Synergism Between Insulin and Low Concentrations of Isoproterenol in the Stimulation of Leptin Release by Cultured Human Adipose Tissue

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The release of leptin by pieces of human adipose tissue incubated in primary culture for 24 or 48 hours in the presence of dexamethasone was reduced by isoproterenol. An inhibition of leptin release was observed at 24 hours in the presence of isoproterenol and was mediated by β_1-adrenergic receptors, since it was blocked by the specific β_1-adrenoceptor antagonist CGP-20712A. The inhibitory effect of 33 nmol/L isoproterenol on leptin release was reversed in the presence of 0.1 nmol/L insulin to a 2-fold stimulation of leptin release. These data suggest that the primary mechanism by which insulin stimulates leptin release is to blunt the inhibitory effects of β_1-adrenergic receptor agonists, and low concentrations of catecholamines actually enhance the stimulation of leptin release by insulin.

THE EXPRESSION OF LEPTIN mRNA is elevated in adipose tissue from morbidity obese individuals.[1,2] The release of leptin over a 2-hour incubation was also greater in adipose tissue from obese women.[3,4] The larger cell size of adipocytes from subcutaneous as compared with omental adipose tissue was associated with an increase in leptin release, as well as leptin mRNA.[4] These data suggest that subcutaneous fat is the major source of leptin release since it also the major fat depot. The finding of more leptin mRNA message in subcutaneous versus omental fat was confirmed by others.[5,6]

The factors that enhance leptin release by adipocytes as they expand in obese individuals are poorly understood, and the hormonal regulation of leptin release by adipose tissue is controversial. Glucocorticoids are the most potent known stimulators of leptin release and leptin mRNA accumulation by human adipose tissue, but the effects are slow in onset.[7] However, it has been argued that the effects of glucocorticoids are not physiological and are restricted to acute pharmacologic dosing.[8] Insulin administration or meal-feeding has no short-term effect on plasma leptin.[9-13] Insulin has actually been reported to inhibit leptin release by human adipocytes incubated for 36 or 48 hours with insulin in the presence of dexamethasone.[14]

Isoproterenol administration reduces plasma leptin within 2 hours in humans.[15,16] Infusion of isoproterenol also elevates plasma insulin, but this effect can be abolished by concurrent infusion of somatostatin without affecting the decrease in plasma leptin or the increase in plasma free fatty acids.[15] These data suggest that the ability of isoproterenol to enhance human adipocyte lipolysis and cyclic adenosine monophosphate accumulation through activation of β-catecholamine receptors may be an important factor in the regulation of leptin release. Trayhurn et al[17] have recently suggested a pivotal role for the sympathetic nervous system in the regulation of leptin biosynthesis.

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The present studies were designed to examine the direct effects of glucocorticoids, isoproterenol, insulin, or their combination on leptin release by human subcutaneous adipose tissue incubated in primary culture for 24 or 48 hours. Our results indicate that the actions of catecholamines and insulin on leptin release are intertwined.

SUBJECTS AND METHODS

Subjects

Subcutaneous adipose tissue was obtained from morbidly obese males or females with a body mass index (BMI) between 40 and 62 Kg/m^2 undergoing elective open abdominal surgery (gastric bypass) under general anesthesia after an overnight fast. The mean age of the patients was 42 years. The study received the approval of the local ethics committee, and all patients involved provided informed consent. None of the patients had significant weight change prior to surgery.

Adipose Tissue Culture

Twenty grams of abdominal subcutaneous adipose tissue was placed in saline at 37°C and immediately transported to the laboratory. The tissue was cut with scissors into small pieces (20 to 30 mg) and mixed with 5 mL incubation buffer. Pieces of adipose tissue were incubated in 50-mL polypropylene tubes containing 5 mL incubation buffer in an upright position in a gyratory water-bath shaker for 48 hours (100 rpm). The buffer for incubation of adipose tissue was Dulbecco's modified Eagle's medium/Ham's F12 (1:1, Sigma No. 2906) containing 17.5 mmol/L glucose, 121 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $CaCl_2$, 25 mmol/L HEPES, 2.4 mmol/L sodium bicarbonate, 10 mg/mL bovine serum albumin, 5 μg/mL ethanolamine, 0.1 ng/mL sodium selenite, 90 μg/mL penicillin G, 150 μg/mL streptomycin sulfate, 50 μg/mL gentamicin, 55 μmol/L ascorbic acid, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. The pH of the buffer was adjusted to 7.4 and the buffer was then filtered through a 0.2-μm filter.

