NEUROPEPTIDE AF AND FF MODULATION OF ADIPOCYTE METABOLISM:
PRIMARY INSIGHTS FROM FUNCTIONAL GENOMICS AND EFFECTS
ON β-ADRENERGIC RESPONSIVENESS

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ß-AR, ß-adrenergic receptor; BRL37344, sodium-4-{2-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl} phenoxyacetate sesquihydrate (RR,SS distereoisomer); CGP20712A, (±)-(2-(3-carbamoyl-4-hydroxyphenoxy)ethylnamino)-3-(4-(1-methyl-4-trifluormethyl-2-imidazol)phenoxy)-2-propanol methane sulfonate; \[^{125}\text{I}][\text{CYP}, (+)-[^{125}\text{I}] iodocyanopindolol; DMEM: Dulbecco’s modified Eagle’s medium; EDTA: ethylene diamine tetra acetate; G3PDH, glycerol-3-phosphate dehydrogenase; GTP\gamma S, guanosine 5’-O-(3-thiotriphosphate); HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; ICI118551, erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ISO, (-)-isoproterenol; NPAF: neuropeptide AF; NPFF: neuropeptide FF; NPFF-R(s): NPFF or NPAF receptor(s); PBS: phosphate buffered saline; PCR: polymerase chain reaction; RT: reverse transcription; RU: resonance unit; full names of gene abbreviations are given in table 1.
Summary

The presence of a neuropeptide AF and FF receptor (NPFF-R2) mRNA in human adipose tissue (Elshourbagy et al., *J Biol Chem* 275: 25965-25971, 2000) suggested these peptides, principally recognized for their pain modulating effects, may also impact on adipocyte metabolism, an aspect which has not been explored previously. Our aim was thus to obtain more insights into the actions of these peptides on adipocytes, an approach initially undertaken with a functional genomics assay. First we showed that 3T3-L1 adipocytes express both NPFF-R1 and NPFF-R2 transcripts, and that NPAF binds adipocyte membranes with a nanomolar affinity as assessed by surface plasmon resonance technology. Then, and following a 24 h treatment with NPFF or NPAF (1 µM), we have measured using real-time quantitative RT-PCR the mRNA steady state levels of already well characterized genes involved in key pathways of adipose metabolism. Among the 45 genes tested, few were modulated by NPFF (~10%) and a larger number by NPAF (~27%). Interestingly, NPAF increased the mRNA levels of β2- and β3-adrenergic receptors (AR), and to a lesser extent those of β1-ARs. These variations in catecholamine receptor mRNAs correlated with a clear induction in the density of β2- and β3-AR proteins, and in the potency of β-AR subtype-selective agonists to stimulate adenylyl cyclase activity. Altogether, these data show that NPFF-R1 and NPFF-R2 are functionally present in adipocytes and suggest that besides their well described pain modulation effects, NPAF and to a lesser extent NPFF, may have a global impact on body energy storage and utilization.
Introduction

The amidated Neuropeptide FF (NPFF)\(^1\) and Neuropeptide AF (NPAF), often referred as to mammalian FMRFamide-like peptide or morphine modulating peptides, have long been recognized for their pain modulation effects as well as for their fundamental role in opioid analgesia and tolerance development (for review see (1, 2)). However, in line with original identification of the molluscan neuropeptide FMRFamide and analogues as cardioexcitory agents (3, 4), these peptides have been shown to produce several other peripheral effects. In particular, NPFF and/or NPAF modulate cardiac and vascular function (5, 6), insulin and somatostatin secretion (7, 8), food intake (9), adrenal aldosterone production (10), or body temperature (11).

The gene encoding the precursor for these peptides has been recently described in human, rat, mouse, and bovine species (12, 13); the two peptides are encoded as a single copy by the same precursor. In the rat, expression of the NPAF and NPFF precursor is limited to discrete regions of the central nervous system including the hypothalamus, medulla and dorsal horn of the spinal chord (13). In human, NPAF and NPFF precursor gene is expressed in various peripheral organs as well as in nervous central regions with highest levels in the cerebellum (14). Detection of pulsatile secretion of NPFF in human plasma suggests the peptides could act as hormones (15) and is relevant to the various peripheral actions reported for these peptides.

Two human receptors for NPFF and NPAF have very recently been identified (14, 16, 17). The first reported human receptor for NPFF and NPAF, initially called HLWAR77, is a previous orphan seven transmembrane domain G-protein coupled receptor which when stably expressed in HEK293 exhibit similar nanomolar affinity for both NPAF and NPFF (14). Bonini et al. (16) as well as Hinuma et al. (17) identified another receptor, sharing 78% amino-acid identity with the precedent, with nanomolar affinity for NPAF and NPFF, called NPFF1. Bonini et al. (16) also identified the receptor characterized by Elshourbagy and collaborators and named it NPFF2. These NPAF/NPFF receptors will be called herafter NPFF-R1 for NPFF1, and NPFF-R2 for HLWAR77 or NPFF2. Tissue distribution analysis of NPFF-R mRNAs in human revealed a wide expression in various brain areas and little or no expression in other tested peripheral organs (14, 16). In the central and peripheral nervous systems, NPFF-R1 mRNA levels are the highest in spinal chord, hippocampus and amygdala (16), whereas NPFF-R2 mRNA are the highest in
cingulate gyrus and the lowest in cerebellum (14). In human peripheral organs, NPFF-R1 transcripts are found at low levels in lung and small intestine (16) whereas NPFF-R2 expression is virtually restricted to adipose tissue with the exception of placenta. Although the restricted and limited expression in peripheral organs do not reconcile multiple reported peripheral effects of NPAF or NPFF and may suggest the existence of additional receptor subtype(s) (1), it highlights possible modulatory metabolic effects of these neuropeptides on adipose tissue, an aspect which has never been reported or explored.

