) and Cyt, according to slight modifications [22] of the protocol of Simpson et al. [23]. HDMs are enriched with endoplasmic reticulum and may also contain PM and LDM markers, whereas LDMs contain Golgi apparatus, *trans*-Golgi network and endosomes [24]. All fractions were stored at -70 °C. Protein concentrations were measured in each fraction by the Bradford method.

Quantification of ApN or leptin

Aliquots from subcellular adipose tissue or cell fractions (2.5–10 μ g of protein), culture medium (30 μ l) or serum (0.5 μ l) were solubilized in modified Laemmli buffer, and then subjected to SDS/PAGE and immunoblotting using a monoclonal antibody directed against ApN {ANOC 9108 [25]; 1:6500 dilution in Tris-buffered saline with 0.05 % (v/v) Tween 20; [18]}. Although initially raised against human ApN, this antibody also recognizes mouse (and bovine) ApN due to high interspecies homology (see Figure 2F). After reaction with a secondary antibody (anti-mouse IgG-horseradish peroxidase), the blots were treated with enhanced chemiluminescence (ECL® Plus; Amersham Pharmacia) and subjected to autoradiography. Two forms of ApN were

detected on Western blots: one that migrates like recombinant ApN (rApN; Linco Research, St. Charles, MO, U.S.A.) as a 30 kDa band, and one that is found in plasma that migrates as a 32 kDa band (see Figure 2F). These signals could not be detected when blots were re-probed with an isotype-matched irrelevant antibody used instead of ANOC 9108 (results not shown). The absorbance of each ApN band was scanned individually using a densitometer as described previously. ApN concentrations in samples were then calculated from a linear standard curve generated by increasing amounts of mouse rApN (like that shown in Figure 2F). As rApN generates as a 30 kDa signal, it should, in theory only be used to quantify the 30 kDa species in samples. However, in practice, it was used as a standard for both forms of ApN. Because ANOC 9108 cross-reacted markedly with foetal calf serum (FCS), and human cultures were performed in the presence of FCS, ApN secretion could not be reliably quantified in human experiments and was measured in mice only.

Leptin levels were measured in adipocyte fractions by RIA, using a commercially available kit (RIA mouse leptin kit; Mediagnost, Reutlingen, Germany); samples (100 μ l) were run in duplicate.

Presentation of the results

Results are the means $\pm$ S.E.M. for the indicated numbers of patients, mouse adipose tissue pools (*in vitro* experiments) or individual mice (*in vivo* study). Comparisons between two conditions were made using two-tailed unpaired or paired Student's *t* test, as appropriate. Comparisons of at least three conditions were carried out by ordinary or repeated ANOVA followed by the Newman–Keuls (comparisons of all pairs) or Dunnett's tests (all versus control) as appropriate. Differences were considered statistically significant at $P < 0.05$.

RESULTS

In human visceral adipose tissue, cAMP induced a 65% fall in the levels of ApN mRNA (approx. 4.5 kb transcript) after 12 h of culture. Isoproterenol, a pure β -adrenergic agonist, already inhibited ApN mRNA by approx. 30% after 8 h (Figure 1).

The effects of cAMP and, subsequently, of β -adrenoceptor activation were characterized further in 10 h cultured mouse adipose explants. In the basal state, the mouse ApN gene (approx. 1.3 kb transcript) was expressed more strongly in visceral than in inguinal (subcutaneous) fat, whereas cyclophilin mRNA and 18 S rRNA levels were similarly expressed (Figure 2A). The presence of cAMP for 10 h caused a 60–70% decrease in ApN mRNA in both depots, without affecting cyclophilin and 18 S parameters (Figure 2A).

