

# Secretion of neuropeptide Y in human adipose tissue and its role in maintenance of adipose tissue mass

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**Kos K, Harte AL, James S, Snead DR, O'Hare JP, McTernan PG, Kumar S.** Secretion of neuropeptide Y in human adipose tissue and its role in maintenance of adipose tissue mass. *Am J Physiol Endocrinol Metab* 293: E1335–E1340, 2007. First published September 4, 2007; doi:10.1152/ajpendo.00333.2007.—NPY is an important central orexigenic hormone, but little is known about its peripheral actions in human adipose tissue (AT) or its potential paracrine effects. Our objective was to examine NPY's role in AT, specifically addressing NPY protein expression, the effect of NPY on adipokine secretion, and the influence of insulin and rosiglitazone (RSG) on adipocyte-derived NPY in vitro. Ex vivo human AT was obtained from women undergoing elective surgery [age:  $42.7 \pm 1.5$  yr (mean  $\pm$  SE), BMI:  $26.2 \pm 0.7$  kg/m<sup>2</sup>;  $n = 38$ ]. Western blot analysis was used to determine NPY protein expression in AT depots. Abdominal subcutaneous (AbSc) adipocytes were isolated and treated with recombinant (rh) NPY, insulin, and RSG. NPY and adipokine levels were measured by ELISA. Our results were that NPY was localized in human AT and adipocytes and confirmed by immunohistochemistry. Depot-specific NPY expression was noted as highest in AbSc AT ( $1.87 \pm 0.23$  ODU) compared with omental (Om;  $1.03 \pm 0.15$  ODU,  $P = 0.029$ ) or thigh AT (Th;  $1.0 \pm 0.29$  ODU,  $P = 0.035$ ). Insulin increased NPY secretion (control:  $0.22 \pm 0.024$  ng/ml; 1 nM insulin:  $0.26 \pm 0.05$  ng/ml; 100 nM insulin:  $0.29 \pm 0.04$  ng/ml; 1,000 nM insulin:  $0.3 \pm 0.04$  ng/ml;  $P < 0.05$ ,  $n = 13$ ), but cotreatment of RSG (10 nM) with insulin (100 nM) had no effect on NPY secretion. Furthermore, adipocyte treatment with rh-NPY downregulated leptin secretion (control:  $6.99 \pm 0.89$  ng/ml; 1 nmol/l rh-NPY:  $4.4 \pm 0.64$  ng/ml; 10 nmol/l rh-NPY:  $4.3 \pm 0.61$  ng/ml, 100 nmol/l rh-NPY:  $4.2 \pm 0.67$  ng/ml;  $P < 0.05$ ,  $n = 10$ ) but had no effect on adiponectin or TNF- $\alpha$  secretion. We conclude that NPY is expressed and secreted by human adipocytes. NPY secretion is stimulated by insulin, but this increment was limited by cotreatment with RSG. NPY's antilipolytic action may promote an increase in adipocyte size in hyperinsulinemic conditions. Adipose-derived NPY mediates reduction of leptin secretion and may have implications for central feedback of adiposity signals.

adipokines; insulin; rosiglitazone; leptin

NEUROPEPTIDE Y (NPY), A 36-AMINO ACID PEPTIDE, established as an important appetite regulator with orexigenic properties, represents one of the most potent appetite stimulants in the brain. NPY is also known (4) to have peripheral functions, such as the regulation of angiogenesis, vasoconstriction, mood regulation, and fertility, with NPY receptors noted in the peripheral tissues. Previous studies have examined the function of NPY in the periphery, particularly examining adipose tissue (AT).

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Such studies have established that intracerebroventricular injection of NPY appears to mediate upregulation of lipoprotein lipase (LPL) and its activity. LPL is a key enzyme in lipogenesis, thus highlighting the antilipolytic potential of NPY (2); additionally, in vitro studies examining cultured adipocytes (22) note similar findings. NPY activity in cellular metabolism appears to be mediated through binding to transmembrane domain G protein-coupled receptors, of which several subtypes exist in humans (NPY Y<sub>1</sub>, Y<sub>2</sub>, Y<sub>4</sub>, and Y<sub>5</sub>) (29). Although to date the role of most of these receptors in human AT is poorly understood, binding studies (3) have suggested that Y<sub>1</sub> receptors may mediate the antilipolytic effect of NPY in AT.

