Biochemical and Biophysical Research Communications 377 (2008) 29–34

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RFamide neuropeptides inhibit murine and human adipose differentiation

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ARTICLE INFO

Article history:
Received 4 September 2008
Available online 25 September 2008

Keywords:
RFamide neuropeptides
3T3 adipogenesis
Human adipogenesis
Id3 expression

ABSTRACT

RFamide neuropeptides NPFF and NPAF affect gene expression in mature 3T3-L1 adipocytes but their role on adipogenesis is unknown. Here, we show that NPFF, NPAF, and NPSF inhibited the differentiation of 3T3-F442A preadipocytes in a concentration-dependent manner, but had no effect on 3T3-L1 adipogenesis. All three neuropeptides also blocked the adipose differentiation of normal and lipoma-derived human preadipocytes. The antiadipogenic effect of RFamide neuropeptides was linked with the overexpression of Id3 gene and the inhibition by NPAF remained after neuropeptide removal and further incubation of 3T3 cells with adipogenic medium. Our results show that NPFF, NPAF and NPSF negatively affect adipogenesis and suggest that these compounds participate in the regulation of the adipose tissue development by the central nervous system.

Adipose tissue is strongly influenced by the nervous system [1]. The sympathetic innervation of adipose depots is the principal initiator of lipid mobilization in mammals [2] and a regulator of fat cellularity [3]. Sensory innervation of adipose tissue has also been documented, suggesting a possible role of sensory neural fibers in conveying information on the degree of adiposity to the brain [4].

Besides the sympathetic and sensory adipose innervation, many circulating factors serving as efferent and afferent signals has been described to mediate multiple aspects of brain–adipose communication [5–8]. A lot of neural factors acting on adipose tissue, however, are still poorly known, and advances in their study would extend our knowledge of obesity and associated co-morbidities. RFamide neuropeptides, initially described in molluscan [9], constitute a family of small peptides widely distributed in animal phyla [10]. NPFF, NPAF and NPSF are mammalian neuropeptides expressed in discrete regions of the central nervous system including hypothalamus, medulla, and the dorsal horn of spinal cord [11,12]. These neuropeptides, mainly recognized by their roles in pain modulation and opioid analgesia [12,13], also modulate cardiac and vascular function [14], insulin and somatostatin secretion [15], aldosterone production [16], body temperature [17], and food intake [10,18]. The recognition of pulsatile secretion of NPFF in human plasma suggests a hormonal role for these peptides [19].

RFamide neuropeptides act through NPFF-R1 and NPFF-R2 G protein-coupled receptors [20–22], and the expression of NPFF-R2 in adipose tissue [21] suggests a role for RFamide neuropeptides in the metabolism and/or development of this tissue. Although it has been shown that NPFF and NPAF neuropeptides alter gene expression in mature 3T3 L1 adipocytes [23], their effect on adipogenesis remains unexplored. Therefore, the central aim of this study was to evaluate the effects of NPFF, NPAF and NPSF on the *in vitro* differentiation of murine and human preadipocytes. Our work show that RFamide neuropeptides inhibit adipogenesis and suggest that these compounds participate in the regulation of the adipose tissue development by the central nervous system.

Materials and methods

3T3 cell culture. 3T3-F442A preadipocytes were differentiated with DMEM (GIBCO BRL) containing 7% calf serum (CS; GIBCO BRL), 5 μg/ml insulin and 1 μM D-biotin, the adipogenic medium (AM), or maintained under non-adipogenic medium (NAM; DMEM containing 5% domestic adult cat serum, 5 μg/ml insulin and 1 μM D-biotin) [24]. 3T3-L1 adipogenesis was induced in confluent preadipocytes with AM supplemented with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.25 μM dexamethasone for 48 h and then incubating cultures in DMEM supplemented with 7% CS and 5 μg/ml insulin. Effects of NPAF, NPFF and NPSF neuropeptides on 3T3 adipogenesis were assayed in 7–14 d postconfluent cultures maintained at 37 °C in a humidified 95% air, 5% CO_2 atmosphere with medium changes every other day. All medium supplements except sera were from Sigma–Aldrich.