For such purpose we have designed an assay aimed at providing insights into the biological effects of NPAF or NPFF on adipocytes. We used as a model the mouse 3T3-L1 preadipose cell line which mimics the morphological, metabolic, and hormonal features of adipose tissue development (18). After having verified that 3T3-L1 differentiated fat cells express NPFF-R1 and NPFF-R2, mature 3T3-L1 adipocytes have been exposed to NPAF or NPFF stimulation and mRNA steady state levels of about fifty genes, which products are involved in key pathways of adipose metabolism, were measured by quantitative real-time RT-PCR. The well characterized tested genes are implicated in the six following pathways: insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation and secretion of factors. With such an approach, one could evidence that NPAF, and to a lesser extent NPFF, modulate in adipocytes a finite number of genes among those tested. The products of these target genes are involved in several facets of adipocyte biology. Interestingly, we observed that NPAF induces the mRNA levels of the three β-adrenergic receptor (β-AR) subtypes. In order to evaluate the consequences of modulation of β-AR transcripts, we focused the last part of our work on the characterization of NPAF effects on β-AR protein expression and functional responsiveness. In agreement with variations in mRNA levels, the neuropeptide increases β2- and β3-AR populations. This was paralleled by a higher potency of β2- or β3-AR-selective agonists and of (-)-epinephrine to stimulate adenyl cyclase activity. This fine regulation may represent a physiological mechanism by which in fat cells, the neuropeptide could promote the vascular over the neuronal adrenergic control of cAMP-dependent biological events.
Materials and Methods

Cell culture

3T3-L1 cells (ATCC number CL-173) were grown and differentiated at 37°C in an atmosphere of air/CO₂ (90:10, v/v) in Dulbecco's modified Eagle's medium (Life Technologies) with 4.5 g/l of D-glucose, 10% fetal calf serum, penicillin/streptomycin (50 U penicillin/50µg streptomycin per ml of medium). Two days after reaching confluence, cells were induced into differentiation with a two day incubation in DMEM/10% fetal calf serum containing insulin (1 µg/ml), dexamethasone (0.25 µM) and isobutyl-methyl-xanthine (0.1 mM) (all from Sigma). Then preadipocytes were shifted in DMEM/10% fetal calf serum supplemented with insulin (1 µg/ml). After ten days, when adipocytes have accumulated numerous lipid droplets as judged by Oil Red O staining (19), cells were placed for 16-18 h in a defined medium consisting of DMEM/Ham’s F12 (1:1, v/v), 4.5 g/l of D-glucose, L-glutamine, penicillin/streptomycin, 5% BSA, then treated for 24 h or 48 h with the indicated concentration of human NPAF (AGEGLNSQFWSLAAPQFa) or human NPFF (SQAFLFQPQRFa) (Bachem). Each experiment was performed 3-5 times.

Interaction of NPAF with membranes from adipocytes assessed by surface plasmon resonance

Membranes from mature 3T3-L1 adipocytes were prepared by hypotonic lysis in HEPES 20 mM, pH 7.5, EDTA 1 mM, in the presence of protease inhibitors, followed by a 500g supernatant centrifugation at 48,000g. Proteins were measured using the Bradford Coomassie dye method using BSA as standard (Pierce). Adipocyte membranes were immobilized on hydrophobic surfaces to allow subsequent measurement by surface plasmon resonance (Biacore) of NPAF binding. For such purpose, the HPA (hydrophobic association) sensor chip surface was cleaned with 50 µl of N-octylglucoside 40 mM in water at a flow rate of 10 µl/mn. After 10 to 30 s, 10 µl of adipocyte membranes (100 µg protein/ml) diluted in PBS were injected onto surface in the sample flow cell at a flow rate of 3 µl/mn. Another flow cell activated with N-octylglucoside too but without membrane served as control. The process of spontaneous fusion of adipocytes membranes to the HPA surface was monitored as the sensorgram reading begins to level out up
to the reaching of a plateau. To allow formation of an homogenous surface, \textit{i.e.}, lipid monolayer, 10 µl of NaOH 10 mM was injected at a flow rate of 10 µl/mn according to manufacturer recommendation (Biacore). Then BSA (10 µl at 0.1 mg/ml) was injected at a flow rate of 10 µl/mn to fill out putative gaps which could have remained on the surface. All experiments were done at 25°C in a continuous flow of PBS, pH 7.4. HPA surface regeneration was achieved with repeated washes with glycine 10 mM at pH 2.0. NPAF was used at indicated concentrations to monitor interaction with adipocyte membranes. Binding curves obtained using two concentrations of NPAF (100 and 1000 nM) were analyzed (BiaEvaluation software), and the data sets were best fitted with a 1:1 Langmuir binding interaction. Non specific binding to membranes was determined using an irrelevant peptide of the same size as NPAF: COOH-GSKGSKGSKGSKGSKGSK-NH2. Experiments were reproduced at least 3 times.

**RNA preparation and real-time quantitative RT-PCR**

Total RNA was prepared as described (20). cDNA was synthesized from 5 µg of total RNA in 20 µl using random hexamers and murine Moloney leukemia virus reverse transcriptase (Life technologies). Design of primers was done using either the Primer Express (Applied Biosystems) or Oligo (MedProbe, Norway) softwares. Real-time quantitative RT-PCR analyses for the genes described in table 1 were performed starting with 50 ng of reverse transcribed total RNA (diluted in 5 µl of 1X Sybr Green buffer), with 200 nM of both sense and antisense primers (Genset) in a final volume of 25 µl using the Sybr Green PCR core reagents in a ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). Fluorescence is generated after laser excitation by bound Sybr Green to double strand DNA. Because we used Sybr Green in measurements of amplification-associated fluorescence for real-time quantitative RT-PCR, it was important to verify that generated fluorescence was not overestimated by contaminations resulting from residual genomic DNA amplification (using controls without reverse transcriptase) and/or from primer dimers formation (controls with no DNA template nor reverse transcriptase). RT-PCR products were also analyzed on ethidium bromide stained agarose to insure that a single amplicon of the expected size was indeed obtained. To measure PCR efficiency, serial dilutions of reverse transcribed RNA (0.1 pg to 200 ng) were amplified, and a line was obtained by plotting cycle threshold (C\text{\textsc{t}}) values as a function of starting reverse transcribed RNA, the slope of which was used for efficiency calculation using the formula $E=10^{(1/slope)}-1$ (21). Ribosomal
18S RNA and GAPDH (which are not modified by treatment with the neuropeptides) amplifications were used to account for variability in the initial quantities of cDNA. Relative quantitation for any given gene, expressed as fold-variation over control (untreated cells), was calculated after determination of the difference between $C_T$ of the given gene A and that of the calibrator gene B (GAPDH) in treated cells ($\triangle C_T = C_{TA} - C_{TB}$) and untreated cells ($\triangle C_T0 = C_{TOA} - C_{TOB}$) using the $2^{-\triangle C_T(1-0)}$ formula (21). GAPDH expression of a control RNA was used as interplate calibrator. $C_T$ values are means of triplicate measurements. Experiments were repeated 3 to 5 times. For quantitation of murine NPFF-Rs, standard curves were determined after amplification of $5 \times 10^2$ to $5 \times 10^6$ copies of purified amplicons generated from 3T3-L1 cDNA using the sense and antisense primers. All primer sequences are presented in table 1.