The time course of the effects of cAMP on ApN gene expression in mouse explants is shown in Figures 2(B) and 2(C). Under basal (control) conditions, a spontaneous decrease of ApN mRNA occurred in both adipose regions (Figures 2B and 2C), whereas cyclophilin and 18 S parameters were again unchanged (results not shown). This decline of ApN mRNA was not due to some potential release of TNF α by explants, because it was unaffected by immunoneutralization of medium with large amounts of anti-TNF α antibody [26] or inhibition of TNF α production by pentoxifylline at a high concentration [26] (Table 1a, no change compared with controls after 10 h of culture). However, this decline was accelerated by cAMP and prevented by actinomycin D, an inhibitor of transcription (Figures 2B and 2C). The inhibitory effect of cAMP on ApN mRNA was detectable from 90 min in visceral fat (i.e. when basal ApN mRNAs were still stable), and 10 h in subcutaneous fat. This

Figure 1 Inhibitory effect of cAMP (A) or isoproterenol (B) on ApN gene expression in human visceral adipose tissue

Explants were cultured in MEM with or without 1 mM dibutylcAMP (cAMP) for 12 h or 10 μ M isoproterenol (ISO) for 8 h. mRNA levels were expressed as percentages of control values (—, i.e. obtained in MEM not supplemented with hormones or agents). Values are the means $\pm$ S.E.M. for 4 (cAMP) or 5 (ISO) subjects. * $P < 0.05$ for the effect of the test agent.

effect of cAMP was partly reversed in the presence of actinomycin D (tested only in visceral fat; Figure 2B) and could not be explained by enhanced lipolysis, since saturated or unsaturated fatty acids did not influence basal ApN gene expression (Table 1b).

Table 1 Effect of inhibiting TNF α action/production (a), and of non-esterified fatty acids (b), on ApN mRNA levels in cultured mouse adipose tissue from visceral and inguinal regions

Tissues from both regions were simultaneously sampled in mice and explants were cultured for 10 h in MEM without (controls) or with the indicated agents. TNF α in medium was immunoneutralized with large amounts of anti-TNF α antibody or TNF α production was inhibited by a high concentration of pentoxifylline as previously described [26]. mRNA levels were expressed as percentages of respective control values obtained in visceral inguinal explants after 10 h of culture. Results are the means $\pm$ S.E.M. for four pools of adipose tissue, each composed of five mice. N.D., not done. Differences versus respective controls, not significant.

	ApN mRNA levels (% of 10 h control values)	
	Visceral	Inguinal
(a) Inhibition of TNF α action or production		
Anti-TNF α antibody (4 μ g/10 ml)	90 $\pm$ 12	119 $\pm$ 14
Pentoxifylline (1 mM)	80 $\pm$ 9	72 $\pm$ 8
(b) Non-esterified fatty acids		
Oleic acid (375 μ M)	105 $\pm$ 11	N.D.
Oleic acid (500 μ M)	114 $\pm$ 14	N.D.
Palmitic acid (500 μ M)	101 $\pm$ 14	N.D.

Figure 2 Effect of cAMP on ApN gene expression and protein secretion from mouse visceral and inguinal adipose tissues

Tissues from both regions were simultaneously sampled in mice, and explants were cultured for up to 10 h in MEM with or without 1 mM cAMP. (A) Representative Northern blot of depot-specific ApN mRNA abundance after 10 h of culture in control conditions (—), and inhibition of ApN mRNA by 10 h cAMP (+) in both depots. Cyclophilin mRNA and 18 S rRNA levels are shown for comparison. (B, C) Time course of the effects of cAMP on ApN gene expression in visceral and inguinal explants cultured for up to 10 h without (○) or with (●) cAMP. In some experiments, cultures were performed with 2 μ M actinomycin D in the absence (◇) or in the presence (◆) of cAMP for 10 h. mRNA levels were expressed as percentages of pre-culture values (i.e. zero time) measured in visceral fat. (D) Western blot of ApN secreted in medium by explants from both regions cultured for 10 h without (—) or with (+) cAMP. Equal volumes (30 μ l) of culture medium were loaded on each lane. (E) Quantification of ApN secreted by visceral explants over the course of the culture. Each ApN species (30 kDa or 32 kDa band) was scanned individually and quantified by using mouse rApN as standards. ApN released in medium was expressed as ng per mg of adipose tissue. (F) Representative standard curve obtained with increasing amounts of mouse rApN. The inset shows the primary antibody used for immunoblotting cross-reacts with mouse (m) or human (h) recombinant (30 kDa) or plasma (32 kDa) ApN, while no signals could be detected in mouse skeletal muscle. Results presented in panels B, C and E are the means $\pm$ S.E.M. for six (three for actinomycin D experiment) pools of adipose tissue, each composed of ten mice. * P < 0.05, ** P < 0.01, *** P < 0.001 for the effect of cAMP versus respective controls (i.e. ○).