Isolation and culture of human preadipocytes. Human adipose tissue samples were obtained at the outpatient plastic surgery service, Hospital General de Ciudad Valles (San Luis Potosí, México), from patients who gave their informed consent. Normal (HNPA) and lipoma-derived (HLPA) subcutaneous adipose samples were extensively washed with sterile PBS added with antibiotics (penicillin G 2000 U/ml and streptomycin sulphate 100 μg/ml), finely

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doi:10.1016/j.bbrc.2008.09.071

chopped, and digested with type II collagenase 3 mg/ml in DMEM containing 1.5% of BSA for 60 min at 37 °C. Collagenase was eliminated by low-speed centrifugation and cell pellets (the stromal-vascular fraction) were resuspended in basal medium (BM; DMEM-F12 [1:1] added with 10% FBS), plated in 60 mm tissue culture dishes, and incubated as described for 3T3 cells. Adipogenesis was induced in confluent cultures with DMEM-F12 (1:1) added with 5% FBS, 100 nM insulin, 100 nM dexamethasone, 0.2 nM triiodothyronine, 1 μM rosiglitazone and 25 μM IBMX for 48 h. Cultures were maintained in induction medium lacking IBMX for at least 21 d under conditions described for 3T3 cells.

Quantitation of adipose differentiation. Adipose differentiation was estimated by quantifying intracellular lipid accumulation with oil red O [25] or by measuring glycerol-3-phosphate dehydrogenase activity (GPDH; EC 1.1.1.6) [24].

Semi-quantitative RT-PCR. Total RNA was isolated using TRIzol® (GIBCO BRL) and quantified by UV spectrophotometry. RNA integrity was assessed by electrophoresis on 2% agarose gels stained with ethidium bromide. RNA (2 μg) was reverse-transcribed in a 25 μl volume reaction containing 4.0 μl RT buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT), 0.5 mM dNTPs, 25 U of RNasin, and 10 U of Moloney murine leukemia virus reverse transcriptase (RT) in the presence of oligo dT for 1 h at 42 °C. RT was omitted in negative control reactions. All reactives were from Promega. Resultant cDNAs were amplified in a 25 μl reaction volume containing 2 μl cDNA, 200 μM dNTP, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase and 12.5 pmoles of specific primers for murine Id3: Inhibitor of differentiation 3 (GenBank Accession No. NM008321) (forward and reverse) 5′-CTGCTACGAGG CGGTGTG-3′ and 5′-CACCTGGCTAAGCTGAGTGC3′ and GAPDH: Glyceraldehyde-phosphate dehydrogenase (**MUSGAPDH**), 5′-TGAA GGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTC CACCAC-3′, or human Id3 (NM002167) 5′-GCCGTGTCCTGAC ACCTC-3′, 5′-TAGAGTTCATAAATCAGGGCAACAG and β-actin (NM001101) 5′-AAGGCCAACCGCAGAAGATG-3′, 5′-CCGGCCAGCCA GGTCCAG-3′. PCR conditions comprised an initial denaturation at 94 °C for 5 min, followed by 17–30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 5 min in a 2720 Thermal Cycler (Applied Biosystems). Amplicons were electrophoresed on 2% agarose gel, stained with ethidium bromide and analyzed with ChemicDoc Eq software (Bio-Rad). The number of cycles was determined within the linear range of amplification of each cDNA.

Statistical analysis. Results are presented as means ± standard deviations. Statistical significance between two groups was determined by one way ANOVA. $P \leqslant 0.05$ was considered significant.

