Regulation of leptin secretion from white adipocytes by insulin, glycolytic substrates, and amino acids

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Am J Physiol Endocrinol Metab 289: E166–E171, 2005. First published March 1, 2005; doi:10.1152/ajpendo.00602.2004.—The aim of the present study was to determine the respective roles of energy substrates and insulin on leptin secretion from white adipocytes. Cells secreted leptin in the absence of glucose or other substrates, and addition of glucose (5 mM) increased this secretion. Insulin doubled leptin secretion in the presence of glucose (5 mM), but not in its absence. High concentrations of glucose (up to 25 mM) did not significantly enhance leptin secretion over that elicited by 5 mM glucose. Similar results were obtained when glucose was replaced by pyruvate or fructose (both 5 mM). L-Glycine or L-alanine mimicked the effect of glucose on basal leptin secretion but completely prevented stimulation by insulin. On the other hand, insulin stimulated leptin secretion when glucose was replaced by L-aspartate, L-valine, L-methionine, or L-phenylalanine, but not by L-leucine (all 5 mM). Interestingly, these five amino acids potently increased basal and insulin-stimulated leptin secretion in the absence of glucose or insulin. Finally, nonmetabolizable analogs of glucose or amino acids were without effects on leptin secretion. These results suggest that 1) energy substrates are necessary to maintain basal leptin secretion constant, 2) high availability of glycolysis substrates is not sufficient to enhance leptin secretion but is necessary for its stimulation by insulin, 3) amino acid precursors of tricarboxylic acid cycle intermediates potently stimulate basal leptin secretion per se, with insulin having an additive effect, and 4) substrates need to be metabolized to increase leptin secretion.

tricarboxylic acid cycle intermediates; metabolism; energy

LEPTIN IS A HORMONE MAINLY SECRETED by mature white adipocytes (45). It acts within specific areas of the hypothalamus to regulate energy expenditure, food intake, and the activity of the sympathetic nervous system, at least in rodents, therefore contributing to the regulation of body weight via a negative feedback loop (1, 9, 12, 14). Its concentration in the plasma is correlated with the total amount of white adipose tissue in the body, consistent with its role as a “lipostatic factor.”

Leptin transcription and secretion are modulated by numerous factors. For example, dexamethasone, peroxisome proliferator-activated receptor-γ agonists, and glucocorticoids are potent stimulators, whereas catecholamines, free fatty acids, and thyroid hormones inhibit leptin secretion (3, 14, 40). Glucose and insulin are also involved in the regulation of leptin secretion under physiological and in vitro conditions. In vivo, starvation or food deprivation are two conditions that decrease both insulinemia and glycemia, as well as plasma leptin levels and leptin transcription in adipose tissue (8, 41). Refeeding or injection of insulin increases plasma glucose, insulin, and leptin concentrations and reverses the decrease of leptin transcription in adipocytes of rodents and humans (17, 36). Leptinemia seems therefore to tightly follow variations of plasma insulin and glucose concentrations, independent of changes in adipose tissue mass.

Some observations have led to the hypothesis that glucose or other energy substrates are the major regulators of leptin secretion, with insulin having a minor role. In male mice, plasma leptin decreases with age and is more correlated with glucose than with insulin concentrations (31). Likely, the correlation between plasma glucose and leptin is stronger than the correlation between plasma insulin and leptin (32). Nevertheless, streptozotocin-induced diabetic rats are hyperglycemic and hypo leptinemic, and injection of insulin reverses this state (13). In vitro, high concentrations of glucose, L-alanine, or other substrates have been reported to stimulate leptin secretion per se at levels similar to those obtained with insulin (21, 31), whereas vanadium and metformin, two agents that increase glucose uptake, decreased or did not change leptin secretion from cultured adipocytes, respectively (32). Besides glucose and insulin, plasma amino acid levels also increase after food intake (10, 29) and have been shown to increase insulin secretion from β-cells (37). Amino acids are also potent regulators of protein synthesis in numerous tissues (15). However, so far, the role of amino acid in leptin secretion by white adipose tissue has scarcely been studied and has been limited to L-alanine and L-leucine (20, 35).

