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In Situ Regulation of Lipolysis by Insulin and Norepinephrine: A Microdialysis Study During Euglycemic-Hyperinsulinemic Clamp

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Lipolytic responsiveness of subcutaneous and epididymal adipose tissue to norepinephrine (NE) was measured by microdialysis before and during a euglycemic-hyperinsulinemic clamp in male Sprague-Dawley rats (280 ± 7 g, n = 8). Microdialysis probes were perfused with standard Krebs-Ringer buffer without (basal condition [BC]) or with NE 10^{-6} mol/L to determine basal and stimulated rates of lipolysis. The dialysate concentration of glycerol was measured (lipolytic index). NE infusion resulted in 3.0- and 4.2-fold increases in glycerol release in abdominal subcutaneous and epididymal adipose tissues, respectively. A euglycemic-hyperinsulinemic clamp at 6 mU/kg · min increased by ninefold the insulinemia (120 ± 9 U/L). Hyperinsulinemia suppressed basal glycerol release by 57% and 42% in subcutaneous and epididymal adipose depots, respectively (BC + I). Lipolytic responses to NE infusion during a euglycemic-hyperinsulinemic clamp (NE + I) were reduced by 45% and 33% in subcutaneous and epididymal adipose tissues, respectively, as compared with BC. Under BC, the lipolytic response to NE was greater in epididymal than in subcutaneous adipose tissue. Physiological levels of insulin regulated basal lipolysis and counteracted adrenergic stimulation of lipolysis to a similar extent in both superficial (subcutaneous) and intraabdominal (epididymal) adipose tissues. Our findings show that lipolysis is more responsive to NE in epididymal than in subcutaneous adipose tissue. The antilipolytic effects of insulin are similar in both superficial and deep intraabdominal adipose tissues. Furthermore, physiological plasma insulin levels cannot fully antagonize the lipolytic effects of NE.

ADIPOSE TISSUE is the main site of lipid storage and plays a critical role in energy homeostasis. Both catecholamines and insulin are the principal hormonal regulators of adipose tissue metabolism. Norepinephrine (NE) has a direct stimulatory effect on lipolysis. This stimulatory effect is mediated through activation of β-adrenoceptors. Conversely, insulin has a marked antilipolytic effect.

Regional differences in the regulation of adipose tissue metabolism are now well documented and widely accepted in humans. Indeed, abdominal adipocytes are more sensitive to the antilipolytic effect of insulin than femoral and omental adipocytes.[1,2] Although android and gynoid forms of obesity are not present in rats, regional differences in morphology and hormonal control of adipose depots have been described. Nutritional and hormonal stimuli affect cell size and cell number of adipose tissues differently depending on their location.[3,4] Differences in glucose metabolism and in control of lipolysis between adipocytes from different regions have been reported.[5,6] Nevertheless, most of the studies on hormonal interactions have been performed on isolated fat cells.

The development of the microdialysis technique allows in situ study of lipolysis regulation. Indeed, it is now possible to measure concentrations of different substrates present in the extracellular fluid. Furthermore, microdialysis can also be used to apply drugs locally, directly in the vicinity of adipose cells, and thus to study in vivo effects.

The objectives of the present study were (1) to determine in vivo and in situ the lipolytic response to NE, (2) to measure the antilipolytic action of insulin in superficial (subcutaneous) and intraabdominal (epididymal) adipose tissues, and (3) to explore possible regional tissular differences in lipolysis production in rats. For these purposes, glycerol release was measured using the microdialysis technique before and during a euglycemic-hyperinsulinemic clamp in subcutaneous and epididymal white adipose tissues.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 280 ± 7 g (IFFA CREDO, L'Arbresles, France) were used in this study. Rats were housed one per cage in a temperature-controlled room (25° ± 1°C) and subjected to a 12-hour light/dark cycle, with lights on at 7 AM. Food and water were available ad libitum, with the commercial diet (UAR A04, Villemoisson, France) containing 23% protein and 4% fat.

Experimental Protocol

At least 5 days before the experiments, rats were anesthetized with halothane inhalation and two polyethylene catheters were inserted and secured, one in the left carotid artery and the other in the right jugular vein. Before beginning the experiments, animals were fasted overnight with free access to water.