It is clear that the physiological role of NPY in the regulation of energy stores cannot be fully understood without examining the complex interactions of key regulators of energy homeostasis: insulin and leptin. Although we understand that the central hypothalamic levels of NPY reflect the nutritional state (28), further analysis with leptin and insulin through intracerebroventricular infusion noted a reduction in NPY mRNA (6). However, little is known about a potential peripheral interaction of insulin, leptin, and NPY in human AT.

Thus, the aim of this study was to examine the protein expression and depot distribution of NPY in human AT and, furthermore, to analyze the influence of insulin on NPY secretion and the effect of NPY on leptin secretion and other adipokines. Finally, the study aimed to establish whether adipocyte-derived NPY secretion is modulated by the peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) agonist rosiglitazone (RSG), which is known to be associated with additional weight gain.

## METHODS

**Subjects.** AT samples were obtained from 38 consenting Caucasian female subjects undergoing elective surgery: mean age  $42.7 \pm 7.3$  yr; body mass index (BMI)  $26.2 \pm 4.5$  kg/m<sup>2</sup> (means  $\pm$  SD). Abdominal subcutaneous (AbSc), omental (Om), and thigh (Th) tissue samples were collected with the approval of the South Birmingham Ethics Committee. Subjects on endocrine therapy (e.g., steroids, hormone replacement therapy, thyroxine) and patients with malignant diseases were excluded.

**Tissue culture and adipocyte isolation.** AT was washed with 1 $\times$  Hanks' balanced salt solution containing penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). The tissue was finely cut, and blood vessels and connective tissue were removed. The tissue was then digested with the same batch of collagenase class 1 (2 mg/ml; Worthington Biochemical, Lakewood, NJ) in 1 $\times$  Hanks' balanced

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salt solution (Gibco-BRL, Life Technologies, Paisley, UK) for 1 h at 37°C in a water bath and shaken at 100 cycles/min at 37°C. After that, the tissue was filtered through a double-layered cotton mesh and adipocytes separated by centrifugation at 360 g for 5 min. The

isolation of adipocytes was performed as previously described by Harte et al. (11), with removal of the upper layer of mature adipocytes from the collagenase-dispersed preparation, which was then washed twice in phenol red-free Dulbecco's modified Eagle's medium (DMEM)-F-12 (Gibco-BRL, Life Technologies). After centrifugation at 360 g for 2 min, adipocytes were cultured in flasks (25 cm<sup>2</sup>) in phenol red-free DMEM-F-12 containing glucose (15 mmol/l), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma, Dorset, UK). Aliquots of 1 ml containing ≈500,000 mature adipocytes were maintained in medium (5 ml/25 cm<sup>2</sup> flask) for 48 h while being treated (see below). After incubation of adipocytes (37°C/5% CO<sub>2</sub>), the conditioned media and adipocytes were separated by centrifugation (360 g for 2 min), and the media were removed, separated into aliquots, and stored at -70°C.

**Protein extraction and assay.** Some of the AT was flash-frozen and stored at -70°C. Tissue was homogenized and extracted with RIPA buffer containing 1× PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 complete miniprotease cocktail (Roche Molecular Biochemicals), and 100 µg/ml *N*-acetyl-Leu-Leu-norleucinal (Calbiochem). Extracted protein was then subsequently quantified via the Bio-Rad Detergent Compatible protein assay kit (Bio-Rad, Hercules, CA). Adipocyte protein samples were also quantified to determine that the observed difference between control and treatment regimen values was not due to adipocyte protein variation between samples.