Aliquots of the medium were taken at 24 and 48 hours and stored at −20°C for measurement of leptin content and lipolysis. The leptin content of 20- to 50-μL aliquots of the incubation medium was determined using radioimmunoassay kits from Linco Research. Adipose tissue was homogenized in 5 mL incubation medium, and aliquots (50 μL) were taken for determination of tissue leptin content. Lipolysis was based on the analysis of glycerol release into the medium and determined on 20- to 50-μL aliquots by the procedure of Boobis and Maughan.[18] Statistical comparisons were made using Student's *t* test.

Materials

Bovine serum albumin powder (Bovuminar lot no. L59410; containing <0.05 fatty acid/mol albumin) was obtained from Intergen (Purchase, NY). CGP-20712A ((±)-[2-(2-carbomyl-4-hydroxy-phenoxy)-ethylamino]-3-[4-l-methyl-4-trifluromethyl-2-imidazolyl)-phe-

noxy]2-propanol methane sulfonate) was a gift from Ciba Geigy (Basel, Switzerland). Isoproterenol, insulin, and other chemicals were from Sigma Chemical (St Louis, MO). All agents were added at the start of the 48-hour incubation period.

RESULTS

The leptin release into the medium over a 48-hour incubation represents newly synthesized leptin, since in adipose tissue from 4 females the initial leptin content was 12 ± 2 (mean ± SEM) ng/g fat. At the end of the 48-hour incubation, the leptin content of the adipose tissue was 9 ± 2 ng/g in the absence of dexamethasone and 47 ± 7 ng/g in the presence of 200 nmol/L dexamethasone. The accumulation of leptin in the medium in the same experiments was 111 ± 13 ng/g in the absence and 351 ± 40 ng/g in the presence of dexamethasone. Thus, for all practical purposes, leptin biosynthesis accounted for the leptin release over a 48-hour incubation.

In the studies shown in Fig 1, the accumulation of leptin in the medium was 60 ng/g fat at 24 hours and there was only a 28% increase in leptin formation due to 200 nmol/L dexamethasone at 24 hours, which was not statistically significant. In contrast, there was a 200% increase in leptin formation with 200 nmol/L dexamethasone versus without dexamethasone at 48 hours ($P < .025$). Leptin formation was not significantly affected at 24 hours in the presence of 25 nmol/L dexamethasone, but at 48 hours, there was a 47% increase in leptin over the control value ($P < .05$). The amount of leptin accumulation in the medium between 24 and 48 hours in dexamethasone-free medium was less than 23% of the accumulation during the first 24 hours. However, basal leptin release was essentially maintained in the presence of 25 nmol/L dexamethasone between 24 and 48 hours (Fig 1).