**Enzyme and binding assays**

3T3-L1 adipocytes were rinsed two times with PBS, then harvested and homogenized at 4°C using a Dounce B pestle (20 strokes) in 1 mM EDTA, 25 mM Tris-HCl, pH 7.5. Homogenates were centrifuged at 100,000g for 30 min at 4°C, and membrane pellets were resuspended in the homogenization buffer and stored at −80°C until use in adenylyl cyclase or binding assays. Protein was assayed (22) using bovine serum albumin as a standard. Cell triglyceride content was determined with the Infinity$^\text{TM}$ triglyceride kit (Sigma).

Adenylyl cyclase (EC 4.6.1.1) activity was measured for 10 min at 35°C in a 50-µl standard assay consisting of 0.1 mM [$\alpha$-32P]ATP (ICN Pharmaceuticals), 1 mM cAMP, 10 mM phosphocreatine, 0.5 unit of creatine phosphokinase, 100 µM GTP (except when GTPγS, NaF, or forskolin were used), 10 mM MgCl₂, 0.2 mM EDTA, and 50 mM Tris-HCl, pH 7.5, with or without a β-adrenergic effector. The reaction was initiated by the addition of crude membranes (20 µg of protein) and terminated as described (23).

Glycerol-3-phosphate dehydrogenase (G3PDH) activity was measured in the cytosolic fraction by recording the initial rate of oxidation of NADH at 340 nm at 25°C (24). The standard mixture contained 50 mM triethanolamine/25 mM HCl buffer, pH 7.5, 1 mM EDTA, 0.13 mM β-NADH, 1 mM dihydroxyacetone phosphate, 1 mM 2-mercaptoethanol and variable amounts of 100,000 g supernatants.

For $[^{125}\text{I}]$CYP binding experiments, membrane aliquots (40-60 µg of protein) were incubated for 30 min at 37°C with $[^{125}\text{I}]$CYP (Amersham Pharmacia Biotech.) with or without
competing ligand in a final volume of 100 µl consisting of 10 mM MgCl₂, 100 µM GTP, 50 mM Tris-HCl, pH 7.5. After dilution in 2 ml ice-cold 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, separation of bound from free radioligand was achieved by vacuum filtration over Whatman GF/C glass fiber disks pre-soaked in 0.3 % polyethyleneimine, followed by three washes with the same buffer. Saturation experiments were performed with [¹²⁵I]CYP concentrations ranging from 5 to 4000 pM. Competition experiments were carried out at 30 pM [¹²⁵I]CYP. Non specific binding was determined in the presence of 100 µM (+)-propranolol and represented 9.4 ± 0.5 % of total binding at 30 pM [¹²⁵I]CYP. CGP20712A was a gift from Novartis (Basel, Switzerland), and ICI118551 was provided by ICI Pharma (France Division, Cergy-Pontoise). All other β-AR ligands were from Sigma. Data from saturation and competition experiments were analyzed with the EBDA and LIGAND programs (Biosoft Elsevier, Cambridge, U.K.).

**Statistical analysis**

Results are presented as mean ± S.E. of at least 3 independent experiments. Statistical significance was assessed by ANOVA followed by Newman-Keuls comparison tests (Statistica, StatSoft Inc.). A $P<0.05$ was considered as the threshold of statistically significance.
Results

The presence of a Neuropeptide AF and FF receptor expression in human adipose tissue suggested that NPAF or NPFF, besides their well documented pain modulating effects, may impact on adipocyte metabolism. Using a well characterized murine preadipose cell line, we first ensured that mature 3T3-L1 adipocytes expressed NPFF-R1 and/or NPFF-R2 and that NPAF binds to adipocyte membranes. Then, in order to gather initial information as regards to the actions of these peptides on adipocytes, we have treated differentiated 3T3-L1 murine adipocytes with the two neuropeptides and measured by real-time quantitative RT-PCR the expression of various genes which products are known to be involved in key pathways of adipose metabolism, including insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation and secretion of factors (Table 1).

NPAF binds to adipocyte membranes

We have analyzed NPAF binding capability on adipocyte membranes using a plasmon resonance technology. For such purpose, adipocyte membranes were immobilized as a monolayer on an hydrophobic sensorchip (see “Material and Methods”), thus giving a substantial increase in resonance units (1677 RU) reflecting effective immobilization (Fig. 1A). Whereas PBS control led only to a 19.9 RU response, NPAF increased resonance response to 43.8 and 138 RU at concentrations of 10 nM and 1 µM, respectively (Fig. 1B). On the contrary, an irrelevant control peptide of the same size as NPAF gave very low signals (1.2 RU and -0.7 RU at 1 and 10 µM, respectively), while in the same experiment NPAF at 1 µM produces a 150 RU response (Fig. 1C). Binding curves were fitted using a 1:1 Langmuir binding interaction model giving a $K_D$ value of immobilized adipocytes membranes for NPAF of 7.7 nM. These data show that NPAF is able to bind specifically to adipocyte membranes, supposedly to NPFF-R1 and/or NPFF-R2, with a nanomolar range affinity.

NPAF and NPFF modulation of gene expression in 3T3-L1 adipocytes

For most of tested genes, PCR efficiencies were close to one (1.1 ±0.07, mean ± S.E., n=21) indicating a doubling of DNA at each PCR cycle, as theoretically expected. It was thus
possible to sort the genes by their relative expression levels compared to a chosen reference, e.g., ribosomal 18S RNA, on the basis of their respective cycle threshold. Data obtained in non-stimulated control adipocytes are presented (Fig. 2). The expression levels of the genes could be scaled between \(10^{-1}\) to \(10^{-8}\)-times that of reverse transcribed r18S RNA, then divided into 3 arbitrary expression levels: high (above \(10^{-3.3}\)), medium (between \(10^{-3.3}\) and \(10^{-5.7}\)), and low (below \(10^{-5.7}\)). These corresponded to PCR cycle threshold ranging from 14 to 34, from GAPDH to ACE, with a cycle threshold of 10 for 18S. The highest levels were obtained for SCD-1, LPL, aP2, G3PDH, or FAS; medium levels for e.g., perilipin, caveolin-1, SR-BI, GLUT1, CD36, HSL, \(\beta3-AR\), PPAR\(\gamma\), UCP2, ABC1, or leptin, and lowest levels for insulin receptor, GLUT4, hexokinase-II, SREBP-1c, or PI3K. Both mNPFF-R1 and mNPFF-R2 were expressed in 3T3-L1 adipocytes at low levels (Fig. 2). Expression levels of mNPFF-R1 and mNPFF-R2 were of similar magnitude in preadipocytes and in adipocytes (not shown).