**NPY Western blot analysis.** Homogenized human AT and isolated AbSc adipocytes were resuspended in RIPA buffer. Twenty micrograms/lane AT protein or 60 µg/lane adipocyte protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with the use of 15% gel (NPY). All samples were heated for 5 min at 95°C in a sample buffer. Prestained molecular weight markers (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used as standards. Samples were electrophoresed at 140 V for 1.5 h. Proteins were transferred from the polyacrylamide gels to PVDF membranes by electroblotting in a vertical transfer apparatus at 100 V for 1 h. Membranes were blocked overnight at 4°C in phosphate-buffered saline containing Tween 20 [PBS-T (PBS + 0.05% Tween 20); Sigma] containing 10% (wt/vol) nonfat milk powder (Marvel, Moreton, Merseyside, UK). Membranes were treated with primary polyclonal goat NPY antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 1:1,000 and the membranes developed with a conjugated anti-goat/sheep horseradish peroxidase secondary antibody in concentrations of 1:80,000 diluted in PBS with 0.5% Tween. The prepro-NPY protein (11 kDa) and mature NPY (4 kDa) were detected by chemiluminescent assay ECL-plus (Amersham, Little Chalfont, UK), which enabled visualization after exposure to X-ray film, and the band intensity was determined by densitometry. Equal loading was ensured with use of the α-tubulin antibody (Abcam, Cambridge, UK). A blocking peptide for NPY (Santa Cruz Biotechnology) was used to demonstrate antibody specificity as recommended by the manufacturer. Essentially, the membrane was incubated with the blocking peptide (1:25) and primary antibody in PBS at 4°C overnight and, after washes on PBS-T, treated with secondary antibody as described above.

**Treatment of AbSc adipocytes with insulin and RSG to assess NPY secretion.** AbSc adipocytes were treated with recombinant human (rh) insulin expressed in *Escherichia coli* (1:100 and 1,000 nM; Sigma,