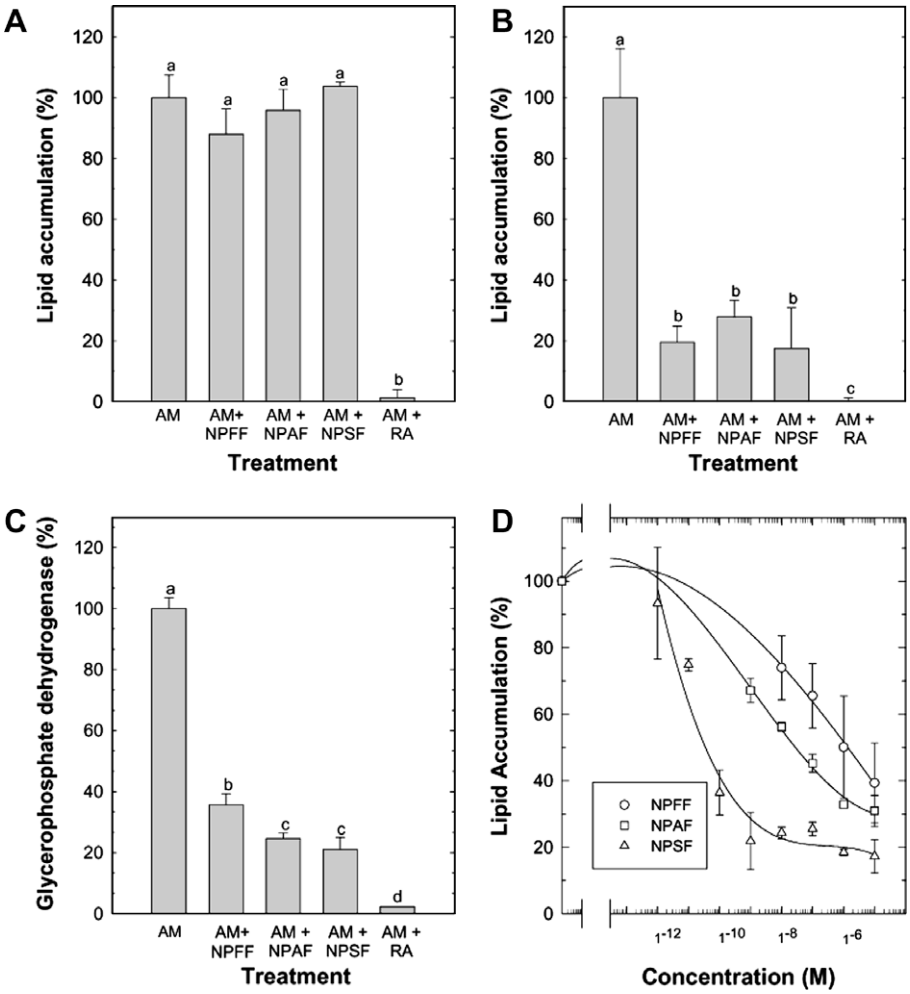

Fig. 1. NPAF, NPFF, and NPSF effect on 3T3 adipogenesis. 3T3-L1 (A) and 3T3-F442A preadipocytes (B–D) were induced to differentiation in presence of 1 μM NPAF, NPFF or NPSF(A–C) or the indicated neuropeptide concentrations (D). Control cultures received adipogenic medium (AM) added with retinoic acid (RA), a proved adipogenic inhibitor. Lipid accumulation was quantified after seven days of postconfluence in cell monolayers stained with Oil red O (A,B,D) or GPDH activity assayed in cytosolic extracts (C). Bars represent the average of three independent experiments each performed in triplicate ± standard deviation. Lowercase letters denote significant differences ($P \leqslant 0.05$).

Results

NPAF, NPFF, and NPSF differentially affect the adipogenesis of 3T3 preadipose cell lines

To assess the effect of RFamide neuropeptides on adipogenesis we induced the differentiation of 3T3-L1 and 3T3-F442A preadipocytes in the presence of 1 μM of NPAF, NPFF, or NPSF. None of these neuropeptides affected 3T3-L1 adipogenesis (Fig. 1A) but all of them markedly blocked the adipose differentiation of 3T3-F442A cells, as judged by decrements in lipid accumulation (Fig. 1B) and GPDH activity (Fig. 1C). All three neuropeptides exhibited a concentration-dependent inhibitory effect on 3T3-F442A lipid accumulation when evaluated at concentrations ranging 1 pM to 10 μM. NPSF showed the strongest antiadipogenic potency with an estimated IC_{50} of 1 nM. NPAF exerted antiadipogenesis with an IC_{50} of 0.04 μM and NPFF presented an IC_{50} of 1.2 μM (Fig. 1D). The antiadipogenic effects of the RFamide neuropeptides were not the result of cytotoxicity because these compounds did not inhibit the cell growth of 3T3 preadipocytes at any of the tested concentrations (not shown).

