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Differential regulation of atrial natriuretic peptide– and adrenergic receptor–dependent lipolytic pathways in human adipose tissue

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Abstract

The aim of the study was to investigate the regulation affecting the recently described atrial natriuretic peptide (ANP)–dependent lipolytic pathway in comparison with the adrenergic lipolytic cascade. We studied in vivo the effect of a euglycemic-hyperinsulinemic clamp on the changes occurring in the extracellular glycerol concentration (EGC) of subcutaneous adipose tissue (SCAT) during ANP or epinephrine perfusion in a microdialysis probe. Homologous desensitization and the incidence of hyperinsulinemia on the ANP- and catecholaminergic-dependent control of lipolysis were also investigated in vitro on fat cells from SCAT.

When perfused in SCAT, epinephrine and ANP promoted an increase in EGC; the EGC increase was significantly lower during the clamp. The reduction of epinephrine-induced lipolysis was limited (18%) when phentolamine (an α_2-adrenergic receptor [AR] antagonist) was perfused together with epinephrine. Unlike the effect of epinephrine, the response to ANP observed during the second perfusion was reduced by 32%. The increase in extracellular guanosine 3′,5′-cyclic monophosphate concentration, which reflects ANP activity, was also reduced during the second perfusion. Desensitization of the lipolytic effects of ANP was observed in vitro after a 2-hour period of recovery, while the effects of a β-AR agonist or of epinephrine were unchanged. Insulin was without any effect on ANP-induced lipolysis and α_2-AR–mediated antilipolysis, while it reduced β-AR–induced lipolysis.

The ANP-dependent lipolytic pathway undergoes desensitization in vitro and in situ. Insulin had no inhibitory effect on either ANP- or α_2-AR–dependent pathways, while it counteracted the β-AR pathway.

1. Introduction

Regulation of human adipose tissue lipolysis by catecholamines is related to their action on fat cells through plasma membrane adrenergic receptors (ARs). Catecholamines activate lipolysis through β-ARs and inhibit it through α_2-ARs. The coordinated stimulation of both receptors modulates intracellular cyclic adenosine monophosphate (cAMP) levels, which enable the activation of a cAMP-dependent protein kinase, resulting in the phosphorylation and activation of hormone-sensitive lipase (HSL) [1]. We reported the existence of a novel lipolytic pathway in human fat cells that involves the cardiac-derived peptide hormones, atrial natriuretic peptide (ANP) and brain natriuretic peptide. Natriuretic peptides (NPs) act through a fat cell plasma membrane receptor (NPR-A subtype) bearing an intrinsic guanylyl cyclase activity and operate through a guanosine 3′,5′-cyclic monophosphate (cGMP)–dependent pathway [2,3]. Natriuretic peptides are potent lipolytic agents on isolated fat cells from subcutaneous adipose tissue (SCAT), specifically in primates [4]. Intravenous infusion of human ANP in human beings promotes a strong lipid-mobilizing effect independently of sympathetic nervous system activation [5]. A

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similar response occurred when ANP was perfused through a microdialysis probe inserted in the subjects' SCAT [6,7].

Various in vitro and in vivo studies have shown that catecholamines induce desensitization of adipose tissue lipolysis in human beings. Desensitization of catecholamine-induced lipolysis after prior β-adrenergic agonist stimulation has been reported in human fat cells. Catecholamine infusion or aerobic exercise induces desensitization of the β-AR–dependent lipolytic pathway [8,9]. Putative changes in ANP-dependent lipolytic effects have never been reported in human beings until now.

Insulin, the main antilipolytic hormone in human beings, can modulate lipid mobilization in adipose tissue and plasma nonesterified fatty acid availability in physiological states such as starvation or during postprandial periods [10]. Inhibition of lipolysis by insulin involves the following 2 mechanisms: (1) the activation of a fat cell–specific phosphodiesterase-3B (PDE-3B), which hydrolyzes cAMP [11], and (2) desensitization of the β-AR–dependent responses [12]. A lower phosphorylation state of the HSL, resulting from a decrease in the cAMP-dependent protein kinase activity, has also been mentioned. Moreover, it was suggested that insulin could promote dephosphorylation of both the basal and regulatory sites of HSL through a cAMP-independent pathway, related to activation of a protein phosphatase [13]. In a previous investigation, using isolated human fat cells, we have shown that the activation of the fat cell–specific PDE-3B by short-term exposure to insulin leads to an inhibition of isoproterenol-induced lipolysis, while the lipolytic effect of ANP is preserved [6].

It is unknown if ANP-dependent effects undergo desensitization and are resistant to insulin. An in vivo comparison was made after sequential epinephrine and ANP infusion in a microdialysis probe implanted in SCAT. Using the same time course of treatment, we performed a comparative study of ANP- and isoproterenol-induced desensitization after a 1-hour treatment with agonists and a 2-hour washout on human fat cells in vitro. Finally, because the incidence of long-term insulin treatment on ANP-dependent lipolytic pathways is unknown, exposure of human fat cells to insulin was performed in vitro for 1 hour. Subsequently, the lipolytic effect of ANP was evaluated and that of catecholamines was used as a reference. In an in vivo study performed on healthy men and using the microdialysis method, we evaluated the effect of hyperinsulinemia (experimental euglycemic-hyperinsulinemic clamp) on the in situ response of SCAT to a perfusion of ANP or epinephrine.

2. Materials and methods

2.1. In vivo studies

2.1.1. Subjects

Eight healthy young men aged 22.7 ± 1.9 years (range, 21-27 years), with a mean body mass index of 23.7 ± 1.8 kg/m^2 (range, 20.7-25.7 kg/m^2), who had not been submitted to any pharmacological or nutritional protocol before the study were recruited. All had a stable weight during the previous 3 months. Selection of the subjects was based on a screening evaluation of detailed medical history, a physical examination, and several blood chemistry analyses. The Ethical Committee of the Third Faculty of Medicine of Charles University approved the study. All the subjects gave their informed consent for the experimental conditions after detailed explanation. The investigations were performed in the Diabetes Center of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic.

