Regulation of leptin secretion from white adipocytes by free fatty acids

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Cammisotto, Philippe G., Yves Gélinas, Yves Deshaies, and Ludwik J. Bukowiecki. Regulation of leptin secretion from white adipocytes by free fatty acids. Am J Physiol Endocrinol Metab 285: E521–E526, 2003. First published May 7, 2003; 10.1152/ajpendo.00052.2003.—Norepinephrine stimulates lipolysis and concurrently inhibits insulin-stimulated leptin secretion from white adipocytes. To assess whether there is a cause-effect relationship between these two metabolic events, the effects of fatty acids were investigated in isolated rat adipocytes incubated in buffer containing low (0.1%) and high (4%) albumin concentrations. Palmitic acid (1 mM) mimicked the inhibitory effects of norepinephrine (1 μM) on insulin (10 nM)-stimulated leptin secretion, but only at low albumin concentrations. Studies investigating the effects of the chain length of saturated fatty acids [from butyric (C4) to stearic (C18) acids] revealed that only fatty acids with a chain length superior or equal to eight carbons effectively inhibited insulin-stimulated leptin secretion. Long-chain mono- and polyunsaturated fatty acids constitutively present in adipocyte triglyceride stores (oleic, linoleic, γ-linolenic, palmitoleic, eicosapentanoic, and docosahexanoic acids) also completely suppressed leptin secretion. Saturated and unsaturated fatty acids inhibited insulin-stimulated leptin secretion with the same potency and without any significant effect on basal secretion. On the other hand, inhibitors of mitochondrial fatty acid oxidation (palmitoxirate, 2-bromopalmitate, 2-bromocaproate) attenuated the stimulatory effects of insulin on leptin release without reversing the effects of fatty acids or norepinephrine, suggesting that fatty acids do not need to be oxidized by the mitochondria to inhibit leptin release. These results demonstrate that long-chain fatty acids mimic the effects of norepinephrine on leptin secretion and suggest that they may play a regulatory role as messengers between stimulation of lipolysis by norepinephrine and inhibition of leptin secretion.

insulin; albumin; saturated and unsaturated fatty acids; β-oxidation

LEPTIN, THE PRODUCT OF THE ob gene, is a hormone mainly secreted by white adipocytes (1, 14, 15, 41). Binding of leptin to its hypothalamic receptors alters various messengers that regulate energy expenditure, food intake, and the activity of the sympathetic nervous system, at least in rodents (25). Plasma leptin levels are tightly correlated with the total amount of white fat in the body. Leptin has therefore been considered as a “lipostatic factor” contributing to the regulation of body weight via a negative feedback loop (10).

Several hormones can modulate leptin transcription and secretion in vivo and in vitro, the most important being insulin and norepinephrine (11, 16, 26). In vivo, starvation or food deprivation decreases plasma leptin concentrations and leptin transcription in adipose tissue. These changes are closely associated with decreased plasma insulin concentrations, activation of the sympathetic nervous system, increased lipolysis, and elevated plasma free fatty acid levels. Refeeding or injection of insulin reverses the decrease of plasma leptin concentrations and leptin transcription in white adipocytes (26). Similarly to starvation, cold exposure activates the sympathetic nervous system, increases the levels of circulating norepinephrine and free fatty acids, and decreases plasma insulin concentrations (30, 38). These effects of cold exposure can be mimicked in vivo by administration of norepinephrine or by treatment with β-agonists in mice (21, 22) and humans (29, 37).

In vitro studies using isolated adipocytes have shown that insulin acutely stimulates leptin secretion and that β-agonists such as norepinephrine, isoproterenol, or CL-316243 exert a strong inhibitory effect on insulin-stimulated leptin secretion (5, 33). A wide range of agents, such as lipolytic hormones (ACTH and TSH), inhibitors of phosphodiesterases (caffeine, theophylline, IBMX, imazodan, milrinone, and amrinone), adenylyl cyclase modulators ( forskolin and pertussis toxin), and nonhydrolyzable cAMP analogs, all suppressed insulin-stimulated leptin secretion and concomitantly stimulated lipolysis and fatty acid release (5). On the other hand, insulin is known to be a strong antilipolytic hormone that increases the transcription of lipogenic enzymes (6, 12). These observations suggest, but do not demonstrate, that fatty acids play a messenger role by mediating the inhibitory effects of lipolytic agents on insulin-stimulated leptin secretion (2, 39).