The aim of the present study was to determine the respective importance of metabolic substrates and insulin on leptin secretion from white adipocytes. Various glycolytic substrates and amino acids entering either glycolysis or the tricarboxylic acid cycle were assessed for their ability to modulate leptin secretion in the presence or absence of insulin. We report herein for the first time that glycolytic substrates are necessary to maintain basal leptin secretion but are not sufficient per se to stimulate leptin secretion to the same extent as does insulin. Interestingly, several amino acid precursors of tricarboxylic acid cycle intermediates also maintained basal leptin secretion but were also able to acutely and potently stimulate leptin secretion in the absence of insulin. Addition of insulin has an additive effect, suggesting that insulin and amino acids stimulate leptin secretion by two independent pathways.

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ENERGY SUBSTRATES IN LEPTIN SECRETION

METHODS

Chemicals. Fatty acid-free BSA, collagenase (type II, lot 107H8649), d-glucose, pyruvate, D-fructose, L-alanine, L-glucose, L-leucine, L-valine, L-glutamate, L-glutamine, L-phenylalanine, L-histidine, L-arginine, L-methionine, L-aspartate, L-glucose, D-glutamate, D-leucine, and 2-amino-2-norbornane carboxylic acid (BCH) were all obtained from Sigma Chemical (St. Louis, MO). Insulin (Humulin R) was purchased from Eli Lilly (Toronto, ON, Canada).

Animals. Male Wistar rats were obtained from Charles River and were housed in individual cages at 25°C with a 12:12-h light-dark cycle. They were fed a pelleted stock diet (5075; Charles River Rodent Animal, distributed by Ralston Products, Woodstock, ON, Canada) containing 57.3% carbohydrates, 18.1% protein, and 4.5% fat; the remaining 20.1% was composed of vitamins, minerals, and fiber. Food and water were available ad libitum. The mean body mass of the rats used in the present experiments was 300 ± 15 g.

Adipocyte isolation. Adipocytes were isolated from epididymal fat pads by a slight modification of Rodbell’s (34) method. Briefly, rats were killed by decapitation, and their epididymal fat pads were removed and placed in Krebs-Ringer bicarbonate (KRB) buffer of the following composition: 120 mM NaCl, 4.75 mM KCl, 1.2 mM KH2PO4, 1.12 mM MgSO4, 2.5 mM CaCl2, 25 mM NaHCO3, 1 mM pyruvate, 20 mM HEPES, and 1% fatty acid-free BSA, pH 7.4 (1% KRB). The minced tissue was incubated in fresh medium containing 1 mg/ml collagenase at 37°C for 15–20 min with a shaking frequency of 150 cycles/min. At the end of incubation, the cells were filtered through a 500-μm nylon filter (Nitex) and diluted with 5 ml of 1% KRB. The floating cells were washed four times with the same medium and preincubated at 37°C for 15 min (shaking frequency of 40 cycles/min). They were then washed three times with warm (37°C) KRB containing 4% fatty acid-free BSA (4% KRB) and devoid of glucose, pyruvate, or any other energy substrates. Finally, cells were incubated for 2 or 4 h in the presence of substrates or agents to be tested, under the same conditions (unless otherwise specified), with a final cell concentration of 3–5 × 10⁵ cells/ml KRB (4%). At the end of incubation, adipocytes were allowed to float, and the infranatants were frozen at −20°C for leptin and glycerol measurements.

Leptin assays. Leptin concentrations were determined by RIA using a rat leptin detection kit available from Linco Research (St. Charles, MO). This kit utilizes a guinea pig anti-rat leptin serum and 125I-labeled rat leptin label to determine the level of leptin in plasma or tissue culture media. Purified recombinant rat leptin is provided to carry out a standard curve.

