

# Functional and Pharmacological Characterization of the Natriuretic Peptide-Dependent Lipolytic Pathway in Human Fat Cells

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## ABSTRACT

A lipolytic pathway involving natriuretic peptides has recently been discovered in human fat cells. Its functional characteristics and the interactions of the atrial natriuretic peptide (ANP)-induced effects with adrenergic and insulin pathways were studied. Characterization of the action of ANP antagonists, i.e., A71915, anantin, S-28-Y (Ser-28-Tyr, a synthesized peptide), and HS-142-1 (a microbial polysaccharide), was performed. Lipolytic assays and intracellular cGMP and cAMP determinations were performed on isolated fat cells. Cell membranes were used for binding studies. At low concentrations ANP and isoproterenol [ $\beta$ -adrenergic receptor ( $\beta$ -AR) agonist] exerted additive lipolytic effects. The  $\alpha_2$ -AR pathway did not interfere with that of ANP. Lipolytic effects of ANP were unaltered by a 2-h pretreatment of fat cells with insulin, whereas  $\beta$ -AR-induced lipolysis was reduced. Homologous desensitization occurred for ANP-dependent lipolytic pathways. Dendroapsis na-

triuretic peptide exhibited a similar maximal effect but a 10-fold higher lipolytic potency than ANP and mini-ANP (the shortest form of ANP). The antagonist A71915 exhibited competitive antagonistic properties with a  $pA_2$  value of 7.51. Anantin displayed noncompetitive antagonism and exerted an inhibitory action on basal and  $\beta$ -adrenergic receptor-induced lipolytic response. S-28-Y exhibited antagonist potencies toward ANP-induced lipolysis and behaved as a partial lipolytic agonist with a lower  $pD_2$  value ( $7.4 \pm 0.2$ ) than ANP ( $9.4 \pm 0.3$ ). HS-142-1 exerted the weakest antagonistic effects. The results demonstrate that ANP-dependent effects do not interfere with  $\beta$ - and  $\alpha_2$ -adrenergic pathways in human fat cells. They are unaffected by insulin pretreatments of fat cells but undergo desensitization. In the search of potent and specific natriuretic peptide receptor-A antagonist, in the human fat cell, A71915 was the only reliable one found.

Natriuretic peptides ANP, BNP, and C-type natriuretic peptide (NPs) are a family of peptides that exert potent diuretic, natriuretic, and vasodilator activities (Athanasopoulos and Dennis, 1991; Levin et al., 1998). They are synthesized as prohormones in the mammalian heart, stored as prohormones, and then released into the plasma after cardiomyocyte stretch. ANP sequence comprises 28 amino acids with a Cys<sup>7</sup>-Cys<sup>23</sup> bridge, which enables binding to atrial natriuretic peptide receptors (NPRs) (Levin et al., 1998). ANP binds with high affinity to human fat cell membranes, and we have recently demonstrated that NPs are

potent lipolytic agents in human and in nonhuman primate fat cells only (Sengenés et al., 2000, 2002a). Among the members of the NP family, the relative order of lipolytic potencies is ANP > BNP > C-type natriuretic peptide. The lipolytic activity of ANP and BNP is mediated by specific fat cell-plasma membrane receptors (NPR-A) bearing a guanylyl cyclase activity. They operate via a cGMP-dependent pathway and independently of phosphodiesterase-3B inhibition (Sengenés et al., 2000). We have shown that intravenous perfusion or local infusion of ANP in a microdialysis probe increases extracellular glycerol concentration in human subcutaneous adipose tissue (Galitzky et al., 2001; Sengenés et al., 2002a).

In physiological conditions such as during physical exercise, both catecholamines (after sympathetic nervous system

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**ABBREVIATIONS:** ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; NP, natriuretic peptide; NPR, natriuretic peptide receptor; AR, adrenergic receptor; DNP, dendroapsis natriuretic peptide; KRBA, Krebs Ringer bicarbonate buffer containing bovine serum albumin; S-28-Y, Ser-28-Tyr; HS-142-1, polysaccharide isolated from culture broth of *Aureobasidium* sp.

activation) (Kjaer et al., 1987) and ANP and BNP (originating from the heart) (Ohba et al., 2001; Huang et al., 2002) are released. Both catecholamines and NPs are putatively involved in the control of lipid mobilization in humans (Moro et al., 2003). Moreover, insulin, the potent antilipolytic hormone, is able to counteract catecholamine-induced lipolysis in fat cells (Carey, 1998; Moberg et al., 1998); its incidence on ANP pathways is unknown.

The present study had two aims. One was devoted to the exploration of the putative crossover existing between the lipolytic and antilipolytic pathways. The second concerns the search for a suitable antagonist active in human fat cells. The first part of the study was designed to determine whether the lipolytic effects of NPs and catecholamines exhibit additive or potentiating effects on human fat cells and whether long-term exposure (2-h) of the fat cells to insulin changes the relative proportions of the lipolysis brought about via the two lipolytic pathways. The time courses of the production of the intracellular messengers (cAMP and cGMP, respectively) and of lipolysis were studied during activation of  $\beta$ -adrenergic receptors ( $\beta$ -ARs) and NPR-A receptors in fat cells. Moreover, since it is known that  $\beta$ -adrenergic lipolytic pathways are submitted to homologous desensitization, we investigated whether ANP-induced desensitization occurred under conditions known to alter  $\beta$ -AR-mediated effects.

Second, since agonists and antagonists represent valuable tools to explore the physiological roles of NPs in humans, the action of agonists and antagonists at human fat cell NPRs was pharmacologically characterized. Two agonists, the dendroaspis natriuretic peptide (DNP), isolated from the venom of the green mamba snake and the mini-ANP (the smallest form of ANP) were tested. Finally, the antagonistic capacities of peptides often considered to act as NPR-A antagonists on various tissues were evaluated on isolated human fat cells. The first one was A71915 (Delporte et al., 1992; Nachshon et al., 1995); a peptide derived from ANP possessing two unnatural amino acids in positions 8 and 26 (cyclohexylalanine and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, respectively). The second one, named anantin, was isolated from a strain of *Streptomyces coeruleus* (Weber et al., 1991; Wyss et al., 1991). The third, named S-28-Y (Minamitake et al., 1990), is a synthetic peptide in which ANP amino acids Cys<sup>7</sup> and Cys<sup>23</sup> were replaced by unnatural amino acids (penicillamine and D-penicillamine, respectively). We also evaluated the antagonistic property of HS-142-1, a microbial polysaccharide isolated from the culture broth of *Aureobasidium* sp. (Morishita et al., 1991). Using various cell or tissue models, these three compounds have been shown to display some NPR-A binding properties and potencies to inhibit ANP-induced cyclic GMP generation (Von Geldern et al., 1990). The action of these agents of natural or synthetic origin was investigated in functional assays (lipolysis and cGMP production) on isolated human fat cells and in binding studies on human fat cell membranes, to determine the best competitive antagonist(s) that could be of interest for further clinical use. Our results indicated that anantin exhibited unspecific properties, S-28-Y acted as a partial agonist, and HS-142-1 was only a poor inhibitor of ANP-induced lipolysis. Finally, compound A71915 was the only

valid antagonist available and can be proposed for further clinical use.