2.1.2. Experimental protocol

The subjects entered the hospital at 8:00 AM after an overnight fast and were maintained in the supine position during the experimental period. An indwelling polyethylene catheter was inserted into the antecubital vein of each arm. At 8:30 AM, microdialysis probes (Carnegie Medicine, Stockholm, Sweden) of 20×0.5 mm and 20 000-MW cutoff were inserted percutaneously after epidermal anesthesia (200 μL of 1% lidocaine, Roger-Bellon, Neuilly-sur-Seine, France) into the SCAT at a distance of 10 cm immediately to the right of the umbilicus. The probes were connected to a microperfusion pump (Harvard Apparatus, SARL, Les Ulis, France) and perfused with Ringer's solution (139 mmol/L sodium, 2.7 mmol/L potassium, 0.9 mmol/L calcium, 140.5 mmol/L chloride, 2.4 mmol/L bicarbonate, 5.6 mmol/L glucose). Ethanol (1.7 g/L) was added to the perfusate to estimate changes in the blood flow, as previously described [14]. After a 30-minute equilibration period, a 30-minute fraction of dialysate was collected at a flow rate of 0.5 μL/min. Then, the perfusion flow rate was set at 2.5 μL/min for the remaining experimental period. The calibration procedure using various perfusion rates was applied for interstitial glycerol concentration determination in SCAT as previously described by our group [15,16]. This simplified but relevant and less time-consuming method was selected in this study. The estimated extracellular glycerol concentration (EGC) was calculated by plotting (after log transformation) the concentration of glycerol in the dialysate measured at 0.5 and 2.5 μL/min against the perfusion rates. The EGC found in the present study fits with previous determinations performed in lean subjects [17,18].

2.1.3. Control study

After calibration of the probe, a 30-minute fraction of the outgoing dialysate was collected in all probes. Thereafter, one probe was perfused with 10 μmol/L of ANP for 60 minutes. The second probe was perfused with epinephrine (10 μmol/L) for 60 minutes, and the third probe was perfused with epinephrine in combination with 100 μmol/L of phentolamine (an $\alpha_{1/2}$-adrenergic antagonist). At the end of the above-mentioned perfusions, the probes were perfused with Ringer's solution for 2 hours; 30 minutes before the second perfusion, a 30-minute fraction of the outgoing dialysate was collected in all probes. Then, the 3

probes were perfused with the same concentrations of ANP, epinephrine, or epinephrine in combination with phentolamine, respectively, for 60 minutes.

2.1.4. Euglycemic-hyperinsulinemic clamp study

In the second investigation, the 3 probes were perfused with the same concentrations of ANP, epinephrine, or epinephrine in combination with phentolamine for 60 minutes; then, a euglycemic-hyperinsulinemic clamp was started at the end of the above-mentioned drug perfusion in the probes and performed for 3 hours. During the third hour of the clamp, the perfusions using ANP, epinephrine, and epinephrine plus phentolamine were performed, with concentrations identical to those used before the clamp. Thirty minutes before the second perfusion, a 30-minute fraction of the outgoing dialysate was collected in all probes.

2.1.5. Euglycemic-hyperinsulinemic clamp

The euglycemic-hyperinsulinemic clamp was performed using the method of De Fronzo et al [19]. A catheter for insulin and glucose infusion was inserted into an antecubital vein, and a second catheter for blood sampling was placed in a dorsal vein of the contralateral hand. The hand was kept in a warm box (60°C) to provide arterialization of venous blood. Priming plus continuous infusion of crystalline human insulin (Actrapid Human, Novo, A/S, Bagsvaerd, Denmark), 1 $mU \cdot kg^{-1}$ min^{-1}, was given for 3 hours. Euglycemia, the fasting blood glucose concentration, was maintained by a variable 15% glucose perfusion. The perfusion rate was determined by measurements of arterialized plasma glucose (Beckman Glucose Analyzer, Beckman Instruments Inc, Fullerton, Calif) every 5 minutes. Plasma concentrations of glucose, glycerol, and free insulin were analyzed in the basal state (mean value of 3 samples obtained 70, 65, and 60 minutes before the start of the clamp) and every 60 minutes during the clamp.

2.2. In vitro studies

2.2.1. Subjects

Human SCAT was obtained from 6 normal-weight or moderately overweight women undergoing plastic surgery. Their mean age was 41.7 ± 3.1 years, and their mean body mass index was 24.4 ± 1.3 kg/m^2. The investigation respected the guidelines of the Ethical Committee of the Toulouse University Hospital.

2.2.2. Adipocyte isolation

Isolated adipocytes were obtained, as previously described by Rodbell [20], using collagenase (0.5 mg/mL) digestion of adipose tissue fragments (2-3 g) in Krebs-Ringer bicarbonate buffer containing 10 mmol/L of HEPES, 2% bovine serum albumin, and 6 mmol/L of glucose at pH 7.4 (KRBHA) under shaking at around 200 cycles/min at 37°C. Adipocytes were filtered through a silk screen (250 μm) and washed 3 times with KRBHA buffer to eliminate collagenase.

2.2.3. Experimental protocols

2.2.3.1. Effect of ANP and isoproterenol treatment on ANP- and isoproterenol-induced lipolysis. Using a similar experimental procedure, 500-μL samples of packed isolated fat cells diluted in 3 mL KRBHA buffer were exposed to 1 μmol/L of ANP or 10 μmol/L of isoproterenol for 1 hour at 37°C. Then, adipocytes were washed 3 times to eliminate the drugs and maintained for 2 hours in fresh incubation buffer at 37°C. At the end of this washout period, the cells (2000-3000 cells/assay) were incubated with 5 μL of increasing concentrations of ANP or isoproterenol in a final volume of 100 μL for 90 minutes at 37°C under gentle shaking at 120 cycles/min. Control cells were treated similarly in the absence of any agent.