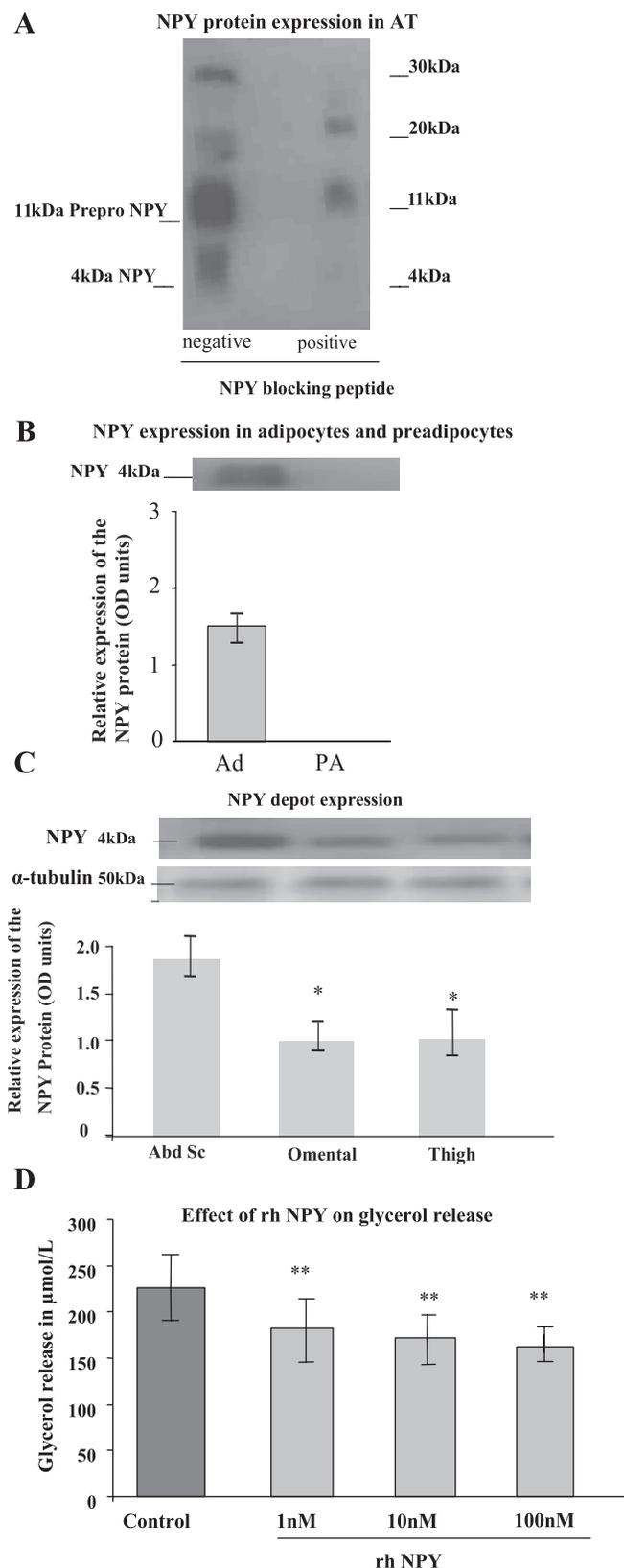


Fig. 1. A: expression of neuropeptide Y (NPY) in abdominal subcutaneous (AbSc) adipose tissue (AT) and addition of an NPY blocking peptide. When NPY blocking peptide is added (positive), the signal for NPY is lost, which demonstrates antibody specificity. B: expression of NPY in human AbSc adipocytes (Ad), but not AbSc preadipocytes (PA), derived from the same patient;  $n = 3$ . C: relative expression of NPY compared with thigh (Th) fat, which was allocated the value of 1;  $n = 8$  for paired AbSc AT and omental (Om) AT samples and  $n = 7$  for paired AbSc AT and Th subcutaneous AT. A representative Western blot is shown at top. \* $P < 0.05$ . D: effect of recombinant human (rh) NPY on glycerol release;  $n = 14$ . \*\* $P < 0.01$ .

Poole, UK), insulin in combination with RSG (10 nmol/l; Glaxo-SmithKline, Harlow, UK), and insulin (100 nmol/l) or RSG alone (10 nmol/l) for 48h. Untreated adipocytes were used as controls and maintained in culture for 48 h. Conditioned medium was assayed using the commercially available NPY ELISA kit (sensitivity 0.04–0.06 ng/ml, intra-assay variation <5%, interassay variation <14%; Bachem Peninsula Laboratories). Medium samples were concentrated 10-fold with use of a centrifuge evaporator.

**Adipocyte treatment with rh-NPY and assessment of glycerol and adipokine release.** AbSc adipocytes were treated for 48 h with 1, 10, and 100 nmol/l of rh-NPY (Sigma-Aldrich, Gillingham, UK). Glycerol, a byproduct of lipolysis, was assessed in the infranatant containing medium. The glycerol content was measured using a commercially available colorimetric kit (Randox Laboratories, Crumlin, UK). The sensitivity of the assay was estimated to be between 0 and 100 mmol/l according to the manufacturer's protocol. The following adipokines were measured with commercial ELISA-based colorimetric kits; leptin ELISA (R & D Systems, Abingdon, UK) has the assay limit of 7.8 pg/ml, intra-assay of 3.2% coefficient of variation, and interassay variability of 4.4%. Adiponectin and TNF- $\alpha$  were measured with the multiplex system (Millipore, Watford, UK) according to the manufacturer's instructions.

**Immunohistochemistry.** The histological specimens were incubated with primary polyclonal NPY antibody (Santa Cruz Biotechnology) in a dilution of 1:400 and developed using Vector VIP peroxidase substrate kit (Vector Laboratories, Peterborough, UK). Rat brain tissue was used as a positive control for NPY staining and obtained from Medical Solutions (Nottingham, UK). Sections were developed using diaminobenzidine (BioGenex). Omission of primary antibody was undertaken to exclude potential bias due to contamination and/or artefacts and utilized as a negative control.

**Statistical analysis.** Statistics were performed using the SPSS version 14 (SPSS UK, Surrey, UK). For assessment of protein expression and secretion, statistical analysis was undertaken with the paired *t*-test when assessing depot expression and ANOVA when assessing cell culture treatments for comparison of control vs. treatments. *P* values <0.05 were considered statistically significant. Values are expressed as means  $\pm$  SE.

## RESULTS

**Characterization of NPY in AT.** Protein studies determined that NPY was detectable in isolated AbSc adipocytes [ $1.51 \pm 0.26$  optical density units (ODU),  $n = 3$ ] but not AbSc preadipocytes, whereas the adipocytes and preadipocytes were derived from the same patient for each assessment ( $n = 3$ ; Fig. 1, A and B). The presence of NPY in AT was further confirmed by immunohistochemistry. Immunohistochemical analysis demonstrated that the NPY antibody (brown) appears to be located in the cytoplasm of adipocytes, consistent with protein expression of NPY within adipocytes. As a positive control for the antibody we utilized rat brain, where the neurotransmitter NPY is abundant (Fig. 2).