## Materials and Methods

**Adipocyte Preparation and Lipolysis Measurement.** Human subcutaneous abdominal adipose tissue (1–2 g) was obtained from 16 normal or moderately overweight women undergoing plastic surgery. Their mean age was  $45.7 \pm 3.5$  years and their mean body mass index was  $24.4 \pm 1.3$  kg/m<sup>2</sup>. The Ethical Committee of Toulouse University Hospital approved the study. Isolated adipocytes were obtained according to the method of Rodbell (1964) by collagenase digestion of adipose fragments in Krebs Ringer bicarbonate buffer containing bovine serum albumin (2 g/100 ml) (KRBA) and glucose (6 mM) at pH 7.4 and by shaking at around 200 cycles/min at 37°C. After digestion, fat cells were filtered through a silk screen and washed three times with KRBA buffer to eliminate collagenase and stroma-vascular elements. Isolated adipocytes were brought to a suitable dilution (2000–3000 cells/assay) in KRBA for lipolysis measurement and incubated with pharmacological agents in a final volume of 100  $\mu$ l for 90 min at 37°C. At the end of the incubation, 20- to 50- $\mu$ l aliquots of the infranatant were taken for glycerol determination (Bradley and Kaslow, 1989) and used as the lipolytic index. For insulin pretreatment of fat cells or desensitization studies, washed isolated fat cells were preincubated for 2 h with the corresponding agent, washed three times with fresh KRBA, and treated with ANP or isoproterenol for 90 min. Control cells were treated in parallel in the absence of agents.

**Determination of cGMP and cAMP Concentrations.** Fat cells were preincubated in 500  $\mu$ l of KRBA at 37°C for various times (5, 10, 15, 20, 30, 60, and 90 min) in the presence of 1  $\mu$ M ANP, 1  $\mu$ M S-28-Y, or 1  $\mu$ M isoproterenol. Addition of a solution of chloroform, methanol, 1 M HCl (2:1:0, v/v) containing 0.5 mM 3-isobutyl-1-methylxanthine (a nonselective phosphodiesterase inhibitor) stopped the reaction. After centrifugation (5000 rpm, 5 min), the aqueous phase of each sample was collected, freeze-dried, and redissolved in the buffer of the enzyme immunoassay kit according to the manufacturer's instructions for cGMP or cAMP determinations.

**Radioligand Binding Assay.** Isolated adipocytes were broken in a hypotonic lysing medium (5 mM Tris, pH 7.5, 5 mM EDTA) containing several protease inhibitors (100  $\mu$ M phenylmethylsulfonyl fluoride, 0.5 mg/ml bacitracin, 1  $\mu$ M aprotinin, 10  $\mu$ M thiorphan). Then crude adipocyte membranes were pelleted by centrifugation (48,000g for 15 min at 4°C). The pellet was washed twice with 10 ml of binding buffer (50 mM Tris, pH 7.5), 5 mM MgCl<sub>2</sub>, 0.1% bovine serum albumin, and an antiprotease cocktail containing 0.5 mg/ml bacitracin, 1  $\mu$ M leupeptin, and 10  $\mu$ M thiorphan. The pellet was finally resuspended in the same buffer at a final concentration of 1 to 2 mg of protein per milliliter and immediately used for binding experiments. Assays were performed in a final volume of 200  $\mu$ l containing 50  $\mu$ l of membrane suspension and 50  $\mu$ l of <sup>125</sup>I-ANP. Nonspecific binding was measured in the presence of 1  $\mu$ M unlabeled ANP. Saturation experiments were carried out under constant shaking for 45 min at 25°C. The incubation was stopped on ice, and the fraction bound was separated by centrifugation (13,000g for 10 min). The pellet was washed twice with 500  $\mu$ l of binding buffer, and the radioactivity retained was counted in a gamma counter.

**Drugs and Chemicals.** Isoproterenol hydrochloride (nonselective  $\beta$ -AR agonist), bovine serum albumin (fraction V), and crude collagenase were obtained from Sigma Chemical (Paris, France). Phentolamine methanesulfonate (Regitine) was obtained from Aventis (Strasbourg, France). Insulin came from Sigma (Saint-Quentin Fallavier, France); enzymes for glycerol assays and complete minitab-let protease inhibitors came from Roche Diagnostics, (Meylan, France). Human ANP (1-28) was from Neosystem (Strasbourg, France); bromo-cGMP was from Alexis Biochemicals (Paris, France). [<sup>3-125</sup>I]iodotyrosyl-ANP came from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). S-28-Y was synthesized by

Neosystem. DNP, mini-ANP, A71915, and anantin came from Bachem Biochimie (Voisins-le-Bretonneux, France). HS-142-1 was provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) Glycerol in incubation medium was analyzed with an ultrasensitive radiometric method (Bradley and Kaslow, 1989). Determination of cAMP and cGMP was performed using enzyme immunoassay kits from Cayman Chemical Company/SPI-BIO (Massy, France).

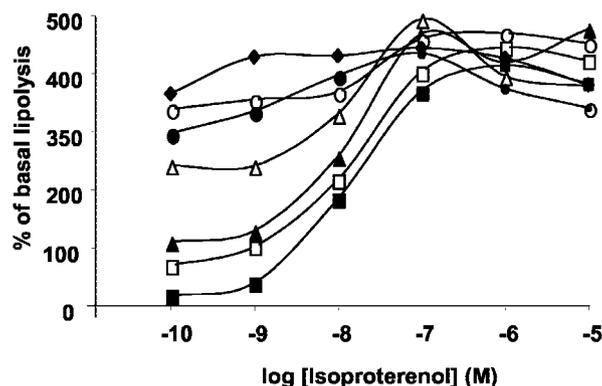
**Data Analysis.** Values are given as means  $\pm$  S.E.M. of *n* separate experiments. Student's paired *t* test was used for comparisons between matched pairs. Unless specified, differences were considered significant when  $P < 0.05$ . The concentration-response curves were fitted by nonlinear regression, and  $EC_{50}$ ,  $pD_2$ , and  $pA_2$  values were calculated using the program Prism (GraphPad Software Inc., San Diego, CA).

