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Inhibition of leptin release by atrial natriuretic peptide (ANP) in human adipocytes

John N. Fain[a,*], Alie Kanu[a], Suleiman W. Bahouth[b], George S.M. Cowan Jr.[c], M. Lloyd Hiler[c]

[a]*Department of Molecular Sciences, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA*
[b]*Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA*
[c]*Department of Surgery, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA*

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Abstract

The addition of atrial natriuretic peptide (ANP) to isolated human adipocytes in primary culture from very obese individuals resulted in an inhibition of leptin release after a 24- or 48-hr incubation. There was also an inhibition of leptin release by isoproterenol (ISO) that was partially reversed by insulin, whereas the inhibition due to ANP was unaffected. Similar results were seen with *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89), which is a cell-permeable inhibitor of protein kinase A. H-89 markedly reduced the effects of ISO on both lipolysis and leptin release without affecting the stimulation of lipolysis or the inhibition of leptin release due to ANP. Inhibition of endogenous nitric oxide formation using N^{ω}-nitro-L-arginine resulted in a 20% increase in leptin release over 48 hr, which suggests that the nitric oxide/cyclic GMP pathway might play a small role in the regulation of endogenous leptin release. Similarly, the addition of the nitric oxide donor (*Z*)-1-[2-aminoethyl)-*N*-(2-aminoethyl)diazen-1-ium-1,2-diolate (DETA NONOate) at 0.1 or 1 μM to explants of human adipose tissue enhanced lipolysis by 29%. Our data demonstrate that the lipolytic effect of ANP is probably secondary to stimulation of cyclic GMP accumulation in human adipocytes, and this is accompanied by an inhibition of leptin release.

Keywords: ANP; Leptin; Lipolysis; Insulin; H-89; Cyclic GMP

1. Introduction

In 1986, Uehlinger *et al*. [1] reported that infusion of ANP elevated plasma free fatty acids by 140% within 30 min in humans. ANP did not stimulate lipolysis in white [2] or brown [3] adipocytes from rats but did elevate cyclic GMP [2]. However, Sengenes *et al*. [4,5] demonstrated in human adipocytes that ANP elevates cyclic GMP as well as lipolysis. The lipolytic effect of ANP differed from that of catecholamines since insulin and A_1 adenosine agonists have no effect on the stimulation of lipolysis by ANP [4].

The intravenous administration of ANP elevated the plasma free fatty acids of obese women, and the response was enhanced after 28 days on a low calorie diet [5]. The natriuretic peptides are clearly able to activate a novel pathway for lipolysis in humans, but whether it is physiologically relevant remains to be demonstrated.

Leptin is a protein hormone secreted primarily by adipocytes, and a lack of functional leptin is a molecular defect in the *ob*/*ob* mouse, which is infertile, obese, and diabetic [6]. Obesity is also seen in humans lacking leptin, but the vast majority of obese individuals have no defects in leptin formation or function [6]. In fact, the level of plasma leptin correlates positively with the extent of obesity in humans. In adipocytes from rodents as well as humans, the stimulation of lipolysis by catecholamines or other lipolytic agents is associated with inhibition of leptin release [6]. All lipolytic agents, except natriuretic peptides, are thought to stimulate lipolysis secondary to the elevation of cyclic AMP. The question arises as to whether it is cyclic AMP or the products of lipolysis that inhibit leptin release. If it is the elevation of cyclic AMP, then an agent that stimulates cyclic GMP formation might not have an inhibitory effect on leptin release. Therefore, the present

* Corresponding author. Tel.: +1-901-448-4343; fax: +1-901-448-7360.
E-mail address: jfain@utmem.edu (J.N. Fain).

Abbreviations: ANP, atrial natriuretic peptide; DETA NONOate, (*Z*)-1-[2-aminoethyl)-*N*-(2-aminoethyl)diazen-1-ium-1,2-diolate; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide; ISO, isoproterenol.

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studies were designed to determine whether the stimulation of lipolysis by ANP is associated with an inhibition of leptin release by human adipocytes.

A second aim of these studies was to determine whether activation or inhibition of the formation of nitric oxide in adipocytes would affect lipolysis and leptin release, since another pathway for cyclic GMP formation involves activation of a soluble guanylate cyclase by nitric oxide. There is evidence for the presence of endothelial nitric oxide synthase in human adipocytes [7].