NPY protein was expressed in AbSc AT ( $1.87 \pm 0.23$  ODU,  $n = 8$ ) which was approximately twofold higher than either paired Om ( $1.03 \pm 0.15$  ODU,  $P = 0.029$ ,  $n = 8$ ) or Th AT ( $1.0 \pm 0.29$  ODU,  $P = 0.035$ ,  $n = 7$ ) (Fig. 1C). AbSc adipocytes treated with rh-NPY were utilized to assess the antilipolytic action of NPY through glycerol release, an index of lipolysis, which was significantly reduced in a dose-dependent manner with addition of rh-NPY (control:  $224 \pm 37$   $\mu$ mol/l; 1 nmol/l NPY:  $182 \pm 32$   $\mu$ mol/l; 10 nmol/l NPY:  $171.1 \pm 26$   $\mu$ mol/l; 100 nmol/l NPY:  $161 \pm 27$   $\mu$ mol/l;  $P < 0.01$ ,  $n = 14$ ; Fig. 1D).

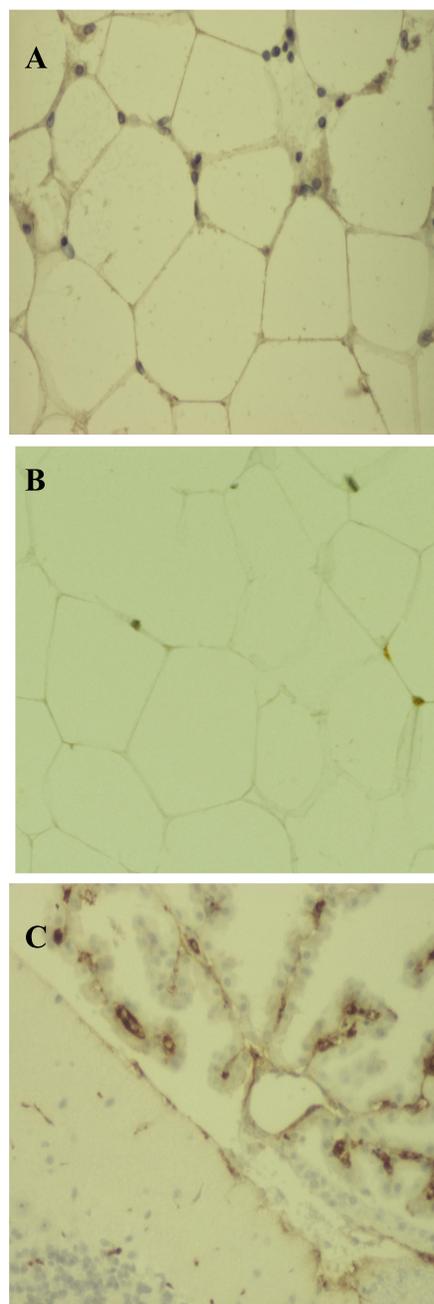


Fig. 2. Immunohistological staining. A: negative staining for human AT (blue). B: staining for NPY in human AT (brown). C: positive control, mouse brain tissue with cerebellum showing brown staining of NPY in cytoplasm (brown) ( $\times 2,000$  magnification).

**Effect of insulin and RSG on NPY secretion from isolated AbSc adipocytes.** Freshly harvested AbSc adipocytes were treated with rh-insulin (1, 100, 1000 nM) to investigate the regulation of NPY by insulin. This experiment showed that NPY was significantly upregulated with insulin treatment across the concentration range (control:  $0.22 \pm 0.02$  ng/ml; 1 nM insulin:  $0.26 \pm 0.05$  ng/ml; 100 nM insulin:  $0.29 \pm 0.04$  ng/ml; 1,000 nM insulin:  $0.3 \pm 0.04$  ng/ml;  $*P < 0.05$ ,  $n = 13$ ; Fig. 3A). This insulin-mediated increment of NPY secretion in AbSc adipocytes when studied in a subpopulation using adipocytes of the same subjects for each treatment ( $n = 7$ ) was