## Results

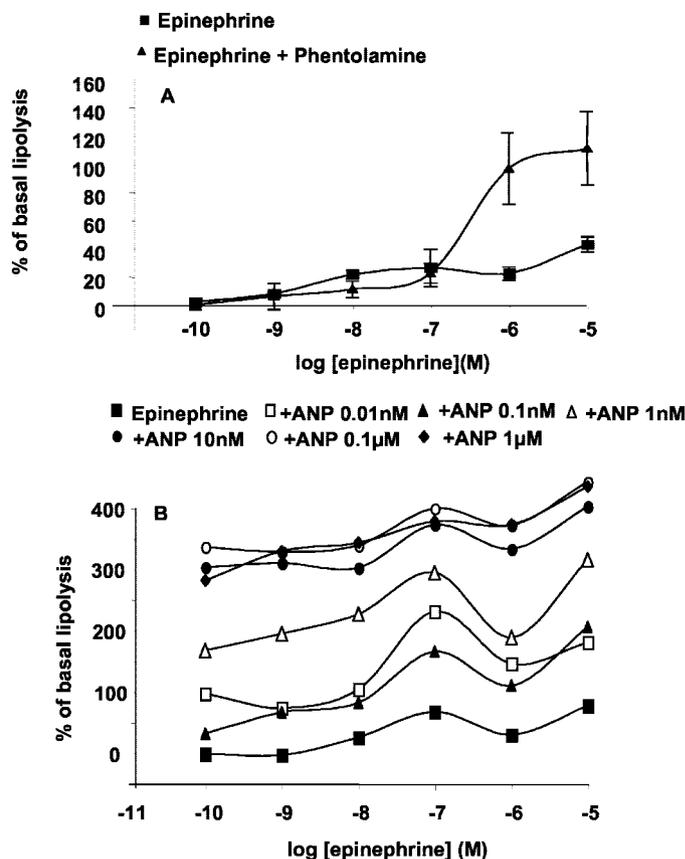
### Interaction between NPR-A and Adrenergic Pathways on Human Fat Cells

To evaluate a possible interaction between ANP and  $\beta$ -AR-dependent pathways (using isoproterenol as an agonist), increasing concentrations of isoproterenol were associated with various increasing concentrations of ANP (Fig. 1). An additive effect of the two compounds on lipolysis was observed at their lowest concentrations. However, the maximal lipolytic effect of isoproterenol was not significantly amplified by addition of maximal concentrations of ANP. The same experiment was performed using the physiological amine epinephrine (Fig. 2). As expected, epinephrine promoted a weak lipolytic effect in subcutaneous human fat cells. This was due to the important  $\alpha_2$ -adrenergic antilipolytic effect, since the response induced by epinephrine was largely enhanced in the presence of the  $\alpha_2$ -adrenergic antagonist phentolamine (10  $\mu$ M). As observed with isoproterenol, ANP-induced lipolysis was strictly additive to that of epinephrine at the lowest concentrations, whereas maximal effects were not additive. Overall, these data suggest the absence of an interaction among NPR-A,  $\beta$ -, and  $\alpha_2$ -adrenergic receptor pathways in human fat cells.

■ Isoproterenol □ +ANP 0.01nM ▲ +ANP 0.1nM △ +ANP 1nM  
● +ANP 10nM ○ +ANP 0.1 $\mu$ M ◆ +ANP 1 $\mu$ M



**Fig. 1.** Effect of increasing concentrations of ANP on glycerol release induced by increasing concentrations of isoproterenol in human fat cells. Lipolysis is expressed as a percentage of spontaneous glycerol production.  $n = 5$ . S.E.M. was below 20% and omitted for clarity. Spontaneous glycerol production was  $0.190 \pm 0.040$   $\mu$ mol glycerol/100 mg lipid/60 min.



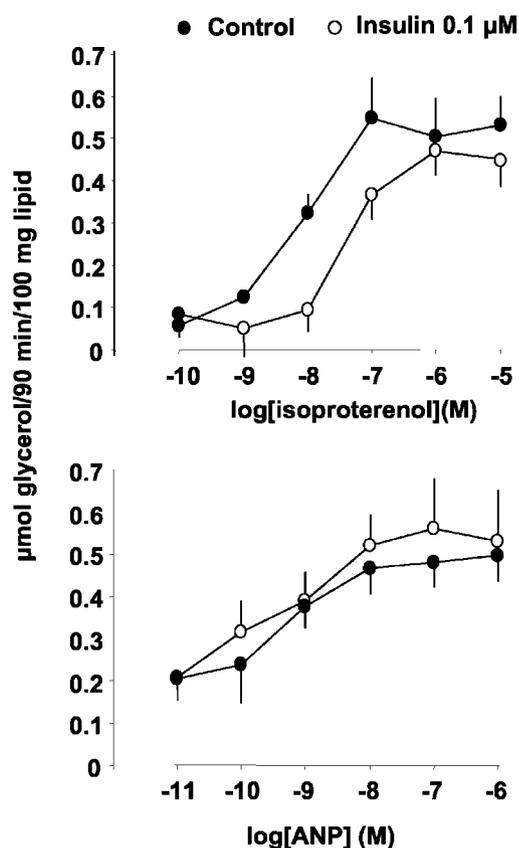
**Fig. 2.** A, effect of phentolamine (10  $\mu$ M) on epinephrine-induced glycerol release in human fat cells. B, effect of increasing concentrations of ANP on glycerol release induced by increasing concentrations of epinephrine.  $n = 5$ . Lipolysis is expressed as a percentage of spontaneous glycerol production; S.E.M. was below 20% and omitted for clarity in the lower part of the figure.

### Interaction between NPR-A and Insulin Pathways on Human Fat Cell

Since it is well known that insulin counteracts  $\beta$ -AR-induced lipolysis, it was necessary to establish whether such interactions could exist with the NP pathway. Figure 3 shows the incidence of a 2-h pretreatment of fat cells with insulin (100 nM) on the lipolytic responses promoted by isoproterenol or ANP. As expected, isoproterenol-induced lipolysis was reduced by insulin pretreatment of fat cells, and the concentration-response curve for isoproterenol was shifted to the right; the  $pD_2$  values were significantly different ( $8.3 \pm 0.4$  and  $7.7 \pm 0.2$ , respectively,  $P < 0.05$ ). In contrast, concentration-response curves of ANP-induced lipolysis were similar in control and insulin-pretreated cells, and  $pD_2$  values did not differ ( $10.1 \pm 0.3$  and  $10.5 \pm 0.5$ , respectively). The lipolytic effects of ANP were unaffected by insulin pretreatment of human fat cells.

### Comparison of the Homologous Desensitization of AR- and ANP-Dependent Lipolytic Pathways

The effect of a 2-h pretreatment of isolated fat cells with isoproterenol or ANP on the lipolytic responses induced by the same agents is reported in Table 1. The  $pD_2$  values for isoproterenol or ANP were significantly reduced when cells were preincubated with the homologous drugs. This result argues for the occurrence of homologous desensitization for both drugs during pretreatment. Moreover, these data con-



**Fig. 3.** Effect of insulin treatment (0.1  $\mu$ M; 2-h) on lipolysis induced by isoproterenol or ANP. Values are means  $\pm$  S.E.M. of five separate experiments.

firm the absence of a cross-interaction between the two pathways. The values of  $pD_2$  for isoproterenol- or ANP-induced lipolysis remained unchanged when cells were preincubated with ANP or isoproterenol, respectively.