RFamide neuropeptides block the differentiation of human normal and lipoma-derived preadipocytes

We then assayed the effect of RFamide neuropeptides on *in vitro* human adipogenesis. Normal (HNPA) and lipoma-derived (HLPA) human subcutaneous preadipocytes were induced to differentia-

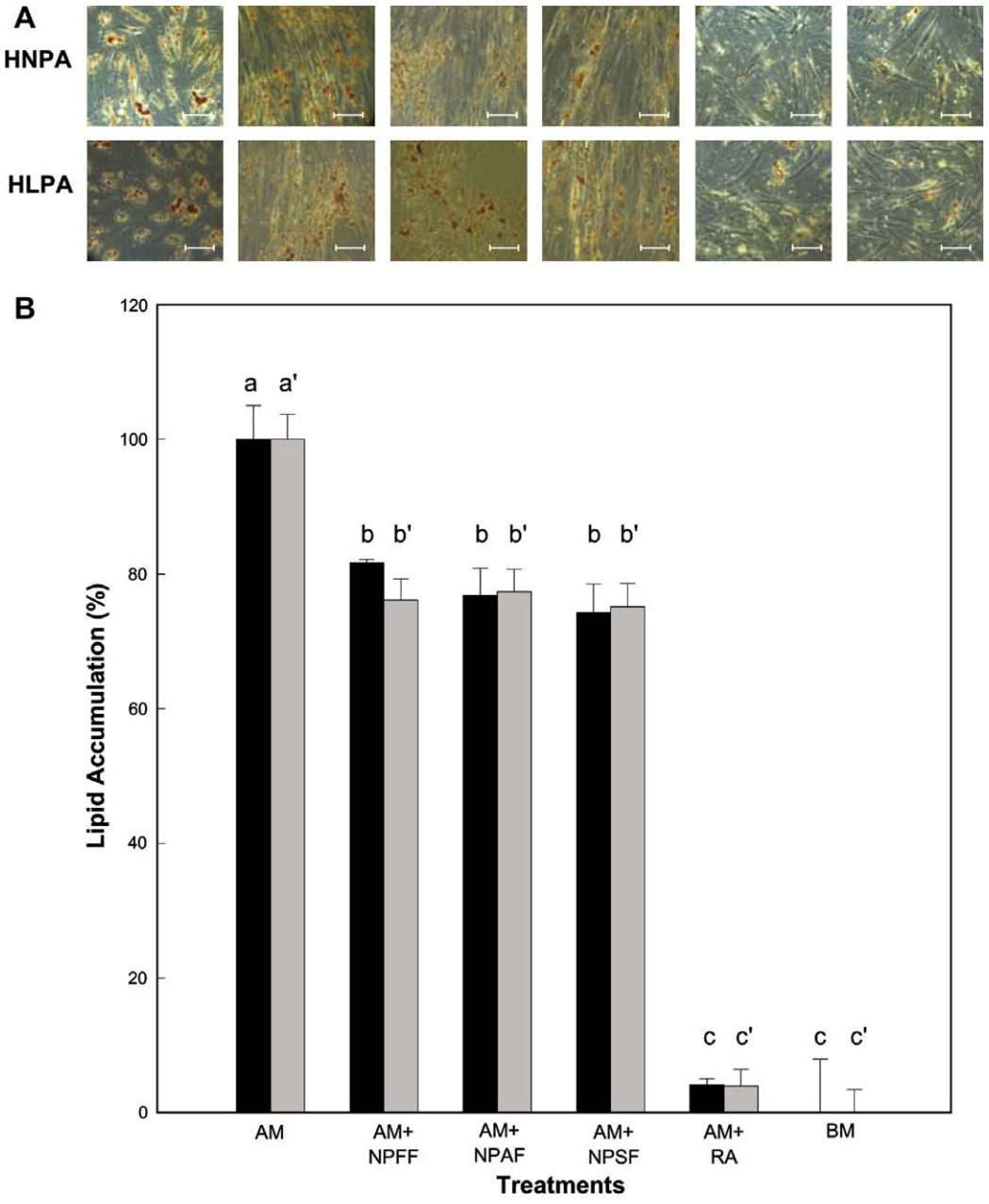

Fig. 2. Effect of NPAF, NPFF and NPSF on the adipose differentiation of human preadipocytes. Human normal (HNPA, ▬) and lipoma-derived preadipocytes (HLPA, ▭) preadipocytes were induced to differentiation with adipogenic medium (AM) in presence of 1 μM NPAF, NPFF, or NPSF. Control cultures received AM added with 1 μM retinoic acid (RA) or maintained in basal medium (BM) as non-adipogenic culture condition. Lipid accumulation was quantified after 21 d of incubation in cultures stained with Oil red O. Bars represent the average of three experiments ± standard deviation. Lowercase letters denote significant differences in the values of the bars between treatments ($P \leqslant 0.05$). Micrographics show Oil red O staining of triglyceride droplets in HNPA and HLPA cells cultivated for 21 d under indicated treatments. Scale bars represent 25 μm.

tion as described under Materials and methods, in the presence of inhibitory concentrations of neuropeptides. In these experiments, HNPA adipogenesis was diminished in 18% by NPFF, in 23% by NPAF and in 26% by NPSF. In the same manner, HLPA adipogenesis was blocked in 24% by NPFF and reduced in 23% and 25% by NPAF and NPSF, respectively, (Fig. 2).