Statistics. The data were analyzed using ANOVA. Values represent means ± SE of a number of individual experiments performed on separate occasions, as indicated in the text.

RESULTS

Effects of glucose and insulin on leptin secretion. It has been reported that high concentrations of glucose (25 mM) maximally stimulate leptin secretion from isolated white adipocytes and that addition of insulin does not further increase this stimulation (21). To further investigate these results, white adipocytes were incubated in a medium containing increasing concentrations of glucose from low (5 mM) to high (25 mM) concentrations, with or without insulin (10 nM). Glucose enters adipocytes through facilitative transporters GLUT1 in basal conditions and GLUT4 in the presence of insulin (16). Several concentrations were tested (5, 10, 15, 20, 25 mM), but, because results for all concentrations were similar, only 5 and 25 mM are shown (Fig. 1). The effects of the complete absence of glucose (or any other substrate) on leptin secretion were assessed in parallel. Incubation times were 2 h (Fig. 1A) or 4 h (Fig. 1B). In the presence of glucose (5 mM), insulin doubled basal leptin secretion (Fig. 1, A and B). When glucose was omitted, basal leptin secretion was markedly decreased, and insulin did not stimulate leptin secretion. On the other hand, high concentrations of glucose (25 mM) did not potentiate basal or insulin-stimulated leptin secretion compared with 5 mM glucose. Results for 2 and 4 h were identical. These results show that pyruvate and fructose act in a way different from glucose on the effects of glucose on basal and insulin-stimulated leptin secretion. These results suggest that glucose maintains basal leptin secretion and is necessary to observe a stimulation by insulin. Furthermore, high concentrations of glucose do not enhance leptin secretion.

Effects of pyruvate and fructose on leptin secretion. To further examine the specificity of glucose on leptin secretion, glucose was replaced by pyruvate (Fig. 2A) or fructose (Fig. 2B). Pyruvate crosses the plasma membrane by passive diffusion. Fructose enters white adipocytes by use of the facilitative transporter GLUT 5 (11). Controls with glucose (5 mM) or no substrates were incubated in parallel. Incubation time was 4 h. Dose-response curves were obtained for pyruvate and fructose, but only results for 5 and 25 mM are shown. Pyruvate mimicked the effects of glucose on basal and insulin-stimulated leptin secretion at all concentrations (Fig. 2A). Fructose also mimicked glucose effects but only at high concentrations. These results show that pyruvate and fructose act in a way similar to glucose on basal and insulin-stimulated leptin secretion. They also suggest that high availability in energy substrates is not sufficient per se to increase leptin secretion, as shown by a lack of dose-response relationship for pyruvate, which can freely cross the plasma membrane.

Effects of amino acids on leptin secretion. Amino acids are catabolized by being converted into glycolysis or tricarboxylic acid cycle intermediates (Table 1). Amino acids enter white...
adipocytes via numerous specific transporters (19, 22, 33). To assess their role on leptin secretion, white adipocytes were incubated in the presence of a fixed concentration of a given amino acid (5 mM) with or without insulin and in the presence or absence of glucose. Controls without amino acids were realized in the same experiments. Dose-response curves of amino acids (5–25 mM) were obtained, but only results for 5 mM are shown. First, L-glycine and L-alanine, two amino acid precursors of pyruvate, maintained basal leptin secretion in a way similar to glucose, but insulin failed to stimulate leptin secretion in their presence alone (Fig. 3). No effect of the amino acids on basal and insulin-stimulated leptin secretion was seen in the presence of glucose. Second, cells were incubated in the presence of L-aspartate, L-leucine, or L-glutamate (5 mM). These three amino acids enter the tricarboxylic acid cycle by the following three different pathways: L-aspartate is a precursor of oxaloacetate, L-leucine a precursor of acetyl-CoA, and L-glutamate is converted to α-ketoglutarate.

