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Inhibition of leptin secretion by insulin and metformin in cultured rat adipose tissue

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Abstract

Leptin's role in the regulation of food intake, energy expenditure and weight control are widely recognized, especially in rodents. Likewise, the potential regulation of leptin secretion by insulin (and vice versa) has been of particular interest insofar as these nutrient signals may have meaningful, even adverse (inter)actions, in diabetes. We used a freshly isolated rat adipose tissue culture model to examine the effect of insulin, metformin and glibenclamide on basal and steroid-stimulated leptin secretion. This model was selected because of its physiologic rates of leptin formation and preservation of potentially significant cell–cell interactions compared to isolated cells. The basal rate of leptin secretion was 3.4 ± 1.2 ng/100 mg tissue/24 h. The addition of 100 nM dexamethasone or 400 nM hydrocortisone stimulated leptin secretion by 3–4 fold over basal (no steroid). Insulin inhibited both basal and steroid-activated leptin secretion by 35–50%. This inhibition was present with either 1 mM pyruvate or 5 mM glucose as a substrate suggesting that glycolysis was not required. Metformin inhibited basal and dexamethasone-stimulated leptin secretion in a dose dependent manner (50% inhibition occurred at 1 mM metformin) while glibenclamide was ineffective. The effect of insulin on isolated fat cells versus fat tissue was tested in parallel. After 24 h in culture, insulin inhibited leptin secretion similarly in both adipose preparations. The addition of 200 nM (−)N6-(2-phenylisopropyl)-adenosine did not alter the results.

Keywords: Leptin; Insulin; Adipose tissue; Metformin

1. Introduction

Leptin, the 16 kDa product of the *ob* gene, is an adipocyte-derived hormone. Its essential role in the regulation of food intake, energy expenditure and total body fat stores is well recognized [1]. Furthermore, diverse neuroendocrine actions for leptin are emerging [2]. A paradigm for leptin secretion, therefore, exists in which blood leptin levels fall when caloric or energy intake is reduced and fat stores decline. Conversely, blood leptin levels would rise with feeding and expanded fat stores which, in turn, would suppress appetite and enhance thermogenesis.

Recognizing the pivotal role of insulin in metabolic homeostasis, the potential and manifold interactions between leptin and insulin are of obvious interest, especially in disease states such as insulin resistant diabetes wherein blood insulin and leptin levels are both frequently elevated. Under normal physiologic conditions, and analogous to leptin, in-

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sulin levels rise with feeding and fall with fasting. Inasmuch as leptin might alter insulin secretion or its peripheral action and, on the other hand, insulin might modify leptin's secretion or action, the interactions of these two hormones could have a profound impact on energy metabolism [3].

We examined one aspect in this complex scenario, namely, whether insulin has direct effects on adipose tissue leptin secretion. The antidiabetic agents metformin and glibenclamide were, furthermore, tested for their potential direct regulation of adipose tissue leptin secretion. We found that insulin causes a direct inhibition of basal and steroid-stimulated leptin secretion in cultured fat explants as well as isolated fat cells; metformin's actions were analogous.

2. Materials and methods

2.1. Materials

Tissue culture media and ingredients were purchased from Life Technologies (Grand Island, NY, USA). General chemicals were from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Animals and tissue culture

Following euthanasia by cervical dislocation at between 9 and 10 a.m., adipose tissue was obtained from the epididymal fat pads of three or four, 150–250 g fed Sprague-Dawley rats. The tissue was excised and placed in sterile Dulbecco's medium (DMEM) containing 20 mM HEPES, pH 7.4, 5 mM glucose, 1% (w/v) bovine serum albumin (BSA), 100 IU/ml penicillin and 100 μg/ml streptomycin. The total tissue was trimmed of visible vessels and blood clots, minced into 2–4 mm^3 pieces, mixed gently and distributed into 1 cm plastic culture wells such that each well contained 100 mg tissue in 1 ml of the above DMEM. All groups were prepared in quadruplicate. The samples were then incubated at 37°C under 5% CO_2 for 24 h and an aliquot of the medium was removed to measure leptin. Fat cells were cultured as noted previously [4]. For experiments in which fat cells versus fat tissue were compared in parallel, epididymal fat tissue from several rats was pooled, minced and divided for either direct culture (explants) or collagenase isolation (cells) prior to culture.