The day of the experiment, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and with small additional doses throughout the experiment as necessary.

First, a 1.5-cm skin incision in the abdominal region was made. Then with the help of a steel-guide cannula, a microdialysis probe was inserted into the inguinal white subcutaneous adipose tissue. On the opposite side of the abdominal region, another 2-cm skin incision was made. After obliquus internus abdominis muscle incision, a second probe was inserted into the epididymal adipose tissue following the same procedure. Correct implantation of the probes was ascertained by visual inspection. Body temperature during the experiment was maintained with a hot plate to prevent changes in whole-body carbohydrate metabolism.[7]

Immediately after implantation, each probe was continually perfused with standard dialysis solution. At first, dialysis probes were rinsed for 30 minutes for equilibration to eliminate the transient adenosine triphosphate concentration due to the initial trauma of implantation.[8] The next two 15-minute fractions were collected and are referred to as the basal condition (BC). Then NE (10^{-6} mol/L) was added to standard dialysis Krebs-Ringer buffer (NE condition) for 30 minutes. NE was removed and at the same time a euglycemic-hyperinsulinemic clamp was started (basal-insulin condition [BC + I]; Fig 2). One hour after beginning the clamp, NE stimulation (10^{-6} mol/L) was repeated (NE-insulin condition [NE + I]) for 60 minutes. The difference between the two stimulations (NE alone and NE + I) allowed evaluation of the antilipolytic effect of insulin.

In a second series of experiments, the lipolytic effect of a second addition of NE was tested. Experimental conditions were exactly as previously described, except that the hyperinsulinemic-euglycemic clamp was not realized.

Microdialysis

Dialysis probes consisted of regenerated cellulose fiber with a 0.5-mm outer diameter, a 10-mm length, and a 20-kd molecular weight cutoff (Carnegie Medicin, Stockholm, Sweden). Before use, probes were perfused with 2 mL Krebs-Ringer buffer, which effectively removed all glycerol in the membrane. During dialysis, the inlet tube of the probes was connected to a perfusion pump and dialysate was collected from the outlet tube into the test tubes, which had been placed in an ice box. Standard dialysis solution was Krebs-Ringer buffer supplemented with ascorbic acid (1 mg/mL), an antioxidant of catecholamines. Ascorbic acid has no influence on in situ lipolysis.[9] During the experiment, NE was added (10^{-6} mol/L) to standard dialysis Krebs-Ringer buffer. Under these experimental conditions, in vitro recovery for catecholamines was greater than 10%, according to the manufacturer's manual.[9] This means that adipose tissues were exposed to 10^{-7} mol/L NE. All solutions perfused were filtered through a 0.45 μm mesh filter. The dialysis perfusion rate was 5.00 ± 0.04 μL/min. Fractions were collected every 15 minutes, except between 45 and 60 minutes, during which fractions were collected every 7.5 minutes to better characterize the lipolytic response to NE. Dialysate fractions were weighed to the nearest 0.1 mg, to correct possible changes in the flow rate of perfusion. Dialysates were assayed for glycerol and glucose content.

Basal Lipolytic Rate During Anesthesia

Basal lipolysis was measured during a 3-hour period of anesthesia (n = 3). Experimental conditions (animals, anesthesia, surgical methods, timing of surgeries, and parameters for microdialysis) were exactly identical to those described previously.

Glycerol Interstitial Recovery

The term recovery is defined as the ratio between the concentration of a substance in the dialysate and the concentration of the same substance in the interstitial fluid outside the probe. Recovery was calculated with the isotopic method for glycerol, according to the method of Jansson et al.[10] This method assumes that the relative loss of labeled substrate across the dialysis membrane into the adipose tissue equals the relative flux of substrates over the membrane into the perfusion liquid. Efflux percent was calculated as $(C_{in}^* - C_{out}^*) \times 100/C_{in}^*$, where C_{in}^* is radioactivity in the perfusate (dpm/mL) before dialysis and C_{out}^* is radioactivity in the perfusate after dialysis. Perfusates were prepared with approximately 20,000 dpm/μL (U-^{14}C)glycerol (Isotopchim; Ganagobie-Peyrus, France). Radioactivity was measured in a liquid scintillation counter (Intertechnique SL3000; Kontron, St Quentin-Yvelines, France).

