British Journal of Pharmacology (2007) 152, 1102–1110
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Phosphodiesterase-5A and neutral endopeptidase activities in human adipocytes do not control atrial natriuretic peptide-mediated lipolysis

C Moro[1,2], E Klimcakova[1,2], M Lafontan[2,3], M Berlan[1,2,4] and J Galitzky[2,3]

[1]*INSERM, U858, Obesity Research Laboratory, Toulouse, France;* [2]*Louis Bugnard Institute IFR31, Paul Sabatier University, Toulouse, France;* [3]*INSERM, U858, Avenir Team 1, Toulouse, France and* [4]*Faculty of Medicine, Laboratory of Medical and Clinical Pharmacology, Toulouse, France*

Background and purpose: Atrial natriuretic peptide (ANP) stimulates lipolysis in human adipocyte through a cGMP signalling pathway, the regulation of which is poorly known. Since phosphodiesterases (PDE) and neutral endopeptidase (NEP) play a major role in the regulation of the biological effects of natriuretic peptides in the cardiovascular and renal systems, we investigated whether these mechanisms could regulate cGMP signalling and ANP-mediated lipolysis in human adipocytes.
Experimental approach: The presence of cGMP-specific PDE and NEP in differentiated pre-adipocytes and in mature adipocytes was evaluated by real-time qPCR and Western blot. The effect of non-selective and selective inhibition of these enzymes on ANP-mediated cGMP signalling and lipolysis was determined in isolated mature adipocytes.
Key results: PDE-5A was expressed in both pre-adipocytes and adipocytes. PDE-5A mRNA and protein levels decreased as pre-adipocytes differentiated (10 days). PDE-5A is rapidly activated in response to ANP stimulation and lowers intracellular cGMP levels. Its selective inhibition by sildenafil partly prevented the decline in cGMP levels. However, no changes in baseline- and ANP-mediated lipolysis were observed under PDE-5 blockade using various inhibitors. In addition, NEP mRNA and protein levels gradually increased during the time-course of pre-adipocyte differentiation. Thiorphan, a selective NEP inhibitor, completely abolished NEP activity in human adipocyte membranes but did not modify ANP-mediated lipolysis.
Conclusions and implications: Functional PDE-5A and NEP activities were present in human adipocytes, however these enzymes did not play a major role in the regulation of ANP-mediated lipolysis.
British Journal of Pharmacology (2007) **152,** 1102–1110; doi:10.1038/sj.bjp.0707485; published online 1 October 2007

Keywords: cGMP signalling; atrial natriuretic peptide; lipolysis; phosphodiesterase

Abbreviations: ANP, atrial natriuretic peptide; HSL, hormone-sensitive lipase; NEP, neutral endopeptidase; NPR, natriuretic peptide receptor; PDE, phosphodiesterase

Introduction

Lipolysis in human adipocytes is an important physiological process to provide energy substrates as fuel for various situations associated with increased energy expenditure. The regulation of lipolysis has been extensively studied in the past 30 years (Arner, 2005; Langin, 2006). And the role of catecholamines in the lipolytic process has been well defined. Lipolysis is activated by catecholamines through stimulation of β-adrenoceptors and inhibited through α_2-adrenoceptor stimulation (Lafontan and Berlan, 1993). The simultaneous activation of both receptors modulates the intracellular cAMP concentration, which activates cAMP-dependent protein kinase, leading to the phosphorylation and activation of the hormone-sensitive lipase (Lafontan and Berlan, 1993). Our group have shown that natriuretic peptides (NP) are potent activators of lipolysis in human adipocytes (it is specifically observed in primate adipocytes) (Sengenès *et al*., 2000, 2002). The physiological relevance of the atrial natriuretic peptide (ANP)-dependent lipolytic pathway has been demonstrated in various conditions in men (Moro *et al*., 2004a, 2006). ANP stimulates adipocyte plasma membrane receptors (NPR-A subtype) bearing an intrinsic guanylyl cyclase activity and increases intracellular levels of cyclic GMP (cGMP) that activates a cGMP-dependent protein kinase (cGK). cGK-mediated phosphorylation of hormone-sensitive lipase stimulates lipolysis (Sengenès *et al*., 2003).

The functional regulation of ANP/cGMP pathway in human adipocytes has been poorly studied so far. It is clear

Correspondence: Dr J Galitzky, INSERM, U858/I2MR, Team 1 Avenir, 37 allées Jules Guesde, Toulouse Cedex 31000, France.
E-mail: jean.galitzky@toulouse.inserm.fr
Received 20 March 2007; revised 20 July 2007; accepted 31 August 2007; published online 1 October 2007

that cGMP turnover is regulated by specific phosphodiesterases (PDE) such as PDE-5, PDE-6, PDE-9 in various cell types (Maurice *et al*., 2003). PDE-5 is encoded by a single gene and PDE-5A is the main cGMP-specific PDE in humans (Soderling and Beavo, 2000). PDE-5A is highly expressed in various tissues such as pancreas, skeletal muscle, heart, thyroid, stomach, small intestine, penis and adrenal cortex (Loughney *et al*., 1998; Soderling *et al*., 1998; Yanaka *et al*., 1998). A rapid and sustained activation of PDE-5A occurs in platelets and vascular smooth muscle cells after nitric oxide (NO) or ANP stimulation (Mullershausen *et al*., 2001). This is followed by a dramatic lowering of cGMP levels. Activation of PDE-5A constitutes a physiological counter-regulatory mechanism to modulate the amplitude and the duration of the cGMP signal. To our knowledge, the presence and the functionality of PDE-5A has never been explored in adipose tissue. Enzymatic processing of the NPs by neutral endopeptidase (NEP) represents an alternative way to regulate the biological action of the peptide. NEP (EC 3.4.24.11), known also as neprilysin, enkephalinase, common acute lymphocytic leukaemia antigen (CALLA) or CD10, is a membrane-bound metallo-endopeptidase widely expressed in the vasculature (Turner, 2003). Increased NEP activity that could induce a biological resistance to NP action has been reported in heart failure patients (Knecht *et al*., 2002). In the present study, we investigated the putative presence and the functional role of PDE-5A and NEP in the regulation of cGMP signalling and in the lipolysis mediated by ANP in isolated human adipocytes.

Material and methods

Subjects

The study was carried out in accordance with the Declaration of Helsinki and under the ethical regulations of Toulouse University Hospital and the National Institute of Health and Medical Research (INSERM). Human subcutaneous adipose tissue was obtained from moderately overweight women undergoing plastic surgery. Their mean age was 34.1 ± 1.4 years and mean body mass index was $26.0 \pm 1.2\,kg\,m^{-2}$. To limit variability between samples, functional studies including cyclic nucleotide production, lipolysis measurement and gene expression were done on the same adipocyte batches.