#### Comparison of the Intracellular Messenger Kinetics and Lipolysis Induced by Isoproterenol and ANP

The time course of the intracellular messenger accumulation induced by NPR-A and  $\beta$ -AR activation (cGMP and cAMP, respectively) was evaluated for 60 min (Fig. 4). Intracellular cGMP concentration in fat cells rose very rapidly and reached a maximal concentration 2.5 min after exposure of the cell to 1  $\mu$ M ANP. Then the cGMP concentration remained stable during the 60-min incubation period. For  $\beta$ -AR activation by 1  $\mu$ M isoproterenol, intracellular cAMP concentrations rose more slowly and reached a maximal value only at 15 to 20 min; then they remained stable for the 60-min incubation period. Despite the differences observed in the kinetics of second messenger production, there was no noticeable incidence on the time course of glycerol release promoted by the two lipolytic agents (Fig. 4).

#### Pharmacological Properties of NPR-A Agonists on Human Fat Cells

Using lipolysis, the comparative effects of two available agonists (DNP and mini-ANP) and ANP were studied (Table 2). The concentration response of the lipolytic effect of mini-ANP was quite similar to that of ANP, and their  $pD_2$  values were identical ( $9.05 \pm 0.2$  and  $8.99 \pm 0.10$ , respectively). DNP and ANP exerted similar maximal lipolytic effects, but DNP had

a 10-fold higher potency than ANP (mean  $pD_2$  value for DNP was  $10.02 \pm 0.28$ ).

#### Pharmacological Properties of NPR-A Antagonists on Human Fat Cells

**Lipolysis Measurements.** Table 3 reports the effects of increasing concentrations of ANP and the four putative antagonists on the spontaneous glycerol release of fat cells (i.e., basal lipolysis). A71915 and HS-142-1 (not shown) did not significantly modify basal lipolysis, whereas anantin induced a concentration-dependent inhibition of lipolysis. Surprisingly, S-28-Y partially increased lipolysis. When compared with the ANP effect, the maximal effect was observed at 1  $\mu$ M.

The effects of A71915 on the ANP-induced lipolysis in human fat cells are illustrated in Fig. 5. A71915 exerted competitive antagonism toward the concentration-response effects of ANP. Analysis of the data allowed the determination of the calculated  $pA_2$  value, which was 7.51.

The effects of anantin on the lipolytic effect of ANP are depicted in Fig. 6. Anantin induced a concentration-dependent and noncompetitive inhibitory effect on ANP-induced lipolysis. At the highest concentration, anantin totally suppressed the lipolytic effect of ANP and also reduced basal lipolysis. Even if anantin exhibits some antagonistic properties toward ANP-effects, it was suspected to possess a non-specific activity on the lipolytic pathway. This was confirmed when studying the effect of anantin on lipolysis induced by compounds acting at  $\beta$ -ARs (i.e., isoproterenol) or drugs activating protein kinases and lipolytic processes, such as bromo-cGMP and dibutyryl-cAMP, which are membrane-permeable cGMP and cAMP analogs. As shown in Table 4, anantin exhibited inhibitory effects at a concentration of 0.1  $\mu$ M, whatever the nature of the lipolytic agent. Indeed, for higher concentrations, the lipolysis returned to basal levels. No significant effect of A71915 was found using similar protocols (not shown).

The effects of S-28-Y on the lipolytic action of ANP are shown in Fig. 7. Since this drug acts as a partial agonist, its concentration-dependent effect was also evaluated against increasing concentrations of ANP. As expected for a partial agonist, S-28-Y partly blocked the effect of the full agonist ANP. The final lipolytic effect, with the concentrations used in the study, was equal to that obtained with S-28-Y alone, as seen in Table 3. cGMP is the second messenger generated after NPR-A activation in human fat cells. Bromo-cGMP increased lipolysis, its effect representing  $47.8 \pm 13.2\%$  of the maximal lipolytic effect of ANP (Sengenès et al., 2000). To assess whether the partial lipolytic effect of S-28-Y is linked to the activation of the NPR-A, we compared the increase of intracellular cGMP induced by 1  $\mu$ M ANP and S-28-Y. The kinetics of the processes were similar, and the effect of S-28-Y on cGMP production was about 50% that of ANP (Fig. 8). For example, after 60 min of incubation, spontaneous cGMP production was  $19 \pm 6$  pmol/100 mg of lipid. In the presence of ANP or S-28-Y, the cGMP concentrations were  $1229 \pm 580$  and  $531 \pm 270$  pmol/100 mg of lipid, respectively. HS-142-1 was a poor inhibitor of ANP-induced lipolysis. A 12% inhibitory effect (toward 0.1  $\mu$ M ANP) only appeared at a concentration of 100  $\mu$ g/ml (not shown).

TABLE 1

Effect of 2-h preincubation (or not) of isolated human fat cells with isoproterenol (0.1  $\mu\text{mol/l}$ ) or ANP (0.01  $\mu\text{mol/l}$ ) on the lipolytic response induced by isoproterenol or ANP

$EC_{50}$  and  $pD_2$  values were calculated from concentration-response curves of isoproterenol ( $10^{-10}$  to  $10^{-5}$  M) and of ANP ( $10^{-11}$  to  $10^{-6}$  M). Values are means  $\pm$  S.E.M. of six separate experiments.  $P$  values were obtained when comparing values obtained with preincubated cells with those from control cells.

	$EC_{50}$	$pD_2$
	<i>nmol/l</i>	
Control cells		
Isoproterenol	$14.2 \pm 0.50$	$8.02 \pm 0.2$
ANP	$0.62 \pm 0.32$	$9.45 \pm 0.20$
Cells preincubated with isoproterenol		
Isoproterenol	$70 \pm 0.22$ ( $P < 0.01$ )	$7.30 \pm 0.18$ ( $P < 0.002$ )
ANP	$1.5 \pm 0.82$ (NS)	$9.35 \pm 0.38$ (NS)
Cells preincubated with ANP		
Isoproterenol	$24 \pm 0.8$ (NS)	$7.79 \pm 0.18$ (NS)
ANP	$2.6 \pm 0.65$ ( $P < 0.05$ )	$8.69 \pm 0.15$ ( $P < 0.01$ )