To test this hypothesis, freshly isolated white adipocytes were incubated in the presence of insulin, norepi-
nephrine, and a wide range of saturated and unsaturated fatty acids. In addition, inhibitors of mitochondrial fatty acid oxidation were used to investigate the mechanisms by which fatty acids may act on leptin secretion. We now report that medium- and long-chain fatty acids (saturated or unsaturated) acutely suppress the stimulatory effects of insulin on leptin secretion. This effect is independent of mitochondrial fatty acid oxidation and of an increase in leptin transcription. Results from this study indicate that fatty acids may mediate the inhibitory effects of norepinephrine and other lipolytic agents on insulin-stimulated leptin secretion.

METHODS

**Chemicals.** Fatty acid-free BSA, norepinephrine, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic, γ-linolenic, conjugated linoleic, palmitolieic, eicosapentaenoic, and docosahexaenoic acids, glycerol, acetoacetate, acetone, β-hydroxybutyrate and collagenase (type II, lot 107H8649), bromocaprate, and bromopalmitate were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Palmoxirate was a generous gift from R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). Insulin (Humulin R) was purchased from Eli Lilly (Toronto, Canada).

**Animals.** Male Wistar rats were obtained from Charles River (St. Constant, Quebec, Canada) and were housed in individual cages at 24°C with a 12:12-h light-dark cycle. The rats received standard Purina chow and water ad libitum. The mean body mass of the rats used in the present experiments was 290 ± 15 g.

**Adipocyte isolation.** Adipocytes were isolated from epididymal fat pads by a slight modification of Rodbell's (32) 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, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5.5 mM glucose, 20 mM HEPES, and 1% fatty acid-free BSA, pH 7.4 (1% KRB). The minced tissue was incubated in 1% KRB containing 0.5 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 1% KRB, preincubated at 37°C for 15 min in 1% KRB (shaking frequency of 40 cycles/min), and washed two times with warm (37°C) KRB containing 4% fatty acid-free BSA (4% KRB). Finally, the cells were incubated in 1% KRB containing 0.5 mg/ml collagenase at 37°C for 2 h (unless otherwise specified) in the presence of hormones or drugs at a concentration of 3–5 × 10⁵ cells/ml 4% KRB. The adipocytes were then allowed to float, and the infranatants were frozen at −20°C for leptin and glycerol measurements.

**Leptin and glycerol assays.** Leptin concentrations were determined by RIA using a kit available from Linco Research (St. Charles, MO). Glycerol was measured using an enzymatic method (40).

**Statistics.** The data were analyzed using ANOVA. Values represent the means ± SE of a number of individual experiments performed on separate occasions (n), as indicated in the text. The half-effective concentrations for the reversal of palmitate inhibition in the presence of increasing albumin concentration (IC₅₀) were determined by computer analysis (Sigma Plot program) of concentration-response curves.