NPAF irreversibly blocks the commitment to adipose differentiation in 3T3-F442A cells

Commitment to terminal adipogenesis is reversibly blocked by retinoids, TGF-β, or TNF-α [26–28] and blocked preadipocytes evolve to terminal differentiation as these inhibitors are removed and cells refed with adipogenic medium. To better characterize the antiadipogenic effect of RFamide neuropeptides, we blocked 3T3-F442A adipogenesis with NPAF 1 μM during 7 d of postconfluence. Then, some cultures were refed with AM or non-adipogenic medium (NAM) for additional 7 d. The lipid accumulation in cultures maintained with NPAF for 14 d (AM + NPAF) was strongly blocked (71%) respect to adipogenic medium control (AM) (Fig. 3), accordingly to our previous results (Fig. 1B–D). NPAF inhibition could not be reverted by refeding cultures with NAM (AM + NPAF → NAM), as expected from the absence of adipogenic signals in this medium [24], but not also when parallel cultures were refed with adipogenic medium (AM + NPAF → AM). Additional control cultures receiving retinoic acid (AM + AR) were totally inhibited in their lipid accumulation, but this inhibition could be largely reverted (49% with respect to the AM control) when some of these cultures were refed with AM (AM + AR → AM), but not when refed with NAM (AM + AR → NAM). Taken together, these results suggest that NPAF inhibits 3T3-F442A adipogenesis by irreversibly blocking the commitment to terminal differentiation and therefore it acts by a different way from most of the adipogenic inhibitors until now described.