In agreement with the depot-specific pattern of mRNA expression, the amount of ApN secreted into the medium by visceral fat exceeded that by inguinal fat (Figure 2D). Regional differences in secretion have already been reported for other adipocytokines [1]. Under our experimental conditions, release of ApN by the inguinal depot was so low that it could not be reliably quantified; this study will therefore focus on ApN secretion by visceral fat. In basal medium, ApN was secreted as a single 32 kDa species that increased in a time-dependent manner. In the presence of cAMP, an additional 30 kDa species was released, and the total amount of ApN (32 + 30 kDa) secreted over 10 h was lower than that accumulated under control conditions (Figure 2E). It is noteworthy that recombinant ApN migrates as a 30 kDa species (Figure 2F). This suggests that the 30 kDa species recovered in the medium may be an immature form of ApN that did not become modified post-translationally.

To examine the influence of cAMP on the different forms of ApN in crude adipose tissue fractions, explants were separated into TMs and Cyt. The 30 kDa species of ApN was recovered in adipose tissue homogenates and Cyt, while the 32 kDa species was present mainly in TMs (Figure 3A). However, when large amounts of protein were loaded, a faint 32 kDa signal was also observed in the homogenate (results not shown). After 10 h of culture in basal medium, ApN levels (in ng/ μ g of protein) were distributed equally between TM and Cyt fractions. cAMP

markedly decreased ApN concentrations in homogenates and Cyt, but those in membranes were spared (Figure 3A). Because of the lower protein yield per mg of tissue in TMs, when ApN was expressed as ng/mg of tissue, the contribution of TMs to the total amount of ApN in whole control tissue (homogenate) was lowered, and yet the relative decrease in ApN levels caused by cAMP remained less pronounced in the TM than in the Cyt fractions (64% and 96% respectively, P < 0.001) (Figure 3B).

Table 2 compares the effects of cAMP on ApN secretion and ApN changes in TM and Cyt from explants cultured for 10 h. Under control (basal) conditions, only the 32 kDa species was secreted, while changes in ApN concentrations occurred in TMs. The decrease of ApN in TMs amounted to 1.2 ng/mg of tissue, whereas virtually no change could be observed in Cyt. The ratio of secreted ApN (32 kDa) to depleted ApN in TMs was approx. 4:1 (i.e. ApN secretion largely exceeded ApN depletion of the membrane compartment), suggesting ongoing synthesis of the protein. After 10 h cAMP exposure, secretion of the 32 kDa species was lower than under control conditions (2.3 ng/mg of tissue compared with 5.1 ng/mg) and depletion of ApN in TMs was more pronounced (1.8 ng/mg compared with 1.2 ng/mg). The ratio of secreted ApN (32 kDa) to depleted ApN in TMs was approx. 1:1, which indicates that the whole amount of ApN initially present in membranes was secreted. The Cyt pool was almost completely emptied by cAMP and the immature form of

Figure 3 Effect of cAMP on ApN levels in homogenate, TMs and Cyt obtained from explants

Visceral adipose tissue was cultured for 10 h in MEM with or without 1 mM cAMP, then fractionated into TM and Cyt fractions. Homogenate, TM or Cyt proteins (2.5 μ g) were loaded on each lane, and autoradiographic signals from Western blots, like that shown in the inset, were quantified by scanning densitometry using rApN as a standard. ApN levels were expressed as ng per mg of protein (**A**) or in ng/mg of tissue (**B**). Results are the means $\pm$ S.E.M. for three pools of adipose tissue, each composed of four or five mice. $^{\circ}P = 0.05$, $^*P < 0.05$, $^{**}P < 0.01$ for the effect of cAMP.

30 kDa was subsequently exported. The ratio of secreted ApN (30 kDa) to depleted ApN in Cyt was approx. 3:5, which suggests intracellular or intra-medium degradation of this species.