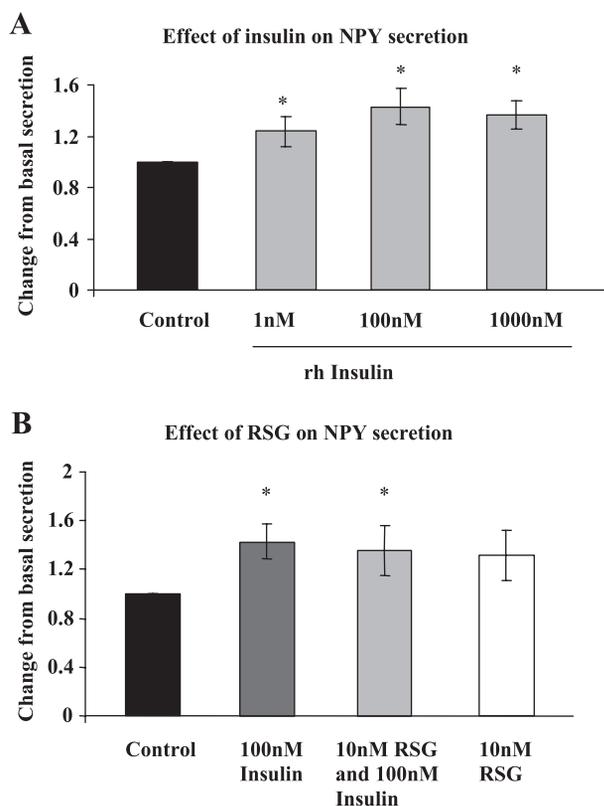


Fig. 3. A: effect of rh-insulin on NPY secretion. nM = nmol/l;  $n = 13$ ,  $*P < 0.05$ . B: the effect of rosiglitazone (RSG) and insulin treatment on the level of NPY secretion from cultured adipocytes;  $n = 7$ ,  $*P < 0.05$ .

reduced with addition of RSG (10 nmol/l) and remained similar to the levels when treated with RSG alone without addition of insulin [insulin alone (100 nmol/l):  $0.39 \pm 0.05$  ng/ml vs. insulin in combination with RSG:  $0.34 \pm 0.04$  ng/ml,  $P < 0.05$ ,  $n = 7$ , and RSG alone:  $0.33 \pm 0.05$  ng/ml compared with basal secretion:  $0.25 \pm 0.02$  ng/ml,  $P =$  not significant,  $n = 7$ ; Fig. 3B].

**The effect of rh-NPY on adipokine secretion.** We did not detect any apparent effect of increasing rh-NPY concentration on adiponectin (Fig. 4B) or TNF- $\alpha$  secretion (Fig. 4C) but noted that increasing NPY concentration reduced the secretion in leptin (control:  $6.99 \pm 0.89$  ng/ml; 1 nM NPY:  $4.4 \pm 0.64$  ng/ml; 10 nM NPY:  $4.3 \pm 0.61$  ng/ml; 100 nM NPY:  $4.2 \pm 0.67$  ng/ml;  $P < 0.05$ ,  $n = 10$ ; Fig. 4A).

## DISCUSSION

This is the first study to demonstrate NPY protein expression in human AT and adipocytes. NPY is expressed not only within various adipose tissue depots but also as a secreted protein in adipocyte-conditioned medium. We further confirmed the presence of NPY in human AT by immunohistochemical staining. The production of neurotransmitters in human AT has previously been suggested by cDNA array studies (30); as such, neurotransmitters like NPY appear to influence peripheral organs not only by innervation but also through local production with potential paracrine effects. Hence, as NPY is produced by fat itself, it may influence fat growth in an autocrine and paracrine manner both directly and indirectly.

We further examined the antilipolytic properties of rh-NPY, which affirmed its status in our study on human adipocytes (3, 4). The AT depot-specific differences in lipolytic ability as well as an alteration of receptor sensitivity-mediated lipolysis in subjects with upper body obesity, e.g., the metabolic syndrome, have previously been identified (13, 18). The identified AT depot differences in NPY protein expression in our study may lead to enhanced depot differences in lipolysis as well as influence adipokines, leading to different rates of their secretion between depots. Subcutaneous AT, which was the depot with the highest NPY expression, makes a substantial contribution to the total body weight of obese patients and contrib-