Controls and conditions were the same as for L-glycine and L-alanine. L-Aspartate, L-leucine, and L-glutamate showed three different patterns of effects on leptin secretion (Fig. 4). In the absence of glucose, L-aspartate mimicked glucose on both basal and insulin-stimulated leptin secretion. L-Leucine alone maintained basal leptin secretion, but insulin failed to stimulate leptin secretion. Surprisingly, L-glutamate alone potently stimulated leptin secretion in the absence of insulin or glucose. In the presence of glucose, these three amino acids stimulated leptin secretion at a level similar to control glucose and insulin. Addition of insulin further increased leptin secretion. Third, three amino acid precursors of acetyl-CoA (L-phenylalanine, L-methionine, and L-valine) stimulated leptin secretion in a way similar to L-aspartate but not to L-leucine (Fig. 5). On the other hand, three amino acid precursors of L-glutamate, namely, L-glutamine, L-arginine, and L-histidine, did not potently stimulate leptin secretion (Fig. 6). To explain these results, we measured levels of \( \text{ob} \) (leptin gene) mRNA. Insulin and amino acids did not change mRNA levels after 2 and 4 h when compared with basal (results not shown).

### Table 1. Amino acids as precursors of tricarboxylic acid cycle intermediates

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Metabolic Intermediates</th>
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</thead>
<tbody>
<tr>
<td>L-Aspartate, L-asparagine</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>L-Glutamate, L-glutamine, L-proline, L-histidine, L-arginine</td>
<td>α-Ketoglutarate</td>
</tr>
<tr>
<td>L-Phenylalanine, L-tyrosine</td>
<td>Fumarate</td>
</tr>
<tr>
<td>L-Isoleucine, L-valine, L-methionine</td>
<td>Succinyl-CoA</td>
</tr>
<tr>
<td>L-Leucine, L-valine, L-phenylalanine, L-methionine</td>
<td>Acetyl-CoA</td>
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Fig. 2. Effects of pyruvate (A) and fructose (B) on leptin secretion. Cells were incubated without substrates (No sub) or in the presence of one of the following substrates: glucose (Glc; 5 mM), pyruvate (Pyr; 5 or 25 mM), or fructose (Frc; 5 or 25 mM), with (filled bars) or without (open bars) insulin (10 nM). Incubation time was 4 h. Bars and vertical lines indicate means ± SE (n = 5). Leptin secretion with or without insulin was compared with 5 mM glucose with or without insulin, respectively. **Significant differences at the level of \( P < 0.01 \).

Fig. 3. Effects of L-glycine and L-alanine on leptin secretion. L-Glycine (5 mM) or L-alanine (5 mM) was added to a medium containing 5 mM glucose (+Glc) or no glucose (-Glc), in the presence (filled bars) or absence (open bars) of insulin (10 nM). Controls without amino acids were realized in parallel. Cells were incubated for 4 h. Bars and vertical lines indicate means ± SE (n = 6). Each condition (with or without glucose, with or without insulin) in the presence of amino acids was compared with its respective control in the absence of amino acids. Significant differences were noted at the level of \( *P < 0.05 \) and \( **P < 0.01 \).

Fig. 4. Effects of L-aspartate, L-leucine, and L-glutamate on leptin secretion. White adipocytes were incubated in a medium containing L-aspartate (5 mM), L-leucine (5 mM), or L-glutamate (5 mM) in the same conditions as in Fig. 3. Incubation time was 4 h. Controls were done in the absence of amino acids. Bars and vertical lines indicate means ± SE (n = 5). Significant differences were noted for each condition [with (+Glc) or without (-Glc) glucose, with or without insulin] in the presence of amino acids compared with respective control, at the levels of \( *P < 0.05 \) and \( **P < 0.01 \).
same experiments, the basal secretion of adiponectin (another adipokine; see Ref. 44) was not affected by insulin or amino acids (results not shown). These results show that some amino acids per se are able to potently stimulate leptin secretion. Insulin has an additive effect, which suggests that insulin and amino acids are acting on leptin secretion at two different metabolic levels. Leptin transcription is not required to observe these stimulations, and the stimulatory effect of insulin and amino acids is specific to leptin.