2.3. Leptin secretion

Leptin was measured with a commercial rat leptin radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, MO, USA). The sensitivity of this commercial assay is 0.5 ng/ml leptin. The intra-assay coefficient of variation was 3.4%.

2.4. Statistics

Fat explant or cell samples, pooled from three or four rats ($n = 1$), were tested ($\pm$ leptin, etc.) in quadruplicate unless otherwise noted. Results are given as a mean $\pm$ S.D. The paired Student's *t*-test was used for statistical analysis.

3. Results

3.1. Fat tissue culture

The viability of the cultured epididymal fat tissue was established by several criteria: (1) The rate of leptin secretion (ng secreted/g tissue/24 h) was comparable to published norms for both human adipose tissue leptin secretion in vivo [5] and cultured rat adipose tissue in vitro [6]. (2) After incubating the fat tissue explants for 24 h, the fat pieces were colla-

Table 1
Effect of insulin on basal and dexamethasone-stimulated leptin secretion from cultured rat adipose tissue

[Insulin] nM	No dexamethasone	With dexamethasone (100 nM)
0	100 ± 0 (5)	100 ± 0 (5)
0.5	84 ± 4 (3)**	85 ± 3 (4)**
1.0	80 ± 13 (4)	73 ± 5 (4)**
100	73 ± 7 (5)**	63 ± 8 (5)**

Rat adipose tissue was incubated in the absence and presence of 100 nM dexamethasone and with increasing concentrations of insulin. Results are expressed as mean ± S.D. (*n*) relative to the control (no insulin). The control rates of leptin formation for the no dexamethasone versus 100 nM dexamethasone groups were 4.1 ± 0.7 versus 11.9 ± 1.4 ng leptin secreted/100 mg tissue/24 h. Statistically significant differences due to insulin are shown (**$P < 0.01$).

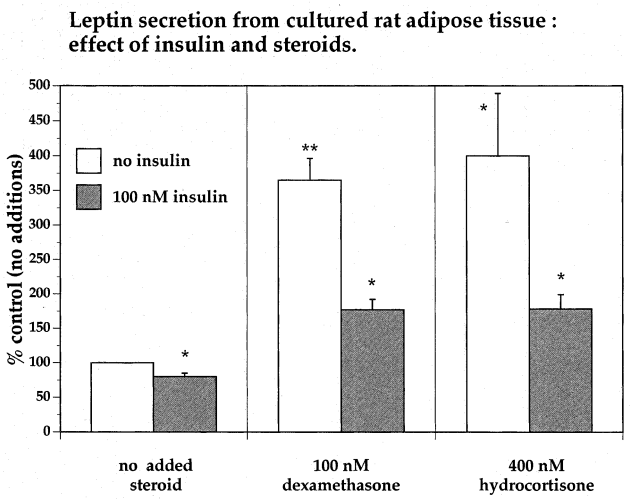

Fig. 1. Leptin secretion from cultured rat adipose tissue: effect of insulin and steroids. Rat epididymal adipose tissue was incubated with (shaded bars) and without insulin (open bars) under three conditions: no added steroid, 100 nM dexamethasone, or 400 nM hydrocortisone. Results are expressed as mean ± S.D. ($n = 4$) relative to the control (no insulin or steroid). The control rate of leptin production was 3.4 ± 1.2 ng/100 mg tissue/24 h. Statistical differences due to steroids alone (open bars, no insulin) are versus the control (*$P < 0.05$, **$P < 0.01$). In each of the three treatment groups, the effect of insulin was tested. These statistical differences are given over the shaded bars (*$P < 0.05$).

genase-isolated to release free cells [7]. These isolated cells were greater than 95% viable by propidium iodide staining. Moreover, using this collagenase technique, both the percent viable cells and the number of cells isolated/100 mg of cultured tissue were no different between the control and treatment groups. (3) Leptin secretion rates were constant throughout the 24 h incubation and linearly related to the amount of tissue added (data not included).