RESULTS

**Effects of norepinephrine, palmitic acid, and albumin on insulin-stimulated leptin secretion.** In previous studies (5), we reported that norepinephrine inhibited insulin (10 nM)-stimulated leptin secretion between 0.1 and 1 μM and concurrently increased lipolysis in the same range of concentrations. This provided a first indication that inhibition of leptin secretion might be metabolically associated with the stimulation of lipolysis. To test whether fatty acids play a messenger role between stimulation of lipolysis by norepinephrine and inhibition of leptin secretion, adipocytes were incubated with insulin (10 nM) in the presence of norepinephrine (1 μM) or palmitic acid (1 μM; Fig. 1). Because albumin strongly binds extracellular fatty acids (4), experiments were carried out at low (0.1%) and high (4%) albumin concentrations. Palmitic acid (1 mM) mimicked the inhibitory effects of norepinephrine (1 μM) on leptin secretion at low but not at high albumin concentrations (Fig. 1). This indicates that albumin, at high concentrations, effectively binds extracellular fatty acids and consequently inhibits their effects on leptin secretion. Therefore, subsequent experiments were carried out at low albumin concentrations. Concentration-response curves carried out in the presence of 0.1% albumin revealed that palmitic acid inhibited insulin (10 nM)-stimulated leptin secretion between 0.1 and 1 mM without significantly affecting basal values (Fig. 2). In fact, the palmitic acid effect critically depended on the ratio of the molar concentrations of palmitic acid over albumin with an IC₅₀ of 4.5 (Fig. 3). This is consistent with the observation that one molecule of albumin has several low- and high-affinity binding sites for long-chain fatty acids (8).

**Effect of fatty acid chain length on insulin-stimulated leptin secretion.** Adipocytes were incubated in a medium containing albumin (0.1%), insulin (10 nM), and

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**Fig. 1.** Effects of norepinephrine (NE), palmitic acid (palm), and BSA on leptin release. Adipocytes were incubated in the presence of insulin (10 nM) and fixed concentrations of norepinephrine (1 μM) or palmitic acid (1 mM) in the presence of low (0.1%) or high (4%) albumin concentrations. Bars and vertical lines indicate means ± SE (n = 5). **Significant differences compared with basal values at the level of P < 0.01.
various saturated fatty acids with a chain length varying from 4 (butyric acid) to 18 carbons (stearic acid).

Concentration-response curves similar to those described for palmitic acid (Fig. 3) were generated for all fatty acids. Results for a fatty acid concentration of 1 mM are given in Fig. 4 (as well as in Figs. 5 and 6), since the maximal effect for all fatty acids occurred at this concentration. Results were expressed as percentages of basal values observed in the absence of insulin.

It can be seen that fatty acids with a chain length equal or superior to eight carbons markedly inhibited insulin-stimulated leptin secretion. These observations indicate that the fatty acid effect is specific for medium- and long-chain fatty acids.

Effects of various mono- and polyunsaturated fatty acids on insulin-stimulated leptin secretion.

To determine the importance of chain unsaturation, adipocytes were incubated in medium containing albumin (0.1%), with various unsaturated fatty acids (C18:1 oleic, C18:2 linoleic, C18:3 linolenic, C16:1 palmitoleic, C20:5 eicosapentaenoic, C22:6 docosahexanoic, conjugated linoleic acids) in the presence of insulin (10 nM; Fig. 5). Concentration-response curves (data not shown) revealed that unsaturated fatty acids exerted their inhibitory effects with IC50 values similar to that observed with palmitic acid (Fig. 3). Thus saturated and nonsaturated fatty acids inhibited insulin-stimulated leptin secretion with the same potency and without any significant effect on basal secretion.

Effects of inhibitors of fatty acid oxidation on insulin-stimulated leptin secretion.

To determine whether fatty acids need to be oxidized by adipose tissue mitochondria to exert their inhibitory action, we used several inhibitors of mitochondrial palmitate carnitine transferase (Fig. 6). Palmitoxirate (100 μM), 2-bromopalmitate (10 μM), or 2-bromocaproate (10 μM) was added at optimal inhibitory concentrations (19, 23), in the presence or absence of insulin (10 nM), palmitate (1 mM), or norepinephrine (1 μM) in a 0.1% albumin medium. All carnitine transferase inhibitors slightly obviated the stimulatory effects of insulin on leptin release without reversing the effects of palmitic acid or norepinephrine. On the contrary, their inhibitory effect on

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**Fig. 2.** Concentration-response curve of the effect of palmitic acid on basal and insulin-stimulated leptin secretion. Adipocytes were incubated in a medium containing albumin (0.1%), insulin (10 nM), and various concentrations of palmitic acid. The incubation conditions were as described under METHODS (n = 5 experiments).