2.2.3.2. Effect of insulin pretreatment on ANP- and adrenergic-receptor–dependent lipolytic and antilipolytic pathways. Packed isolated fat cells (500 μL) were diluted in 3 mL of KRBHA buffer with (or without) 1 μmol/L of human insulin and incubated for 1 hour at 37°C under gentle shaking at 120 cycles/min. The adipocytes were washed 3 times with the same buffer and immediately brought to a suitable dilution (2000-3000 cells/assay) in fresh KRBHA buffer for lipolysis assays. They were incubated with 5 μL of pharmacological agents at the indicated concentrations in a final volume of 100 μL for 90 minutes at 37°C under gentle shaking at 120 cycles/min.

For both protocols, 30-μL aliquots of the infranatant were taken at the end of the incubation period for determination of glycerol (used as a lipolytic index). Total lipid content was determined gravimetrically after solvent extraction.

2.2.4. Drugs and biochemical determinations

The following drugs and chemicals were kindly provided by or obtained from the sources indicated: the nonselective β-AR agonist isoproterenol hydrochloride, the α_2-AR agonist UK14304, epinephrine bitartrate, bovine serum albumin (fraction V), collagenase, human insulin, and forskolin were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Enzymes for glycerol assays and adenosine deaminase (ADA) were from Roche Diagnostics (Meylan, France). The selective α_{2A}-AR antagonist RX821002 was a gift from Reckitt and Colman (Kingston Upon Hull, UK). Phentolamine methanesulfonate (Regitine) was from Avantis (Rueil-Malmaison, France), and human α-ANP (1-28), from Neosystem Laboratories (Strasbourg, France).

Ethanol in dialysate and perfusate was determined with an enzymatic method [21]. Glycerol was determined in plasma and in dialysate using an ultrasensitive radiometric method [22]. Plasma glucose was determined with a glucose oxidase technique (Biotrol, Paris, France), and plasma free fatty acids were assayed with an enzymatic method (Unipath, Dardilly, France). Plasma insulin was measured using a radioimmunoassay kit, Irma (Immunotech,

Prague, Czech Republic). Extracellular cGMP concentration was determined using a radioimmunoassay kit (Cayman Chemical Company, Ann Arbor, Mich), the detection limit was 4 pmol/mL and intra-assay variability was less than 5%.

2.2.5. Statistics

All the values are given as means ± SEM. A statistical comparison of the values was performed using 2-way analysis of variance for repeated measures with the experimental conditions (control vs euglycemic-hyperinsulinemic clamp) as factors of the analysis. Subsequently, the effects of drug perfusions were analyzed in each experimental condition using 2-way analysis of variance with time as the factor of the analysis and were followed by a Bonferroni-Dunett post hoc test. Values were considered statistically significant when $P < .05$. Statistical analyses were performed using software packages (Statview 4.5 and Supernova 1.11, Abacus Concepts Inc, Berkeley, CA). Paired t test was used for statistical evaluation of in vitro studies.

3. Results

3.1. In vivo studies

3.1.1. Plasma determinations

The fasting free insulin concentration in plasma was 5.9 ± 1.3 mU/L. Insulin perfusion induced a steady-state insulin level of 79.5 ± 1.9 mU/L, which remained stable during the time of the clamp, with average individual insulin level changes being 4.8% ± 2.1%. The fasting arterial plasma glucose level was 4.22 ± 0.05 mmol/L. The subjects were clamped at their individual fasting glucose levels. The coefficient change for the glucose levels during the third hour was 4.14% ± 0.39%. The glucose disposal rate was calculated from the exogenous glucose infusion rates during the third hour of the clamp, and the value was 9.97 ± 0.48 $mg \cdot kg^{-1}\ min^{-1}$.

3.1.2. Control and euglycemic-hyperinsulinemic clamp studies on the lipid-mobilizing effect of ANP

In the control study (without euglycemic-hyperinsulinemic clamp), EGC values rose from 164 ± 17 to 312 ± 39 μmol/L during the first ANP perfusion. During the second ANP perfusion period (performed after 2 hours of recovery with Ringer's infusion in the probes), ANP promoted a significantly lower EGC increase (from 132 ± 11 to 214 ± 30 μmol/L). The lipid-mobilizing effect of ANP was significantly reduced by 44% ± 3.3% (Fig. 1A). To assess whether the reduction of ANP-induced lipolysis was linked to changes affecting the ANP lipolytic pathway, the amount of cGMP released before and during each perfusion in dialysate was determined. Basal extracellular cGMP concentrations obtained before each ANP perfusion were 5.8 ± 2.5 and 6.2 ± 3.1 pmol/mL, respectively. The first ANP perfusion induced a rise in extracellular cGMP levels to 42.6 ± 5.0 pmol/mL, $P < .001$. The second ANP perfusion induced a lower increase in extracellular cGMP concentration (19.4 ± 3.4 pmol/mL; $P < .001$). Atrial natriuretic peptide–induced cGMP increment in the extracellular fluid was significantly reduced by 54% compared with that observed during the first perfusion ($P < .005$).