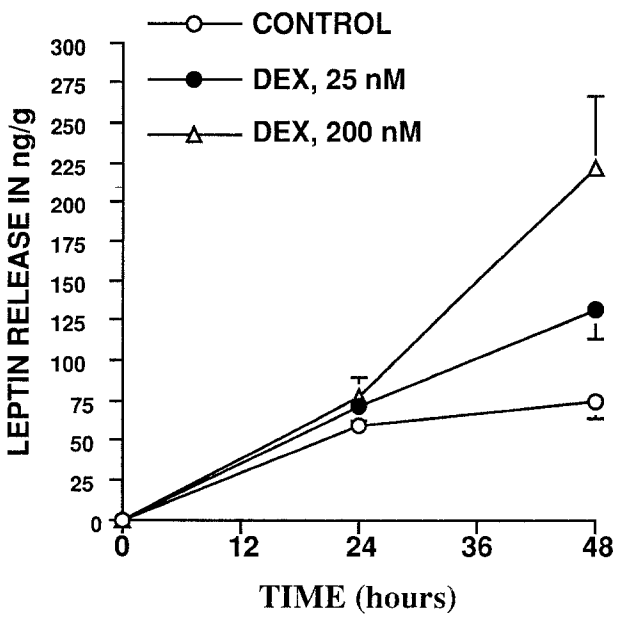


Fig 1. Time course for leptin release by human adipose tissue. Fragments of human subcutaneous adipose tissue (approximately 460 mg) were incubated for 48 hours in 5 mL medium either without (○) or with 25 nmol/L dexamethasone (●) or 200 nmol/L dexamethasone (△). Values for leptin release (ng/g adipose tissue) are the mean ± SEM from 4 females with an average BMI of 50 kg/m² and an average fasting blood glucose of 157 mg/dL.

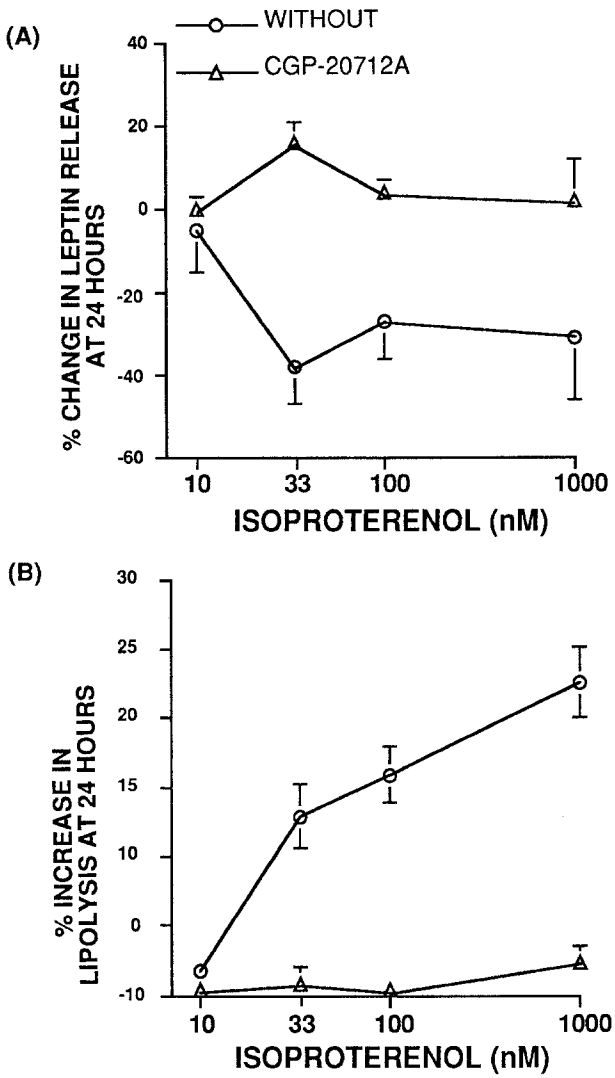
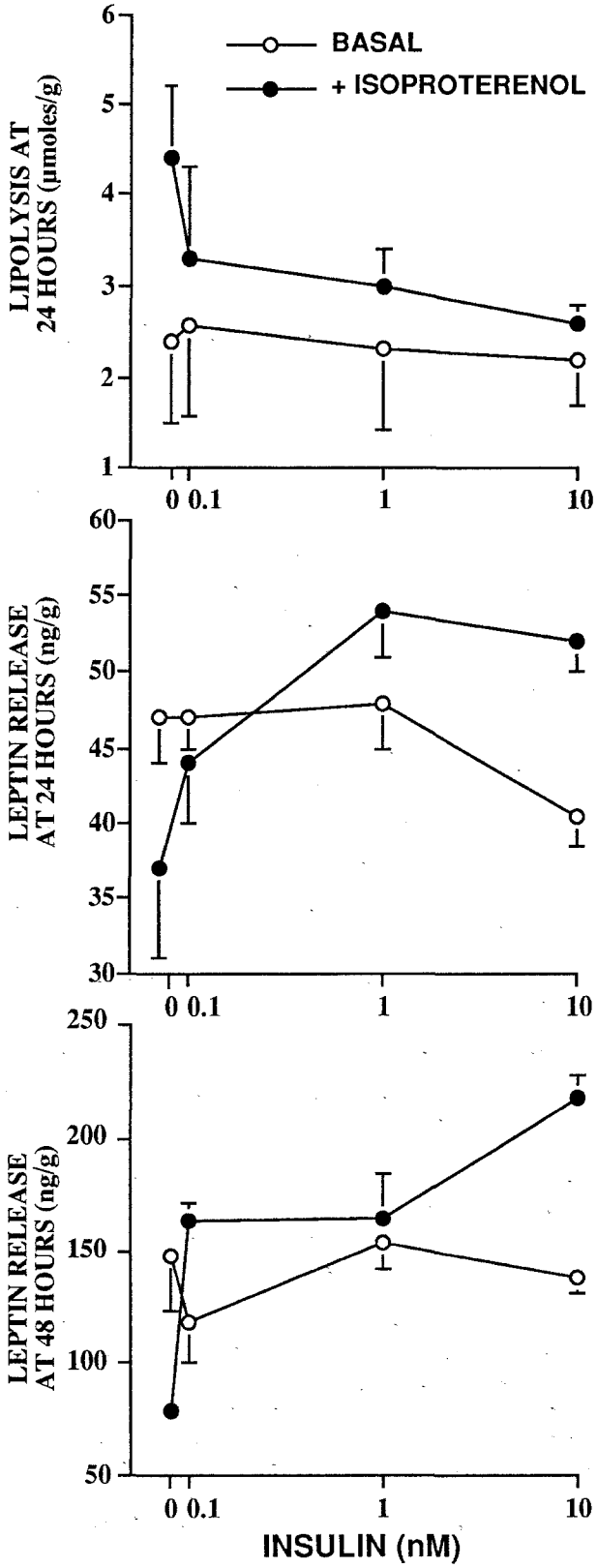