**Effect of nonmetabolizable analogs of amino acids and glucose on leptin secretion.** To determine whether amino acids or glucose needs to be metabolized to impact leptin secretion, L-glutamate, L-leucine, and D-glucose were respectively replaced by their nonmetabolizable analogs D-glutamate, D-leucine and BCH (26), and L-glucose. Conditions of incubation were similar to the previous experiments (Figs. 7 and 8). Nonmetabolizable analogs did not stimulate or inhibit leptin secretion, showing that glucose and amino acids must be metabolized to stimulate leptin secretion, rather than passively activating metabolic pathways.

**DISCUSSION**

The aim of the present study was to examine the respective importance of energy substrates and insulin on leptin secretion from white adipocytes. Several glycolytic substrates and amino acids were tested as to their impact on leptin secretion in the presence or absence of insulin. It was found that glycolytic substrates (glucose, fructose, or pyruvate) are necessary to maintain both basal and insulin-stimulated leptin secretion and that high concentrations of substrates do not further increase the latter. These conclusions are based on the following observations. Adipocytes incubated in a medium devoid of substrates secrete leptin at a rate of 2 ng·10⁻⁶ cells·2 h⁻¹ and are...
insensitive to insulin. Addition of glucose, fructose, or pyruvate doubled leptin secretion to 4 ng·10^{-6}·cells·2·h^{-1}, independent of their concentration. Addition of insulin again doubled leptin secretion (to 8 ng·10^{-6}·cells·2·h^{-1}), also independent of substrate concentration (Fig. 1). Basal and insulin-stimulated leptin secretion rates are similar to those in previous reports using freshly isolated adipocytes (4, 20, 21, 35). Because insulin increases transport of hexoses inside cells (5), it can be argued that, under basal conditions, hexose entry is a limiting factor that prevents an increase in metabolism necessary to increase secretion. Nevertheless, pyruvate can freely cross the plasma membrane, and high concentrations of pyruvate did not stimulate leptin secretion above basal levels. These results disagree with a previous report that described a strong stimulation of leptin secretion by glycolytic substrates in the absence of insulin, with no additive effect of insulin (20). These differences may stem from the following differences in experimental conditions: 1) their cell isolation was conducted in a medium containing L-glutamine, which is a condition suitable for β-cells but not for white adipocytes (23); 2) addition of substrates appeared to stimulate basal secretion as we reported (Figs. 1 and 2); and 3) compared with our own results (Fig. 1), their cells were most probably unresponsive to insulin. Our results are in accord with the essential role of insulin in the stimulation of leptin secretion (3, 4, 35) and suggest that insulin controls a metabolic step necessary for the stimulation of leptin secretion. Indeed, it was previously shown that insulin-stimulated leptin secretion from white adipocytes requires de novo synthesis, independent of changes in leptin mRNA levels (4, 21). On the other hand, insulin is known to be a potent activator of pyruvate dehydrogenase (6). Conversion of pyruvate to acetyl-CoA may be a key regulator, providing energy for de novo leptin biosynthesis (21). Activity of pyruvate dehydrogenase in the presence and absence of insulin remains to be assessed in our conditions.