When 3T3-L1 cells were exposed to NPFF 1\(\mu M\) for 24 h, the expression of only four genes was significantly affected: c/EBP\(\alpha\), PI3K, GLUT1, and ACE expression were increased by a factor of \(~2\)-3 (Fig. 3A). By contrast, NPAF significantly modulated the expression of twelve genes among those tested (Fig. 3B). These are mainly genes coding for transcription factors (1.5- to 5-fold induction in C/EBP\(\alpha\), C/EBP\(\beta\), Id3, SREBP-1c, SREBP-2 mRNA levels), or for proteins involved in glucose transport (GLUT1 and GLUT4) and insulin signaling (insulin receptor and PI3K) or for a secreted factor (cardiotrophin-1). Interestingly, NPAF also increased the mRNA steady state levels of all the three \(\beta\)-ARs subtypes.

To verify that the inducing effect of NPFF or NPAF on the expression of several adipocyte genes did not result from a general promoting action of the peptides on the level of adipocyte differentiation, we also tested two classical biochemical markers of terminal adipose maturation, i.e., cell triglyceride content and G3PDH specific activity (25). As shown in table 2, neither NPAF nor NPFF exerted any significant effect on 3T3-L1 adipocyte triglyceride content or G3PDH activity. This observation, in addition with the absence of NPAF or NPFF effect on the levels of a large panel of adipocyte mRNAs (Fig. 3), support the view that the induction by the peptides of a finite number of transcripts rather corresponds to a specific modulating effect on these targets than to a more general action on adipocyte maturation level.

Thus, while NPFF only modulated the expression of a limited number of genes, NPAF was able to increase the abundance of a larger variety of transcripts. To further document the
effect of NPAF on adipocytes, we decided to focus our study onto a more limited number of proteins that ensure key roles in adipocytes. We chose to more extensively characterize NPAF effect on the β-adrenoceptor subtype expression and function, which are chiefly governed by mRNA abundance. Indeed, several studies in 3T3 preadipose cell lines have shown that variations in β1, β2-, or β3-adrenoceptor gene expression in response to various hormones, cytokines and nutrients are accompanied by the corresponding changes in protein binding capacity and function (20, 26-31). Moreover, the major physiological importance of the β-adrenergic system in adipocyte is well established: in this cell type, β-adrenoceptors not only mediate lipolysis, but are also involved in the negative and positive control of lipogenesis and thermogenesis, respectively (review in (32)).

**Regulation by NPAF of β-ARs density and functional expression**

Saturation and competition binding experiments were carried out on membrane fractions to determine the levels of β-AR subtype density in 3T3-L1 adipocytes cultured in the absence or in the presence of 1 µM NPAF for 48 h. In agreement with the results of real-time RT-PCR analysis, NPAF up-regulated the β3-AR (Table 3). The density of β3-ARs, corresponding to the $B_{max}$ of the low affinity component for $[^{125}\text{I}]$CYP (33), was increased by about 60 % after NPAF exposure ($P<0.02$). The amount of high affinity sites that represented the sum of β1- and β2-ARs was slightly and not significantly induced in the presence of NPAF. No difference in the $K_D$ values of the two binding classes for the radioligand could be detected between control and NPAF-treated cells.

$[^{125}\text{I}]$CYP competition binding experiments against β-AR subtype-selective ligands were also performed to determine the relative proportions of β1- and β2-ARs. These competition studies were carried out at a low (30 pM) $[^{125}\text{I}]$CYP concentration, at which no significant occupancy of the β3-AR by $[^{125}\text{I}]$CYP occurs given the poor affinity of the β3-AR for this radioligand. Under these conditions, competition of $[^{125}\text{I}]$CYP with β1- or β2-AR subtype-selective antagonists allowed estimation of the relative proportions of each of the two β-AR subtypes, with no significant interference of the β3-AR population. Analysis of the displacement curves of $[^{125}\text{I}]$CYP by the β1-AR-selective antagonist CGP20712A or by the β2-AR-selective antagonist ICI118551 gave concordant results (Table 4). In the presence of NPAF there was a
4.5-7-fold induction in β2-AR density. By contrast NPAF only caused a very weak but significant 8-13% increase in β1-AR population.

To determine the functional consequences of β-AR subtype regulation by NPAF, adenylyl cyclase activity was measured in response to various β-AR agonists on membranes from control or NPAF-treated adipocytes. We first compared the relative potencies of the catecholamines (-)-isoproterenol, (-)-norepinephrine, and (-)-epinephrine to activate adenylyl cyclase between control 3T3-L1 adipocytes and cells exposed for 48 h to 1 μM NPAF (Table 5). There was no significant difference in maximal catecholamine-induced adenylyl cyclase activity between control and NPAF-treated cells. Likewise, NPAF did not modify the potencies of (-)-isoproterenol and of (-)-norepinephrine to activate adenylyl cyclase (similar EC50 values in control and NPAF-exposed cells). By contrast, (-)-epinephrine, known to preferentially stimulate the β2-AR subtype, has a higher potency (i.e., a decreased EC50 value) in NPAF-treated cells as compared to control cells. Further investigations were also performed with β1-, β2-, and β3-AR-selective agonists (Table 5). The potency of the β1-AR-selective agonist (+)-dobutamine remained unaffected after exposure to NPAF. On the opposite, the β2-AR-selective agonist (+)-fenoterol and the β3-AR-selective agonist (+)-BRL37344 were significantly more potent in NPAF-exposed 3T3-L1 adipocytes than in control fat cells.