3.2. *Leptin secretion: the effect of steroids, insulin and substrate*

Basal rates of leptin formation were 3.4 ± 1.2 ng secreted/100 mg tissue/24 h. The addition of pharmacological amounts of steroid (100 nM dexamethasone) increased leptin formation 3.7 fold. Importantly, 400 nM hydrocortisone, also stimulated leptin production to a similar extent over basal (Fig. 1). In each case, basal- or steroid-stimulated, 100 nM insulin inhibited leptin secretion by 35–50% compared to its respective pair without insulin. The inhibition of leptin secretion by insulin was, furthermore, dose dependent (Table 1). Neither leptin secretion nor the effect of insulin was altered by the addition of the non-hydrolyzable adenosine agonist (−)N6-(2-phenylisopropyl)-adenosine (data not included).

The effect of various culture medium substrate conditions on basal and steroid-induced leptin was examined. The first comparison was between 5 mM glucose (DMEM) versus 25 mM glucose (DMEM). Rates of leptin secretion in the absence of added steroid (basal conditions) were 3.4. ± 1.2 versus 4.2 ± 1.2 ng secreted/100 mg tissue/24 h ($n = 4$) in the presence of 5 versus 25 mM glucose, respectively ($P < 0.005$). There was no difference, however, when these same groups (5 versus 25 mM glucose) were compared in the presence of 100 nM dexamethasone

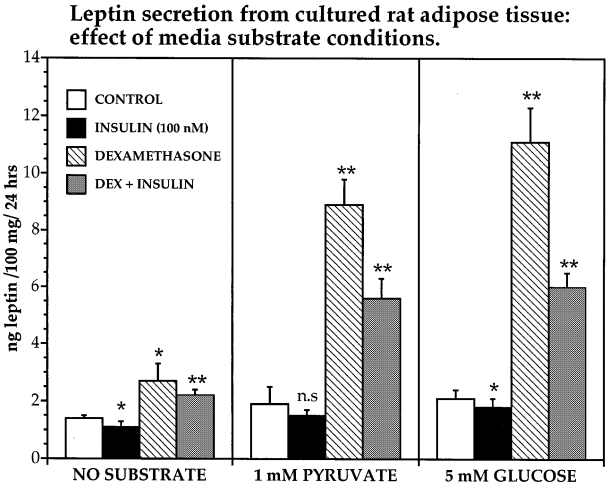

Fig. 2. Leptin secretion from cultured rat adipose tissue: effect of medium substrate conditions. Rat adipose tissue was incubated under three medium substrate conditions (with no added substrate, 1 mM pyruvate, or 5 mM glucose). In each of these three groups, four conditions were tested as indicated in the legend: control (no steroid or insulin added – open bars), 100 nM insulin (black bars), 100 nM dexamethasone (hatched bars), and 100 nM insulin with 100 nM dexamethasone (shaded bars). Within each of the three medium substrate groups, statistical differences are presented relative to the control (no insulin or dexamethasone) within that set (*$P < 0.05$, **$P < 0.01$, n.s. means not significant). The difference between the insulin+dexamethasone versus dexamethasone pairs in each medium set is not shown (for clarity) but was $P < 0.05$ for the 'no substrate group' and $P < 0.01$ for both the '1 mM pyruvate' and '5 mM glucose' groups. Data are given as mean ± S.D. ($n = 3$) and statistical comparisons are by paired t-test.