**Fig. 3.** Effect of the molar ratio palmitic acid/albumin on insulin-stimulated leptin secretion. Adipocytes were incubated in medium containing insulin (10 nM), palmitic acid (1 mM), and increasing concentrations of BSA (0.1–4%). The molar ratio palmitic acid/albumin was calculated using 55,000 as the albumin molecular weight. The incubation conditions were as described under METHODS (n = 6).

**Fig. 4.** Inhibition of insulin-stimulated leptin secretion by saturated FFAs of various chain lengths. Adipocytes were incubated in the presence of insulin (10 nM) and 1 mM of one of the following saturated fatty acids: butyric (C4), caproic (C6), caprylic (C8), capric (C10), lauric (C12), myristic (C14), palmitic (C16), and stearic (C18) acids. Albumin concentration in the medium was 0.1%. Results were expressed by taking each respective fatty acid basal as 100% inhibitory values. The incubation conditions were as described under METHODS (n = 5).
The present results demonstrate that long-chain fatty acids, without the need to be oxidized by adipose tissue mitochondria, exert their inhibitory action on leptin secretion.

DISCUSSION

The present results demonstrate that long-chain fatty acids, the principal products of activated lipolysis, acutely inhibit insulin-stimulated leptin secretion from isolated adipocytes (Figs. 1–6). We have previously shown that insulin-stimulated leptin secretion can be inhibited by a wide variety of agents known to increase intracellular cAMP concentrations either by stimulating its production at the adenylate cyclase level (catecholamines, lipolytic hormones, pertussis toxin, or forskolin), by inhibiting its degradation by phosphodiesterases (methylxanthines, imazodan, milrinone, or amrinone), or by mimicking its action (nonmetabolizable cAMP analogs; see Ref. 5). Without exception, all these agents stimulated lipolysis in the range of concentrations at which they inhibited insulin-stimulated leptin secretion. These observations, combined with the present results, strongly indicate that fatty acids mediate the inhibitory effects of lipolytic agents on insulin-stimulated leptin secretion.

The fact that concentrations of albumin similar to those found in plasma (4%) inhibit the effects of palmitic acid (1 mM) indicates that circulating fatty acids (the concentration of which varies at the millimolar level) have little influence on leptin secretion, at least directly (Figs. 1 and 3). This is supported by several in vivo studies in humans, which failed to demonstrate any inhibitory effects of fatty acids on plasma leptin concentrations (35, 37). It is more likely that an intracellular increase in fatty acids, generated in consequence of activated lipolysis, causes the inhibition of leptin secretion. In the present studies, the intracellular increase was mimicked by adding fatty acids in the presence of low albumin concentrations. In fact, we previously demonstrated that it is possible to dynamically titrate the effects of fatty acids on brown adipose tissue thermogenesis with albumin (4). The effects of fatty acids were not directly proportional to the extracellular fatty acid concentration but to the molar ratio of fatty acid concentration to albumin concentration. In agreement with these observations, we found that palmitic acid inhibits insulin-stimulated leptin secretion with an IC50 value of the molar ratio of palmitic acid concentration to albumin concentration equal to 4.5 (Fig. 3). This value is also compatible with the fact that a single molecule of albumin possesses several binding sites for fatty acids with different affinities (18). In addition, fatty acid-binding proteins other than albumin are also present inside the cells. Therefore, it is likely that the final fatty acid effect depends on the concentration and the localization of intra- and extracellular binding proteins (aP2 and others) as well as on the properties of membrane-associated fatty acid transport proteins such as CD36 (8, 36).

There are several possible mechanisms by which fatty acids may regulate leptin secretion. These mechanisms must include the facts that the fatty acid effect depends on the concentration and the localization of intra- and extracellular binding proteins (aP2 and others) as well as on the properties of membrane-associated fatty acid transport proteins such as CD36 (8, 36).