To ascertain the physiological relevance of the NPAF-induced changes in the potency of (-)-epinephrine, (+)-fenoterol, and (+)-BRL37344 to stimulate adenylyl cyclase, 3T3-L1 adipocytes were exposed for 48 h to various concentrations of NPAF ranging from 1 nM to 1 μM. Then, adenylyl cyclase activity was tested in response to increasing concentrations of each of the three β-AR agonists (Table 6). NPAF clearly provoked a dose-dependent increase in the potency of (-)-epinephrine, (+)-fenoterol, and (+)-BRL37344 to activate adenylyl cyclase. This effect was statistically significant from 10 nM NPAF for (-)-epinephrine and (+)-BRL37344, and from 1 nM NPAF for (+)-fenoterol. The maximal effect was obtained at 100 nM of the neuropeptide. The concentration of NPAF giving a half-maximal effect on β-adrenergic potency was in the 2-3 nM range for the three β-AR agonists. This concentration is in agreement with the estimated KD value drawn from plasmon resonance technology binding experiments on adipocyte membranes (Fig. 1). These observations strongly support the view that the NPAF-induced modulation in catecholamine responsiveness is of physiological relevance.
In control and NPAF-treated adipocytes, we also measured adenylyl cyclase activity in response to maximal concentrations of G protein or adenylyl cyclase effectors. As shown in table 7, NPAF exposure did not modulate adenylyl cyclase activity in response to the G protein effectors GTPγS and NaF, or to the adenylyl cyclase activator forskolin.

Taken together, our [125I]CYP binding studies and adenylyl cyclase experiments support the view that NPAF modulates β-AR subtype expression and β-AR subtype responsiveness in 3T3-L1 adipocytes.
Discussion

The presence of a substantial expression of a Neuropeptide AF and FF receptor mRNA in human adipose tissue (14) suggested these two neuropeptides, principally recognized for their pain modulating effects (reviewed in (1, 2)), may also impact on adipocyte metabolism. The goal of the present study was thus to investigate the effects of these peptides on adipocyte function using the murine preadipose 3T3-L1 cell line as a model.

We have first demonstrated that NPAF binds to 3T3-L1 adipocytes membranes with a nanomolar affinity (7 nM) (Fig. 1), similar to that reported for the human or rat cloned NPFF-R2 (14, 16, 34, 35), and that both NPFF-R1 and NPFF-R2 mRNAs were expressed in 3T3-L1 adipocytes (Fig. 2). Thus, 3T3-L1 adipocytes appear to be a good model to study the effects of these neuropeptides on adipocyte function. For such purpose, we have designed a functional genomics assay aimed at obtaining a rather wide picture of NPAF or NPFF putative actions on adipose metabolism using 3T3-L1 adipocytes. This assay relies on the quantitation after stimulation by both neuropeptides of mRNA steady state levels of already known and well characterized genes implicated in key pathways of adipose metabolism. With such an approach we have obtained relevant primary information as regards to the action of these peptides on adipocyte metabolism. Based on these results we have further investigated in more details the inducing effect of NPAF on β-adrenergic receptor subtype expression and function.

Our findings underline that whereas NPFF has discrete action on adipocyte gene expression, NPAF exerts much wider effects. Indeed, NPFF affected the expression of four genes only: GLUT1, PI3K, C/EBPα, and ACE, all of which were increased. By contrast, NPAF modulates that of twelve genes, corresponding to ~27% of tested genes. Among these are transcription factors involved in the regulation of maintenance of adipocyte phenotype and/or differentiation which all are increased: C/EBPα, C/EBPβ, and Id3 (36, 37). At this point it should be noted that the effects of NPAF or NPFF cannot be ascribed to an action on adipocyte differentiation as some known gene markers of adipocyte differentiation are not modified (e.g., aP2, FAS, LPL, G3PDH) while others are (e.g., CEBPα, GLUT4, β3-AR). Furthermore, two classical biochemical markers of adipocyte maturation level, cell triglyceride content and G3PDH specific activity, remain unaltered in the presence of NPAF or NPFF (Table 2). Other genes
altered by NPAF treatment encode products involved in insulin-mediated regulation of gene expression, SREBP-1c (reviewed by (38)) or cholesterol metabolism, SREBP-2 (39). Although SREBP-1c and SREBP2 are increased by NPAF treatment, it is interesting to note that their known gene targets are not modulated, such as FAS (40), SCD-1 (41), LDL-R (42), LPL (43), or HMGCoA reductase genes (44). It can be suggested that site-1 and site-2 proteases which, in conditions of sterol depletion, are instrumental regulator of SREBPs release from endoplasmic reticulum (reviewed in (45)), are not modulated by NPAF. By inference one can speculate that NPAF induction of SREBPs might synergize with states of cholesterol depletion, in the turning on activation of cholesterol and lipogenic genes. Of interest too, NPAF or NPFF treatment of adipocytes lead to an increase in mRNAs from secreted products such as ACE and cardiotrophin-1, both involved in the control of blood pressure and cardiac remodelling or hypertrophy (46, 47). In line with the well recognized status of adipose as a secretory organs (reviewed in (48, 49)), this study shows that 3T3-L1 adipocytes expressed two additional secreted products: cardiotrophin-1 and apelin, a ligand for APJ receptor (50).

Messenger RNA levels of the three β-AR subtypes were also increased, but at various levels, by NPAF exposure. While there was about a two-fold induction in β2- and β3-AR mRNA abundance, β1-AR transcripts were much more weakly enhanced by the neuropeptide treatment. Two major arguments prompted us to further investigate the potential relationship between the variations in β-AR subtype transcripts and those of the corresponding proteins and functions: i) First in adipocytes, β-ARs play a pivotal physiological role and mediate pleiotropic functions of catecholamines. For instance adipocyte β-ARs not only activate lipolysis and thermogenesis, but also exert a negative control on lipogenesis and glucose transport (32). Thus, modulation of β-AR expression could have consequences on different metabolic pathways of fat cells. ii) Second, several studies have previously reported a good correlation between the levels and β-AR mRNAs, β-AR proteins, and β-AR sensitivity (20, 26-31).

In agreement with the results on β-AR subtype transcripts, we observed a preferential increase in β2- and β3-AR populations, while the induction in β1-AR density appeared very weak (Tables 3 and 4). Overall, the regulation of β2- and β3-AR number is accompanied by an increased potency (decreased EC_{50} values) of the β2-AR-selective (fenoterol) and of the β3-AR-selective (BRL-37344) agonists to stimulate adenylyl cyclase (Table 5). Likewise, among the
three tested catecholamines (-)-isoproterenol, (-)-norepinephrine, and (-)-epinephrine), only (-)epinephrine had an increased potency to activate adenylyl cyclase in NPAF-exposed as compared to control cells, in agreement with its higher ability to stimulate the β2-AR subtype. Following NPAF exposure, the increased potency of (-)-epinephrine, (+)-fenoterol, and (+)-BRL37344 to stimulate adenylyl cyclase was detectable at low concentrations of the neuropeptide (Table 6). The half-maximal effect of NPAF was observed at nM concentrations that are in close agreement with the affinity of the receptor for its endogenous ligand, thus suggesting that the modulation of adipocyte catecholamine responsiveness may have physiological implications. Furthermore, adenylyl cyclase activity in response to optimal concentrations of G-protein or adenylyl cyclase effectors was not affected by NPAF exposure (Table 7). While we cannot exclude an effect of the neuropeptide distal to the β-ARs, both data converge to suggest that NPAF can modulate β-AR responsiveness through a differential regulation of β-AR subtype expression. It also noticeable that despite the accessory expression of β2-ARs in mature 3T3-L1 adipocytes, even after NPAF exposure, the up-regulation of this β-AR subtype caused by the neuropeptide is sufficient to induce an increased β2-adrenergic responsiveness. This observation is in line with studies showing that as compared to the β1-AR, the β2-AR has a higher intrinsic efficacy to stimulate adenylyl cyclase (51) and a greater degree of both physical and functional agonist-promoted coupling with Gs (52).