Isolation of adipose tissue stromal cells and mature adipocytes and pre-adipocyte differentiation

Stromal cells and adipocytes from human adipose tissue were obtained by collagenase digestion as described previously (Hauner and Entenmann, 1991). After digestion, the suspension was centrifuged (200 *g*, 10 min), filtered (210 μm filter) and adipocytes were washed three times with PBS. The pellet containing the stroma vascular fraction was incubated for 10 min in erythrocyte-lysing buffer (155 mM NH_4Cl, 5.7 mM K_2HPO_4 and 0.1 mM EDTA), resuspended in PBS/2% fetal calf serum (FCS) and sequentially filtered through 100, 70 and 40 μm filters. The stromal cells were then resuspended in DMEM/F12 supplemented with 10% FCS and plated at 60 000 cells cm^{-2}. After incubation (37 °C) for 24 h, the medium was changed for medium consisting of DMEM/F12 supplemented with 33 μM biotin, 17 μM pantothenate and 50 μg ml^{-1} gentamycin (basal medium) in the presence of 66 nM insulin, 1 nM triiodothyronine, 100 nM cortisol, 10 μg ml^{-1} human transferrin (adipogenic medium) and, for the first 3 days, 1 μg ml^{-1} ciglitazone (PPAR-γ agonist). After the 3-day priming period, the cells were cultured in the adipogenic medium. Pre-adipocytes were collected in RLT lysis buffer (Qiagen, Courtaboeuf, France) at various stage of differentiation (days (D), −3, 0, 5, 10) and stored at −80 °C until RNA analyses.

Kinetics of intracellular cAMP and cGMP in response to IBMX treatment

One volume of packed adipocytes was exposed to 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX, non-selective PDE inhibitor) in 3 ml Krebs Ringer bicarbonate HEPES 10 mM and albumin 0.2 g ml^{-1} (KRBHA) buffer for 30 min at 37 °C. Then, adipocytes (2000–3000 cells per assay) were incubated with 5 μl of either 1 μM of ANP or 10 μM of isoprenaline in a final volume of 100 μl KRBHA at 37 °C under gentle shaking (120 cycles min^{-1}) in a water bath. Control cells were treated similarly in the absence of IBMX. The reaction was stopped at various times (0, 5, 10, 20 and 30 min), and then cAMP and cGMP were extracted in a mixture of chloroform/methanol/HCl 1N (2:1:0.1, v/v) containing 0.5 mM of IBMX. After centrifugation (3000 *g*, 5 min), the aqueous phase of each sample was collected, freeze-dried and redissolved in the EIA buffer (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Kinetics of intracellular cGMP and lipolysis in response to sildenafil

One volume of packed adipocytes was exposed to 10 μM of sildenafil (PDE-5 selective inhibitor) in 9 volumes of KRBHA buffer for 30 min at 37 °C. Then, adipocytes (2000–3000 cells per assay) were incubated with 5 μl of 10 nM of ANP in a final volume of 100 μl at 37 °C under gentle shaking (120 cycles min^{-1}) in a water bath. Control cells were treated similarly in the absence of sildenafil. Then, 50 μl aliquots of medium were collected at various times (0, 5, 10, 20, 30, 45, 60, 75 and 90 min) for determination of glycerol and non-esterified fatty acids (NEFA). Afterwards, the reaction was stopped in ice and cGMP was extracted as described previously in the paragraph above.

Effect of sildenafil on ANP-mediated cGMP production and of various PDE-5 inhibitors on lipolysis

After collagenase digestion, adipocytes were incubated in the presence of 10 μM of sildenafil in 3 ml KRBHA buffer for 30 min at 37 °C. Control cells were treated similarly in the absence of sildenafil. Then, adipocytes (2000–3000 cells per assay) were incubated with 5 μl of increasing concentrations of ANP in a final volume of 100 μl for 90 min at 37 °C under gentle shaking (120 cycles min^{-1}) in a water bath. Then, aliquots of medium and cell lysates were collected for glycerol, NEFA and cGMP determination. The effects of various PDE inhibitors such as vardenafil and zaprinast

(PDE-5 selective inhibitors) or OPC 3911 (PDE-3 selective inhibitor) were tested by performing concentration–response curves on isolated adipocytes as described above. Their effects at 10 μM on ANP-mediated lipolysis were studied after 30 min of pretreatment like for sildenafil.

Gene-expression analysis

Gene expression was performed on the stroma vascular fraction (see above) before and during the time course of adipose differentiation (D-3, D0, D5, D10), and on mature adipocytes. Total mRNA and reverse transcription were performed as described previously (Arvidsson *et al*., 2004). Real-time quantitative PCR (qPCR) was performed on ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A set of primers was designed for PDE-3B using the software Primer Express 1.5 (Applied Biosystems) and used at a final concentration of 300 nM with SYBR-Green-based chemistry. To verify that genomic DNA was not amplified, qPCR was performed on reverse transcription reactions with no addition of reverse transcriptase. Primers and probes for NEP, PDE-5A, PDE-6 and PDE-9 were obtained from Applied Biosystems using TaqMan probe-based assays. For each primer pair, a standard curve was obtained using serial dilutions of human adipose tissue cDNA prior mRNA quantification. We used 18S ribosomal RNA (Ribosomal RNA Control TaqMan Assay kit, Applied Biosystems, Foster City, CA, USA) as control to normalize gene expression. Each sample was performed in duplicate and 10 ng of cDNA were used as a template for real-time PCR. When the difference between the duplicates was above $0.5C_t$, qPCR was performed again.

Neutral endopeptidase activity assays

For determination of NEP activity in mature adipocytes, approximately 20 ml of packed cells were lysed in 5 mM EDTA, 5 mM Tris–HCl at pH 7.4. Crude membranes were pelleted by centrifugation (45 000 *g*, 15 min at 4 °C) and resuspended in NEP buffer (50 mM Tris–HCl, pH 7.4). NEP activity was measured in 96-well black fluorometric plate by adding 50 μl of crude membrane homogenate to wells containing 25 μl of NEP buffer. Plates were warmed (37 °C, 30 min) and then 25 μl of *N*-dansyl-D-alanyl-glycyl-*p*-nitrophenylalanyl-glycine (DAGNPG, Sigma, France) fluorogenic substrate was added to each sample to obtain final concentrations from 0 to 500 μM DAGNPG. The plate was then incubated for 60 min at 37 °C and the fluorescence read every 5 min at λ_{ex}: 355/λ_{em}: 538 nm excitation in a fluorometer (Fluoroskan Ascent FL, Labsystems, France). Standard curves were generated using *N*-dansyl-D-Ala-Gly in buffer. Homogenate protein was measured with the BCA protein assay kit (Perbio Science, France), and product synthesis was corrected for protein. NEP inhibition was performed using increasing concentrations of thiorphan in the presence of 100 μM DAGNPG.