Blood Flow Changes During Microdialysis

Blood flow changes around the microdialysis probe were measured according to the method of Hickner et al[11] using ethanol at 20 mmol/L. Ethanol rapidly diffuses through the membrane probe. Hickner et al[11] showed that ethanol clearance from the perfusion medium is related to local blood flow. Ethanol concentration was measured using an enzymatic spectrophotometric technique.[12] Changes in blood flow are presented as a percentage of baseline, ie, (outflow/inflow) × 100.

Euglycemic-Hyperinsulinemic Clamp

Euglycemic-hyperinsulinemic clamps were performed according to the method of Kraegen et al.[13] Rats were infused with human neutral insulin (Actrapid HM 40 U/mL; Novo Nordisk, Boulogne, France) at 6 mU/kg · min (in 0.9% saline and 1% bovine serum fatty acid–free albumin) at a flow rate of 16.6 μL/min for 2 hours to achieve plasma insulin concentrations through the physiological range of insulinemia. Blood samples of 25 μL were taken at 5-minute intervals to determine glycemia, which was maintained constant at 6 mmol/L by an adjusted infusion of glucose (20%) at a variable rate using a peristaltic roller pump. Delivery rate of the pump was controlled after each clamp. This permitted calculation of the glucose infusion rate ([GIR] milligrams of glucose per kilogram per minute) during the second hour of the glucose clamp; mean GIR plateaued at 60 to 120 minutes without evidence of a further increase in the glucose requirement.

One hundred microliters of blood were taken at 0, 60, and 120 minutes of the clamp for insulin measurement. Hematocrits were

also determined on 25-μL blood samples at 0 and 120 minutes of the clamp.

Glucose, Glycerol, and Insulin Analysis

Ten microliters of plasma and dialysate samples were analyzed for glucose content on a Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Plasma insulin concentration was measured by a double-antibody radioimmunoassay (Kit CIS Biointernational, Gif sur Yvette, France) on 50 μL using a human insulin standard. Intraassay and interassay coefficients of variation were 13.7% and 8%, respectively.

Glycerol level was measured on 10 μL dialysate using an ultrasensitive kinetic bioluminescence assay.[14] Adenosine triphosphate and luciferin-luciferase were from Bio Orbit (Turku, Finland) and glycerokinase was from Sigma (La Verpillère, France). Before use, glycerokinase was purified by chromatography using Sephadex G-50, as previously described.[15] Luminescence was measured using a 1250 luminometer from Kontron Instruments.

Statistical Analysis

All data are expressed as the mean ± SEM. Values were examined with a two-way ANOVA using the StatView program of the MacIntosh system (Apple, Les Ulis, France). A Fisher protected least-significant difference post hoc test was used for group comparisons. Statistical significance was set at P less than .05.

RESULTS

Euglycemic-Hyperinsulinemic Clamp

Plasma glucose reduction, induced by insulin perfusion, was prevented by a progressive perfusion of glucose solution (Fig 1A). The rate of glucose infusion required to maintain euglycemia (6 mmol/L) was 15.0 ± 1.4 mg/kg · min (Fig 1B). Insulin perfusion at 6 mU/kg · min resulted in a ninefold increase in plasma concentration (14.0 ± 1 mU/mL under BC *v* 118.5 ± 8.5 mU/mL at the end of the clamp). Steady-state plasma insulin concentration was reached less than 60 minutes after beginning the clamp (Fig 1C).

Lipolytic Response to NE of Subcutaneous Adipose Tissue

Dialysate was collected every 15 minutes. Microdialysis started after a 30-minute equilibration period following implantation of the dialysis probe. Under these conditions, basal glycerol level measured over a 30-minute period was found to be constant. Addition of NE to the perfusate caused a rapid and significant increase (threefold, $P < .05$) in the dialysate glycerol concentration. The maximum concentration of glycerol (53 ± 4 μmol/L) in the dialysate was reached less than 15 minutes after addition of NE to the perfusate.