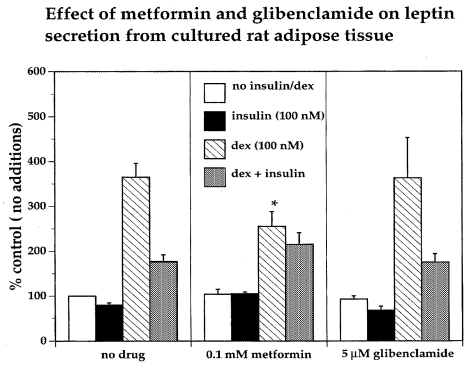

Fig. 3. Effect of metformin and glibenclamide on leptin secretion from cultured rat adipose tissue. Rat adipose tissue was incubated under three conditions: in the absence of added drug or with either 0.1 mM metformin or 5 μM glibenclamide. In each of the three conditions, the fat tissue was also incubated with and without 100 nM insulin and 100 nM dexamethasone as shown in the legend and described in Fig. 2. Results are expressed as mean ± S.D. ($n=4$) relative to the control (no drug, insulin, or dexamethasone). The control rate of leptin formation was 3.9 ng/100 mg fat tissue/24 h. The only statistically significant effect due to a drug was caused by metformin in the presence of dexamethasone (*$P<0.05$).

(the rates of leptin secretion were 15.1 ± 8.9 versus 16.7 ± 10.4 ng/100 mg tissue/24 h, ($n=4$) respectively).

The culture medium was next adjusted to contain either 1 mM pyruvate (no glucose), 5 mM glucose (without pyruvate) or no added glucose or pyruvate in DMEM (plus other basic medium additions as described in Section 2), see Fig. 2. In each of these three experimental groups, leptin secretion was examined with and without insulin and in the absence and presence of 100 nM dexamethasone. Fig. 2 demonstrates that the inhibitory effect of insulin on leptin secretion is present in each group except one (the 1 mM pyruvate group without added steroid).

3.3. Comparison of leptin secretion from cultured fat cells versus fat tissue

In separate experiments, we compared leptin secretion from cultured fat cells versus fat tissue in the presence of 100 nM dexamethasone and with and without 100 nM insulin. After 4 and 24 h in culture, insulin inhibited leptin secretion from minced fat tissue by 34 ± 13% and 17 ± 13%, respectively. In fat cells, however, insulin stimulated leptin secretion by 27 ± 10% at 4 h but inhibited leptin secretion by 31 ± 10% at 24 h. Of importance, there was no change in fat cell counts throughout the 24 h incubation.

3.4. Leptin secretion: the effect of metformin and glibenclamide

Fig. 3 shows the effect of 0.1 mM metformin and 5 μM glibenclamide on adipose tissue leptin secretion. In each of the three panels in Fig. 3, leptin secretion is examined with and without insulin and in the absence and presence of 100 nM dexamethasone. The results are expressed relative to the control (no added drug, insulin or steroid). The effect of insulin and steroid are comparable with the results presented in Fig. 1. Therefore, only statistically significant comparisons for the action of metformin or glibenclamide are presented. As is shown in Fig. 3, only metformin caused a statistically significant inhibition of dexamethasone-stimulated leptin secretion. Metformin did not augment the inhibitory action of insulin on leptin secretion. Fig. 4 demonstrates the dose response curve for the action of metformin on basal and dexamethasone-stimulated leptin secretion.

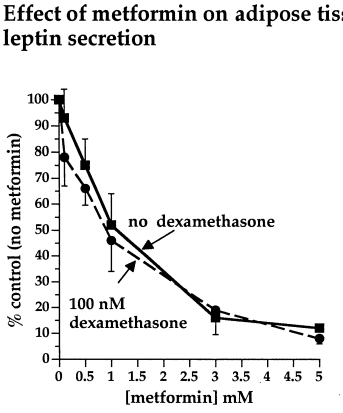

Fig. 4. Dose response for effect of metformin on adipose tissue leptin secretion. Rat adipose tissue was cultured in the presence and absence of 100 nM dexamethasone and with increasing amounts of metformin. Results are given as mean ± S.D. ($n=4$) relative to the controls (no metformin, with or without dexamethasone). The rate of leptin formation was 2.3 ± 0.8 versus 8.0 ± 3.2, ($n=4$) in the no steroid versus 100 nM dexamethasone groups (without metformin), respectively. Metformin caused significant inhibition of leptin secretion ($P<0.05$) at concentrations greater than 0.5 mM (both with and without dexamethasone). At 0.1 mM metformin, only the dexamethasone-stimulated leptin secretion was significantly inhibited ($P<0.05$).