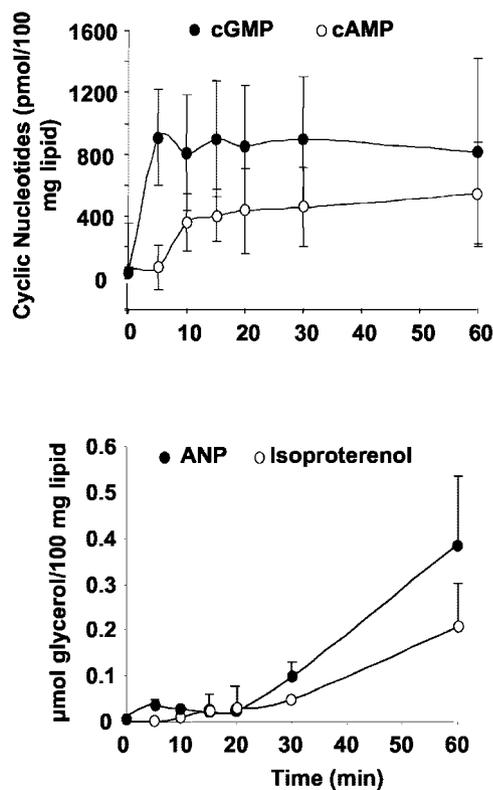


Fig. 4. Time course of the intracellular messengers produced by NPR-A and  $\beta$ -AR activation (cGMP and cAMP, respectively) and of lipolysis on isolated fat cells. ANP was used at 1  $\mu\text{M}$  and isoproterenol at 10  $\mu\text{M}$ . Values are means  $\pm$  S.E.M. of five separate experiments.

**Binding Studies.** ANP receptors were quantified on membrane preparations by saturation experiments using  $^{125}\text{I}$ -ANP as the ligand. In a previous study, we demonstrated that specific binding of  $^{125}\text{I}$ -ANP was saturable and of high affinity (i.e.,  $B_{\text{max}}$ ,  $400 \pm 38$  fmol/mg of protein;  $K_D$ ,  $72.9 \pm 16$  pmol/l). Nonspecific binding, defined in the presence of 1  $\mu\text{M}$  ANP, represented about 10% of the total radioactivity bound at equilibrium (Sengenès et al., 2002b). Competition curves are depicted in Fig. 9. The four compounds displaced 100% of bound  $^{125}\text{I}$ -ANP with equilibrium dissociation constants ( $IC_{50}$ ) of  $0.46 \pm 0.09$ ,  $83 \pm 40$ ,  $270 \pm 99$ , and  $350 \pm 150$  nM for ANP, A71915, S-28-Y, and anantin, respectively. HS-142-1 exerted a very weak competitive effect toward  $^{125}\text{I}$ -ANP binding. The drug did not displace  $^{125}\text{I}$ -ANP binding at concentrations from 0.1

to 10  $\mu\text{g/ml}$ . A 30 to 35% inhibition only occurred at high concentrations (100  $\mu\text{g/ml}$ , not shown).

## Discussion

We have demonstrated that natriuretic peptides (ANP and BNP) are potent activators of lipolysis in human fat cells (Sengenès et al., 2000, 2002b, 2003). They also exert a lipid-mobilizing effect when infused by the venous route in humans (Galitzky et al., 2001). The present study was carried out to assess the putative interactions between the pathways that control human fat cell lipolysis, namely ANP, catecholamines, and insulin. In physiological conditions, such as during physical exercise, the sympathetic nervous system is activated, releasing both epinephrine and norepinephrine (Kjaer et al., 1987). ANP output from the heart is simultaneously observed depending on exercise intensity (Ohba et al., 2001), whereas insulin release is usually reduced (Stich et al., 2000).

Neither the physiological or pathological roles of NPs in the control of lipid mobilization have actually been evaluated in humans due to the lack of relevant NPR-A antagonists. Thus, we investigated the ability of various, previously described natriuretic peptide antagonists to antagonize ANP-induced lipolysis in human fat cells. Some antagonist properties of these compounds have been previously assessed in different cells models (Zamir et al., 1995; Carvajal et al., 2001) or tissues (Von Geldern et al., 1990) using binding assays or cGMP production as relevant criteria. In a previous study, we characterized NPR-A binding sites on human fat cell membranes (Sengenès et al., 2000, 2002b) and demonstrated that the lipolytic activity of ANP involves plasma membrane NPR-A guanylyl cyclase activation followed by an increase in intracellular cGMP (Sengenès et al., 2000). These parameters were also used in the present study to define the antagonistic properties of the compounds.

The absence of interaction between the lipolytic pathways involving ANP and  $\beta$ -ARs is demonstrated. Strictly additive effects were observed between ANP- and isoproterenol-induced lipolysis at the lowest concentrations used (Fig. 1), whereas the maximal lipolytic effect of isoproterenol was not significantly amplified by ANP. The result suggests that the cGMP increment is largely in excess of that needed to maximally stimulate lipolysis. Considering the relationship between cAMP and lipolysis induced by catecholamines, the cAMP increment is also known to be largely in excess of that required to maximally stimulate lipolysis. These two inde-

TABLE 2

Effects of ANP, DNP, and mini-ANP on spontaneous basal lipolysis on isolated human fat cells

Values are expressed in percent variation of spontaneous basal lipolysis ( $0.185 \pm 0.052 \mu\text{mol glycerol}/100 \text{ mg lipid}/60 \text{ min}$ ) evaluated by glycerol concentration in the incubation medium. Values are means  $\pm$  S.E.M. of five separate experiments.

	log [Concentration]				
	M				
	10	9	8	7	6
ANP	63.2 $\pm$ 18*	125. $\pm$ 32*	228 $\pm$ 126*	289 $\pm$ 103*	295 $\pm$ 93*
DNP	194 $\pm$ 15*	244 $\pm$ 43*	293 $\pm$ 63*	331 $\pm$ 61*	322 $\pm$ 70*
Mini-ANP	58 $\pm$ 32*	92 $\pm$ 38*	298 $\pm$ 107	304 $\pm$ 78*	280 $\pm$ 90*

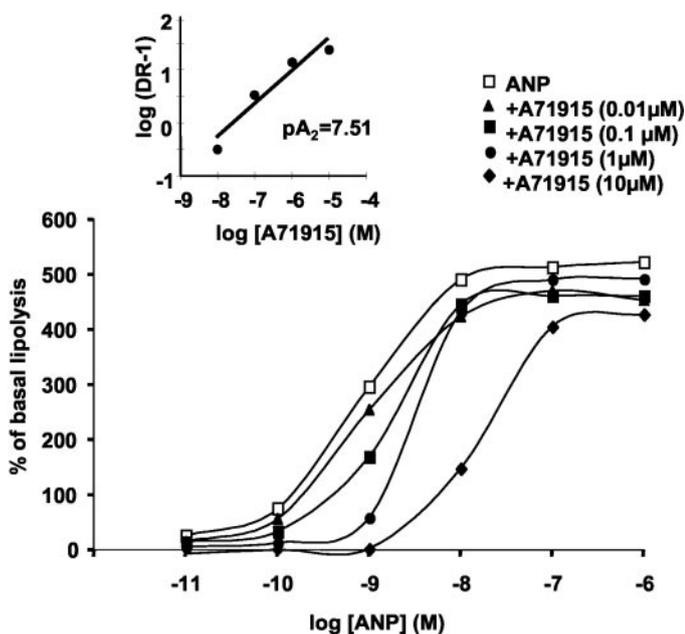
\* Significant when compared with basal lipolysis ( $P < 0.05$ ).