Then NE was removed from the perfusate, and simultaneously the euglycemic-hyperinsulinemic clamp was started. As soon as NE was removed from the perfusate, glycerol concentration decreased within 30 minutes to values less than the initial basal values (BC + I = 43% of BC; Fig 2). Addition of NE during the second hour of the euglycemic-hyperinsulinemic clamp resulted in a fourfold increase in glycerol concentration in the dialysate as compared with basal values during the clamp ($P < .05$, BC + I *v* NE + I; Fig 2). Thus, the reduction in the lipolytic effect of NE, due

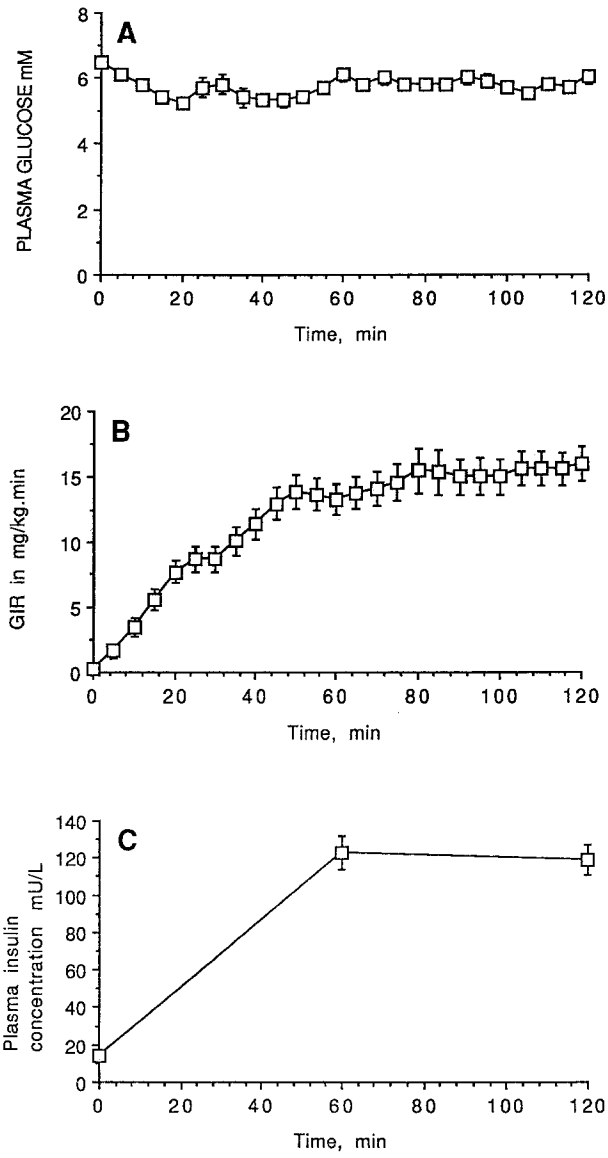

Fig 1. (A) Plasma glucose concentration during 2 hours of insulin infusion in 12 individual rats. (B) GIR necessary to maintain blood glucose at 6 mmol/L. (C) Plasma insulin concentration during the clamp. Values are the mean ± SEM.

to hyperinsulinemia, reached 44% as compared with the first response without insulin infusion ($P < .05$, NE *v* NE + I; Fig 2).

Lipolytic Response to NE of Epididymal Adipose Tissue

The response observed in epididymal adipose tissue was similar to the one seen in the subcutaneous adipose depot. The time responses to either NE alone or NE during the clamp were exactly the same. The only difference concerned the amplitude of glycerol release in response to addition of NE in the perfusate. Indeed, the lipolytic response increased by 4.2-fold as compared with the basal value. This increase is 40% higher than the one observed in subcutaneous adipose tissue.

The euglycemic-hyperinsulinemic clamp resulted in a

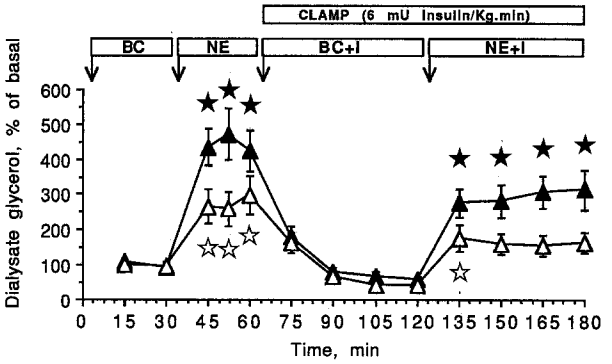

Fig 2. In vivo lipolytic effect of NE under BC and during euglycemic-hyperinsulinemic clamp in abdominal subcutaneous (△) and epididymal (▲) adipose tissues. The difference between the two lipolytic responses corresponds to the antilipolytic effect of insulin. Glycerol is expressed as a percentage of initial basal value. Values are the mean ± SEM (n = 8). Stars indicate significant differences for subcutaneous and epididymal adipose tissues, respectively ($P < .05$), in comparison to BC.