Amino acids are catabolized at the glycolysis and tricarboxylic acid cycle levels (Table 1). Interestingly, they exhibited the following four different types of effect on leptin secretion: 1) amino acids that stimulated poorly or did not stimulate leptin secretion (L-glycine, L-alanine, L-histidine, L-arginine and L-glutamine; Figs. 3 and 6), 2) an amino acid that increased leptin secretion only in the presence of glucose (L-leucine; Fig. 4), 3) an amino acid that mimicked and potentiated glucose action (L-aspartate, L-valine, L-methionine, and L-phenylalanine; Figs. 4 and 5), and 4) an amino acid that stimulated leptin secretion in the absence of glucose or insulin (L-glutamate; Fig. 4). Each of these is discussed below.

First, regarding the amino acids that did not show any or showed only small effects on leptin secretion, one hypothesis is that white adipocytes metabolize these amino acids poorly. For example, glutamine is known to be released rather than being oxidized in white adipose tissue (18). Second, L-leucine has been shown to stimulate protein synthesis independently of insulin by activating the mammalian target of rapamycin pathway (2, 24, 42, 43). We confirmed these studies with regard to leptin secretion but showed that glucose is necessary to observe leptin stimulation by L-leucine (35). This is likely because L-leucine is not a gluconeogenic amino acid. Third, L-aspartate, L-valine, L-methionine, and L-phenylalanine are amino acid precursors of tricarboxylic acid cycle intermediates and are gluconeogenic. Our results suggest that increasing the amount of these intermediates has an overall stimulatory effect on leptin synthesis, but this remains to be assessed. Fourth, L-glutamate has been shown to be a central messenger in the secretion of insulin from pancreatic β-cells (7, 25). In particular, activation of glutamate dehydrogenase is a necessary step (38). According to our results (Fig. 4), it seems that L-glutamate may also have a central role in leptin secretion from white adipocytes, but the precise mechanisms remain to be elucidated. L-Glutamate is known to be involved in numerous metabolic pathways, including transamination, mitochondrial malate/aspartate shuttle, and protein synthesis (28). Our observations on the additive effect of amino acids and insulin are in agreement with previous reports showing that addition of amino acids enhances both maximal insulin responsiveness and insulin sensitivity of the protein synthesis system (27). Of particular interest, this report revealed that amino acids do not modify the insulin receptor binding capacity of insulin, nor do they affect the insulin responsiveness of the glucose transport system. On the other hand, our results suggest that plasma insulin, glucose, and amino acid levels may act together to control leptin secretion from adipose tissue in different physiological conditions (starvation, feeding).

Nonmetabolizable analogs of glucose and amino acids (Figs. 7 and 8) did not stimulate leptin secretion from white adipocytes. This suggests that energy substrates need to be metabolized and raises the possibility that one of their derivatives may activate a metabolic pathway. Interestingly, BCh, the L-leucine analog, increases insulin secretion in β-cells by allosteric activation of glutamate dehydrogenase (26, 39). Indeed, white adipocytes and pancreatic β-cells, while considered as endocrine cell types, are morphologically very different (hormone content, size of vesicles, etc.) and secrete hormones in a different pathway (regulated vs. constitutive; see Refs. 4, 21, and 25). All of these observations suggest that leptin and insulin secretions are differentially regulated at the metabolic level. On the other hand, insulin, amino acids, and glycolytic substrates did not increase basal adiponectin secretion from white adipocytes, which suggests that, within adipocytes themselves, the release of adipokines may follow different metabolic pathways.

In conclusion, glycolytic substrates sustain basal leptin secretion. Insulin is a potent stimulator of leptin secretion from white adipocytes. Several amino acids can enhance leptin secretion in the absence of insulin, most probably by stimulating adipocyte metabolism at the level of the tricarboxylic acid cycle. At the moment, we can only formulate a hypothesis to explain how amino acid stimulated leptin secretion. Further studies are clearly needed, for instance, to determine the activity of tricarboxylic acid cycle enzymes and the rate of synthesis of amino acid derivatives. In addition, it will be interesting to elucidate how glycolytic substrates, amino acids, and insulin are interrelated with other agents known to increase or inhibit leptin secretion and transcription.

GRANTS

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REFERENCES