The main roles ensured by NPAF and NPFF concern their antiopioid properties in the central nervous system. Peripheral effects of these peptides are poorly documented, particularly their potential interaction with catecholamine responsiveness. However it has been previously reported that NPFF/AF receptors are present in the rat heart, and that peripheral administration of NPFF modulates blood pressure and heart rate (5). Overall, the authors have suggested that these peripheral cardiovascular responses could be mediated by functional interactions between adrenergic and NPFF/AF systems. In adipose tissue, it is thus conceivable that NPAF, through its own receptors, could mediate a modulation in β2- and β3-AR expression. Especially, the induction in β2-AR density caused by NPAF will increase the potency of epinephrine as compared to norepinephrine for stimulating the adenylyl cyclase system, and could have consequences on several cAMP-dependent processes in adipocytes, including lipolysis, thermogenesis, and anti-lipogenesis. As regards to the dual innervation and vascularization of white and brown adipose tissues, this switch in β-AR expression may privilege the vascular (i.e.,
by epinephrine) over the sympathetic control (i.e., by norepinephrine) of energy expenditure in situations that could increase NPAF levels. It has been reported that neuropeptide FF, and possibly NPAF which is encoded by the same gene precursor (12, 13), is secreted in the plasma in a pulsatile manner suggesting the peptides could act as hormones (15). Under conditions of chronic increase in plasma NPAF, it can be anticipated that modifications of adipocyte catecholamine responsiveness could occur.

Gene profiling of a limited number of informative genes has revealed a fruitful approach to achieve initial gain of knowledge concerning NPAF and NPFF action on adipocytes. The dynamics of quantitative RT-PCR, which is of about one million between the highest and the lowest tested gene, is far above other current gene expression measurement techniques. The separation of gene expression into three arbitrary high, medium, and low expression levels also stresses that genes encoding enzyme involved in the key functions of adipocytes, i.e., storage and synthesis of lipids and sterols are predominantly expressed.

In conclusion, based on the fact that a neuropeptide FF and AF receptors are substantially expressed in adipose tissue, we have explored NPAF and NPFF effects on adipocytes primary starting with a functional genomics approach. This relied on the analysis of a wide panel of well characterized genes involved in key pathways of adipocyte metabolism. Gene expression and functional data converged and showed these peptides affect several adipocyte metabolic pathways, including catecholamine responsiveness. Thus, besides their well described pain modulation effects, NPAF and to a lesser extent NPFF may have global impact on body energy storage and utilization.
Reference List


Aknowledgements

We wish to thank Drs. Jean-François Faivre, and Jacques Pairault for helpful advices and discussions, and Drs Isabelle Dugail and Philippe Robert for reviewing the manuscript.
Legends of Figures

Figure 1: NPAF binds to adipocyte membranes in a biosensor assay
Adipocyte membranes were immobilized on HPA hydrophobic surfaces to allow subsequent measurement of NPAF binding by surface plasmon resonance (Biacore). The process of spontaneous fusion of adipocytes membranes to the HPA surface was monitored as the sensorgram reading begins to level out up to the reaching of a plateau (panel A). NPAF was used at indicated concentrations to monitor interaction with adipocyte membranes (panel B). The figure displays a single representative experiment. Non specific binding to membranes was determined using the 18 amino-acid peptide COOH-GSKGSKGSKGSKGSKGSK-NH2 (panel C). Experiments were reproduced at least 3 times. For more details, see “Materials and Methods” and “Results” sections.

Figure 2: Snapshot of gene expression levels in 3T3-L1 adipocytes relative to r18S.
The relative expression levels of the indicated genes have been compared to that of ribosomal 18S RNA. Mean data obtained in unstimulated control adipocytes are presented (n=3 to 5). The expression levels of the genes have been scaled between $10^{-2}$- to $10^{-10}$-times that of reverse transcribed r18S RNA, then divided into 3 arbitrary expression levels each of $10^{-2.33}$ wide: high, medium and low. NPFF-R1 and NPFF-R2 are indicated with arrows.

Figure 3: Variation in gene expression in 3T3-L1 adipocytes upon treatment with NPAF and NPFF
3T3-L1 mature adipocytes were treated with 1 µM NPFF (panel A) or NPAF (panel B) for 24 h. Total RNA was prepared and mRNA steady state levels of well characterized genes implicated in the six following pathways of adipocyte metabolism were measured by quantitative RT-PCR: insulin signaling and glucose metabolism, lipoprotein and cholesterol metabolism, energy storage, energy degradation, gene transcription regulation and secretion of factors. Results are presented as fold increase over control values using the $2^{-\Delta\Delta CT}$ formula. Data presented are means ± S.E. of 3 to 5 independent experiments. For additional details see “Materials and Methods” section. Significant variation over control assessed by one-way ANOVA are labelled as * for $P<0.05$ and ** for $P<0.01$. 