TABLE 3

Effects of ANP, A71915, anantin, and S-28-Y on spontaneous basal lipolysis in isolated human fat cells

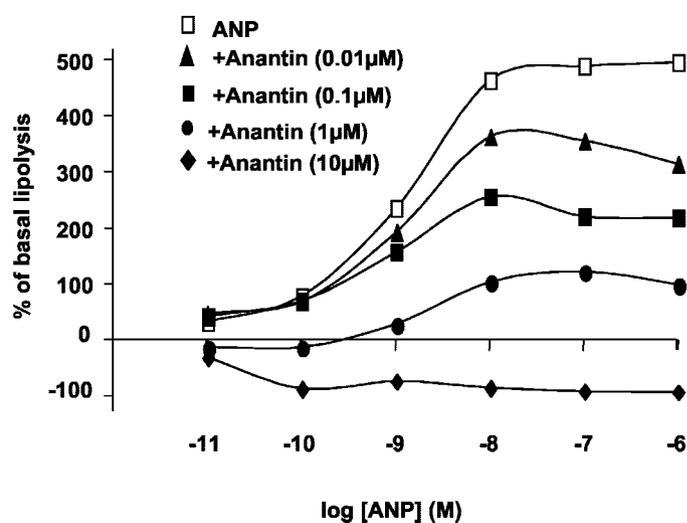
Values are expressed in percentage of variation of the spontaneous basal lipolysis ( $0.175 \pm 0.065 \mu\text{mol}/100 \text{ mg lipid}/60 \text{ min}$ ) evaluated by glycerol concentration in the incubation medium. Values are means  $\pm$  S.E.M. of five separate experiments.

	log [Concentration]				
	M				
	10	9	8	7	6
ANP	69 $\pm$ 10*	247 $\pm$ 84*	424 $\pm$ 174*	457 $\pm$ 183*	437 $\pm$ 184*
A71915	—	8.1 $\pm$ 2.6	5.2 $\pm$ 3.3	5.2 $\pm$ 3.2	-0.1 $\pm$ 4.3
Anantin	—	12 $\pm$ 9.8	-17 $\pm$ 24	-42 $\pm$ 29*	-85 $\pm$ 3*
S-28-Y	—	37 $\pm$ 9*	84 $\pm$ 29*	192 $\pm$ 84*	245 $\pm$ 103*

\* Significant when compared with spontaneous lipolysis ( $P < 0.05$ ).

**Fig. 5.** Antagonism of ANP-induced lipolysis by A71915 on human fat cells.  $n = 6$ . Lipolysis is expressed as a percentage of spontaneous glycerol production. Values are means of six separate experiments. S.E.M. was below 20% and omitted for clarity. The insert shows the corresponding Schild plot derived from the mean curves.

pendent pathways lead to the final phosphorylation/activation of hormone-sensitive lipase. The saturation of the response confirmed that this enzyme is the true rate-limiting step of lipolysis. In addition, the activation of the  $\alpha_2$ -AR-dependent antilipolytic pathway did not modify the lipolytic response initiated by ANP in human fat cells. These points are important since during some physiological situations, like physical activity, activation of both the sympathetic nervous system (release of norepinephrine and epinephrine) but also of ANP release (from the heart) occurs, which can con-



**Fig. 6.** Antagonism of ANP-induced lipolysis by anantin on human fat cells. Lipolysis is expressed as a percentage of spontaneous glycerol production. Values are means of five separate experiments. S.E.M. was below 20% and omitted for clarity.

tribute to the lipolytic response and lipid mobilization in vivo. This double contribution was suspected since, when using the in situ microdialysis method, part of the exercise-induced lipid mobilization was found to be resistant to the  $\beta$ -AR antagonist propranolol (Hellstrom et al., 1996). In addition, a number of studies have demonstrated that oral treatment with  $\beta$ -AR antagonists only partially suppressed the exercise-induced lipid mobilization (Bulow, 1981; Cosenzi et al., 1995). Finally, under efficient oral  $\beta$ -AR blockade, which initiates a strong ANP release during exercise (Berlin et al., 1993a,b), a recent study in our laboratory has shown that the lipid mobilization promoted by exercise in subcutaneous adipose tissue was related to the action of ANP (Moro et al., 2003).

Results reported in the present paper focus on the differ-

TABLE 4

Effect of anantin on lipolysis induced by 0.1  $\mu\text{mol/l}$  isoproterenol, 100  $\mu\text{mol/l}$  dibutyryl-cAMP (Db-cAMP), or 100  $\mu\text{mol/l}$  bromo-cGMP (Br-cGMP) on isolated human fat cells

Values are expressed in percentage of variation of the basal lipolysis evaluated by glycerol concentration in the incubation medium. Values are means  $\pm$  S.E.M. of four separate experiments.

	log [Anantin]					
	M					
	0	9	8	7	6	5
Isoproterenol	197 $\pm$ 35	199 $\pm$ 35	203 $\pm$ 34	189 $\pm$ 35*	-17 $\pm$ 2*	-98 $\pm$ 1*
Db-cAMP	98 $\pm$ 38	102 $\pm$ 56	97 $\pm$ 53	62 $\pm$ 25*	16 $\pm$ 3*	-89 $\pm$ 1*
Br-cGMP	42 $\pm$ 11	38 $\pm$ 10	26 $\pm$ 13*	20 $\pm$ 12*	-6 $\pm$ 211*	-99 $\pm$ 3*

\* Significant when compared with lipolysis induced by isoproterenol, Db-cAMP, and Br-cGMP alone ( $P < 0.05$ ).