Id3 is overexpressed during the inhibition of adipogenesis by NPAF and NPSF

To gain insight into the molecular mechanisms by which RFamide neuropeptides inhibit adipogenesis, we evaluated the effects of NPAF and NPSF on the expression of Id3 gene, a transcription factor that inhibits adipogenesis [29] and which expression is induced by NPAF in terminally differentiated adipocytes [23]. Id3 mRNA was clearly expressed by growing 3T3-F442A preadipocytes (preconfluence) and this expression was totally abolished as the cells get committed to terminal differentiation (48 h of postconfluence) and remained undetectable when the cells became terminally differentiated (Fig. 4A, AM), as reported [29]. On the contrary, NPAF maintained the expression of Id3 mRNA in 3T3-F442A cells during the commitment and terminal differentiation stages whereas NPSF induced a marked 2- or 3-fold overexpression of the transcription factor in both freshly committed and terminally differentiated 3T3F442A. NPFF and NPAF neuropeptides also increased Id3 mRNA expression in human adipocytes. HNPA and HLPA strains clearly expressed Id3 at preconfluence and this expression was reduced (HNPA) or maintained (HLPA) after adipose induction (21 d of postconfluence in AM) NPAF induced a modest but significant increased of Id3 expression (1.2-fold) in HNPA and a robust overexpression (1.8-fold) in HLPA. On the other hand, NPSF markedly induced the expression of Id3 in both strains, 1.7-fold in HNPA and 2-fold in HLPA.

Discussion

RFamide neuropeptides NPFF and NPAF, mainly known by their role in pain modulation and tolerance to opioids [12,13], alter the gene expression of terminally differentiated 3T3-L1 adipocytes [23], but their role on adipogenesis remains unexplored. We addressed this question inducing 3T3 adipogenesis in the presence of NPFF, NPAF, and NPSF. None of these neuropeptides affected the lipid accumulation in 3T3-L1 cells, in agreement with previous evidence showing that neither NPAF nor NPFF affect the activity of GPDH in terminally differentiated 3T3-L1 adipocytes [23]. On the contrary, all three neuropeptides markedly blocked the lipid accumulation and GPDH activity in the related preadipose cell line 3T3-F442A. Neuropeptides displayed a diverse inhibitory capacity, in all the cases clearly concentration-dependent: NPSF showed the strongest antiadipogenic potency with an estimated IC_{50} of 1 nM. NPAF exerted its inhibitory effect with an IC_{50} of 0.04 μM and NPFF presented an IC_{50} of 1.2 μM. RFamide neuropeptides also blocked the adipogenesis of normal and lipoma-derived human subcutaneous preadipocytes, although in a lesser extent than murine adipogenesis and with similar potency among the three neuropeptides. NPFF, NPAF and NPSF, assayed at 1 μM each, diminished lipid accumulation of HNPA and HLPA in 20% respect to adipogenic control. This last result emphasizes the physiological significance of the antiadipogenic effect of RFamide neuropeptides.

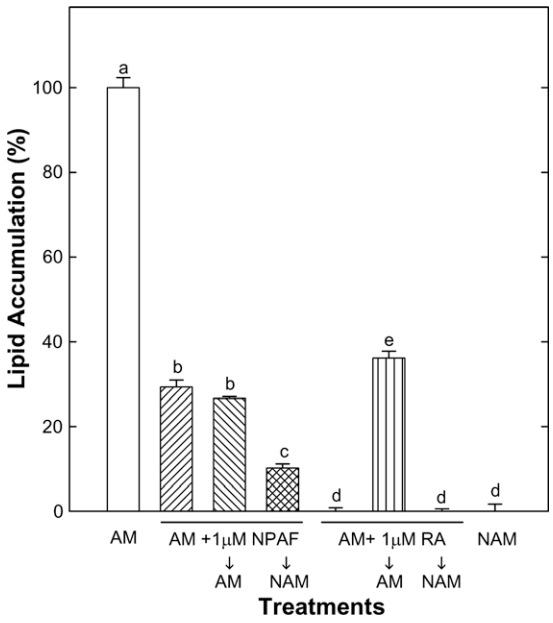

Fig. 3. Irreversible antiadipogenic effect of NPAF on the 3T3-F442A adipogenesis. 3T3-F442A preadipocytes were induced with adipogenic medium (AM, ▭) or AM added with 1 μM NPAF (▨, ▨, ▨). Control cultures received AM added with retinoic acid (RA, ▭, ▥, ▦), a reversible inhibitor of the adipogenesis, or were maintained in non-adipogenic medium (NAM, ■). Seven days later, some cultures treated with NPAF or RA were refed with AM (▨, ▥) or NAM (▨, ▦) for seven additional days to evaluate the reversibility of inhibitory effects. Lipid accumulation was quantified in monolayers stained with Oil red O. Each bar represents the average of three experiments ± standard deviation. Lowercase letters denote significant differences between treatments ($P \leqslant 0.05$).