reduction of basal glycerol release, reaching 40% of the value measured during BC (BC + I *v* BC; Fig 2). Addition of NE to the perfusate during the euglycemic-hyperinsulinemic clamp resulted in a threefold increase as compared with the initial basal value (NE + I *v* BC). Thus, the reduction of the lipolytic response to NE due to hyperinsulinemia reached 33% as compared with the first response without hyperinsulinemia (NE + I *v* NE; Fig 2).

Lipolytic Response to Repeated Addition of NE

In a different series of experiments, we tested the lipolytic effects of repeated addition of NE. Experimental conditions were exactly as those previously described except that the hyperinsulinemic-euglycemic clamp was omitted. The first addition of NE resulted in 2.5-fold and 4.5-fold increases in dialysate glycerol concentrations in subcutaneous and epididymal adipose depots, respectively (Fig 3). The second addition of NE produced exactly the same increases in dialysate glycerol concentrations (2.5-

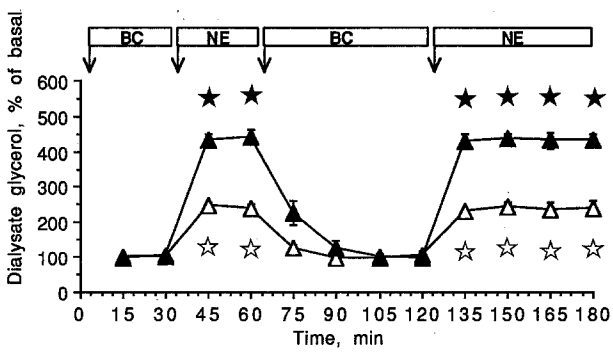

Fig 3. In vivo lipolytic effects of repeated additions of NE in abdominal subcutaneous (△) and epididymal (▲) adipose tissues. Glycerol is expressed as a percentage of initial basal value. Values are the mean ± SEM; when not shown, SEMs were smaller than the symbol (n = 3). Stars indicate significant differences for subcutaneous and epididymal adipose tissues, respectively ($P < .05$), in comparison to BC.

and 4.5-fold in subcutaneous and epididymal adipose depots, respectively).

Interstitial Glycerol Recovery

Interstitial glycerol recovery in subcutaneous adipose tissue was 12.8% ± 3.7% under BC. It did not change significantly following addition of NE (12.0% ± 2.4%). No significant change was observed during the euglycemic-hyperinsulinemic clamp in either the absence or presence of NE (9.4% ± 1.8% and 10.9% ± 1.1%, respectively).

Under BC, glycerol recovery in epididymal adipose tissue was 10.3% ± 3.2%. NE addition did not change glycerol recovery (9.4% ± 2.6%). Glycerol recovery slightly decreased during the euglycemic-hyperinsulinemic clamp; nevertheless, these differences were not significant (7.1% ± 2.2% BC + I and 7.0% ± 2.0% NE + I).

Basal Lipolysis During Anesthesia

Control experiments were designed to measure the effect of long-term anesthesia on the basal lipolytic rate. Results showed that periodic injections of low-dose pentobarbital to maintain anesthesia did not affect the basal lipolytic rate (Fig 4).

Changes in Local Blood Flow

Local blood flow around the probes was unchanged throughout the experiment in both subcutaneous and epididymal adipose tissues (Fig 5). This demonstrates that NE concentration was not sufficiently high to modify local blood flow. At the end of the hyperinsulinemic-euglycemic clamp, local blood flow slightly increased in subcutaneous and epididymal adipose tissues (8.3% and 6.8%, respectively). These differences did not reach statistical significance.