Before the euglycemic-hyperinsulinemic clamp and during the 60-minute ANP perfusion, EGC induced an increase in EGC values similar to those of the previous control study (171 ± 28 to 340 ± 71 μmol/L). The euglycemic-hyperinsulinemic clamp was started at the end of the first ANP perfusion. Another ANP perfusion was performed after 2 hours of clamp. Basal EGC values were 96 ± 16 μmol/L and rose to 188 ± 29 μmol/L during ANP perfusion (Fig. 1B). The glycerol increase was significantly different when compared with values measured before the

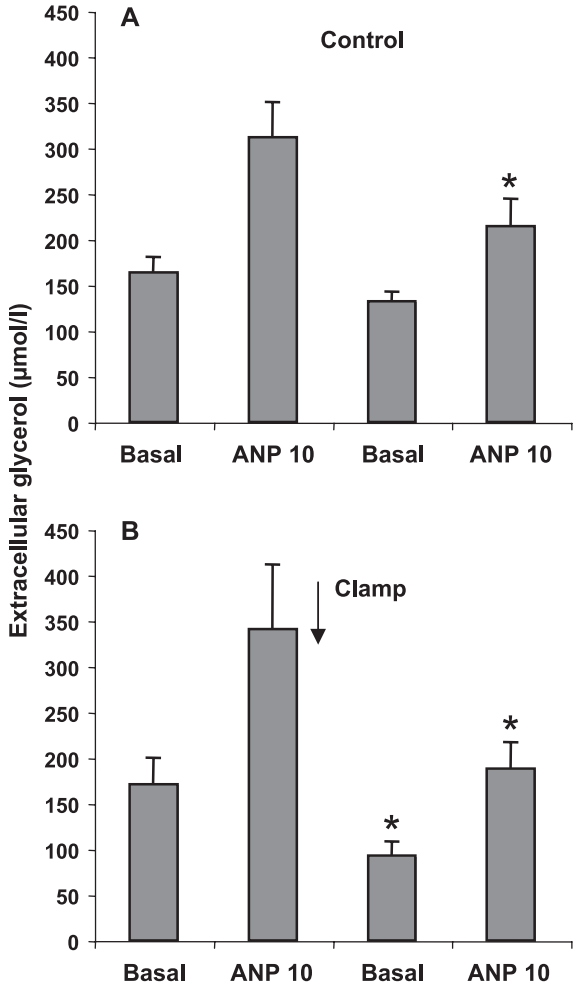

Fig. 1. Lipolytic effect of 2 successive perfusions of ANP on EGC in subcutaneous abdominal adipose tissue (A) during the control study and (B) during the euglycemic-hyperinsulinemic clamp. Atrial natriuretic peptide was applied through the microdialysis probes for 60 minutes. The successive ANP perfusions were separated by 2 hours. Values are means ± SEM of 8 separate experiments. The asterisk indicates $P < .05$ when compared with basal and ANP values obtained during the first perfusion.

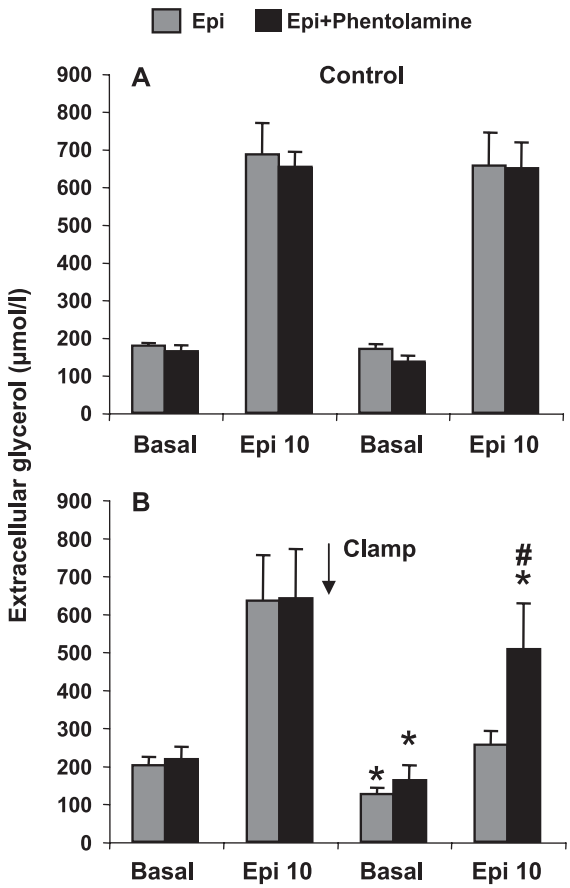

Fig. 2. Lipolytic effect of 2 successive perfusions of 10 μmol/L of epinephrine (Epi 10) alone or associated with 100 μmol/L of phentolamine on EGC in subcutaneous abdominal adipose tissue (A) during the control study and (B) during the euglycemic-hyperinsulinemic clamp. Epinephrine perfusion was applied through the microdialysis probes for 60 minutes. The epinephrine perfusion was separated by 2 hours. Values are means ± SEM of 8 separate experiments. Asterisk indicates $P < .05$ when compared with basal and epinephrine values obtained during the first perfusion; number sign, $P < .05$ when compared with values obtained with epinephrine alone.

clamp. From the calculated increment value (representing the EGC changes during the perfusion), it appeared that the reduction of the lipolytic effect of ANP during hyperinsulinemia was significantly reduced (43% ± 2.5%) to a value that was not different from that found during the control study.

3.1.3. Control and euglycemic-hyperinsulinemic clamp studies of the lipid-mobilizing effect of epinephrine

In the control study, the first epinephrine perfusion (10 μmol/L) promoted an increase in EGC values from 179 ± 8 to 685 ± 83 μmol/L; during the second perfusion (initiated after a 2-hour recovery period), a similar increase in EGC value was again observed, from 169 ± 13 to 655 ± 90 μmol/L (Fig. 2A). The increase in EGC during the first perfusion with epinephrine (plus phentolamine) was similar to that seen without phentolamine (from 164 ± 16 to 653 ± 40 μmol/L). During the second perfusion period, a similar increase in EGC values was again observed, from 136 ±16 to 649 ± 68 μmol/L (Fig. 2A).