2. Materials and methods

Subcutaneous abdominal adipose tissue was obtained from obese female subjects undergoing elective open abdominal surgery (gastric bypass) under general anesthesia after an overnight fast. The mean body mass index (BMI) averaged 48. Each experimental replication involved tissue from a separate individual. The study had the approval of the local Institutional Review Board, and all patients involved gave their informed consent. The patients were fasted overnight prior to surgery but had not been on any type of dietary restriction just prior to surgery.

Twenty to eighty grams of abdominal subcutaneous adipose tissue was transported immediately to the laboratory. The handling of tissue and cells was done under aseptic conditions. The tissue was cut with scissors into small pieces (20–30 mg). All the studies utilized explants of adipose tissue that had been incubated in buffer plus albumin for approximately 30 min to remove diffusible factors and blood cells. At the conclusion of the 30-min incubation, the tissue explants were centrifuged for 30 s at 400 *g* at room temperature in order to remove erythrocytes and pieces of tissues containing insufficient adipocytes to float. The explants were separated from the medium plus the sedimented cells and resuspended in fresh buffer. The explants (80–100 mg/mL) were then incubated for 48 hr in suspension culture under aseptic conditions.

Adipocytes were obtained by collagenase digestion, and the cells and medium were separated from undigested debri by filtration through 200 μm nylon mesh at the end of the 90-min digestion period. The stromal (non-fat) cells were separated from the free adipocytes by centrifugation in 15-mL tubes at 400 *g* for 1 min at room temperature. The adipocytes were resuspended in fresh buffer and centrifuged at 400 *g* for 10 s at room temperature. The medium was removed, and this process was repeated three times.

The culture medium used for the incubation of adipose tissue and adipocytes was Dulbecco's modified Eagle's medium/Ham's F12 (1:1, Sigma No. 2906) containing 17.5 mM glucose, 121 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 25 mM HEPES, 2.4 mM sodium bicarbonate, 10 mg/mL of bovine serum albumin, 5 μg/mL of ethanolamine, 0.1 ng/mL of sodium selenite, 90 μg/mL of penicillin G, 150 μg/mL of streptomycin sulfate, 50 μg/mL of gentamicin, and 55 μM ascorbic acid. The pH of the culture medium was adjusted to 7.4, and then the medium was passed through a 0.2 μm filter. Aliquots of the medium were taken at 48 hr and stored at −20° for measurement of leptin. The leptin content in 20- to 50-μL aliquots of the incubation medium was determined using radioimmunoassay kits from Linco Research, Inc. Lipolysis was measured as glycerol [8]. Cyclic GMP or cyclic AMP was measured, using an acetylation EIA system from Amersham Pharmacia and Assay Design, respectively, in extracts of cells plus 0.5 mL of medium after heating in a boiling water bath for 1 min. The extracts were then frozen and kept at −20° until they were assayed for cyclic GMP or cyclic AMP.

Bovine serum albumin powder (Bovuminar, containing <0.05 mol of fatty acid/mol of albumin) was obtained from Intergen. Bacterial collagenase *Clostridium histolyticum* (Type 1) was obtained from the Worthington Biochemical Corp. (Lot CLS1-4197-MOB3773-B, 219 U/mg). Human synthetic ANP, obtained from Sigma, was dissolved in 0.1% acetic acid at a concentration of 10 μM, and aliquots of this stock solution were stored at −80°. Other chemicals were from Sigma.

3. Results

Leptin release by human adipocytes was inhibited by ANP as well as by ISO in a time-dependent manner (Fig. 1). While neither ISO nor ANP had an effect on leptin release at 4 hr, there was a decrease at 24 and 48 hr.

Insulin reduced the inhibition of leptin release due to ISO by 40% and of lipolysis by 31% at 48 hr, and these effects were statistically significant (Fig. 2). However, insulin did not alter the effect of ANP on leptin release or on lipolysis (Fig. 2). We also looked at lipolysis over the first 2 hr of the studies shown in Fig. 2, and insulin inhibited the increase in lipolysis due to ISO by 26% while having no effect on that due to ANP (data not shown).