In some experiments, we detailed further the effects of cAMP on ApN distribution within the different subcellular membrane compartments (HDMs, LDMs and PMs) in adipocytes isolated from 10 h-cultured explants (Figure 4). Leptin was measured simultaneously for comparison. ApN levels in each adipocyte

Figure 4 Effect of cAMP on ApN (**A**) and leptin (**B**) levels in different subcellular fractions prepared from isolated adipocytes

Visceral explants were cultured for 10 h in MEM without (–, control) or with (+) 1 mM cAMP. At the end of the culture, adipocytes were isolated, and then fractionated by differential ultracentrifugation into HDMs, LDMs, PMs and Cyt. Aliquots of these fractions (5 μ g of protein for ApN, 100 μ l for leptin) were used for adipocytokine determination by Western blotting (ApN, a representative blot is shown in the inset) or RIA (leptin). Data were calculated as absorbance units (ApN) or ng/ml (leptin) normalized to aliquot protein content. ApN or leptin levels were then expressed as percentages of control values in HDM fractions. The 30 or 32 kDa species of ApN are indicated. Absolute values of leptin concentrations in control HDMs were 2.80 ± 0.19 ng/mg protein. Results are the means $\pm$ S.E.M. for three (two for PMs) independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control HDM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of cAMP in a given subcellular fraction.

fraction were calculated as absorbance units/ μ g of protein, and then expressed relative to control values in HDMs. After 10 h of culture in control medium, ApN was most abundant in HDMs, where it was detected as the 32 kDa species. The 30 kDa species was mainly recovered in the Cyt fraction. On the whole, these

Table 2 Comparison of cAMP effects on ApN secretion and ApN changes in subcellular fractions (SCF) from adipose tissue

Visceral adipose explants were cultured for 10 h in MEM without (control) or with 1 mM cAMP, then fractionated into TM or Cyt fractions. Concentrations of the indicated ApN species secreted in medium by explants over 10 h were expressed as ng/mg of adipose tissue. Changes of ApN concentrations in each subcellular fraction (TM or Cyt) over the culture course were calculated as the initial concentrations (i.e. at 0 h) minus those measured after 10 h and expressed as ng/mg of adipose tissue. Changes of ApN in TMs correspond to those of the 32 kDa species, and changes in Cyt to those of the 30 kDa species. The initial concentrations of ApN in TMs (32 kDa) and in Cyt (30 kDa) were 2.1 ± 0.1 and 2.5 ± 0.2 ng/mg of adipose tissue respectively. Ratios of secreted ApN (32 or 30 kDa)/depleted ApN in the related subcellular fraction are also presented. Results are the means $\pm$ S.E.M. for four pools of adipose tissue, each composed of ten mice. $^*P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ for the effect of cAMP.

Conditions	Secretion of the indicated ApN species (ng/mg)		Depletion of ApN in each SCF (ng/mg)		Secretion of the indicated ApN species/depletion of ApN in the related SCF	
	32 kDa	30 kDa	TM	Cyt	32 kDa/TM	30 kDa/Cyt
Control	5.1 ± 0.5	0	1.2 ± 0.3	0	4.2 ± 1.2	–
cAMP	$2.3 \pm 0.2^{\ddagger}$	$1.4 \pm 0.2^{\ddagger}$	$1.8 \pm 0.2^*$	$2.4 \pm 0.9^{\dagger}$	$1.3 \pm 0.1^*$	$0.6 \pm 0.1^{\ddagger}$

Figure 5 Effects of isoproterenol, propranolol or BRL37344 on ApN gene expression and secretion

Visceral adipose tissue was cultured in MEM without (—, control medium) or with the indicated agents. Concentrations of 10 μ M isoproterenol (Iso), 10 μ M isoproterenol + 100 μ M propranolol (Iso + Propra) or 10 μ M BRL37344 (BRL) were added to the medium for 10 h. mRNA levels were expressed as percentages of control values. ApN released into the medium after BRL37344 exposure for 10 h was expressed as ng/mg of adipose tissue. Results are the means $\pm$ S.E.M. for three to six pools of adipose tissue, each composed of four or five mice. * P < 0.05, ** P < 0.01 versus control medium; *** P < 0.001 versus Iso.