Metformin caused a significant reduction in leptin secretion at concentrations greater than 0.5 mM.

4. Discussion

The secretion of leptin by adipose tissue underlies the mechanism by which the status of peripheral energy stores is communicated to the central nervous system (CNS). Overall, the emerging picture is one in which the serum leptin level reflects both a stable index of fat stores (the lipostat theory [8]) and yet may be subject to regulation by other factors. Various hormones, circulating compounds, and neurogenic signals have been examined for their potential ability to regulate adipose leptin secretion, including insulin, cAMP and glucocorticoids. Given the association between obesity and diabetes, we investigated whether insulin or two antidiabetic agents, metformin and glibenclamide, might alter adipose tissue leptin secretion.

To date, there have been numerous in vitro studies examining whether insulin regulates serum leptin concentrations. Meaningful comparisons between these diverse experimental designs requires consideration of many factors, including, the animal and fat depot of origin [9], whether collagenase isolated cells, tissue explants or induced preadipocytes are employed [10,11], the incubation time, and also, whether steroids are added [12,13].

Using collagenase-isolated cells from human fat tissue (no steroids) [12,14,15], exposure to 100 nM insulin, long-term, for 72 h, (two subjects, abdominal fat) [14] or 96 h (six subjects, subcutaneous fat) [15] increased leptin secretion by 900% and 190%, respectively. In contrast, however, using isolated subcutaneous fat cells from surgical biopsies, an inhibitory action of insulin on leptin production was first noted [12]. These investigators found that insulin inhibited dexamethasone-stimulated (but not basal) leptin secretion by about 200% following prolonged culture (after 36–48 h). This result is consistent with ours wherein insulin (with dexamethasone) inhibited leptin secretion from both fat cells and fat tissue at 24 h.

Using freshly isolated rat adipocytes exposure to insulin either acutely (4 h or less) [13,16,17] or over days [18,19] led to increased basal (non-steroid-stimulated) leptin secretion. Other studies, using cultured preadipocytes [20–23] have also found that insulin enhances leptin secretion despite their very low rates of leptin secretion [11].

The effect of insulin on cultured adipose tissue explants (obtained from either rats or humans) differs from the above isolated fat cell studies. For example, using either rat epididymal tissue [24] or human omental and visceral tissue from lean and obese subjects [6], steroid-stimulated but not basal *ob* mRNA or leptin secretion was inhibited 40–50% by 100 nM insulin when these tissues were cultured for 12–48 h. These studies are consistent with our data. In addition, we demonstrate an inhibitory action of insulin on leptin secretion in the absence of steroid as well as with both high-dose (dexamethasone) and physiologic (hydrocortisone) steroids [25] (Fig. 1). Antithetical to these fat explant studies, one study using rat adipose tissue found that 700 nM insulin stimulated basal leptin formation by 80% [16]. This fat explant study differs substantially, however, in that rat epididymal fat was incubated acutely (4 h) and without added steroid. To address this further we measured the effect of insulin on leptin secretion from isolated rat fat cells versus fat tissue at 4 and 24 h in the presence of 100 nM dexamethasone only. While insulin consistently inhibited fat tissue, it caused a brief $27 \pm 10\%$ stimulation of leptin secretion at 4 h. Of importance, however, similar to the fat explants, insulin inhibited fat cell leptin secretion by $31 \pm 6\%$ at 24 h.