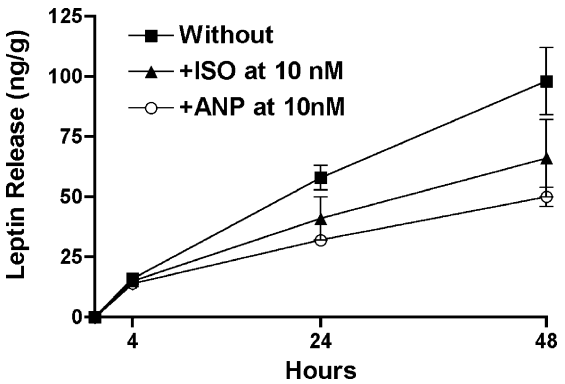

Fig. 1. Effect of ANP or ISO on leptin release by human adipocytes. Human adipocytes (60 mg/mL) were incubated for 48 hr in the absence or presence of 10 nM ANP or 10 nM ISO. Aliquots of the medium were removed at 4, 24, or 48 hr for leptin analysis. Data are the means ± SEM of 4 paired replications.

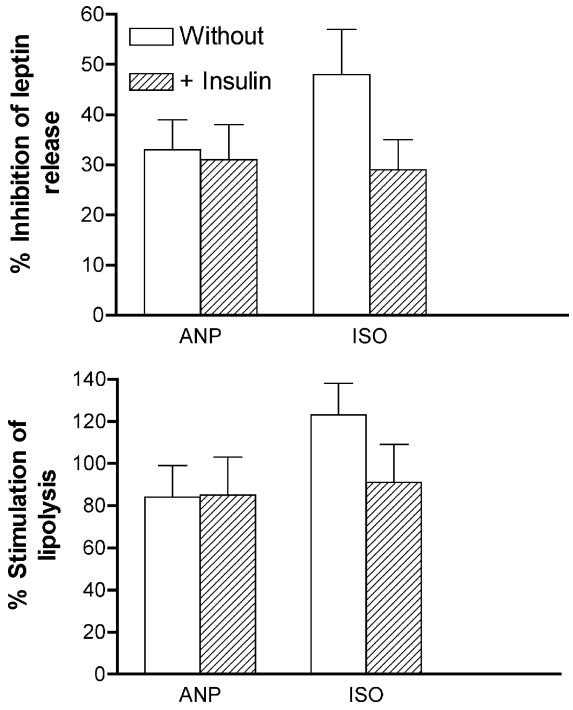

Fig. 2. Effect of insulin on the inhibition of leptin release due to ANP and ISO. Human adipocytes (100 mg/mL) were incubated for 48 hr in the presence of 10 nM dexamethasone in either the absence or presence of 10 nM insulin, 50 nM ANP, or 10 nM ISO, as indicated. Values are the means ± SEM of 10 paired replications. Insulin reduced the inhibition of leptin release due to ISO by 40 ± 12% ($P < 0.01$ by paired comparisons) and the stimulation of lipolysis due to ISO by 31 ± 10% ($P < 0.025$ by paired comparisons). Leptin release was 210 and 249 ng/g, respectively, in the absence and presence of insulin. Lipolysis was 4.6 and 4.2 μmol/g of glycerol, respectively, in the absence and presence of insulin.

The finding that insulin did not inhibit the lipolytic action of ANP was similar to the results found with H-89 (Fig. 3). H-89 is a potent and selective inhibitor of the cyclic AMP-dependent protein kinase and inhibits the stimulation of lipolysis by catecholamines [9]. H-89 (15 μM) reduced the lipolytic action of ISO over a 48-hr incubation but did not affect that of ANP (Fig. 3). A similar effect was seen with respect to leptin release in that H-89 had a significant effect on the inhibition of leptin release by ISO while having no effect on that due to ANP (Fig. 3).

The data in Fig. 4 indicate that as little as 0.3 nM ANP was able to enhance cyclic GMP accumulation in human adipocytes, as well as lipolysis, over a 60-min incubation. In a larger series of experiments shown in Fig. 5, the increase in lipolysis over 10 min induced by 50 nM ANP (+35%) was comparable to that seen with 10 nM ISO (+43%). There was a large increase in cyclic GMP due to ANP, but there was no effect of ISO on cyclic GMP (Fig. 5).

The possible role of endogenous cyclic GMP formation in the regulation of lipolysis is likely to be mediated through nitric oxide, which activates the soluble guanylate cyclase [10]. There is evidence for endothelial (eNOS) and inducible (iNOS) nitric oxide synthase in human adipose tissue, but the level of the mRNA for eNOS in human subcutaneous adipose tissue from obese individuals is at least 15 times higher than that for iNOS [7]. The role of the nitric oxide/cyclic GMP system in basal lipolysis of human adipocytes was examined by inhibiting nitric oxide formation using N^{ω}-nitro-L-arginine [11–13]. The data in Table 1 indicate that 10, 20, or 50 μM N^{ω}-nitro-L-arginine significantly enhanced basal leptin release by 16–28% without having a statistically significant effect on lipolysis due to the wide variability in the data for lipolysis.