Before the euglycemic-hyperinsulinemic clamp, 1-hour perfusion of epinephrine (10 μmol/L) induced an increase in EGC similar to that of the control study (201 ± 22 to 635 ± 118 μmol/L; Fig. 2B). At the end of the perfusion of epinephrine, the euglycemic-hyperinsulinemic clamp was started. Epinephrine perfusion performed during the third hour of the clamp induced an EGC increase that was dramatically lower than that before the clamp. The basal EGC value was 124 ± 16 μmol/L, and the value measured during epinephrine perfusion was 255 ± 37 μmol/L. The effect of epinephrine was significantly reduced by 70%. Perfusion of epinephrine (10 μmol/L) in combination with phentolamine (100 μmol/L) promoted an increase in EGC values similar to that of the control study (217 ± 32 to 639 ± 129 μmol/L; Fig. 2B). A similar perfusion performed during the third hour of the insulin clamp induced an increase in EGC values that was significantly lower than those obtained before the clamp (from 162 ± 39 to 507 ± 120 μmol/L). The effect of 10 μmol/L of epinephrine (plus phentolamine) perfusion was reduced by only 18% during the clamp.

3.1.4. Modifications of local adipose tissue blood flow

The changes in the local adipose tissue microcirculation were evaluated by using the validated ethanol escape method [14]. The ethanol ratio was calculated and taken as an index of ethanol washout. A high ethanol ratio corresponds to low ethanol washout, and this reflects low regional adipose tissue blood flow (ATBF). The average ethanol ratios measured during the period of ANP or catecholamine perfusion are depicted in Fig. 3. Atrial natriuretic peptide perfusion induced a significant increase in blood flow (decrease in outflow-inflow ratios) before and during the euglycemic-hyperinsulinemic clamp. The effect of ANP on blood flow changes was similar in the control and insulin clamp studies. Epinephrine alone tended to induce an increase in the ethanol ratio, but the increase did not reach a significant level. No difference was found between the control study and the euglycemic-hyperinsulinemic clamp study. In the study where epinephrine perfusion was combined with phentolamine, a decrease in ethanol ratio was observed, but no significant difference was found between the control and the euglycemic-hyperinsulinemic clamp study. The relevance of these in vivo observations was questioned and investigated in vitro.

3.2. In vitro studies

3.2.1. Effect pretreatment with ANP and isoproterenol on fat cell lipolysis

To explore if homologous desensitization could occur, isolated fat cells were exposed for 1 hour to 1 μmol/L of ANP or 10 μmol/L of isoproterenol. Control cells were maintained without any drug. After a 2-hour recovery period (after washing the cells to remove agonists and incubation in fresh buffer), a concentration-response study

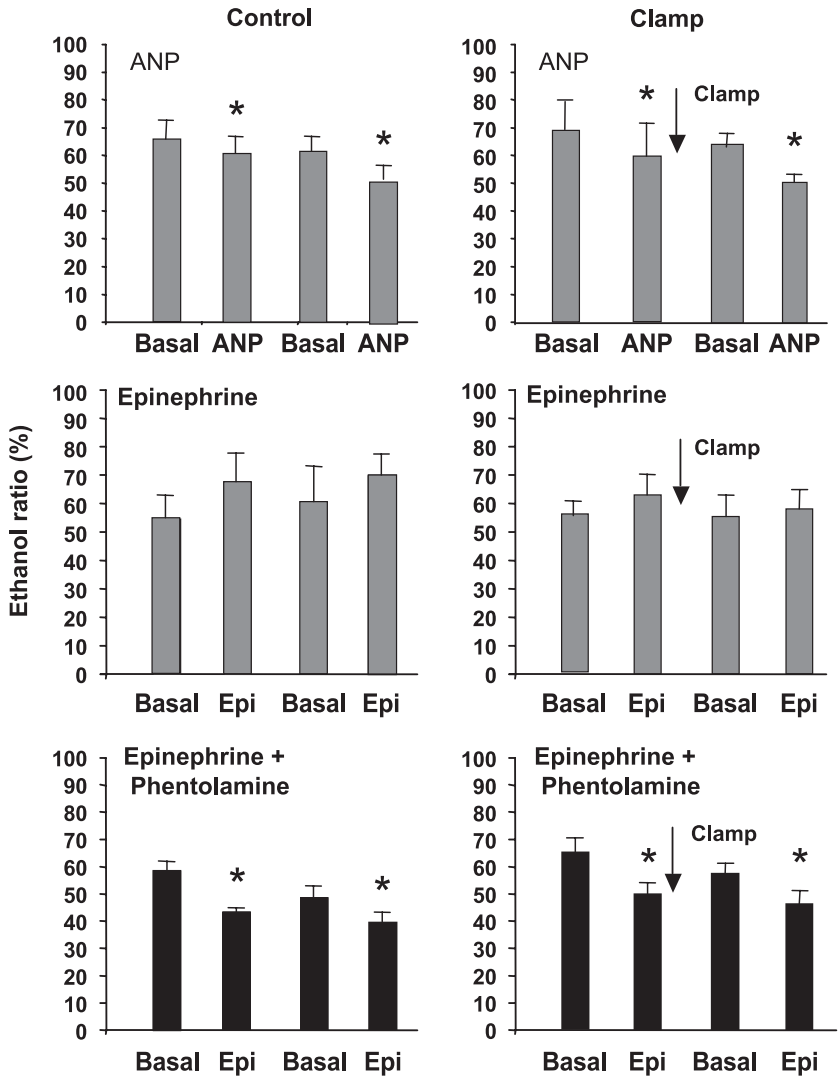

Fig. 3. Change in ethanol outflow-inflow ratio in SCAT during the control study or during the euglycemic-hyperinsulinemic clamp. Upper part, Effects of 2 successive perfusions of ANP (10 μmol/L). Middle part, Effects of 2 successive perfusions of 10 μmol/L of epinephrine (Epi 10). Lower part, Effects of 2 successive perfusions of epinephrine plus 100 μmol/L of phentolamine. Values are means ± SEM of 8 separate experiments. The asterisk indicates $P < .05$ when compared with values obtained before perfusion of the drug.