Table 3 Inhibition of ApN gene expression by various β -adrenergic agonists in visceral adipose tissue

Explants were cultured for 10 h in MEM without (basal) or with increasing concentrations of various β -adrenergic agonists. The inhibitory effects of dobutamine (β_1), fenoterol (β_2) or BRL37344 (β_3) on ApN mRNA levels were measured. The maximal inhibitory response ($I_{\max}$) corresponds to the % decrease of ApN mRNA from basal values, at the maximal effective concentration of the agonist. The concentration of β -adrenergic agonists inducing 50% of maximal inhibition of ApN mRNA (IC_{50}) is expressed as $-\log_{10} IC_{50}$ in M. Results are the means $\pm$ S.E.M. for the numbers of pools of visceral adipose tissue shown in parentheses. Each pool was composed of 20 mice. * P < 0.01, significant response with the indicated agent versus basal conditions; † P < 0.05, significant differences for the IC_{50} of BRL37344 versus dobutamine.

β -Adrenergic agonists	Maximal inhibitory response $I_{\max}$ (% decrease from basal values)	IC_{50} ($-\log_{10} IC_{50}$ in M)
Dobutamine	60.1 $\pm$ 9.8* (5)	5.07 $\pm$ 0.41 (5)
Fenoterol	18.1 $\pm$ 12.6 (5)	5.95 $\pm$ 0.36 (5)
BRL37344	67.0 $\pm$ 7.6* (5)	6.99 $\pm$ 0.40† (5)

findings are consistent with those obtained with the other experimental procedure performed on explants as depicted in Figure 3(A). Importantly, leptin was virtually absent from Cyt and only present in intra- and peri-cellular membranes (Figure 4). Relatively high amounts of leptin were found in PMs, at variance with another study [22], because adipocytes were isolated from 10 h-cultured, not fresh, tissue (M. L. Delporte, unpublished work). cAMP induced quantitative and qualitative changes of ApN within adipocytes. cAMP depleted ApN levels in HDM and Cyt fractions (owing to the small number of experiments, the quantitative modifications in PMs were not statistically significant). cAMP also caused changes in the distribution of ApN species into specific subcellular compartments. Thus after 10 h cAMP exposure, only the 30 kDa species was recovered in HDMs, whereas small amounts of the 32 kDa species were found, in addition to the expected 30 kDa form, in LDMs and PMs. cAMP emptied leptin pools in both microsomal fractions.

The inhibitory effects of cAMP on ApN mRNA and secretion were mimicked by β -adrenergic agonists (isoproterenol, a non-

A. ADIPOSE TISSUE

B. PLASMA

Figure 6 Effect of BRL37344 treatment on adipose tissue ApN gene expression (A) and plasma ApN level (B)

Mice were injected s.c. with BRL37344 (BRL) dissolved in saline (2 mg/kg of body weight, twice at an 8 h interval), while control mice (—) received the vehicle only. Adipose tissue and plasma were collected at the end of the experiment (i.e. 16 h after the first injection). mRNA and plasma levels of ApN were expressed as percentages of values in control mice. The inset shows Western blot of plasma ApN after treatment with BRL or vehicle. Results are the means $\pm$ S.E.M. for five control and five BRL mice. * P = 0.05; † P < 0.05 for the effect of BRL37344. † P < 0.001 versus visceral adipose tissue of respective mice.

specific agonist, or BRL37344, a selective β_3 -agent, both used at 10 μ M concentrations) and prevented by the addition of propranolol, a β -adrenoceptor antagonist. In the presence of

BRL37344, ApN was secreted in medium as two species of 30 and 32 kDa, in ratios (approx. 3:7) similar to those observed with cAMP (compare Figure 5 and Figure 2E).

The inhibitory effects of several selective β -adrenergic agonists on ApN gene expression were compared after 10 h of culture (Table 3). While fenoterol (a β_2 -agonist) had no significant effect, dobutamine (a β_1 -agonist) and BRL37344 produced similar maximal inhibitory responses. However, BRL37344 caused a 50% inhibition of ApN mRNA levels at a concentration (approx. 10^{-7} M) two orders of magnitude lower than that required to achieve a similar effect with dobutamine (approx. 10^{-5} M).

The effects of BRL37344 were tested in mice *in vivo* (Figure 6). Mice were injected s.c. twice with BRL37344, and adipose tissue ApN mRNA and plasma ApN levels were measured after 16 h of treatment. Administration of BRL37344 to mice caused a 30–50% reduction of ApN mRNA levels in visceral and s.c. adipose depots and a 60% decrease of ApN levels in plasma (where only the 32 kDa species was detected).