Table 1
Stimulation of leptin release by N^{ω}-nitro-L-arginine

Nitroarginine	Leptin release (%)	P value	Lipolysis (%)	P value
10	+16 ± 5	<0.01	−20 ± 20	NS
20	+28 ± 5	<0.005	−18 ± 9	NS
50	+20 ± 4	<0.001	−10 ± 12	NS

Human adipocytes (100 mg/mL) were incubated for 48 hr in the presence of 10 nM dexamethasone either without or with the indicated concentrations of N^{ω}-nitro-L-arginine. The effects of nitroarginine are shown as the percent differences ± SEM from control values for 16 paired experiments. NS refers to a P value > 0.05.

We also examined the effects of exogenous nitric oxide on lipolysis and leptin release. DETA NONOate is a nitric oxide donor, with a half-life of 20–23 hr at 37°, that donates 2 mol of nitric oxide per mol of the compound [14]. The compound is active at concentrations in the range of 1–10 μM as a stimulator of cyclic GMP accumulation [15]. The data in Fig. 6 indicate that in isolated explants of human adipose tissue incubated for 48 hr there was a 29% increase in lipolysis due to 0.1 or 1 μM DETA NONOate. However, in the presence of dexamethasone there was an inhibitory effect of DETA NONOate on lipolysis (Fig. 6).

4. Discussion

Khoo *et al.* [16] reported in 1977 that hormone-sensitive lipase purified from chicken adipose tissue was activated by cyclic GMP-dependent kinase purified from bovine lung. Subsequently, Stralfors and Belfrage [17] found similar results using rat hormone-sensitive triglyceride lipase. They demonstrated that the same serine residues on the lipase are phosphorylated by protein kinase A and by cyclic GMP-dependent protein kinase. Jeandel *et al.* [2] found that ANP enhances cyclic GMP accumulation by rat adipocytes without affecting lipolysis. These data suggested that the missing link is the absence of cyclic GMP-dependent protein kinase in rodent adipose tissue. The subsequent demonstration that ANP stimulated lipolysis in human adipocytes suggests that cyclic GMP-dependent protein kinase is present in human adipocytes. While there was initial resistance to accepting a prominent role for cyclic GMP-dependent protein kinase in mediating the effect of cyclic GMP, it is now accepted with regard to signaling in smooth muscle [18].