was performed with both agonists. Preexposure to ANP (Fig. 4A) was followed by a decrease in maximal ANP-induced lipolysis compared with the control cells ($P < .05$). Moreover, median effective concentration values were significantly lower for ANP-pretreated than control fat cells (5.6 ± 1.8 and 1.2 ± 0.3 nmol/L, respectively; $P < .04$). In contrast, the preexposure to isoproterenol (Fig. 4B) followed by a 2-hour recovery period was not associated with a decrease in the lipolytic response to isoproterenol. The median effective concentration values were 60 ± 39 and 51 ± 16 nmol/L for control and isoproterenol-pretreated cells, respectively (not significant). Because short-term desensitization of β-adrenergic responses has previously been reported in human fat cells, it is probable that the isoproterenol effect was recovered during the 2-hour washout period. Desensitization was stronger for ANP, and full recovery was absent.

3.2.2. Effect of insulin pretreatment on the lipolytic response to ANP and AR agonists on fat cells

Basal lipolysis (expressed as micromoles of glycerol released into the medium for 90-minute incubation per 100 mg lipid) was 0.07 ± 0.01 and 0.08 ± 0.01 in control cells and in cells treated for 1 hour with 1 μmol/L of insulin, respectively. In both cases, the addition of 4 μg/mL of ADA, which hydrolyzes adenosine and prevents the stimulation of the antilipolytic adenosine receptors, weakly increased spontaneous glycerol release (0.12 ± 0.05 and 0.13 ± 0.04). Forskolin (100 μmol/L), an activator of adenylyl cyclase, strongly stimulated lipolysis (0.39 ± 0.12 and 0.40 ± 0.11).

The results are depicted in Fig. 5. Insulin pretreatment did not modify the lipolytic effect of ANP (1 nmol/L). The antilipolytic effect of UK14304 (a selective α_2-AR agonist), evaluated on fat cells incubated with ADA or with forskolin,

was also unaffected by insulin. As expected, an increase in lipolysis was observed on human fat cells stimulated by submaximal concentrations of catecholamines (ie, isoproterenol at 1 μmol/L and epinephrine at 10 μmol/L). The weaker increase in the lipolytic effect of epinephrine is linked to the simultaneous activation of the lipolytic β-AR and of the antilipolytic α_2-AR [23]. This point is assessed by the potentiation of the effect of epinephrine after blockade of the α_2-AR by the selective α_2-AR antagonist RX821002 [24,25]. Fig. 5 also shows the effect of insulin on these responses. As expected, the lipolytic response of isolated adipocytes to isoproterenol (1 μmol/L) was significantly reduced in terms of maximal effect (29%) after pretreatment with insulin. In the same experiment, the reduction of the effect of epinephrine (10 μmol/L) by insulin pretreatment was stronger (56%). However, this strong inhibitory effect of insulin on epinephrine-induced lipolysis was partially reversed by the addition of RX821002 (100 μmol/L), which leads to an insulin effect quite similar to that observed with isoproterenol (26%).

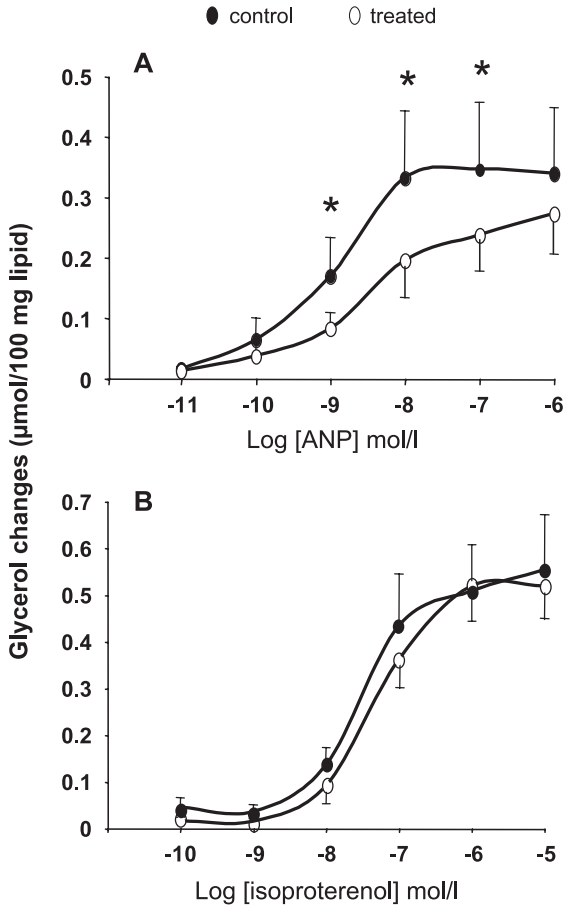

Fig. 4. Lipolytic responsiveness of human fat cells treated 1 hour with (A) 1 μmol/L of ANP and (B) 10 μmol/L of isoproterenol and followed by a 2-hour recovery period. After 1 hour of incubation with the drugs, fat cells were washed 3 times to eliminate isoproterenol or ANP. Finally, the lipolytic assays using concentration-response curves of ANP and isoproterenol were performed after a 2-hour recovery period (incubation of fat cells in fresh buffer without any drug). Control cells were treated similarly in the absence of any agent. Values are means ± SEM of 6 separate experiments. The asterisk indicates $P < .05$ when compared with control values using the paired t test.