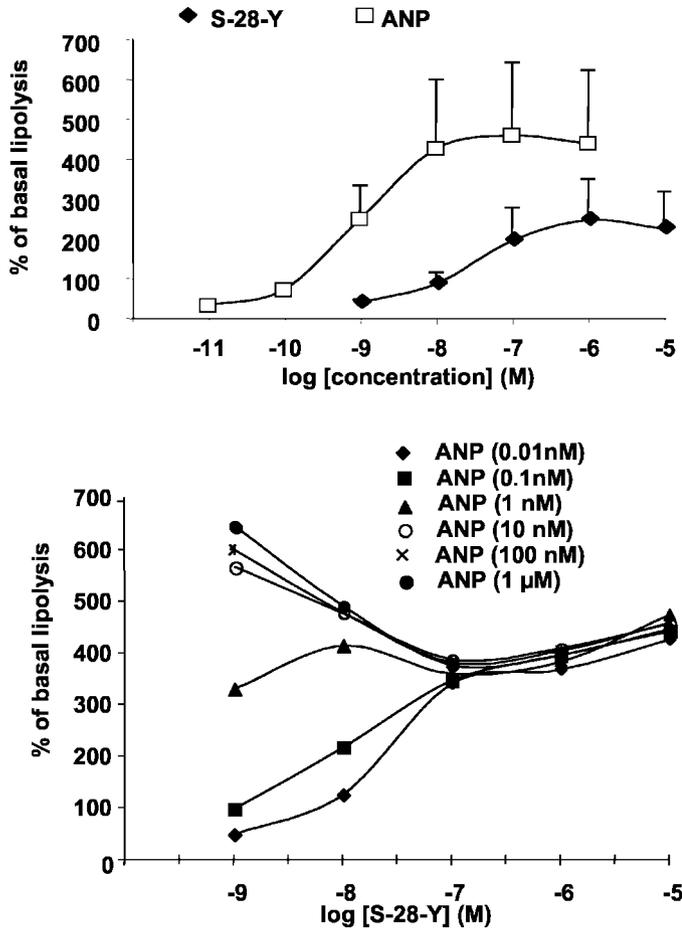


Fig. 7. A, comparative effect of increasing concentrations of S-28-Y and ANP on glycerol release in human fat cells. B, effect of increasing concentrations of S-28-Y on ANP-induced lipolysis. Lipolysis is expressed as a percentage of spontaneous glycerol production. Values are means of five separate experiments. S.E.M. was below 20% and omitted for clarity.

ences existing between both pathways. It was clearly shown that, as known for a long time for  $\beta$ -ARs, the ANP lipolytic pathway exhibits a homologous desensitization after a 2-h pretreatment of fat cells by ANP (Table 1). The desensitization phenomenon does not cross-react between the two pathways since the ANP response was unchanged by  $\beta$ -AR stimulation and conversely. This is a strong argument supporting the complete independence of the two pathways. Differences are also supported by the results obtained with insulin. Major differences exist in the interaction between insulin and the two lipolytic pathways. We previously observed that short-term exposure to insulin did not promote changes in

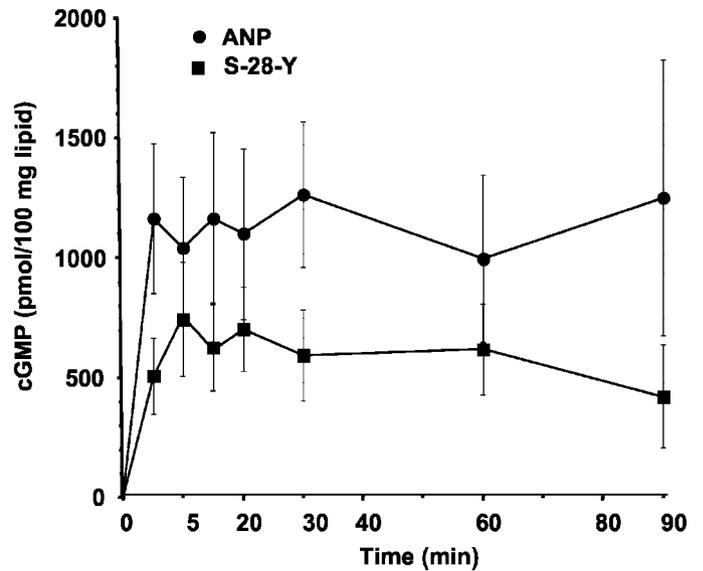


Fig. 8. Time course of 1  $\mu\text{M}$  ANP or S-28-Y on cGMP formation in isolated human fat cells. Values are means  $\pm$  S.E.M. of three separate experiments.

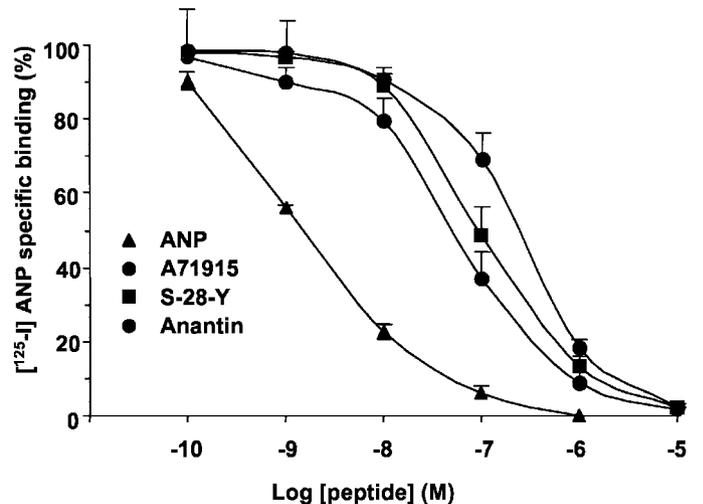


Fig. 9. Competition curves of human  $^{125}\text{I}$ -ANP binding on human fat cell membranes by increasing concentrations of ANP, A71915, S-28-Y, or anantin. Values are means  $\pm$  S.E.M. of five separate experiments.

ANP lipolytic effect in human fat cells (Sengenès et al., 2000). The present data confirm preliminary results and show that long-term exposure of fat cells to insulin dramatically affected the  $\beta$ -AR pathway and lipolysis but did not impair the

ANP pathway. Type 3B phosphodiesterase, the main enzyme involved in insulin action and degradation of cAMP in the adipocyte (Moberg et al., 1998), is not involved in the control of ANP-induced lipolysis.

The kinetics of the production of the intracellular messengers (cAMP and cGMP) was analyzed. The accumulation of these two compounds differs in the human fat cell, the increase in cGMP being faster and higher than that of cAMP. However, the kinetics of the increase in glycerol production was similar in both cases. This result suggests that the limiting effect in the full activation of lipolysis is distal to cGMP and cAMP production. It is well accepted that the final phosphorylation of perilipin and phosphorylation/activation of hormone-sensitive lipase are the main determinants of the overall lipolytic response (Lafontan and Berlan, 1995; Carey, 1998; Londos et al., 1999; Sengenès et al., 2003).

Concerning NPR agonists, DNP, a 38-amino acid peptide is a potent activator of NPR-A receptors and, like ANP and BNP, it induces relaxation and formation of the second messenger cGMP in the artery (Richards et al., 2002). Our results show that DNP is a more potent lipolytic activator in human fat cells since it exhibited a higher  $pD_2$  value than ANP ( $10.1 \pm 0.3$  versus  $8.9 \pm 0.1$ ). The mini-ANP, a smaller form of ANP (reduction of 28 residues to 15), had a similar potency to ANP in stimulating lipolysis.