Western blots

NEP and PDE-5 protein levels were investigated by western blot. Crude pellet membranes were solubilized in buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet *P*-40, 0.1% SDS, 1% Triton X-100, 2% Na deoxycholate and a mix of protease inhibitors (Complete mini, Roche, France) for 30 min at 4 °C. Non-solubilized proteins were removed by centrifugation (10 000 *g*, 5 min). The protein concentration in the supernatant was determined. Proteins (40 μg) were loaded and separated by electrophoresis using 8% SDS-polyacrylamide gel (SDS-PAGE) under denaturing conditions and resolved proteins were electrotransferred on to nitrocellulose membranes. Ponceau staining was performed to verify equal loading of the lanes. Membranes were then blocked (for 90 min) in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% defatted dried milk and incubated overnight with either mouse anti-human CD10 (NEP) antibody (Serotec, Oxford, UK) or rabbit anti-human PDE-5A (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After several washes in TBST nitrocellulose membranes were incubated with the HRP-conjugated goat anti-rabbit or mouse secondary antibody for 60 min. The immunocomplexes were detected using a chemiluminescence reagent kit (ECL plus, GE Healthcare Europe GmbH, Munich, Germany) and acquired on a Chemi-Smart 3000 station (Vilbert & Lourmat, Marne-la-Vallée, France). For western blot-based quantitative analysis, the intensities of immunoreactive bands were determined with Bio1-D software (Vilbert & Lourmat) and normalized to β-actin that was detected on the same membrane after stripping.

Biochemical determinations

The concentration of glycerol in medium was determined using an ultrasensitive radiometric method (Bradley and Kaslow, 1989). NEFA were assessed with an enzymatic method (Unipath, Dardilly, France). Intracellular concentrations of cAMP and cGMP were determined using EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA), the detection limit was 4 pmol ml^{-1} and intra-assay variability was less than 5%.

Statistical analyses

Data are presented as means ± s.e.mean. Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Gene expression during the time-course of differentiation was analysed with a one-way ANOVA followed by Tukey's multiple comparison test. Effect of drug treatment was analysed using a two-way ANOVA (drug concentration and treatment as criteria) followed by Bonferroni's multiple comparison test. The level of significance was set at $P<0.05$.

Results

Expression of PDE-5A in human differentiated pre-adipocytes and mature adipocytes

PDE-5A was expressed in stromal cells including pre-adipocytes. Its mRNA levels decreased during pre-adipocyte differentiation (Figure 1a). Figure 1b depicts a comparative evaluation of mRNA levels of PDE-3B, PDE-5A, PDE-6 and

PDE-9. PDE-3B (the main cAMP phosphodiesterase in human adipocytes) is abundantly expressed whereas PDE-5A is poorly expressed in mature adipocytes. The gene expression ratio between PDE-3B and PDE-5A is about 1/750. PDE-6 and -9 were not detectable. Figure 1c shows both a representative immunoblot of PDE-5 and quantification of PDE-5 protein levels normalized to β-actin levels during the time course of pre-adipocyte differentiation. This graph shows that PDE-5 protein level drops during the time course of differentiation as already shown for gene expression (ANOVA, $P=0.02$).

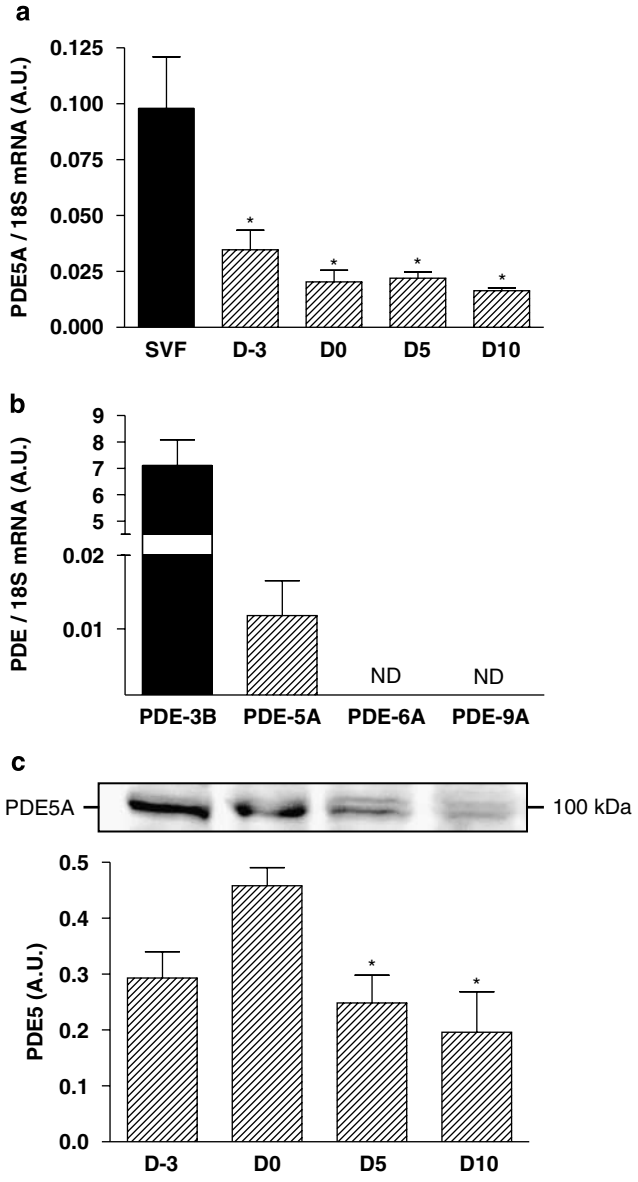

Figure 1 (**a**) Change in mRNA levels of the PDE-5A during the time course of differentiation (days (D) −3, 0, 3, 5,10). *$P<0.05$ compared to stroma vascular fraction (SVF); $n=6$. (**b**) Gene expression of different PDE isoforms specific for cGMP and of PDE-3B, the main camp-specific PDE in human mature adipocytes ($n=5$). (**c**) Change in PDE-5 protein level during adipocyte differentiation; normalized to β-actin. Inset is a representative western blot of one experiment. *$P<0.05$ compared to D0; $n=4$; ND: non-detectable; AU: arbitrary units.

Effect of IBMX on the kinetics of cAMP and cGMP in human adipocytes

The methyl xanthine, IBMX, has been described as a potent inhibitor of PDE-3B. To verify the specificity of PDE-5A in the regulation of cGMP, a comparative study was conducted on mature adipocytes. IBMX treatment did not change baseline cGMP concentrations (1.7 ± 0.2 versus 2.3 ± 0.2 pmol ($100\,mg^{-1}$) lipid for control and IBMX respectively). Also cGMP increases induced by ANP remained unaffected by IBMX whatever the incubation time (Figure 2a). In contrast, IBMX markedly increased, as expected, baseline (12.0 ± 2.0 versus 50.0 ± 11.9 pmol ($100\,mg^{-1}$ lipid) for control and IBMX respectively, $P=0.01$) and isoprenaline-stimulated cAMP concentrations (Figure 2b).