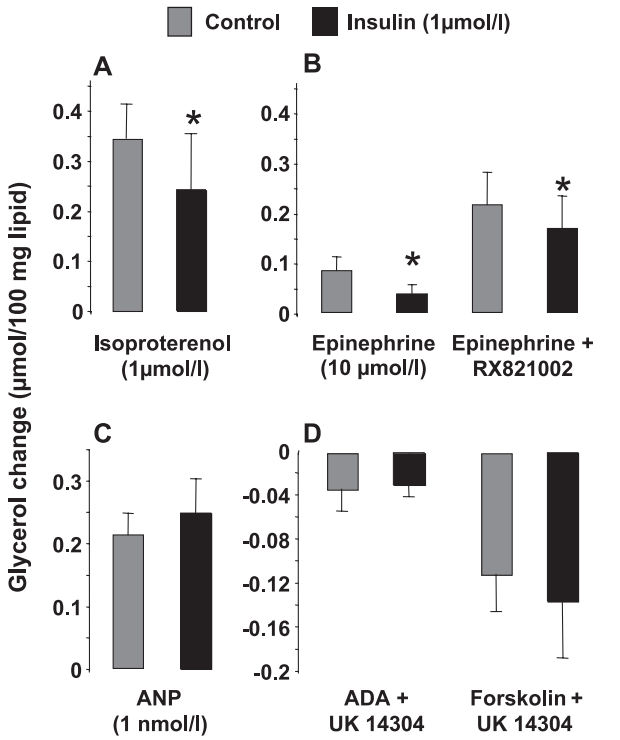

Fig. 5. Effect of a 1-hour treatment of human fat cells by 1 μmol/L of insulin on the regulation of lipolytic activity induced by β-AR and α_2-AR agonists and ANP. Fat cells were incubated for 1 hour with 1μmol/L of insulin, washed 3 times, and immediately incubated (without recovery) with the pharmacological agents over a 90-minute period. A, Effect of isoproterenol. B, Effect of epinephrine alone or in the presence of the α_2-AR antagonist RX821002. C, Effect of ANP. D, Effect of the α_2-AR agonist UK14304 on lipolysis induced by ADA, an enzyme-suppressing adenosine produced by fat cells, or by an activator of adenylyl cyclase, forskolin. Values are means ± SEM of 6 separate experiments. The asterisk indicates $P < .05$ when compared with values obtained with untreated cells using paired t test.

4. Discussion

The present study highlights the homologous desensitization of ANP-dependent effects on lipolysis and lipid mobilization. A desensitization of the ANP-dependent pathway, consecutive to prior exposure of the adipose cells to ANP, is shown in isolated fat cells in vitro. Desensitization of ANP action also occurred in vivo after ANP perfusion. The comparison of the effects of insulin on both adrenergic- and NP-dependent pathways in the regulation of lipolysis and lipid mobilization reveals the striking differences in both lipid-mobilizing pathways. The data underline the lack of counterregulatory action of insulin on the ANP-dependent lipolytic pathway and the α_2-AR–mediated antilipolytic response. The counterregulatory action of

insulin on the β-AR pathway was confirmed in our experimental conditions and validates the protocols.

Catecholamines and insulin have been described for a long time to regulate fat cell lipolysis by modulation of intracellular cAMP levels. Physiological amines, such as epinephrine and norepinephrine, are known to exert a dual action on fat cells, mediated by activation of lipolysis through β-AR stimulation and inhibition of lipolysis through α_2-AR activation [23,26]. An α_2-AR activation blunts adipose tissue lipolysis in healthy subjects, the effect being stronger in the obese [27]. Moreover, epinephrine, which exhibits the highest affinity for α_2-ARs [25], impairs exercise-induced lipid mobilization in the SCAT of healthy and obese patients [18]. More recently, we have identified a new lipolytic pathway that involves NPs (ANP, brain NP) in human adipocytes [6]. Atrial natriuretic peptides acting through NPR-A receptor stimulation promote cGMP production, cGMP-dependent kinase I activation, perilipin phosphorylation, and, finally, HSL phosphorylation and activation of lipolysis [28]. The lipolytic effect of ANP is independent of cAMP production and PDE-3B inhibition [6]. This new pathway seems to be involved in the physiological activation of lipid mobilization during physical exercise in human beings [29]. Thus, integrated control of lipid mobilization depends on a functional balance between β-AR– and α_2-AR–mediated effects on one hand and/or ANP-dependent pathways on the other. To perform an integrated approach of regulatory events affecting both pathways, we tested whether the ANP-dependent pathway undergoes desensitization. Changes in ANP effects were compared with those obtained with catecholamines used in similar experimental conditions. It was also investigated whether in vitro and in vivo changes in insulin level in the adipocyte environment could influence both pathways.

Various in vitro studies have shown that catecholamines, after β-AR stimulation, induce desensitization of β-AR–mediated lipolysis in rodent and human fat cells. Catecholamine infusion or aerobic exercise induces desensitization of β-AR–dependent lipolytic pathways [8,9]. For example, a desensitization of catecholamine-induced lipolysis consecutive to prior perfusion of epinephrine either intravenously or through a microdialysis probe has been described [9]. However, some discrepancies exist in the literature depending on the concentration of the agents, durations of treatments, and length of recovery periods after agonist administration. In our control study performed on isolated human fat cells treated with isoproterenol (Fig. 4), it is demonstrated that pretreatment with isoproterenol, followed by a 2-hour recovery period, does not lead to desensitization of the β-AR–mediated response. It is probable that under our experimental conditions the human fat cells have recovered from the 1-hour desensitizing challenge performed with isoproterenol during the 2-hour washout period. The phenomenon occurs in vivo because a lack of desensitization was also observed when the epinephrine perfusions were separated by at least 2 hours (Fig. 2). Indeed, when epinephrine perfusion was performed in the control condition, there was no significant difference in EGC increase between the 2 separate perfusions (Fig. 2).