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Table 1: Primer sequences for genes involved in key pathways of adipose metabolism

The abbreviation of the genes, full name, accession number or locus, and corresponding primer numbers, and 5’ to 3’ nucleotide sequences of the sense and antisense primers are presented.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Full name</th>
<th>Accession number/locus</th>
<th>Primer number (S/AS)</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin I converting enzyme</td>
<td>MUSACEA</td>
<td>875/876</td>
<td>CTCCCTGGGCGCTGTACCT</td>
<td>GCTCATGAAATATCGGATGCTAG</td>
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<td>pro-pro angiopoietin</td>
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<td>899/900</td>
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<td>GGCTGCTGGCAATTTCTTCTCT</td>
</tr>
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<td>798/799</td>
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</tr>
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<td>fatty-acid binding protein</td>
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<td>733/734</td>
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<td>ACACATTCCACCCACAG</td>
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<td>beta1-adrenoceptor</td>
<td>MUSADRR</td>
<td>893/894</td>
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<td>GAGACTGATAGTGAGGGTTCG</td>
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<td>pro-pro angiopoietin</td>
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<td>fatty-acid binding protein</td>
<td>MUSLBP</td>
<td>733/734</td>
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<td>beta1-adrenoceptor</td>
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<td>betacatenin</td>
<td>MMACTBR2</td>
<td>45/46</td>
<td>CAGACGACCTGGCAATG</td>
<td>GCGAGATGAGTGAGGTCCAG</td>
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Table 2: Absence of NPAF or NPFF effect on 3T3-L1 adipocyte triglyceride content and on glycerol-3-phosphate dehydrogenase activity

Cells extracts (homogenates and 100,000 g supernatants) were prepared from control and NPAF- or NPFF-treated (1 µM for 48 h) 3T3-L1 adipocytes, and were tested for triglyceride content (in nmol/dish) and glycerol-3-phosphate dehydrogenase (G3PDH) activity. (in nmol NADH/min/mg of protein). Data were also normalized to the corresponding control values. Results are expressed as mean ± S.E. of eight independent experiments.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Triglyceride content</th>
<th>G3PDH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/dish</td>
<td>% of control</td>
</tr>
<tr>
<td>Control</td>
<td>548.7 ± 40.6</td>
<td>100</td>
</tr>
<tr>
<td>NPAF</td>
<td>570.3 ± 50.2</td>
<td>104</td>
</tr>
<tr>
<td>NPFF</td>
<td>568.6 ± 50.6</td>
<td>103.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>nmol NADH/min/mg</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>505.0 ± 17.1</td>
<td>100</td>
</tr>
<tr>
<td>NPAF</td>
<td>484.1 ± 12.8</td>
<td>96</td>
</tr>
<tr>
<td>NPFF</td>
<td>481.8 ± 13.3</td>
<td>95.5</td>
</tr>
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</table>
Table 3: Characterization of $[^{125}\text{I}]$CYP binding sites in membranes from control and NPAF-exposed 3T3-L1 adipocytes

Membranes from control and NPAF-treated (1 µM for 48 h) 3T3-L1 adipocytes were tested in $[^{125}\text{I}]$CYP saturation binding experiments using a wide range of concentrations (5-4000 pM) of the radioligand. Scatchard analysis of the data with the EBDA/LIGAND program was used to calculate the $K_D$ and the $B_{\text{max}}$ values of the high- ($\beta_1$- and $\beta_2$-ARs) and the low ($\beta_3$-AR) affinity sites for $[^{125}\text{I}]$CYP. Results are expressed as mean ± S.E. of six separate experiments. The percentage of each affinity binding class is indicated in parentheses after the $B_{\text{max}}$ values.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>High affinity ($\beta_1$- and $\beta_2$-ARs)</th>
<th>Low affinity ($\beta_3$-AR)</th>
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<tr>
<td></td>
<td>$K_D$</td>
<td>$B_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>pM</td>
<td>fmol/mg</td>
</tr>
<tr>
<td>Control</td>
<td>35.4 ± 5.6</td>
<td>11.4 ± 2.1 (26.5%)</td>
</tr>
<tr>
<td>NPAF</td>
<td>39.6 ± 4.9</td>
<td>14.2 ± 1.9 (22.2%)</td>
</tr>
</tbody>
</table>

*, $P<0.02$, NPAF-treated versus control adipocytes
Table 4: Competition of [125I]CYP against β1- and β2-AR selective antagonists in membrane from control and NPAF-exposed cells

Membranes were prepared control and NPAF-treated (1 μM for 48 h) 3T3-L1 adipocytes. Competition binding experiments were performed at 30 pM [125I]CYP in the absence or in the presence of various concentrations of CGP20712A and ICI118551, β1- and β2-AR-selective antagonists, respectively. Data from displacement of [125I]CYP binding by these subtype-selective ligands were used to calculate the $K_i$ values for each affinity component. The corresponding $B_{\text{max}}$ values were derived from total β-AR density (β1- plus β2-Ars) drawn from [125I]CYP saturation experiments (see table 3), and taking into account the percentage of each affinity component (indicated in parentheses after the $B_{\text{max}}$ values) obtained from competition experiments. Results are expressed as mean ± S.E. of six separate experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Selectivity</th>
<th>Binding affinity (related β-AR subtype)</th>
<th>Control</th>
<th>NPAF</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$K_i$ ($nM$) $B_{\text{max}}$ (fmol/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGP20712A</td>
<td>β1 High (β1-AR)</td>
<td>9.2 ± 1.2 11.0 ± 0.2 (96%)</td>
<td>10.1 ± 1.0</td>
<td>12.4 ± 0.6 (87%)$^*$</td>
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<tr>
<td></td>
<td>β2 Low (β2-AR)</td>
<td>919 ± 320 0.4 ± 0.2 (4%)</td>
<td>704 ± 178</td>
<td>1.8 ± 0.6 (13%)$^*$</td>
</tr>
<tr>
<td>ICI118551</td>
<td>β2 High (β2-AR)</td>
<td>1.6 ± 0.7 0.3 ± 0.2 (3%)</td>
<td>1.3 ± 0.5</td>
<td>2.2 ± 0.2(15%)$^{***}$</td>
</tr>
<tr>
<td></td>
<td>β1 Low (β1-AR)</td>
<td>118 ± 15 11.1 ± 0.2 (97%)</td>
<td>172 ± 25</td>
<td>12.0 ± 0.2 (85%)$^{**}$</td>
</tr>
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</table>

*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, NPAF-treated versus control adipocytes.
Table 5: Relative potencies of various β-AR agonists for stimulating adenylyl cyclase activity in control and NPAF-treated 3T3-L1 adipocytes

Adenylyl cyclase activity was measured in membranes from control and NPAF-exposed (1 µM for 48 h) 3T3-L1 adipocytes and in response to increasing concentrations of the indicated β-AR agonists. EC50 values (in µM) are the concentrations of each ligand required for a half-maximal stimulation of adenylyl cyclase. Vmax values (in pmol cAMP/min/mg of protein) are expressed as agonist-stimulated over basal adenylyl cyclase activity. Results represent the mean ± S.E. of 5-12 independent experiments. Basal adenylyl cyclase activity was 6.7 ± 1.0 and 6.8 ± 1.1 pmol cAMP/min/mg of protein in control and NPAF-exposed cells, respectively.