Fig 2. Inhibition by CGP-20712A of isoproterenol effects on lipolysis and leptin release. Fragments of adipose tissue (500 mg) were incubated for 24 hours in 5 mL medium with 25 nmol/L dexamethasone either without or with 500 nmol/L CGP-20712A in the presence of the indicated concentration of isoproterenol. Values are the % change ± SEM (n = 3) *v* the 24-hour basal value, which was 65 ng/g leptin in both the absence and presence of CGP-20712A, while basal lipolysis was 2.5 μmol/g in the absence and 2.3 μmol/g in the presence of CGP-20712A. The mean BMI was 42 kg/m² and blood glucose 164 mg/dL.

While a 48-hour incubation was required for the demonstration of significant effects of glucocorticoids on leptin, it was possible to observe inhibitory effects of isoproterenol on leptin accumulation at 24 hours (Fig 2A). The maximal inhibition of leptin formation at 24 hours required only 33 nmol/L isoproterenol, which stimulated lipolysis to about 50% of the level found with 100 nmol/L isoproterenol (Fig 2B). Human adipose tissue contains β_1- and β_2-adrenergic receptors.[19] The inhibition of leptin release and stimulation of lipolysis appear to be mediated by β_1-adrenergic receptors (Fig 2), since the effects of nanomolar concentrations of isoproterenol on both parameters were

abolished in the presence of CGP-20712A, a specific β_1-adrenergic antagonist.[20] We used 0.5 μmol/L CGP-20712A because this concentration inhibits β_1-adrenergic receptors without any effect on β_2-adrenergic receptors.[21]



Leptin formation by human adipose tissue is exquisitely sensitive to insulin, since low concentrations of insulin (0.1 nmol/L) reversed the inhibition of leptin release by 33 nmol/L isoproterenol in adipose tissue incubated for 24 or 48 hours (Fig 3). In the experiments depicted in Fig 3, there was a 57% increase in leptin formation due to isoproterenol in the presence of 10 nmol/L insulin, as compared with a 47% decrease due to isoproterenol in the absence of insulin, at 48 hours.