Unlike other adipogenic inhibitors [26–28], RFamide neuropeptides did not completely inhibit 3T3 adipogenesis. This fact, and the lower antiadipogenic capacity of neuropeptides on human preadipocytes, raises the possibility that the assayed neuropeptides are not the active form of the neurotransmitter for adipose tissue. Neuropeptides NPA-NPFF and EFW-NPSF, putatively produced from the same gene than NPFF and NPSF, display higher affinities by their binding sites in spinal cord neurons than initially described peptides [30]. Additionally, new RFamide neuropeptides with orexigenic and peripheral effects had been recently described [31,32]. The effect of these newly described RFamide neuropep-

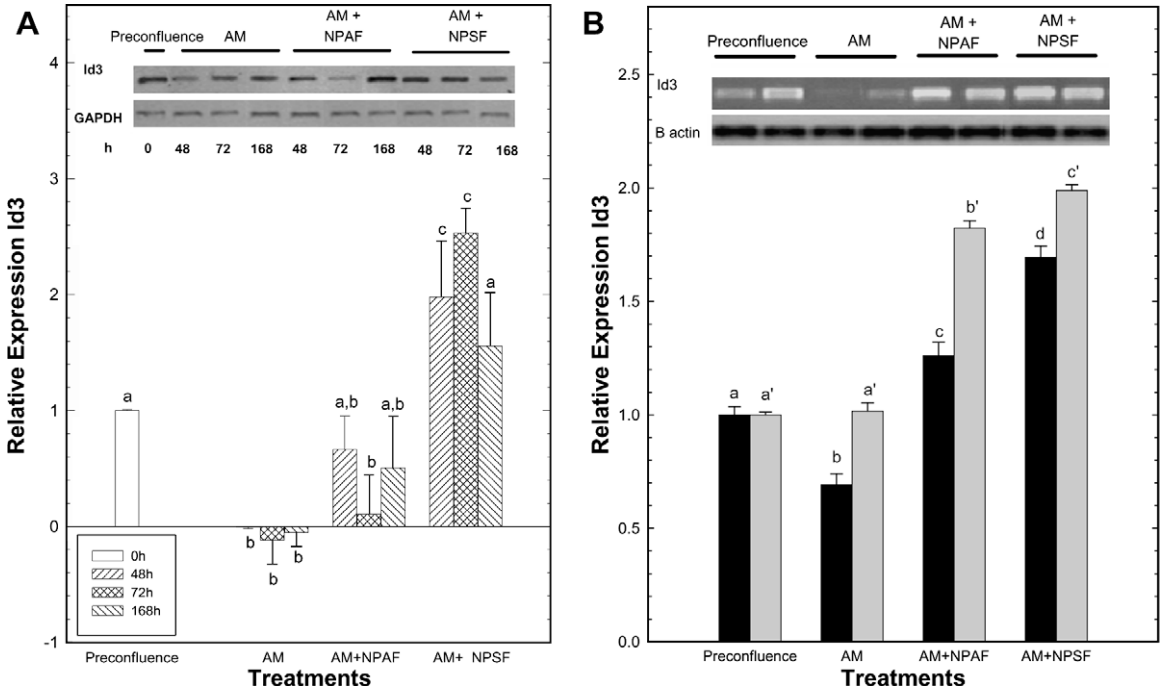

Fig. 4. Effect of NPAF and NPSF on Id3 gene expression in murine and human preadipocytes. 3T3-F442A (A) and human normal (HNPA, ▬) or lipoma-derived preadipocytes (HLPA, ▭) (B) were differentiated with adipogenic medium (AM) in the presence of 1 μM NPAF or 1 nM NPSF for 48 (▨), 72 (▩), 168 h (▧) (murine cells) or along 21 d (human cells). Total RNA from each cell type was analyzed by RT-PCR using oligonucleotides specific for murine or human Id3 gene. Amplification products were quantified by densitometry in relative units respect to GAPDH (3T3 cells) and β-actin (human cells). Bars represent the average of three experiments ± standard deviation. Lowercase letters denote significant differences between treatments ($P \leqslant 0.05$).

tides on murine and human adipogenesis needs to be addressed in future studies.