DISCUSSION

cAMP and β -agonists exert a dual (pre- and post-translational) negative effect on ApN secretion in cultured mouse adipose explants. The inhibitory effect was reproduced in mice *in vivo* and in humans *in vitro*.

In the basal state, mouse ApN mRNA levels decreased spontaneously in both adipose tissue (visceral and subcutaneous) sites, whereas 18 S and cyclophilin parameters did not change, arguing against a non-specific decrease of the mRNAs. Although TNF α may potentially be released by explants and inhibit ApN gene expression [27,28], the decrease of ApN mRNA was unaffected by immunoneutralization of the medium with anti-TNF α antibody or inhibition of TNF α production. It was also unaffected when BSA was replaced by FCS in the culture medium (results not shown), ruling out some serum-starved mechanisms. Moreover, it could not reflect glucose deprivation, as glucose concentrations in the medium remained stable, or any other nutritional deficiency, because of the short culture time. This decrease was, however, prevented by actinomycin D, and thus could require ongoing transcription and ensuing synthesis of an inhibitory protein that should act only by destabilizing the mRNA. Similar conclusions have been reached in humans both *in vivo* and *in vitro*, suggesting that this factor may be part of a negative feedback loop by which fat mass itself controls its own ApN production [18,29].

cAMP accelerated the spontaneous decline of the mRNAs. Its effect was unrelated to enhanced lipolysis, but was substantially reversed by actinomycin. cAMP could destabilize the messengers or inhibit the transcription of the ApN gene via direct or indirect mechanisms. If cAMP was only directly destabilizing ApN mRNAs, one would expect no change in the 'blunted' levels of the mRNA when cAMP and actinomycin D were combined. As this was not the case, other mechanisms must be involved. cAMP could directly inhibit ApN gene transcription. No cAMP-response element ('CRE') has been identified in the promoter of the human or mouse ApN gene, but this does not rule out the possibility that the transcription factors activated by cAMP may bind to non-conventional sites [30,31]. Eventually cAMP could act indirectly, possibly through enhanced transcription of the inhibitory protein mentioned above.

In the basal state, a 32 kDa form of ApN was secreted progressively into the medium and subsequently exported in blood. Its sustained secretion may involve ongoing synthesis, as described for leptin [32, 33], and sorting from a vesicular, mainly

HDM compartment, a subcellular fraction that contains mostly endoplasmic reticulum [23]. Small amounts of ApN were also associated with downstream membrane localizations (i.e. LDMs and PMs). Our results are thus consistent with those of Bogan et al. [34], who showed, by deconvolution immunofluorescence microscopy in 3T3-L1 cells that ApN partially co-localized with a resident protein of the endoplasmic reticulum while some ApN staining was present in peripheral storage vesicles. Trafficking into the adipocytes appears to be complex; in particular, secretion of some adipocytokines (leptin, adipon and ApN) may involve both constitutive and regulated exocytosis (i.e. non-canonical pathways) [22,34]. In our experiments, a substantial amount of ApN, in its 30 kDa form, was consistently recovered with the Cyt fraction, although we took the utmost care to avoid vesicular linkage. Importantly, under the same conditions, leptin was confined mainly to microsomes ([24] and the present study) and was virtually absent from this Cyt fraction, which may thus comprise a novel adipocytokine storage pool. This 30 kDa species of ApN was likely to represent an immature form of the protein that did not become modified post-translationally. First, this immature form, unlike the 32 kDa one, was not fated to be exported (i.e. not found in medium or blood, at least under 'normal' or basal conditions). Secondly, its relative molecular mass (M_r) is similar to that of rApN, which rules out the possibility that it may represent a breakdown product of the native protein [3]. Thirdly, during metabolic pulse-chase experiments, a small but reproducible increase in the M_r of ApN has been observed after 20 min of chase, as shown by SDS/PAGE [34]. This is likely to represent hydroxylation of collagen-domain lysine and proline residues, and glycosylation [2,13,35], by analogy to similar modifications in the structurally related mannan-binding protein, which increases from M_r 24000 to 26000 during maturation [36].