Fig. 5. Inhibition of insulin-stimulated leptin secretion by unsaturated fatty acids. Adipocytes were incubated in the presence of 10 nM insulin, 1 μM norepinephrine (NE), or 1 mM of one of the following fatty acids: oleic (C18:1), linoleic (C18:2), γ-linolenic (C18:2), conjugated linoleic (CLA), palmitoleic (C16:1), eicosapentaenoic (C20:5), and docosahexaenoic (C22:6) acids. Albumin concentration in the medium was 0.1%. Inhibition of fatty acids and norepinephrine was compared with basal. **P < 0.01.

Fig. 6. Effect of inhibitors of mitochondrial fatty acid transport and oxidation on the inhibitory effect of palmitate and norepinephrine on insulin-stimulated leptin secretion. Adipocytes were incubated in the presence of insulin (10 nM), palmitic acid (1 mM), or norepinephrine (1 μM) and one of the following agents: palmoxirate (X; 100 μM), 2-bromopalmitate (BrP; 10 μM), or 2-bromocaproatate (BrC; 10 μM). Albumin concentration in the medium was 0.1%. Effects of palmoxirate, 2-bromopalmitate, or 2-bromocaproatate were compared with their respective controls (bars without these inhibitors). *P < 0.05 and **P < 0.01.
these agents reversed the inhibitory effects of norepinephrine, palmitic acid (Fig. 6), or other saturated or unsaturated fatty acids (results not shown). On the contrary, all three inhibitors slightly inhibited basal and insulin-stimulated leptin secretion (Fig. 6), indicating that leptin exocytosis, similarly to insulin exocytosis (19), depends on some degree of oxidation of long-chain fatty acids. However, although it is likely that some fatty acids serve as an energy source for leptin secretion, mitochondrial fatty acid oxidation does not appear to represent a metabolic step that is absolutely required for observing the inhibitory effects of fatty acids. Consistent with these observations, concentration-response experiments (data not shown) revealed that ketone bodies (acetooacetate, β-hydroxybutyrate, and acetone) and glycerol, added at concentrations varying between 5 and 10 mM, did not significantly affect insulin (10 nM)-stimulated leptin secretion.

There are several other mechanisms by which fatty acids may inhibit insulin-stimulated leptin secretion. First, fatty acids may specifically bind mitochondrial uncoupling proteins (UCP) present in white adipocytes (3). In brown adipocytes, it is well established that fatty acids bind UCP-1, increase the permeability of the inner mitochondrial membrane to protons, and consequently stimulate thermogenesis. Although UCP-1 is not detectable in white adipocytes, a regulatory effect of fatty acids on other UCPs (UCP-2, -3, or others) remains to be tested.

Another possibility is that fatty acids directly affect either transcription or translation of leptin mRNA. It has been reported that, in the pancreas, palmitate inhibits the transcription of the insulin gene (31). Likewise, the inhibitory role of polyunsaturated fatty acids on numerous tissue gene transcription factors is very well documented (7, 28). Using Northern blots, we have found that, under the present conditions, insulin, norepinephrine, or fatty acids do not affect leptin mRNA levels (data not shown). This suggests that the short-term regulation of leptin secretion principally occurs at the posttranscriptional level. This finding was not unexpected because of the short period of time (2 h) within which leptin secretion was influenced by hormones and fatty acids. It should be emphasized that the mechanisms by which fatty acids affect leptin secretion might be different in the short and long term. Indeed, in cell culture experiments, it has been reported that some fatty acids inhibit basal leptin transcription in the absence of insulin or other stimulating agents (17, 27, 34). Our work is the first to provide extensive data on the acute effects of fatty acids on leptin secretion using a wide range of fatty acids naturally present in adipose tissue triglycerides (24).

In summary, the present results demonstrate that fatty acids mimic the effects of norepinephrine on leptin secretion. This effect is specific for medium- and long-chain fatty acids and does not depend on their degree of saturation. The fatty acid action does not directly require their oxidation. Consequently, long-chain fatty acids may play an important metabolic role as messengers between the hormonal activation of lipolysis and the final inhibition of leptin secretion from white adipocytes.

DISCLOSURES

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