Effect of PDE-5 inhibition on the kinetics of cGMP, ANP-mediated cGMP production and lipolysis in human adipocytes

The effect of sildenafil, a potent and selective PDE-5 inhibitor, was tested on cGMP level and lipolysis induced by ANP in human mature adipocytes (Figure 3). In the

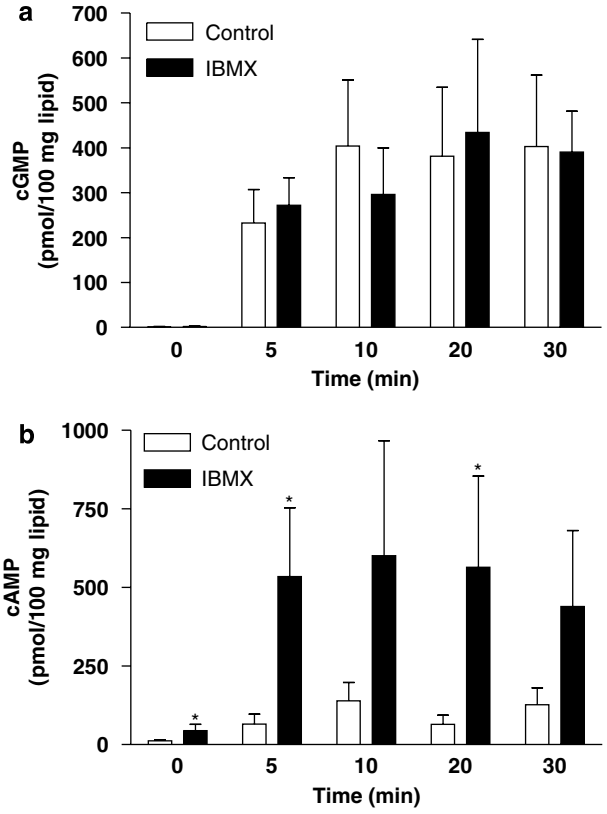

Figure 2 (**a**) Changes in intracellular concentrations of cGMP (expressed in pmol ($100\,mg^{-1}$ lipid)) in response to a stimulation with ANP (1 μM) in presence or absence of the non-selective PDE inhibitor IBMX (0.5 mM) in human adipocytes ($n=5$). (**b**) Changes in intracellular concentrations of cAMP (expressed in pmol ($100\,mg^{-1}$ lipid)) in response to a stimulation with isoprenaline (10 μM) in presence or absence of the non-selective PDE inhibitor IBMX (0.5 mM) in human adipocytes. *$P<0.05$, compared to control; ($n=3$).

control condition, a rapid increase in intracellular cGMP was observed (about 400–500 times over baseline values), with maximal level at 10 min, followed by a progressive reduction after 30 min incubation (Figure 3a). In presence of sildenafil, the rise in cGMP was similar during the first 30 min. The level of cGMP then started to decrease but remained higher than in the control experiment. Calculated areas under the curve were $29\,663 \pm 2323$ and $41\,068 \pm 4194$ ($P = 0.06$) for control and sildenafil, respectively. However, when looking at the time-course of ANP-dependent lipolysis, assessed by glycerol (Figure 3b) and FFA (Figure 3c), no differences were found between control and sildenafil conditions. As previously shown during the cGMP kinetic experiment, baseline cGMP concentrations were unaffected by sildenafil treatment, 1.9 ± 0.4 versus 1.7 ± 0.2 pmol (100 mg^{-1} lipid) for control and sildenafil respectively (Figure 4a). Intracellular cGMP increased dose-dependently with increasing concentrations of ANP (Figure 4a). Pre-treatment with sildenafil potentiated ANP-induced cGMP production significantly at 1 nM. Pre-incubation of adipocytes with three different PDE-5 selective inhibitors (sildenafil, vardenafil and zaprinast) did not modify baseline lipolysis at any concentration tested up to 10 μM, while OPC3911, a potent PDE3 selective inhibitor, stimulated baseline lipolysis in a concentration-dependent manner (Figure 4b). It is to be noted that sildenafil and vardenafil exhibited a weak non-specific lipolytic effect at high doses of 100 μM. Moreover, ANP-mediated lipolysis (evaluated by the glycerol release) remained unaffected in presence of the PDE-5 inhibitors (Figure 4c).

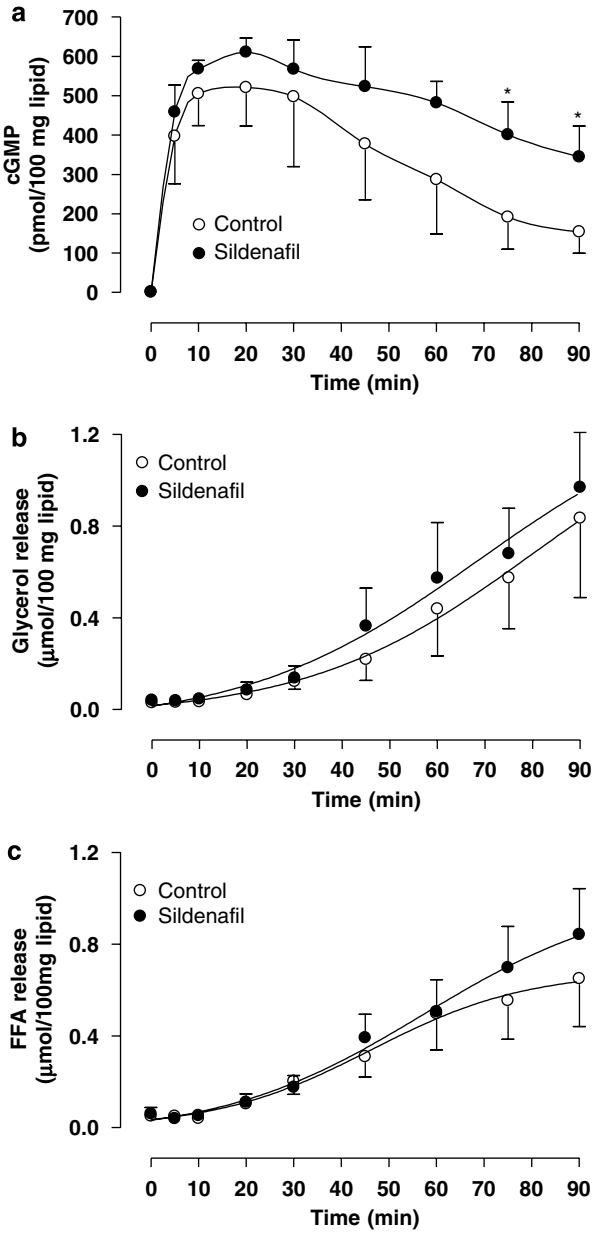

Figure 3 (**a**) Changes in intracellular concentrations of cGMP (expressed in pmol (100 mg^{-1} lipid)) in response to stimulation with ANP (10 nM) in the presence or absence of a selective PDE-5 inhibitor (sildenafil, 10 μM) in human adipocytes ($n = 4$). Time course of glycerol (**b**) and FFA (**c**) release in response to stimulation with ANP (10 nM) in the presence or absence of a selective PDE-5 inhibitor (sildenafil, 10 μM) in human adipocytes ($n = 6$). *$P < 0.05$, compared to control.