After the studies shown in Figs 1 to 3 were completed, we compared the rate of leptin accumulation in both the absence and the presence of 200 nmol/L dexamethasone by adipose tissue from 5 obese normoglycemic males, 6 obese hyperglycemic females, and 19 obese normoglycemic females. Leptin release was significantly lower in adipose tissue from hyperglycemic females or normoglycemic males. The BMIs were not significantly different between the 3 groups. We have found no correlation between the BMI (40 to 62 kg/m^2) and leptin release over 48 hours in adipose tissue from 29 markedly obese individuals (unpublished data, July 1999). Although leptin release was low in tissue from normoglycemic males or hyperglycemic females, the percentage increase in leptin release due to dexamethasone was unimpaired (Table 1).

For the studies shown in Table 2, we examined the effect of insulin and isoproterenol on leptin release by adipose tissue from 7 normoglycemic females in the presence of 200 nmol/L dexamethasone. The data in Table 2 show a decrease in leptin release due to isoproterenol (33 nmol/L) at 48 hours in the presence of 200 nmol/L dexamethasone of $-22\% \pm 6\%$ based on paired analysis ($P < .025$). However, lipolysis was not significantly enhanced by 33 nmol/L isoproterenol. Insulin (0.1 nmol/L) significantly inhibited lipolysis by -19% and -23% in the absence and presence of isoproterenol, respectively. There was a 104% increase in leptin release by 0.1 nmol/L insulin in the presence of isoproterenol that was statistically significant. In the absence of isoproterenol, the increase in leptin release due to insulin was only 20% and is not statistically significant (Table 2).

DISCUSSION

The tissue culture medium used for these experiments contained 17.5 mmol/L glucose, which represents a hyperglycemic condition (315 mg/dL). The complete omission of glucose from the medium reduced leptin release over 48 hours in the presence of 200 nmol/L dexamethasone by only $14\% \pm 3\%$ (mean ± SEM of 8 experiments, data not shown). These data do not agree with the suggestion that glucose metabolism regulates leptin secretion by rat adipocytes cultured for 96 hours in a Matrigel medium.[22] However, what Mueller et al[22] actually demonstrated is that inhibitors of glucose transport or of glycolysis markedly reduced leptin secretion. No effects of

Fig 3. Stimulation of leptin release by insulin in the presence of isoproterenol. Fragments of adipose tissue (500 mg) were incubated for 48 hours in the presence of 25 nmol/L dexamethasone in the absence (○) or presence of 33 nmol/L isoproterenol (●) both in the absence and presence of the indicated concentration of insulin. (A) Lipolysis at 24 hours; (B) leptin release at 24 hours; (C) leptin release at 48 hours. Values are the mean ± range of 2 paired experimental replications from 2 different individuals with an average BMI of 40 kg/m^2. Blood glucose was 105 mg/dL in 1 individual and 291 mg/dL in the other.

Table 1. Comparison of Leptin Release by Adipose Tissue From Obese Normoglycemic Females, Hyperglycemic Females, and Normoglycemic Males

Group	No. of Individuals	BMI (kg/m²)	Fasting Blood Glucose (mg/dL)	Leptin Release Over 48 Hours (ng/g)	
				Without Dexamethasone	+200 nmol/L Dexamethasone
Normoglycemic females	19	46 ± 1	96 ± 2	118 ± 7	265 ± 36
Hyperglycemic females	6	50 ± 3	202 ± 27	70 ± 13†	152 ± 41*
Normoglycemic males	5	52 ± 3	104 ± 8	64 ± 15†	129 ± 33*

NOTE. Fragments of human subcutaneous adipose tissue (400 mg) were incubated for 48 hours in 5 mL medium in the absence or presence of 200 nmol/L dexamethasone. Values are the mean ± SEM for the indicated number of individuals. Statistically significant differences in leptin release by adipose tissue from normoglycemic males or hyperglycemic females compared with normoglycemic females based on an unpaired *t* test are indicated.

*$P < .05$.

†$P < .01$.

omission of glucose from the medium were reported by Mueller et al.[22] Our data indicate that there are alternative sources of energy in human adipose tissue to support leptin secretion over a 48-hour incubation.