Taken together, the above-cited studies and ours support the concept that while insulin appears to have an early stimulatory effect on leptin secretion, the long-term in vitro action of insulin on leptin secretion is inhibitory ([6,12,24], and this study), particularly in the presence of steroids. Perhaps the short-term stimulation is related to the release of preformed leptin [13,16]. Since cyclohexamide attenuates the inhibition of *ob* mRNA in rat fat [24], it may be that long-term exposure to insulin permits sufficient accumulation of an intracellular inhibitor to manifest this action. It is also compelling to consider that paracrine actions, perhaps related to either local proteases or the uptake or release of soluble regulators from either fat cells or their surrounding stromal cells [10] might play a role in modulating the effect of insulin on leptin secretion. These actions would be best appreciated using intact fat tissue as

opposed to dispersed cells. Along these lines, we tested whether the 24 h extracellular conditioned medium from fat cells versus fat tissue contained a soluble, insulin-responsive factor that could degrade recombinant leptin. None, however, was detected (data not included). Since we found that insulin inhibited both fat cells and tissue at 24 h, paracrine etiologies are less likely in the long-term.

We show that steroid-stimulated leptin secretion was present in: the absence of glucose, and with either 5 mM glucose, 25 mM glucose, or 1 mM pyruvate as a substrate (Fig. 2). Since the results with pyruvate and glucose were equivalent, glycolysis is not essential for the regulation of leptin secretion. Our findings differ from those obtained using gel matrix-cultured rat adipocytes [19] in which insulin treatment and/or the attendant increase in glucose metabolism were felt to augment leptin secretion. It is impossible to reconcile differences between our study and one with such differing methodology. Furthermore, final cell counts and absolute rates of leptin production are not provided [19]. Our data, furthermore, suggest that while hyperglycemia per se does not have a major effect on maximally stimulated adipose tissue leptin secretion, it may increase basal secretion (23% in these in vitro studies).

Metformin and glibenclamide were tested to determine whether two antidiabetic agents with dissimilar mechanisms of action might affect adipose tissue leptin secretion. Metformin inhibited both basal and dexamethasone stimulated leptin secretion in a dose dependent manner (Fig. 4), while glibenclamide was ineffective. The ED_{50} for the observed inhibition by metformin was 1 mM. This concentration is higher than the in vivo therapeutic range for metformin (12–100 μM [26] but well within the in vitro experimental test range [27–29]. Insofar as metformin can affect adipocyte intracellular metabolism [26,30], this antidiabetic agent may alter leptin secretion by the same intracellular mechanism proposed above. Recently, and consistent with our data, metformin was shown to decrease serum leptin concentrations in obese women with polycystic ovary syndrome [31]. Not surprisingly, glibenclamide, a sulfonylurea whose primary in vivo action is at the Beta cell, did not alter fat tissue leptin secretion (Fig. 3). The increase in serum leptin found in NIDDM patients treated with glibenclamide [32], could be related to increased insulin, increased weight or improved glycemic control (data not presented by the authors). Also, while leptin and insulin both increased during this drug trial [32], it remains undetermined whether changes in leptin resistance contributed independently to the rise in serum leptin.

In summary, a cultured fat explant model was used to study the regulation of adipose tissue leptin secretion. Insulin and metformin caused a dose dependent inhibition of fat tissue leptin secretion both with and without added steroids. This inhibition was present with various substrates including glucose and pyruvate suggesting that intrinsic glycolysis was unnecessary for the response. These fat explant studies, in concert with the previous studies using cultured fat tissue [6,24], demonstrate an inhibitory action of insulin on leptin secretion. While the mechanism for this inhibition remains unknown, protein synthesis appears to be required [24]. Taken together, these cultured explant studies also suggest that paracrine factors may be crucial in modulating the acute inhibitory effect of insulin on leptin secretion since studies in isolated fat cells have largely derived an opposite conclusion. Along these lines, autocrine effects of leptin on adipocytes have been increasingly recognized [33–36].

Finally, it is reasonable to propose that, at least in the early phases of fat accretion, a direct inhibition of leptin secretion by insulin could be an evolutionary survival advantage for mammals that rely on erratic food supplies by maximizing fat accretion (reducing satiety) when food is available.

Acknowledgements

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