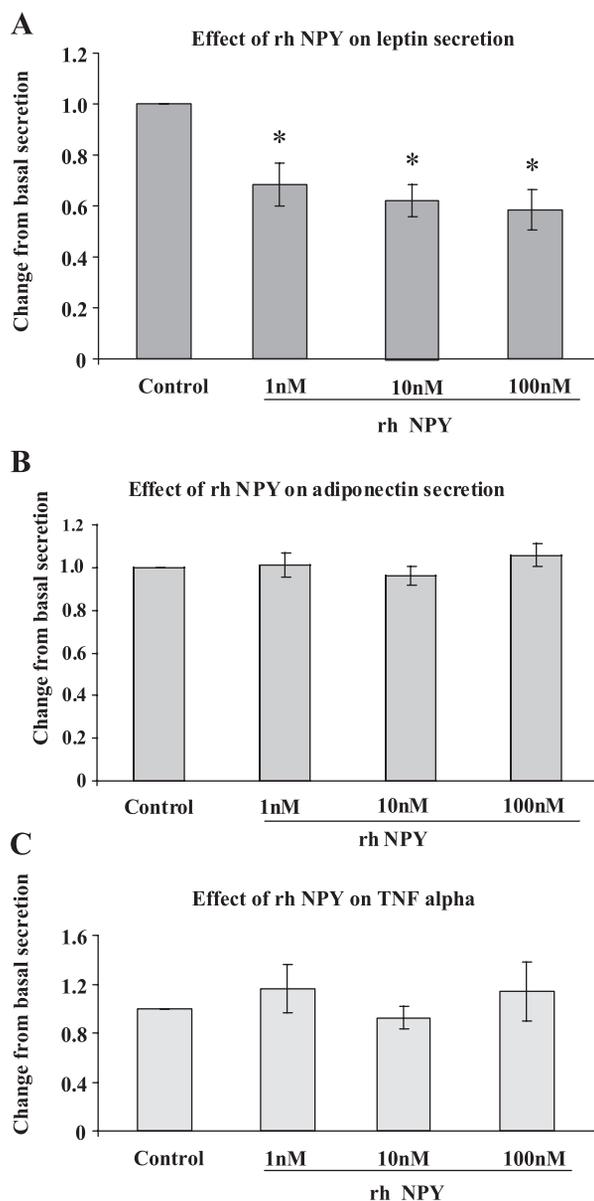


Fig. 4. A: AbSc adipocytes treated with rh-NPY and the relative change of leptin secretion compared with basal secretion in the control medium with adjusted value of 1;  $n = 10$ ,  $*P < 0.05$ . B: AbSc adipocytes treated with rh-NPY and the relative change of secretion of adiponectin compared with basal secretion of control medium with adjusted value of 1;  $n = 9$ ,  $P =$  not significant (NS). C: AbSc adipocytes treated with rh-NPY and the relative change of TNF- $\alpha$  secretion compared with basal secretion in the control medium with adjusted value of 1;  $n = 9$ ,  $P =$  NS.

utes to more than 50% of total body fat (12). Therefore, subcutaneous AT has considerable potential to contribute to the total AT-mediated endocrine action.

In addition to the antilipolytic nature of NPY in AT, we also observed that insulin could mediate NPY release. This may have considerable implications in the hyperinsulinemic state associated with the metabolic syndrome and type 2 diabetes mellitus (T2DM). NPY inhibits lipolysis in human adipocytes, which could translate into further AT growth and may, in part, explain the weight gain observed in patients with T2DM, which is exacerbated with initiation of insulin therapy. However, this hypothesis would require further confirmation by *in vivo* experiments. The peripheral antilipolytic effect of insulin and the peripheral effect of insulin on NPY secretion described in this study appear to contrast with central insulin regulation, whereby insulin inhibits NPY gene expression (9) and promotes weight loss. However, although these observations may appear contradictory, an opposing action of insulin in the brain compared with its peripheral action is well described (23), thus demonstrating the complexity of peripheral and central action in the appetite and energy homeostasis pathways. This apparent contradiction may be partly explained with differential central and peripheral effects, with insulin promoting surplus energy storage in the periphery, thereby mediating fat accumulation. However, centrally, energy intake may be limited through the reduction of appetite induced by hypothalamic action of insulin on NPY. Thus NPY may have a biological role in promoting weight gain through both central and peripheral mechanisms.