The search for NPR-A antagonists has not been fully developed since blockade of NPR-A receptors has never been proposed for therapeutic purposes. The development of antagonists is of interest to clarify the physiological role of this lipolytic pathway in humans. Indeed, the use of  $\alpha_2$ -AR antagonists, using the microdialysis method, permitted the role of fat cell  $\alpha_2$ -ARs in pathophysiological situations to be assessed in man (Stich et al., 2000). Such an approach could be of interest in the NP field. The main goal of this study was to screen for a potent antagonist for further studies in humans. It could be useful to demonstrate that ANP released by the heart is physiologically involved in the control of lipid mobilization during exercise or in other physiological and pathological situations (Ogawa and Kazuwa, 1995; Kalra and Tigas, 2002). For these reasons, we investigated the action of various antagonists on ANP-induced glycerol production by human fat cells as well as on  $^{125}$ I-ANP-specific binding. A71915 was chosen among a family of ANP analogs shown to interfere strongly with ANP binding sites (assessed by binding studies) and to induce very weak cGMP production (Delporte et al., 1992). It appeared that A71915 had no effect by itself on basal glycerol production (Table 3) and competitively antagonized ANP-induced lipolysis (Fig. 5). The calculated  $pA_2$  value of 7.51 obtained on fat cells does not differ from the value reported in cells expressing a recombinant form of human NPR-A. The second natural compound studied (anantin, isolated from *Streptomyces coeruleus*) has previously been described as a competitive interactive drug on ANP binding sites from bovine adrenal cortex and inhibited the ANP-induced increase in intracellular cGMP in bovine aorta smooth muscle cells (Von Geldern et al., 1990). It can also reduce the increase in cGMP produced by ANP or BNP in pregnant guinea pig myometrium (Carvajal et al., 2001). In our hands, anantin potently counteracted the lipolytic effect of ANP, and the displacement of concentration-response curves suggested that it behaves as a noncompetitive antagonist although some inverse agonism cannot be completely

excluded (Fig. 6). Nevertheless, two other sets of experiments question inverse agonism, since they suggested a lack of specificity or possible toxicity of this compound in human fat cells. First, anantin exhibited a potent suppressive effect on spontaneous fat cell lipolysis (Table 3) and lipolysis stimulated by a  $\beta$ -AR agonist or with dibutyryl-cAMP (Table 4). The effect of bromo-cGMP (an analog of cGMP, the intracellular messenger linked to the NPR-A lipolytic pathway) was also blocked. Taken together these results suggest that anantin exhibits major and deleterious nonspecific (and even cytotoxic) effects on human fat cells in addition to its previously described antagonist property toward NPR-A receptors. This property seems to be specific to the human fat cell model, since it was not found in other previously used cell types. Indeed, anantin blocks the ANP-induced human sperm acrosome reaction without affecting the acrosomal exocytosis or sperm motility (Rotem et al., 1998).

The third compound tested was a synthetic, conformational, restricted analog of human NP containing L- and D-penicillamine in the place of cysteine residues at positions 7 and 23. This drug (named S-28-Y) displayed powerful binding potencies (100%) at NPR-A and induced low activity (3%) on cGMP accumulation in rat vascular smooth muscle cells when compared with ANP (Minamitake et al., 1990). In human fat cells, S-28-Y exhibited a clear partial agonist effect (around 50%) when compared with ANP (Table 1) with a lower  $pD_2$  value ( $7.38 \pm 0.25$ ) than ANP ( $9.40 \pm 0.31$ ). In addition, S-28-Y partially increased cGMP accumulation in the adipocyte. In theory, partial agonists are defined as drugs that produce a submaximal tissue response and that could competitively block the effects of agonists with high intrinsic efficacies. We showed that S-28-Y exhibits partial antagonist potency toward the lipolytic effect of ANP on fat cells (Fig. 7), and that increasing lipolytic effects of ANP were progressively impaired by the increase in S-28-Y concentrations. HS-142-1 is a microbial polysaccharide different from natural agonists of NPR. HS-142-1 is an antagonist of NPR-A and NPR-B and also inhibited the production of cGMP in cells containing these receptors (Poirier et al., 2002). It has been suggested that because of its structure, HS-142-1 is more likely an allotropic antagonist than a competitive one (Poirier et al., 2002) which binds to a site different from the binding domain of the endogenous agonist. We found that HS-142-1 exerts a very weak competitive effect toward ANP; a 30 to 35% inhibition of  $^{125}$ I-ANP binding occurred only at high concentrations (100  $\mu$ g/ml) with a weak incidence on ANP-induced lipolysis.

In summary, our data show that the two main pathways controlling human fat cell lipolysis, i.e., the well known pathway mediated by  $\beta$ - and  $\alpha_2$ -ARs and activated by catecholamines and the recently discovered ANP-dependent pathway, do not interact. We also demonstrated that insulin was unable to modulate the lipolytic effect of ANP, even after relatively long-term treatment. Hyperinsulinemia impairs  $\beta$ -AR-dependent lipid-mobilizing effects in humans (Stich et al., 2003). Resistance to the antilipolytic action of insulin is an important characteristic of the ANP pathway that could have major pathophysiological importance. Dysregulation of natriuretic peptide production, as occurring in cardiac diseases, will lead to chronic activation of lipolysis without counteraction by insulin and generate lipid disorders. Homologous desensitization revealed by the in vitro studies could

have relevance if they occur in vivo and could limit excessive ANP-related lipid mobilization. Moreover, the potential contribution of NPs in the lipid mobilization of human subcutaneous adipose tissue could have physiological repercussions (Bulow, 1981). It has been shown that exercise-induced glycerol output from umbilical enervated fat deposits in paraplegic spinal cord injured subjects (injury level T3-T5) remained as high as in clavicular fat deposits (Stallknecht et al., 2001). The authors concluded that sympathetic nerve activity is not so important for the exercise-induced increase in subcutaneous adipose tissue lipolysis without proposing an alternative pathway; NPs could be reasonable candidates. We recently demonstrated that a part of exercise-induced lipolysis in subcutaneous adipose tissue (50%) was not related to catecholamines and that under efficient oral  $\beta$ -AR blockade, which initiates strong ANP release, the lipid mobilization promoted in subcutaneous adipose tissue by exercise was essentially related to ANP action. This observation reveals the physiological relevance of the ANP pathway (Moro et al., 2003). Thus, the use of selective antagonists of fat cell NPR-A is a fundamental tool to clearly assess the role of NP in the control of lipolysis and lipid mobilization in various physiological or pathological situations. The present results show that A71915 is the only good candidate available to date. Nevertheless, its low  $pA_2$  value of A71915 could limit its local use in investigations based on the microdialysis method in man. At the present time, since no therapeutic applications have emerged in humans in the natriuretic peptide field, the development of NPR-A antagonists for human applications has been poorly investigated.

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