We were unable to confirm that there is a glucosamine-sensitive pathway that directly regulates leptin release.[23] The addition of 17.5 mmol/L glucosamine to human adipose tissue incubated for 48 hours in the presence of 4 mmol/L glucose and 200 nmol/L dexamethasone had no effect on leptin release (−8% ± 8%, mean ± SEM of 6 paired experiments). Our data indicate that the metabolism of glucose is not required to sustain leptin release by human adipose tissue incubated in primary culture for 48 hours in an enriched medium containing albumin and amino acids, as well as other possible sources of energy.

We found a substantial conversion of glucose to lactate (54 ± 3 μmol lactate accumulated/1 g adipose tissue over a 48-hour incubation, mean ± SEM for 14 different obese females) in the presence of 17.5 mmol/L glucose. Lactate formation was little affected by 200 mmol/L dexamethasone (0% ± 4% change) but was significantly increased by 0.1 nmol/L insulin (+9.2% ± 1.5%) in the same experiments. There was little accumulation of lactate in the absence of added glucose (data not shown), which suggests that the ability of adipocytes to secrete leptin does not depend on glucose metabolism. However, it cannot be excluded that the effects of insulin and isoproterenol on leptin release are dependent on the metabolism of glucose, since the effects of these agents were examined in the presence of 17.5 mmol/L glucose.

Table 2. Stimulation of Leptin Release by Insulin Is Enhanced in the Presence of a Low Concentration of Isoproterenol

Isoproterenol (nmol/L)	Basal	+Insulin 0.1 nmol/L	% Change Due to Insulin Addition	Statistical Significance
Leptin release (ng/g)				
0	438 ± 80	525 ± 90	+20 ± 10	$P < .1$
33	344 ± 86	702 ± 124	+104 ± 35	$P < .025$
Lipolysis (μmol/g)				
0	6.6 ± 1.2	5.2 ± 0.7	−19 ± 6	$P < .025$
33	7.3 ± 0.7	5.6 ± 0.8	−23 ± 5	$P < .01$

NOTE. Fragments of human subcutaneous adipose tissue (400 mg) were incubated for 48 hours in 5 mL medium in the presence of 200 nmol/L dexamethasone and insulin or isoproterenol as indicated. Basal values are the mean ± SEM of 7 paired experimental replications from 7 different females with a mean BMI of 43 kg/m² and a fasting blood glucose of 103 mg/dL. The % changes due to insulin are the mean ± SEM of the paired differences, and significance based on the paired *t* test is indicated.

A 20% to 27% decrease in plasma leptin at 2 hours has been reported in humans infused with 24 ng/kg/min isoproterenol.[15,16] Our in vitro studies demonstrate a similar decrease in leptin release in adipose tissue incubated for 24 hours with 33 nmol/L isoproterenol (Table 2). While the stimulation of leptin release by insulin was only 20% in the absence of isoproterenol, it was possible to observe a 104% increase due to 0.1 nmol/L insulin in the presence of 33 nmol/L isoproterenol. Donahoo et al[15] found an increase in plasma insulin from a basal value of 0.023 nmol/L to 0.035 nmol/L at 2 hours after infusion of 8 mg/kg/min isoproterenol, and the increase in insulin release was blocked in the presence of somatostatin. However, the infusion of somatostatin itself reduced plasma leptin at 2 hours by 20%, while 8 mg/kg/min isoproterenol reduced leptin by 16%.[15]

In humans, the decrease in plasma leptin over a 72-hour fast was blocked by concurrent infusion of glucose, thus preventing the decrease in plasma insulin.[24] The decrease in plasma leptin was near-maximal within the first 24 hours of starvation.[24] The primary effect of insulin on leptin release by human fat may be to reverse the inhibitory effect of catecholamines, as postulated by Trayhurn et al.[17] The addition of only 0.1 nmol/L insulin at the start of a 48-hour incubation reversed the inhibition of leptin release by isoproterenol or forskolin, suggesting that these effects are readily demonstrable with physiological concentrations of insulin. Furthermore, leptin release was enhanced to a greater extent by insulin in the presence of a low concentration of isoproterenol versus its absence (Table 2). This might be due to the enhanced availability of free fatty acids released during lipolysis, especially arachidonate, for conversion to products that stimulate leptin release.