In addition, we further investigated the effects of rh-NPY on the secretion of other adipokines, such as adiponectin, that may influence both peripheral and central action (16). However, in this instance, NPY did not elicit any significant effect on adiponectin or on TNF- $\alpha$  release. Therefore, in this context it is uncertain whether NPY affects fat tissue-mediated inflammatory pathways, and further assessment of downstream mechanisms is required. The analysis on the impact of rh-NPY on adipokines noted that leptin secretion was reduced by rh-NPY treatment. Such a reduction in leptin may be necessary to enable NPY to have a significant effect on fat accumulation via its antilipolytic function (27). Additionally, NPY and leptin appear to have opposing actions on AT, with NPY being adipogenic and leptin antiadipogenic (7). Furthermore, leptin has also been shown to upregulate preadipocyte proliferation; therefore, a reduction in its expression by NPY may promote increased mature fat cell size, paralleled with its antilipolytic action on fat cells (25). However, a downregulation of leptin secretion by NPY may also have implications for central sensing of adiposity signals. Central action of leptin is known to promote peripheral sympathetic activity (21) and enable feedback of leptin on AT mass regulation. Also, coculture of adipocytes with sympathetic neurons has demonstrated decreased leptin release, in support of a central feedback mechanism (23). Previous studies, however, have shown an upregulation of leptin by NPY in cultured adipocytes (5, 21), but in these studies experimental conditions differed greatly with the use of either sheep adipocytes, administration of NPY by intravenous route, or use of preadipocyte cell lines (21), which may account for such variations.

Finally, we assessed the effect of the thiazolidinediones (TZDs), such as RSG, which are known insulin sensitizers with

$\beta$ -cell-preserving action (19), on NPY secretion. TZDs have previously been shown (20) to increase circulating adiponectin and as such enhance cardioprotective properties as well as improve insulin sensitivity. However, the clinical use of TZDs is also associated with weight gain, and PPAR $\gamma$  activation is known (8) to stimulate fatty acid storage through enhanced adipocyte differentiation and increased LPL activity. In our current study, RSG appeared to limit the insulin-stimulated NPY secretion in adipocytes. Such a finding regarding RSG has also been observed for other adipokines such as plasminogen activator inhibitor-1, resistin, and angiotensin II in a similar adipocyte culture system (10, 11, 17, 26). Such an effect of the insulin sensitizer to limit insulin action may appear surprising; however, the insulin-sensitizing action of PPAR $\gamma$  and its agonists in AT is still unclear, and it has been suggested that such action may be limited to skeletal muscle and liver (15), with RSG only indirectly improving insulin sensitivity in AT through a favorable change in adipokine profile (26). Furthermore, it should also be noted that the effect of TZDs to reduce insulin-stimulated NPY may be quite different in AT from obese and diabetic patients than AT collected from lean subjects, as in this study.

The present study has not proven that fat-derived NPY can enter the blood stream; however, this may potentially explain the observed elevated circulating NPY levels in chronically obese subjects and T2DM patients (1). Thus, the role of adipose-derived NPY and its contribution to circulating and central regulatory action remains to be established through further *in vivo* studies. AT-derived NPY could significantly raise circulating NPY in obesity due to the sheer volume of adipocytes and circulating NPY that is known to cross the blood-brain barrier (14). If a significant contribution to circulating NPY is confirmed, AT-derived NPY may contribute to central appetite regulation, although it should be acknowledged that the levels of NPY in the cerebrospinal fluid are at much higher levels.

In summary, this study has demonstrated that NPY is expressed in human AT and secreted by adipocytes. Furthermore, NPY is upregulated by insulin *in vitro*, although release of the adipogenic NPY in response to insulin may, in part, explain the weight gain associated with hyperinsulinemia. However, the insulin-induced NPY secretion is limited by RSG, which by itself is adipogenic. NPY does not appear to affect adipokines such as adiponectin or TNF- $\alpha$ , suggesting that NPY does not stimulate inflammatory pathways. However, NPY does appear to downregulate leptin secretion, which may be part of a local feedback mechanism allowing adipocyte accumulation that would otherwise be opposed by hyperleptinemia.

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