cAMP induced both quantitative and qualitative changes in ApN secretion. It decreased the total amount of ApN secreted by inducing a marked depletion of tissue ApN protein and mRNA levels. It also promoted sorting of the immature 30 kDa form by emptying both the Cyt pool, which otherwise remained unaltered, and the HDM fraction that normally did not contain this species. The latter abnormality may be due to cAMP-induced redistribution of ApN species into the different vesicular compartments (30 kDa form targeted to HDMs, small aberrant 32 kDa amounts found in LDMs and PMs). This 'mistargeting' may result from impairment of protein maturation that otherwise provides the code for ultimate protein destination [37]. Whether other agents or hormones that negatively regulate ApN also promote sorting of an immature form of the protein is still unresolved. A single band of ApN has been detected by Western blot analysis of medium samples from 3T3-L1 adipocytes treated with TNF α or dexamethasone [28]. However, this does not rule out the possibility that the two ApN species could be secreted, since extended running times for gel electrophoresis are required to detect small differences in M_r . Moreover, differences in culture models (3T3-L1 compared with mature adipose tissue) or in the antibody used may also contribute.

Figure 5 shows that, like cAMP, β -adrenoceptor activation inhibited ApN gene expression in cultured mouse explants, in agreement with recent results obtained in 3T3-L1 cells [12]. ApN gene down-regulation was dependent on β_1 - and β_3 -adrenoceptors, and was mainly driven by the latter. These findings are consistent with functional studies on β -adrenoceptor subtype-mediated lipolysis or inhibition of *ob* gene expression in white and brown adipocytes of rodents [38–40]. Like cAMP, β_3 -adrenergic stimulation also inhibited ApN secretion and split protein release into two species of different molecular masses.

On the whole, the effects *in vitro* of BRL37344 were reproduced *in vivo*. Thus administration s.c. of BRL37344 to mice caused a 30–50% decrease in ApN mRNA levels in both adipose tissue sites, and a concomitant decrease in plasma ApN levels. However, only the mature 32 kDa form of ApN, and not the 30 kDa one, was detected in blood. One may speculate that the immature 30 kDa species was less stable and thus more prone to degradation, a hypothesis consistent with our results *in vitro*. This is reminiscent of the reduced stability of non-hydroxylated collagen resulting from impaired peptidyl hydroxylation attributed to vitamin C deficiency and responsible for many of the clinical findings of scurvy [41]. It is noteworthy that calculated concentrations of circulating BRL37344 (on the basis of mean mouse circulating volume and mean s.c. resorption) averaged approx. 10^{-5} M, a concentration close to that found to be efficient *in vitro*.

Lastly, we extended some of our results to humans. cAMP exerted a marked inhibition on ApN mRNA in human visceral adipose explants, in agreement with the decreased content and secretion of the adipocytokine reported in human preadipocytes [42]. We evaluated further the effects of β -adrenoceptor stimulation, which have not been performed in human fat. Owing to limited tissue availability, only isoproterenol was tested. ApN mRNA was negatively regulated by the β -agonist, which supports an inhibitory effects of catecholamines on the human gene.

Epidemiological studies strongly suggest that stress influences the development and progression of carotid and coronary atherosclerosis [43,44]. Stress and catecholamines could therefore contribute to decrease plasma ApN levels. In this case, hypo-adiponectinaemia would be a novel link between stress and atherosclerosis. On the other hand, reducing adiponectinaemia may also be a novel mechanism by which catecholamines affect fuel homeostasis and insulin sensitivity. Both β -adrenergic agonists and ApN increase thermogenesis and lipid oxidation [3,5,45]. In addition to β -adrenoceptor desensitization, catecholamines' inhibition of ApN production and maturation may be another negative feedback loop to limit fuel and energy spillover. Catecholamines also induce insulin resistance. Their ability to inhibit an insulin-sensitizing adipocytokine, ApN, may impair insulin signalling further [12], thereby potentially contributing to the pathogenesis of insulin resistance and the insulin resistance syndrome.

In conclusion, β -adrenergic stimulation inhibited ApN production and maturation. This may have an important role in stress-induced atherogenesis, fuel homeostasis and the insulin resistance syndrome.

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