Russell et al[25] reported that 7 nmol/L insulin increased leptin release after 1 day, but not after 3 days, of culture of subcutaneous adipose tissue in medium without added dexamethasone, albumin, or serum. After 3 days of culture, an effect of insulin on leptin release was found in the presence of 25 nmol/L dexamethasone.[25] More recently, Ricci and Fried[26] found that 10,000 nmol/L isoproterenol reduced leptin release by 30% over a 3-hour incubation of human subcutaneous adipose tissue. Leptin release was also diminished even in the presence of insulin and dexamethasone by isoproterenol after a 24-hour incubation. Our data and those of Russell et al[25] are in

contrast to the inhibitory effect of insulin on leptin release reported in adipose tissue pieces[7] and in human adipocytes[14] incubated in medium containing 10% fetal calf serum.

Most in vitro studies on leptin release have used cut pieces of intact human adipose tissue.[3,4,7,25,26] Moreover, we have shown that there is a substantial loss (40%) of 18S RNA if adipocytes derived by collagenase digestion of cut pieces of rat adipose tissue are incubated in primary culture for 24 hours.[27] The loss of 18S was much less in cut pieces of rat adipose tissue after 24 hours.[28] Our basal release of leptin into the medium by pieces of human adipose tissue over the first 24 hours of incubation (47 to 77 ng/g) is comparable to that reported by Russell et al[25] of about 90 ng/g fat/24 h. Klein et al[29] reported that the in vivo rate of leptin release by male (BMI = 27 Kg/m^2) was approximately 60 ng/g fat/24 h. The serum leptin level is lower in males versus females with the same BMI.[30-32] The rate of leptin release was lower for omental adipose tissue in primary culture from males versus females.[32] We confirmed this finding of a decreased release of leptin using human subcutaneous adipose tissue from males (Table 1). However, we found that dexamethasone increased leptin release to approximately the same extent in subcutaneous adipose tissue from normoglycemic males and normoglycemic females. In contrast, Casabiell et al[32] reported no stimulation of leptin release by dexamethasone in omental adipose tissue from males. Whether this is due to differences in the fat depots remains to be demonstrated.

The release of leptin over 48 hours in the studies shown in Table 1 was 118 ng/g in adipose tissue from 19 normoglycemic markedly obese women in the absence of dexamethasone and 265 ng/g in the presence of dexamethasone. We found that in adipose tissue from markedly obese females with hyperglycemia, leptin release was reduced by about 40% in both the absence and presence of added dexamethasone despite a slightly but not significantly elevated BMI (Table 1). These data are in agreement with those of Sinha et al,[33] who found a 26% reduction in plasma leptin in obese individuals with type 2 diabetes compared with obese individuals without diabetes. The nocturnal elevations in plasma leptin were exactly the same in obese individuals with or without diabetes despite the higher blood glucose in diabetic individuals.[33] The nocturnal increase in leptin is well established[33-35] but is accompanied by decreases in both plasma cortisol and insulin, which is paradoxical since both are stimulators of leptin release by adipose tissue. Since the only known fast-acting regulator of leptin release is catecholamines, it may be that the increase in leptin is secondary to a decrease in catecholamine release at nerve endings in adipose tissue, since plasma levels of catecholamines are lowest at night.[36,37] These data are in agreement with but do not prove the hypothesis that the nocturnal elevation in serum leptin may be secondary to the decrease in sympathetic activity.

In conclusion, our data indicate that low concentrations of isoproterenol, via activation of β_1-adrenergic receptors, are able to inhibit leptin release in the absence of insulin. There was only a small stimulation of leptin release by insulin (0.1 to 10 nmol/L) in fragments of human subcutaneous adipose tissue incubated in primary culture for 48 hours in the absence of isoproterenol. However, in the presence of a low concentration of isoproterenol that acts as an adrenergic agonist, insulin stimulated leptin release to greater extent than insulin alone.

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