Expression of NEP in human differentiated pre-adipocytes and mature adipocytes

The neutral endopeptidase (NEP) is a membrane-bound metallo-endopeptidase involved in the hydrolysis of many circulating peptides. We studied the expression of NEP in differentiated pre-adipocytes and mature adipocytes. Figure 5a shows that the NEP was extensively expressed in mature adipocytes, comparable with mRNA levels for PDE-3B, which is an abundant transcript in human adipocytes. In addition, a progressive increase of NEP mRNA level was observed during pre-adipocyte differentiation (Figure 5b). We investigated NEP protein levels by western blot during the time course of pre-adipocyte differentiation. Figure 5c shows a representative immunoblot of NEP and a graph of its quantification normalized for β-actin in four separate experiments. In line with the gene-expression data, NEP protein levels gradually increased during the time course of pre-adipocyte differentiation (ANOVA, $P = 0.05$).

Determination of a specific NEP activity in human adipocytes

We measured NEP activity on human adipocyte membranes using a fluorescent substrate. The estimated V_{max} was 1.80 ± 0.06 nmol min^{-1} mg^{-1} protein and the K_m was 191 ± 28 μM. NEP activity was inhibited in a dose-dependent manner in presence of thiorphan, a specific NEP inhibitor (Figure 6a). Despite a high NEP activity in human adipocyte membranes, its inhibition did not modify the lipolytic effect of increasing doses of ANP, assessed by glycerol release (Figure 6b). Finally to delineate the possible functional effect of PDE-5A and NEP, ANP-mediated lipolysis was evaluated after pretreatment of mature adipocyte with a combination sildenafil (10 μM) + thiorphan (1 μM). As shown in Figure 6c, simultaneous inhibition of the two enzymes did not modify the lipolytic effect of increasing doses of ANP, assessed by glycerol release.

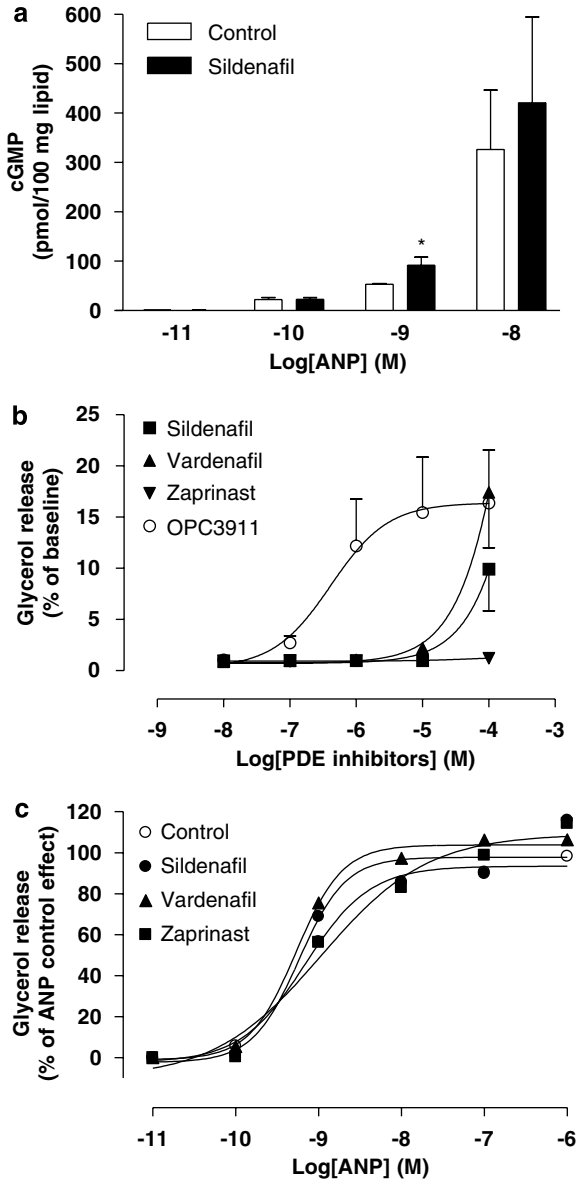

Figure 4 (**a**) Intracellular concentrations of cGMP in response to increasing doses of ANP in the presence or absence of a selective PDE-5 inhibitor (sildenafil, 10 μM) in human mature adipocytes ($n=4$). (**b**) Concentration–response lipolytic effect of PDE3 inhibitor (OPC3911) and PDE-5 inhibitors (sildenafil, vardenafil, zaprinast) in human mature adipocytes ($n=5$). (**c**) Concentration–response lipolytic effect of ANP in human adipocyte in presence or absence of 10 μM of PDE-5 inhibitors (Sildenafil, Vardenafil and Zaprinast). Mean s.e. have been omitted for clarity $^*P<0.05$, compared to control.

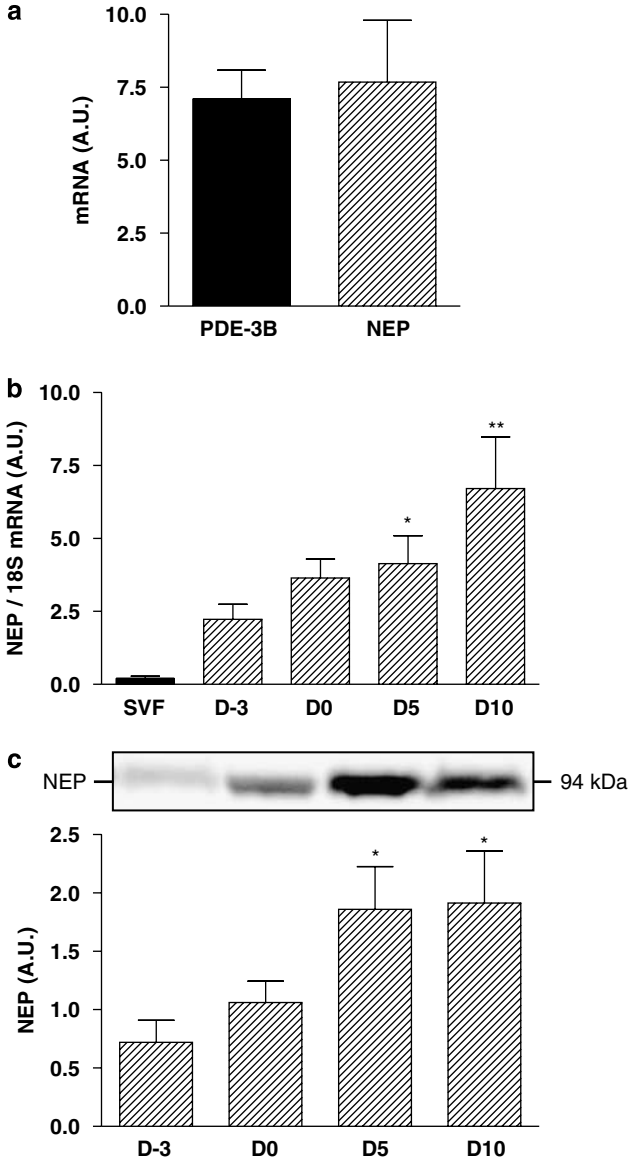

Figure 5 (**a**) Gene expression of NEP and PDE-3B in human mature adipocytes ($n=5$). (**b**) Change in mRNA level of the NEP during the time-course of differentiation. $^*P<0.05$, $^{**}P<0.01$ compared to the stroma vascular fraction (SVF); $n=6$; AU: arbitrary units. (**c**) Change in NEP protein level during pre-adipocytes differentiation ($n=4$). Inset is a representative western blot of one experiment. $^*P<0.05$ compared to D-3; AU: arbitrary units.