<table>
<thead>
<tr>
<th>β-AR agonist</th>
<th>Control</th>
<th>NPAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (µM)</td>
<td>Vmax (pmol cAMP/min/mg)</td>
</tr>
<tr>
<td>(-)-Isoproterenol</td>
<td>0.71 ± 0.08</td>
<td>35.9 ± 3.9</td>
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<tr>
<td>(-)-Norepinephrine</td>
<td>4.26 ± 0.32</td>
<td>38.1 ± 4.7</td>
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<tr>
<td>(-)-Epinephrine</td>
<td>4.07 ± 0.62</td>
<td>31.3 ± 5.4</td>
</tr>
<tr>
<td>(+)-Dobutamine</td>
<td>2.18 ± 0.27</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>(+)-Fenoterol</td>
<td>6.94 ± 0.31</td>
<td>13.8 ± 1.5</td>
</tr>
<tr>
<td>(+)-BRL37344</td>
<td>0.98 ± 0.17</td>
<td>20.0 ± 1.5</td>
</tr>
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</table>

*, P<0.05; **, P<0.01, NPAF-treated versus control adipocytes.
Table 6: Dose-dependent effect of NPAF on the potency of several β-AR agonists to stimulate adenylyl cyclase

Cell extracts were prepared from control 3T3-L1 adipocytes and from cell exposed for 48 h to various concentrations (1-1000 nM) of NPAF. Adenylyl cyclase activity was then measured in response to increasing concentrations of (-)-epinephrine, (+)-fenoterol, and (+)-BRL37344. EC<sub>50</sub> values (in µM) is the concentration of each β-AR agonist required for a half maximal stimulation of adenylyl cyclase. V<sub>max</sub> values (in pmol cAMP/min/mg of protein) are expressed as agonist-stimulated over basal adenylyl cyclase activity. Results represent the mean ± S.E. of 6 independent experiments. Basal adenylyl cyclase activity was 8.6 ± 0.4, 8.7 ± 0.7, 8.0 ± 0.5, 8.6 ± 0.6, and 8.6 ± 0.5 pmol cAMP/min/mg in control cells and in cells treated with 1, 10, 100, and 1000 nM NPAF, respectively.

<table>
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<tr>
<th>NPAF Concentration (nM)</th>
<th>β-AR agonist</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
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<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-)-Epinephrine</td>
<td>µM</td>
<td>pmol cAMP/min/mg</td>
<td>µM</td>
<td>pmol cAMP/min/mg</td>
<td>µM</td>
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<tr>
<td>0 (control)</td>
<td></td>
<td>4.56 ± 0.54</td>
<td>39.4 ± 3.5</td>
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<td>3.37 ± 0.44</td>
<td>41.5 ± 5.0</td>
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<td>25.2 ± 2.0</td>
<td>0.39 ± 0.06</td>
<td>24.9 ± 2.4</td>
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<td></td>
<td>3.40 ± 0.31*</td>
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<td>100</td>
<td></td>
<td>2.62 ± 0.25*</td>
<td>43.3 ± 5.8</td>
<td>5.84 ± 0.18**</td>
<td>27.5 ± 3.7</td>
<td>0.29 ± 0.02**</td>
<td>26.0 ± 3.9</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>2.88 ± 0.27*</td>
<td>46.1 ± 5.1</td>
<td>5.84 ± 0.54**</td>
<td>27.4 ± 3.1</td>
<td>0.29 ± 0.02**</td>
<td>28.6 ± 3.5</td>
</tr>
</tbody>
</table>

*, P<0.05; **, P<0.01, NPAF-treated versus control adipocytes.
Table 7: Absence of NPAF effect on adenylyl cyclase stimulated by G-protein or adenylyl cyclase effectors

Membranes were prepared from control adipocytes or from adipocytes exposed for 48 h to 1 µM NPAF. Adenylyl cyclase in response to an optimal concentration of each indicated effector was determined. Results are expressed as effector-stimulated over basal adenylyl cyclase activity, and represent the mean ± S.E. of six independent experiments. Basal adenylyl cyclase activity was similar in control and NPAF-treated cells (see table 5).

<table>
<thead>
<tr>
<th>Effector</th>
<th>Adenylyl cyclase activity (over basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>pmol cAMP/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>GTPγS (100 µM)</td>
<td>52.6 ± 2.9</td>
</tr>
<tr>
<td>NaF (10 mM)</td>
<td>105.4 ± 21.8</td>
</tr>
<tr>
<td>Forskolin (100 µM)</td>
<td>378.9 ± 51.2</td>
</tr>
</tbody>
</table>
Figure 1

A

N-octylglucoside

adipocytes membrane

NaOH

BSA

1677 RU

injection time

Control Flow Cell

B

response (RU)

1000 nM

NPAF

10 nM

PBS

C

response (RU)

CTRL 10 µM

CTRL 1 µM

PBS

Figure 1
Expression relative to 18S

Figure 2
Figure 3A

% variation vs control

- PPARγ1&2
- C/EBPα
- C/EBPβ
- Id1
- Id2
- Id3
- SREBP-1a
- SREBP-1c/ADD1
- SREBP-2
- Ins Recep
- PI3K
- Akt/PKB
- hexokinase II
- GLUT1
- GLUT4
- FAS
- LPL
- aP2
- PLTP
- G3PDH
- perilipin
- SCD-1
- UCP2
- UCP3
- β1-AR
- β2-AR
- β3-AR
- LDL-R
- SR-BI
- CD36
- VLDL-R
- HMGC0A Rd
- caveolin-1
- caveolin-2
- ACE
- angiotensinogen
- pre-pro apelin
- cardiotrophin-1
- leptin
- PAI-1
- RPL 19
- β-actin
- GAPDH

* indicates significant difference compared to control.
% variation vs control

- PPARγ1&2
- C/EBPα
- C/EBPβ
- Id1
- Id2
- Id3
- SREBP-1a
- SREBP-1c/ADD1
- SREBP-2
- Ins Recep
- PI3K
- Akt/PKB
- hexokinase II
- GLUT1
- GLUT4
- FAS
- LPL
- aP2
- PLTP
- G3PDH
- perilipin
- SCD-1
- UCP2
- UCP3
- β1-AR
- β2-AR
- β3-AR
- LDL-R
- SR-BI
- CD36
- VLDL-R
- HMGCoA Rd
- caveolin-1
- caveolin-2
- ACE
- angiotensinogen
- pre-pro apelin
- cardiotrophin-1
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- RPL 19
- β-actin
- GAPDH
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