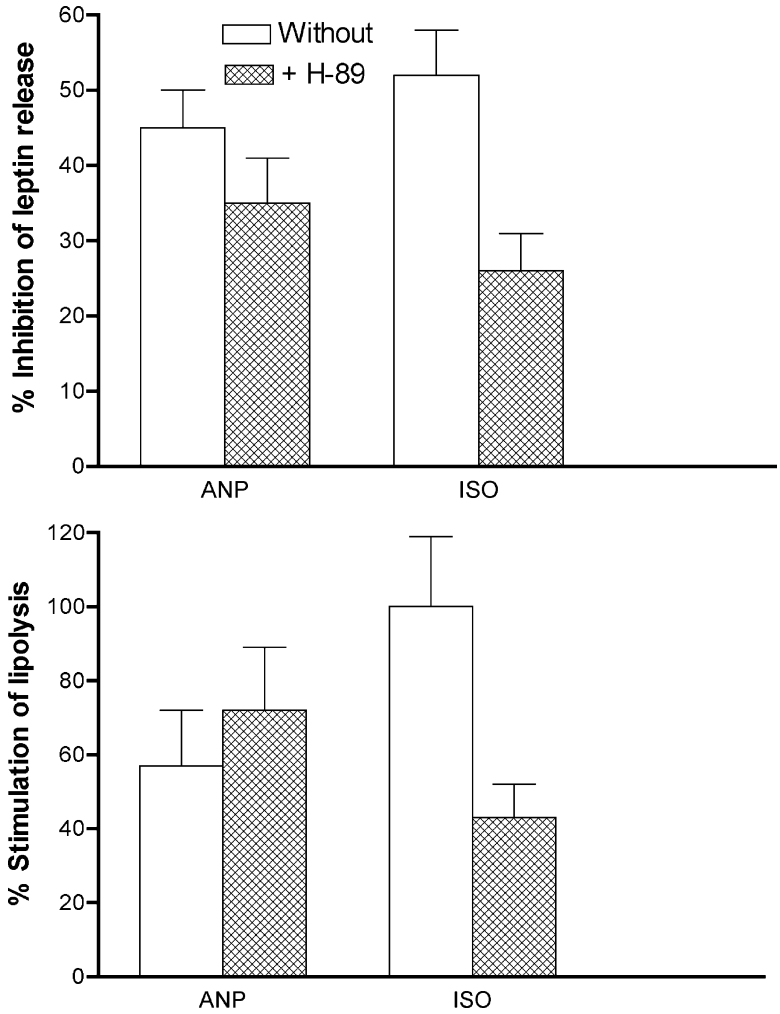

Fig. 3. Effect of an inhibitor of cyclic AMP-dependent protein kinase on the stimulation of lipolysis and the inhibition of leptin release by ANP and ISO. Human adipocytes (100 mg/mL) were incubated for 48 hr in the presence of 10 nM dexamethasone in either the absence or presence of 15 μM H-89, 10 nM ISO, 50 nM ANP, or the combination. Data are the means ± SEM of 10 paired replications. Leptin release was 184 and 147 ng/g, respectively, in the absence and presence of H-89. Lipolysis was 6.5 and 6.6 μmol/g, respectively, in the absence and presence of H-89. There was no statistically significant effect of H-89 on the responses due to ANP. The 26% reduction in the inhibition of leptin release by ISO was statistically significant based on paired comparisons ($P < 0.005$), as was the 57% reduction in the stimulation of lipolysis by ISO ($P < 0.025$).

The present data clearly demonstrated that leptin release by human adipocytes is inhibited by ANP and suggest that this is secondary to the activation of lipolysis, which is also activated by ISO. The lipolytic action of ISO differs from that of ANP in that there is no stimulation of cyclic GMP accumulation and the lipolytic effect of ISO is inhibited by N^6-cyclopentyl adenosine [4], insulin (Fig. 2 and [4]), and H-89 (Fig. 3). Insulin is believed to activate a cyclic AMP phosphodiesterase [19], which lowers the amount of cyclic AMP available to activate protein kinase A. Adenosine analogs are thought to inhibit the formation of cyclic AMP secondary to activation of inhibitory guanine nucleotide binding proteins that reduce the activation of adenylate cyclase by catecholamines [20,21]. In contrast, H-89 is a specific inhibitor of protein kinase A, which phosphorylates the hormone-sensitive lipase and other proteins involved in the activation of lipolysis by cyclic AMP [9]. Thus, it is hardly surprising that H-89 and insulin do not affect the inhibition of leptin release by ANP.

Most prior studies on the role of endogenous nitric oxide formation on lipolysis have used rodent adipocytes where there is no evidence that cyclic GMP activates lipolysis [22,23]. However, in human adipocytes, increases in nitric oxide formation presumably activate guanylate cyclase, which results in an elevation in cyclic GMP. The cyclic GMP-dependent protein kinase then phosphorylates the hormone-sensitive lipase and possibly other targets of the kinase. There is evidence for the presence of mRNA for several subunits of the soluble guanylate cyclase and two isoforms of the cyclic GMP-dependent protein kinase in human adipocytes [24]. ANP enhances lipolysis in human adipocytes, suggesting a possible role for nitric oxide as an activator of lipolysis in humans and this is supported by our findings in the presence of the nitric oxide donor DETA