Discussion

In the present study, we investigated the presence and the functional role of PDE-5 and NEP in human adipocytes obtained from moderately overweight women. We identified mRNA expression, protein and functional activity of both enzymes in human adipocytes. However, they do not play a major role in the regulation of ANP-mediated lipolysis. These results raise the question of the mechanism that regulates ANP lipolytic pathway in human adipocytes.

The atrila and brain NPs (ANP and BNP) regulate blood pressure and cause natriuresis, diuresis, vasorelaxation and inhibition of the renin–angiotensin–aldosterone system (Potter *et al*., 2006). Their biological effects are mediated through cell-surface guanylyl cyclase receptors (NPR-A and NPR-B). ANP and BNP bind preferentially to NPR-A receptors. We have previously demonstrated that the NPs, including ANP and BNP, also exert a potent control of human adipocyte lipolysis through the activation of the type A guanylyl cyclase receptor (Sengenès *et al*., 2000; Moro *et al*., 2004b). Lipolysis is mediated by a cGMP-dependent pathway that induces the phosphorylation of hormone-sensitive lipase and perilipin A (Sengenès *et al*., 2003). cGMP has

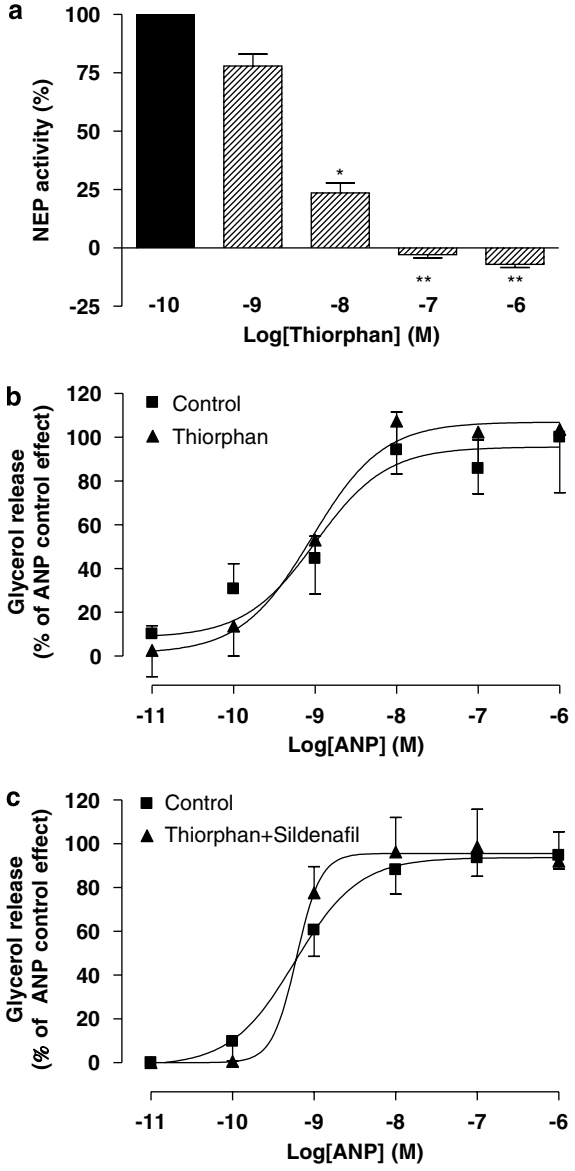

Figure 6 (**a**) Concentration-dependent inhibition of human adipocyte membranes NEP activity by a selective NEP inhibitor (thiorphan) ($n=4$). (**b**) Concentration–response lipolytic effect of ANP in human adipocyte in presence or absence of a selective NEP inhibitor (thiorphan, 1 μM) ($n=11$). (**c**) Concentration–response lipolytic effect of ANP in human adipocytes in presence or absence of a cocktail containing the selective NEP inhibitor (thiorphan, 1 μM) and the selective PDE-5 inhibitor (sildenafil 10 μM) ($n=3$). (**b** and **c**) Values are expressed in percentage of the maximal lipolytic effect of ANP in the control experiment. *$P<0.05$, **$P<0.01$ compared to control.

been established as an important modulator of tissue function (such as vascular smooth muscle tone) acting through a dynamic balance between its synthesis by guanylyl cyclases and its enzymatic degradation by PDEs (Murthy, 2001).

PDEs differ in their structure, tissue distribution and affinities for cyclic nucleotides. PDE-1, PDE-2 and PDE-3 display different substrate affinity for cAMP and cGMP (Beavo, 1995; Maurice *et al*., 2003) and PDE-3B is the main PDE regulating cAMP turnover in human adipocytes, with its activity being stimulated by the antilipolytic hormone insulin (Degerman *et al*., 1990). This enzyme is often described as cGMP-inhibitable in various cell types but we have failed to demonstrate any effect of cGMP on PDE-3 activity, or any molecular interaction between cAMP and cGMP signalling pathways in human adipocytes in previous studies (Sengenès *et al*., 2000; Moro *et al*., 2004b). PDE-4, PDE-7 and PDE-8 are highly specific for cAMP whereas PDE-5, PDE-6 and PDE-9 are highly specific for cGMP (Murthy, 2001; Maurice *et al*., 2003). PDE-5 was previously demonstrated as a specific and potent phosphodiesterase of cGMP in various animal and human tissues (Rybalkin *et al*., 2002, 2003b). PDE-5A is the major isoform of PDE-5 involved in vascular, uterus and small intestine muscle function. The potential role of PDE-5A in the regulation of cGMP signal and lipolysis had never been studied in human adipocytes so far.