The neural actions of RFamide neuropeptides are mediated by two G protein-coupled receptors, NPFF-R1 and NPFF-R2 [20–22], to which neuropeptides bind with distinct affinity. All three neuropeptides exhibit high affinity for NPFF-R1 (EC_{50} of 15.6 nM for NPFF, 24.9 nM for NPAF and 12 nM for NPSF) whereas NPFF and NPAF display high affinity for NPFF-R2 (2.0 and 4.7 nM, respectively) and NPSF shows poor affinity for this receptor (EC_{50} = 560 nM) [22]. The different antiadipogenic capacity of RFamide neuropeptides, however, cannot be explained solely on the base of their affinities for these receptors. 3T3-L1 cells, expectedly expressing both receptors [23] are not affected by neuropeptides. Although NPFF-R1 or NPFF-R2 expression has not been documented in 3T3-F442A cells, the antiadipogenic capacity of NPFF, NPAF, and NPSF on this cell line does not correlate with the similar affinities of these neuropeptides for NPFF-R1 and the low affinity of NPSF for NPFF-R2 [22]. Additionally, primary human preadipocytes, putatively expressing mainly NPFF-R2 [20] are similarly blocked by neuropeptides showing high or low affinity for this receptor. Taken together, our results suggest that NPFF-R1 or NPFF-R2 receptors do not participate in the antiadipogenic actions of RFamide neuropeptides and that an alternative signaling pathway could mediate the effect of these neuropeptides on the metabolism and development of adipose tissue. Such an alternative pathway could be mediated by additional neuropeptide receptor isoforms resulting from alternative splicing of NPFF-R1 and NPFF-R2 receptor genes [33] or by a distinct type of receptors [34].

Our results also show that NPAF irreversibly blocked the commitment to terminal adipogenesis since NPAF-blocked 3T3-F442A preadipocytes did not reinitiate the adipose differentiation when refed with fresh adipogenic medium lacking neuropeptide. This result suggests that NPAF antiadipogenesis is qualitatively different from those reported for antiadipogenic compounds such as retinoic acid, TGF-β or TNF-α. All these compounds inhibit adipogenesis while they are present in the culture medium and their effects cease as they are retired and cultures refed with fresh adipogenic medium. Our finding of the irreversible antiadipogenic effect of NPAF makes these neuropeptides a valuable tool to study the molecular events mediating the commitment to the terminal adipose differentiation. Further studies are needed to evaluate the reversibility of the antiadipogenesis by additional RFamide neuropeptides.

The antiadipogenesis of NPAF and NPSF was accompanied by the overexpression of Id3, an helix-loop-helix transcription factor whose expression in 3T3 preadipocytes declines as they become committed to terminal differentiation and whose constitutive expression prevents 3T3 adipogenesis [29]. Thus, Id3 overexpression induced by RFamide neuropeptides sufficiently explains the antiadipogenic effect of these compounds, without cancelling the possibility that they can also affect to the expression of additional transcription factors regulating adipogenesis.

In conclusion, we showed that NPFF, NPAF, and NPSF neuropeptides inhibit murine and human adipogenesis and that NPAF irreversibly blocks the commitment to terminal adipose differentiation. Our results suggest that RFamide neuropeptides could be important mediators in the neural regulation of adipose tissue development. Further studies will allow the possible participation of RFamide neuropeptides on pathologies associated to adipose tissue.

Acknowledgments

This work was supported by CONACYT (Grant 2004-C01-45804). MLHH was endowed with graduate fellowships from CONACYT (172319). We thank to Dr. M.A. Kalixto-Sánchez for providing human adipose samples and C. Rangel del Camino and R. Mejia-Elizondo for technical assistance.

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