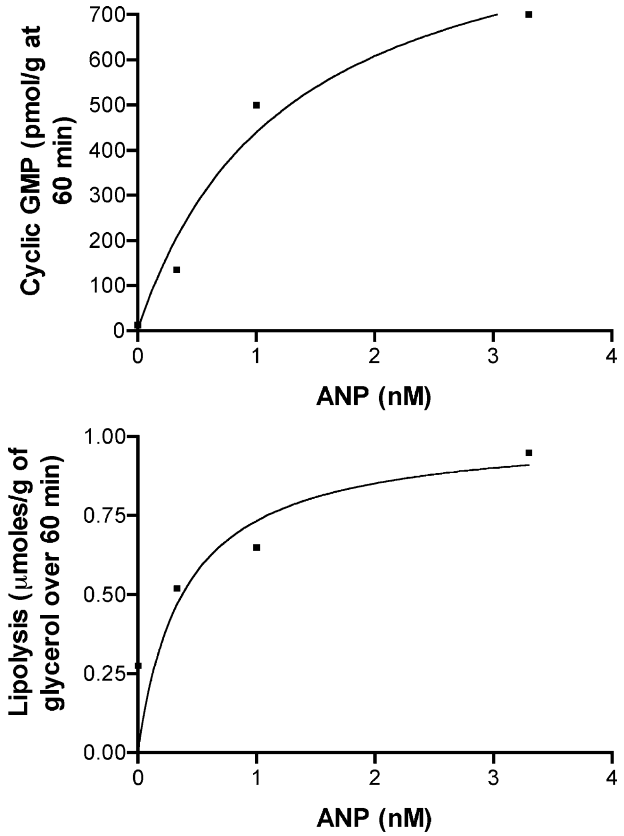

Fig. 4. Stimulation of cyclic GMP accumulation and lipolysis by ANP. Human adipocytes (100 mg/mL) were incubated for 60 min in the absence or presence of the indicated concentrations of ANP. Values are the means of a single experiment done in duplicate.

NONOate. We could see either inhibition of lipolysis in the presence of dexamethasone or stimulation of lipolysis by DETA NONOate in the absence of dexamethasone. Andersson *et al*. [25] reported that after 1 hr of treatment with an inhibitor of nitric oxide formation there was a stimulation of adipocyte lipolysis, based on microdialysis studies of human subcutaneous adipose tissue. They also observed an inhibition of ISO-stimulated lipolysis by

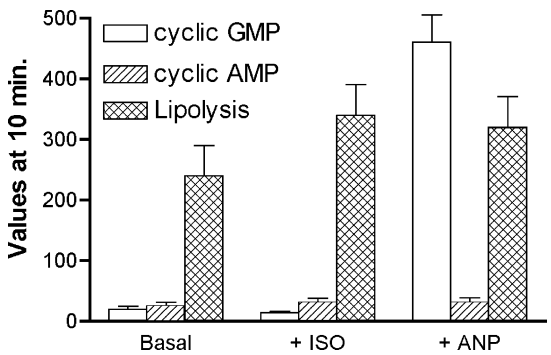

Fig. 5. Effect of ANP and ISO on cyclic GMP, cyclic AMP, and lipolysis. Human adipocytes (100 mg/mL) were incubated for 10 min without or with 10 nM ISO or 50 nM ANP. Values are the means ± SEM of 19 experiments for lipolysis (nmol/g), while those for cyclic GMP (pmol/g) and cyclic AMP (pmol/g) are from 5 experiments.

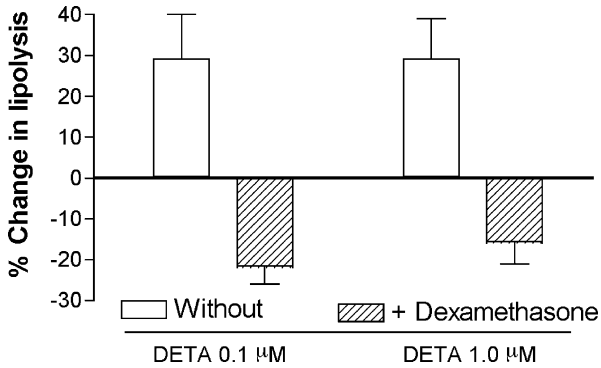

Fig. 6. Effect of DETA NONOate on lipolysis in the absence and presence of dexamethasone. Explants of human adipose tissue (100 mg/mL) were incubated for 48 hr in the absence or presence of 10 nM dexamethasone and/or DETA NONOate. Basal glycerol release averaged 9.4 and 8.2 μmol/g, respectively, in the absence and presence of dexamethasone. Values are means ± SEM of the paired differences in 8 experiments, and all were statistically significant ($P < 0.05$).

human adipocytes over a 2-hr incubation in the presence of millimolar concentrations of nitroglycerine or when the gas phase was nitric oxide [25]. These data indicate that there may be inhibitory effects of nitric oxide on human adipocyte lipolysis that oppose the ability of nitric oxide to activate guanylate cyclase.

In conclusion, our results indicate that ANP inhibits leptin release in isolated human adipocytes from obese individuals. The inhibition of leptin release appears to be secondary to elevations in cyclic GMP that activate lipolysis through mechanisms insensitive to insulin or H-89.

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