We report here that, in contrast to PDE-3B, PDE-5A is poorly expressed in mature adipocytes (Figure 1b) and that its level of expression decreases during adipocyte differentiation (Figure 1a). In addition, the mRNAs for PDE-6 and PDE-9 are not detectable in mature adipocytes. PDE-5 protein levels fell during the time course of pre-adipocyte differentiation in agreement with the gene-expression data (Figure 1c). Then, the functional role of the PDE-5A was studied in human adipocytes. cGMP rates of production strongly (about 500 times over baseline) and rapidly (about 5 min) increase in response to ANP stimulation. The cGMP signal is downregulated after 20 min of incubation. This effect was partly reversed under selective PDE-5 inhibition by sildenafil (Figure 3a). These results are in favour of the presence of a functional PDE-5 in human adipocytes the activation of which downregulates cGMP levels. It has been demonstrated that activation of the PDE-5 requires phosphorylation on a Ser-92 residue (Mullershausen *et al*., 2003). This effect could be mediated by cGMP-dependent protein kinase (cGK). Additionally, according to Rybalkin *et al*. (2003a), cGMP might directly stimulate PDE-5 activity through interplay with the N-terminal regulatory GAF (mammalian cGMP-binding phosphodiesterase, Anabaena adenylyl cyclases, *Escherichia coli* FhlA) domain. Pharmacological blockade of the enzyme by sildenafil increases smooth muscle cell relaxation and this inhibitor is clinically approved for the treatment of erectile dysfunction in humans (Corbin and Francis, 2002; Corbin *et al*., 2002). The IC_{50} of sildenafil for PDE-5 is in the range of 4 nM and this compound is about 4000 times more selective for PDE-5 than PDE-3. Other highly selective PDE-5 inhibitors have been developed lately such as vardenafil and tadalafil for the treatment of erectile dysfunction in humans. In human adipocytes, selective inhibition of PDE-5 using various inhibitors (sildenafil, vardenafil and zaprinast) did not change baseline- and ANP-mediated lipolysis (Figures 4b and c), while selective inhibition of PDE-3 by OPC3911 dose-dependently stimulated baseline lipolysis (Figure 4b). It is to be noted that high concentrations of vardenafil and sildenafil (100 μM) could increase lipolysis probably by n non-specific mechanism involving PDE-3B inhibition. Indeed, even if the selectivity of sildenafil for PDE-5 is

4000-fold higher than for PDE-3, sildenafil is likely to inhibit about 50% of the PDE-3 activity at concentrations higher than 10 μM. Together, this suggests that even if PDE-5 modulates cGMP turnover in human adipocytes, it does not play a major role in the regulation of lipolysis.

Hydrolysis by NEP represents another pathway of regulation of the biological action of ANP. NEP is a membrane-bound glycoprotein of 94 kDa that belongs to the family of zinc metallo-peptidases (Turner, 2003). This enzyme is widely expressed in the vasculature and inactivates a wide range of peptides and peptidic neuro-hormones such as enkephalins, neurotensin, bradykinin, glucagon, adrenomedullin and the NPs. Racecadotril, which is rapidly metabolized to its active metabolite thiorphan, is a NEP inhibitor used in clinical practice for the treatment of acute diarrhoea. Thus, it has been found that ANP degradation could be affected by various soluble proteases or by those linked to the membrane of blood cells or renal cells of the proximal tubule (Walter *et al*., 1997). Interestingly, increased NEP activity is believed to contribute to the resistance of the physiological effect of NP. We hypothesized that the presence of NEP in human adipocytes might regulate the lipolytic effect of ANP. In the present study, we observed a significant level of expression of NEP in human mature adipocytes, close to that of PDE-3B (Figure 5a). The enzyme is expressed in pre-adipocytes and its level of expression increases during differentiation of pre-adipocytes (Figure 5b). Additionally, western blotting analyses revealed a gradual increase in NEP protein levels during the time course of pre-adipocyte differentiation (Figure 5c). A high NEP activity was found in human adipocyte membranes and it was dose-dependently inhibited by the selective NEP inhibitor thiorphan (Figure 6a). NEP is the primary enzyme responsible for the degradation of ANP (Mishima *et al*., 2002). It is well documented that pharmacological inhibition of NEP increases *in vitro* and *in vivo* the half-life of NP and extends their biological action. However, maximal blockade of adipocyte NEP activity with thiorphan did not modify ANP-mediated lipolysis (Figure 6b), even in the presence of PDE-5 blockade (Figure 6c).

We can finally conclude that adipocyte NEP activity does not acutely regulate ANP-mediated lipolysis in humans. One possible explanation is that supra-physiological concentrations of ANP used in lipolytic assays did not permit the observation of a subtle regulation of lipolysis by the NEP. In addition, the substrate affinity (Km) of NEP for various peptides ranges from 50 to 200 μM, which is much higher than the maximum lipolytic effect of ANP in human adipocytes. In the present study, adipose tissue was obtained from moderately overweight women. Several studies have suggested that there is a reduced biological activity of NP in obesity, maybe due to increased clearance of the peptides (Sarzani *et al*., 2004; Wang *et al*., 2004). A negative correlation between circulating NP levels and the body mass index has been shown. The potential role of the NP clearance receptor, NPR-C, has been proposed in this event. Indeed, NPR-C in highly expressed in human adipose tissue and could contribute to increased clearance of circulating or adipose tissue NP in obesity (Sarzani *et al*., 1996, 2004). On the basis of our results, one can hypothesize that, in adipose tissue, NEP activity might also increase in obesity and contribute to the clearance of circulating NP.

Taking into account that the functional regulation of ANP lipolytic pathway in human adipocytes does not involve either PDE-5A or NEP, another regulatory process has to be proposed. It has been previously demonstrated that the change in the phosphorylation state of NPR-A and NPR-B modulate their activity and that dephosphorylation induces their desensitization (Potter and Garbers, 1992; Potthast and Potter, 2005). This apparent desensitization mechanism of NP receptors is in contrast to other cell-surface receptors like adrenoceptors that are desensitized by phosphorylation. We have observed that exposure of adipocytes to ANP leads to homologous desensitization of ANP-dependent lipolysis and lipid mobilization *in vitro* and *in vivo* (Moro *et al*., 2005). When compared to adrenoceptors, we also observed that this desensitization process occurs over a longer period of time with ANP compared to catecholamines, and we attributed this phenomenon to a decrease in the intrinsic guanylyl cyclase activity of the NPR-A (Potter and Garbers, 1992), which does not appear to involve internalization of the receptor into the cell (Fan *et al*., 2004). Finally, homologous desensitization of the NPR-A receptor might represent an efficient short-term regulatory mechanism of ANP-mediated lipolysis in human adipocytes.

In conclusion, the present study shows evidence for functional PDE-5A and membrane-bound NEP enzyme activities in human adipocytes. However, these enzymes do not play a major functional role in the regulation of ANP lipolytic pathway in human adipocytes. Increased expression and/or activities of these enzymes could contribute to reduced circulating levels of NP and blunted ANP-mediated lipolysis in obesity. Thus, it would be of interest to assess the functionality of these enzymes in human adipocytes obtained either from obese or morbidly obese subjects or from heart failure patients in whom plasma NP levels are dramatically increased.