DISCUSSION

Among the advantages of microdialysis, it may be noted that delivery of catecholamines through the probe induces a local stimulation of adipose tissue and has no effect on the circulating concentration of the drug. Another major advantage of the microdialysis technique is that it integrates all

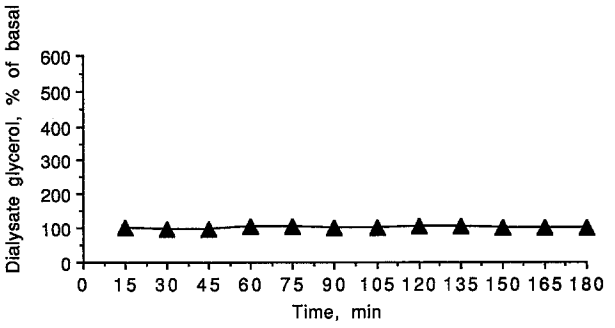

Fig 4. In vivo basal lipolysis in abdominal subcutaneous adipose tissue during 3 hours. Values are the mean ± SEM (n = 3). Constancy of basal values demonstrates that long-term anesthesia did not alter basal lipolytic rate.

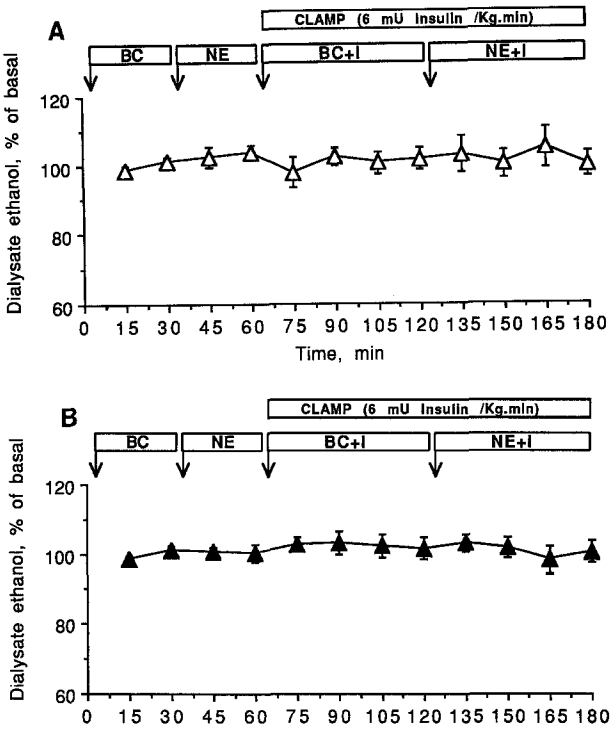

Fig 5. Blood flow monitoring in abdominal subcutaneous (A) and epididymal (B) adipose tissue. Changes in blood flow are expressed as a percentage of initial basal value. Values are the mean ± SEM (n = 5). No significant differences for subcutaneous and epididymal adipose tissues were observed in comparison to BC.

the regulatory systems present in the body, even those not already known.

The present study shows a marked difference in lipolytic responsiveness to catecholamines between the two adipose depots studied. Indeed, the lipolytic response to NE was 40% higher in epididymal than in subcutaneous adipose tissue (Fig 2). Previous studies on isolated adipose cells have noted such differences between subcutaneous adipose tissues from different locations and omental adipocytes.[1,16-18] More recently, differences in basal and fasting glycerol production between two subcutaneous adipose tissues, abdominal and femoral, were described in humans, using the microdialysis technique.[10] The present study reports for the first time differences in lipolytic responsiveness under in vivo and in situ conditions in rats.

The mechanisms leading to regional differences in the regulation of lipolysis are not fully understood. It is likely that the balance in α- and β-adrenoceptors among different adipose depots and/or the increase in the number of β-adrenoceptors play a significant role.[19-22] Indeed, it has been shown that a small reduction in the number of cell-surface β-adrenoceptors in human fat cells causes a large change in catecholamine sensitivity.[23] Furthermore, differences in the concentration of and responsiveness to sex steroid hormones could also partly explain regional differences in the regulation of lipolysis.[24] On the other hand, postreceptor effects such as coupling between adrenoceptor and adenylate cyclase, activation of hormone-sensitive lipase (HSL) by cyclic adenosine monophosphate, or breakdown of cyclic adenosine monophosphate by phosphodiesterase do not seem to be responsible for regional lipolytic responses.[22] Recently, variations in the expression of HSL among anatomically distinct adipose depots have been shown.[25] The increase in expression of HSL mRNA in internal adipose tissues (1.4-fold greater than in peripheral adipose tissue) is on the same order of magnitude as the increase in lipolytic rate in response to NE (seen in the present study, 40%). Our in vivo results support the idea that differences in HSL expression among different adipose tissues explain the variations in rates of stimulated lipolysis.