An opposite response was observed with ANP. In vitro studies on isolated fat cells revealed that prior exposure to ANP noticeably reduced ANP-induced lipolysis, even after the 2-hour washout after exposure (Fig. 4). The clear reduction of the lipolytic action of ANP observed after the 2-hour recovery period demonstrates that the ANP-dependent pathway undergoes desensitization in vitro. The time course of desensitization and recovery differs for isoproterenol and epinephrine. Desensitization of ANP-related responses and mechanisms has been previously reported in other cell types. This phenomenon could be attributed to a decrease in the intrinsic guanylyl cyclase activity of the NPR-A [30] or to down-regulation of NPR-A on the fat cell membrane [31]. Natriuretic peptide–induced desensitization paradoxically occurs after dephosphorylation of NPR-A when compared with most G-protein–coupled receptors that are desensitized by phosphorylation of receptors as described for the β-AR [30]. As will be discussed later, in vivo studies have confirmed the in vitro observations and shown the occurrence of desensitization of the ANP-dependent pathway in SCAT after a previous ANP perfusion (Fig. 1). Homologous desensitization revealed by the in vitro and in vivo studies could have relevance and could limit excessive ANP-related lipid mobilization.

Another point is that a 1-hour in vitro pretreatment of fat cells with insulin was without any effect on ANP-dependent lipolysis. It is probable that insulin pathways do not interfere with ANP pathways. Resistance to the antilipolytic action of insulin is an important characteristic of the ANP pathway that could have major pathophysiological importance. Deregulation of NP production, as occurring in cardiac diseases, will lead to chronic activation of lipolysis, without counteraction by insulin, and generate lipid disorders. In contrast to ANP action, and as expected, β-AR–mediated effects were reduced by insulin treatment. Insulin counteracted isoproterenol-induced lipolysis as well as epinephrine-induced lipolysis, the maximal lipolytic activity being lower with epinephrine (Fig. 5). It is considered that the inhibitory effect of insulin is related to a decrease in intracellular cAMP level consecutive to the activation of a PDE-3B, a reduction of β-AR number on fat cell membrane, and a desensitization of the β-AR pathway. The lack of effect of insulin on the α_2-AR pathway is explainable by the absence of action of insulin on both the expression and activity of these receptors [12].

Because the ANP-induced EGC increase was lower during the second perfusion both in the control study and during the euglycemic-hyperinsulinemic clamp study, it could be concluded that hyperinsulinemia had no specific effect on ANP-induced lipolysis. In vitro investigations have shown that preincubation of fat cells with insulin led to an inhibition of isoproterenol-induced lipolysis, while ANP kept its full lipolytic potency (Fig. 5). The data clearly

indicate independence between ANP- and the β-AR–dependent pathways, the first one being insensitive to the action of insulin. Reduction in ANP-induced EGC increase both in the control study and during hyperinsulinemia could be attributed to a desensitization of the ANP pathway. It was also found that extracellular cGMP concentration (reflecting local ANP action on fat cells) was significantly reduced in the control study, indicating an acute decrease in ANP biologic efficacy. In vivo results fully confirmed the findings of in vitro studies.

Experiments with epinephrine were performed, parallel to ANP, to delineate the relative contributions to lipid mobilization and differential regulation of both pathways in the same patients. For this purpose, we perfused a submaximal concentration of epinephrine (10 μmol/L) in the microdialysis probe [16]. The results confirm those obtained in a previous study in another group of subjects and validate the reproducibility of our protocol [32]. During the euglycemic-hyperinsulinemic clamp, epinephrine-induced lipid mobilization was dramatically reduced, while it remained unaffected in the control study (Fig. 2). Insulin-related deregulation of the functional balance existing between α_2-ARs and β-ARs has previously been analyzed in detail [32].

Any interpretation of in situ studies must consider the changes that could occur in local ATBF because any change occurring in local ATBF can influence EGC [33]. We estimated the changes in local ATBF with the ethanol escape method, a nonquantitative approach that does not allow absolute determination of local blood-flow changes. However, this validated method gave us information on changes in ATBF occurring during the perfusion of pharmacological agents [15,34] or in some physiological conditions [16]. Because insulin by itself, at the concentrations used, does not change ATBF, as previously shown [35], we can conclude that changes in local ATBF are mainly due to the drugs perfused in the probes. In our studies, it was observed that ANP induces vasodilatation (as indicated by the decrease in outflow-inflow ratio) in both experimental conditions (Fig. 3). Epinephrine alone, regardless of the experimental conditions, tended to decrease blood flow; conversely, in the presence of phentolamine, epinephrine increased blood flow when perfused at 10 μmol/L. Atrial natriuretic peptide–induced vasodilatation could reduce the increase in EGC values and explain the lower increment in EGC values with ANP when compared with epinephrine. It is impossible to determine the amount of ANP and epinephrine crossing the microdialysis probe and truly acting at the fat-cell level. For this reason, submaximal doses of ANP (10 μmol/L) and epinephrine (10 μmol/L) were perfused in the microdialysis probes. In vitro calibration studies with iodine 125-ANP have shown that only 3% to 4% of the ANP (compared with 25%-30% of epinephrine) crosses the probe (not shown).

In conclusion, in human fat cells, the NP-dependent lipolytic pathway exhibits homologous desensitization. Atrial natriuretic peptide–dependent lipolysis undergoes desensitization in vitro but also in situ. Homologous desensitization of NP effects has previously been described in several NPR-A–transfected cell lines, and our results suggest that this process occurs in human fat cells too. The time course of desensitization and recovery differs for isoproterenol and epinephrine. As previously shown, insulin treatment had no inhibitory effect on either ANP- [6,36] or α_2-AR–dependent pathways [32], while it counteracted the β-AR pathway. This comparative study of both lipid-mobilizing pathways confirms the major role played by hyperinsulinemia in the regulation of the functional α_2-AR/β-AR balance and pinpoints the originality of the ANP pathway in human adipose tissue. Further investigations have to be designed to clarify the mechanisms of the homologous desensitization affecting the NP pathway. In addition, it could also be of interest to investigate the possible physiological and/or pathological relevance of ANP-mediated desensitization in human SCAT biology.

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