Acknowledgements

We greatly thank Marie-Adeline Marquès for skilled technical assistance. We also thank Dr Jean-Jacques Benarous and Dr Eric Jougla for their help with obtaining the adipose tissue samples.

Conflict of interest

The authors state no conflict of interest.

References

Arner P (2005). Human fat cell lipolysis: biochemistry, regulation and clinical role. *Best Pract Res Clin Endocrinol Metab* **19**: 471–482.

Arvidsson E, Viguerie N, Andersson I, Verdich C, Langin D, Arner P (2004). Effects of different hypocaloric diets on protein secretion from adipose tissue of obese women. *Diabetes* **53**: 1966–1971.

Beavo JA (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* **75**: 725–748.

Bradley DC, Kaslow HR (1989). Radiometric assays for glycerol, glucose, and glycogen. *Anal Biochem* **180**: 11–16.
Corbin JD, Francis SH (2002). Pharmacology of phosphodiesterase-5 inhibitors. *Int J Clin Pract* **56**: 453–459.
Corbin JD, Francis SH, Webb DJ (2002). Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology* **60**: 4–11.
Degerman E, Smith CJ, Tornqvist H, Vasta V, Belfrage P, Manganiello VC (1990). Evidence that insulin and isoprenaline activate the cGMP-inhibited low-Km cAMP phosphodiesterase in rat fat cells by phosphorylation. *Proc Natl Acad Sci USA* **87**: 533–537.
Fan D, Bryan PM, Antos LK, Potthast RJ, Potter LR (2004). Downregulation does not mediate natriuretic peptide-dependent desensitization of NPR-A or NPR-B: guanylyl cyclase-linked natriuretic peptide receptors do not internalize. *Mol Pharmacol* **67**: 174–183.
Hauner H, Entenmann G (1991). Regional variation of adipose differentiation in cultured stromal-vascular cells from the abdominal and femoral adipose tissue of obese women. *Int J Obes* **15**: 121–126.
Knecht M, Pagel I, Langenickel T, Philipp S, Scheuermann-Freestone M, Willnow T *et al*. (2002). Increased expression of renal neutral endopeptidase in severe heart failure. *Life Sci* **71**: 2701–2712.
Lafontan M, Berlan M (1993). Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res* **34**: 1057–1091.
Langin D (2006). Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. *Pharmacol Res* **53**: 482–491.
Loughney K, Hill TR, Florio VA, Uher L, Rosman GJ, Wolda SL *et al*. (1998). Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterase. *Gene* **216**: 139–147.
Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR *et al*. (2003). Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* **64**: 533–546.
Mishima T, Tanimura M, Suzuki G, Todor A, Sharov VG, Tanhehco EJ *et al*. (2002). Effects of chronic neutral endopeptidase inhibition on the progression of left ventricular dysfunction and remodeling in dogs with moderate heart failure. *Cardiovasc Drugs Ther* **16**: 209–214.
Moro C, Crampes F, Sengenes C, De Glisezinski I, Galitzky J, Thalamas C *et al*. (2004a). Atrial natriuretic peptide contributes to physiological control of lipid mobilization in humans. *FASEB J* **18**: 908–910.
Moro C, Galitzky J, Sengenes C, Crampes F, Lafontan M, Berlan M (2004b). Functional and pharmacological characterization of the natriuretic peptide-dependent lipolytic pathway in human fat cells. *J Pharmacol Exp Ther* **308**: 984–992.
Moro C, Polak J, Hejnova J, Klimcakova E, Crampes F, Stich V *et al*. (2006). Atrial natriuretic peptide stimulates lipid mobilization during repeated bouts of endurance exercise. *Am J Physiol Endocrinol Metab* **290**: E864–E869.
Moro C, Polak J, Richterova B, Sengenes C, Pelikanova T, Galitzky J *et al*. (2005). Differential regulation of atrial natriuretic peptide- and adrenergic receptor-dependent lipolytic pathways in human adipose tissue. *Metabolism* **54**: 122–131.
Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, Koesling D (2003). Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J Cell Biol* **160**: 719–727.
Mullershausen F, Russwurm M, Thompson WJ, Liu L, Koesling D, Friebe A (2001). Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. *J Cell Biol* **155**: 271–278.
Murthy KS (2001). Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem J* **360**: 199–208.
Potter LR, Garbers DL (1992). Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization. *J Biol Chem* **267**: 14531–14534.
Potter LR, Abbey-Hosch S, Dickey DM (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev* **27**: 47–72.
Potthast R, Potter LR (2005). Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptors. *Peptides* **26**: 1001–1008.
Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, Beavo JA (2002). Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem* **277**: 3310–3317.
Rybalkin SD, Rybalkina IG, Shimizu-Albergine M, Tang XB, Beavo JA (2003a). PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. *EMBO J* **22**: 469–478.
Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA (2003b). Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* **93**: 280–291.
Sarzani R, Dessi-Fulgheri P, Paci V, Espinosa E, Rappelli A (1996). Expression of natriuretic peptide receptors in human adipose tissue and other tissues. *J Endocrinol Invest* **19**: 581–585.
Sarzani R, Strazzullo P, Salvi F, Iacone R, Pietrucci F, Siani A *et al*. (2004). Natriuretic peptide clearance receptor alleles and susceptibility to abdominal adiposity. *Obes Res* **12**: 351–356.
Sengenès C, Berlan M, de Glisezinski I, Lafontan M, Galitzky J (2000). Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J* **14**: 1345–1351.
Sengenès C, Bouloumié A, Hauner H, Berlan M, Busse R, Lafontan M *et al*. (2003). Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem* **278**: 48617–48626.
Sengenès C, Zakaroff-Girard A, Moulin A, Berlan M, Bouloumié A, Lafontan M *et al*. (2002). Natriuretic peptide-dependent lipolysis in fat cells is a primate specificity. *Am J Physiol Regul Integr Comp Physiol* **283**: R257–R265.
Soderling SH, Bayuga SJ, Beavo JA (1998). Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J Biol Chem* **273**: 15553–15558.
Soderling SH, Beavo JA (2000). Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr Opin Cell Biol* **12**: 174–179.
Turner AJ (2003). Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries. *Biochem Soc Trans* **31**: 723–727.
Walter M, Unwin R, Nortier J, Deschodt-Lanckman M (1997). Enhancing endogenous effects of natriuretic peptides: inhibitors of neutral endopeptidase (EC.3.4.24.11) and phosphodiesterase. *Curr Opin Nephrol Hypertens* **6**: 468–473.
Wang TJ, Larson MG, Levy D, Benjamin EJ, Leip EP, Wilson PW *et al*. (2004). Impact of obesity on plasma natriuretic peptide levels. *Circulation* **109**: 594–600.
Yanaka N, Kotera J, Ohtsuka A, Akatsuka H, Imai Y, Michibata H *et al*. (1998). Expression, structure and chromosomal localization of the human cGMP-binding cGMP-specific phosphodiesterase PDE5A gene. *Eur J Biochem* **255**: 391–399.