Insulin perfusion at 6 mU/kg · min resulted in a plasma insulin concentration equal to 118 mU/L, ie, the range of physiological values.[26] Steady-state plasma insulin concentration was already reached 60 minutes after the beginning of insulin perfusion at 120 mU/L. Stability in the rate of glucose infusion required to maintain euglycemia suggests that plasma insulin plateaued 50 minutes after the beginning of insulin perfusion. The present findings show that the physiological increase in plasma insulin reduces both basal and stimulated lipolysis, but cannot totally counteract the lipolytic effects of 10^{-7} mol/L NE. This is in accordance with a previous report.[27] Since the second addition of NE without hyperinsulinemia resulted in a lipolytic response identical to the first one (Fig 3), the decreased lipolytic response to NE during the hyperinsulinemic-euglycemic clamp can only be attributed to the antilipolytic effect of insulin. Although the lipolytic response to catecholamines was much higher in epididymal than in subcutaneous adipose tissue, there was no difference in the antilipolytic effect of insulin. Indeed, stimulated lipolysis was inhibited to a similar extent (~40%) in both adipose tissues.

Under our experimental protocol, anesthesia was a necessity. Indeed, microdialysis probes have to be implanted under anesthesia. Long-term implantation of microdialysis probes in adipose tissues of awake animals leads to several problems, among which the main ones are that the probes may move out of the adipose tissue or may be rapidly broken when animals are moving. The possibility that the anesthetic may alter lipolytic responses of adipose tissues differently according to their location has to be considered. Several arguments reject this hypothesis. First, the fact that basal lipolysis remained constant throughout a 3-hour anesthesia suggests no direct effect of the anesthetic on lipolysis. Indeed, although the concentration of anesthetic remained high enough to keep the animals asleep, it fluctuated, and in the case of interaction with lipolysis, this would have modified basal lipolysis, which was not observed. Second, in vitro studies have shown differences in the lipolytic response to NE of adipocytes from different locations (ie, in the absence of anesthetic).[1,2] Third, in the case of a differential response to the anesthetic, differences in the antilipolytic effect of insulin between adipose depots would have been observed, which was not the case.

Under our experimental conditions, we found no vasodilatory effect of NE. Although this may be unexpected at first, it can be easily explained. Indeed, the concentration of NE used in the present study was physiological. NE rapidly loses its lipolytic and vasodilatory effects because of dilu-

tion in the interstitial fluid, which is proportional to the distance from the probe. It was not a problem to measure the lipolytic response, since it was measured in the vicinity of the probe. On the other hand, blood flow also depends on events happening farther from the probe. Since blood flow was not increased farther from the probe, the locally increased blood flow rapidly disappeared. Similarly, we found no significant effect of insulin on blood flow. This is in agreement with data from the literature in humans.[28]

In the present study, in vivo recovery rates of glycerol under BC in the two adipose tissues were not significantly different. Since we did not find any significant difference in in vivo glycerol recovery, the data presented in Fig 2 were not corrected for probe efficiency. Under BC, absolute concentrations of glycerol in the extracellular space were 166 ± 22 and 149 ± 20 μmol/L in subcutaneous and epididymal tissues, respectively. This demonstrates that there was no difference in the basal lipolytic rate among intraabdominal and subcutaneous adipose tissues. Our values are in agreement with data reported in the literature, ie, 185 ± 20 and 160 ± 15 μmol/L in human adipose tissues.[10]

In conclusion, the present microdialysis study shows that epididymal adipose tissue is more responsive to NE than subcutaneous adipose tissue. Physiological plasma insulin concentrations cannot totally counteract the lipolytic action of NE. The antilipolytic effect of insulin is of the same magnitude in both epididymal and subcutaneous adipose tissues.

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