Leptin Induces Nitric Oxide-Mediated Inhibition of Lipolysis and Glyceroneogenesis in Rat White Adipose Tissue

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Abstract

Leptin is secreted by white adipose tissue (WAT) and induces lipolysis and nonesterified fatty acid (NEFA) oxidation. During lipolysis, NEFA efflux is the result of triglyceride breakdown, NEFA oxidation, and re-esterification via glyceroneogenesis. Leptin’s effects on glyceroneogenesis remain unexplored. We investigated the effect of a long-term treatment with leptin at a physiological concentration (10 μg/L) on lipolysis and glyceroneogenesis in WAT explants and analyzed the underlying mechanisms. Exposure of rat WAT explants to leptin for 2 h resulted in increased NEFA and glycerol efflux. However, a longer treatment with leptin (18 h) did not affect NEFA release and reduced glycerol output. RT-qPCR showed that leptin significantly downregulated the hormone-sensitive lipase (HSL), cytosolic phosphoenolpyruvate carboxykinase (Pck1), and PPARγ genes. In agreement with its effect on mRNA, leptin also decreased the levels of PEPCK-C and HSL proteins. Glyceroneogenesis, monitored by [1-14C] pyruvate incorporation into lipids, was reduced. Because leptin increases nitric oxide (NO) production in adipocytes, we explored the role of NO in the leptin signaling pathway. Pretreatment of explants with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester eliminated the effect of leptin on lipolysis, glyceroneogenesis, and expression of the HSL, Pck1, and PPARγ genes. The NO donor S-nitroso-N-acetyl-DL penicillamine mimicked leptin effects, thus demonstrating the role of NO in these pathways. The inverse time-dependent action of leptin on WAT is consistent with a process that limits NEFA re-esterification and energy storage while reducing glycerol release, thus preventing hypertriglyceridemia. J. Nutr. 141: 4–9, 2011.

Introduction

Leptin, synthesized and secreted by adipocytes, plays an important role in the central regulation of body weight (1). Leptin plasma concentrations are found between 1 and 10 μg/L in lean humans and rodents and are strongly and positively correlated with fat mass (2,3). This hormone also has important metabolic and neuroendocrine functions (4). Leptin acts predominantly in the hypothalamus with resulting effects on appetite (5). Leptin receptors are expressed in peripheral tissues, including adipocytes, suggesting that direct effects of this hormone can occur independently of central pathways (6–8). In isolated adipocytes, leptin reduces expression of enzymes involved in fatty acid synthesis, fatty acid synthase (FAS), and acetyl CoA carboxylase (9,10), while it upregulates the expression of genes encoding acyl CoA oxidase, carnitine palmitoyl transferase-1, and uncoupling protein-2 (9,11). One of the important roles of leptin is to affect adipocyte lipolysis. Fruhbeck et al. (12,13) originally demonstrated the acute lipolytic effect of this hormone on glycerol release from isolated adipocytes when administered in vivo to mice. It was further shown that nitric oxide (NO) was a potential regulator of leptin-induced lipolysis (14,15). More recently, we demonstrated that leptin acutely induced NO synthase (NOS) III phosphorylation with a resulting increase in NO production in isolated adipocytes (16). The lipolytic process is the result of the sequential hydrolysis of stored triacylglycerol by adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase.

1 Supported by the Institut National de la Recherche Médicale at the Université Paris Descartes. The Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la recherche and the Association de Recherche sur le Cancer have awarded fellowships to F.N. and G.P., respectively.
3 Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.
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6 Abbreviations used: ATGL, adipose triglyceride lipase; BSA, bovine serum albumin; Db-cAMP, dibutyryl cyclic adenosine monophosphate; FAS, fatty acid synthase; GyK, glycerol kinase; HSL, hormone sensitive lipase; L-NAME, Nω-nitro-L-arginine methyl ester; NEFA, nonesterified fatty acid; NO, nitric oxide; NOS, nitric oxide synthase; PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase; SNAP, S-nitroso-N-acetyl-DL penicillamine; WAT, white adipose tissue.
However, there is no proportional release of glycerol and nonesterified fatty acids (NEFA) to the blood because of the occurrence of intra-cellular NEFA re-esterification while glycerol is liberated (17). NEFA re-esterification requires glycerol-3-phosphate synthesis from lactate, pyruvate, or certain amino acids as the endpoint of a pathway named glyceroneogenesis (18,19). Glyceroneogenesis induction in adipose tissue results in decreased NEFA output, whereas its repression restores NEFA release (20–22). The cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK-C) is the key enzyme in this metabolic pathway (18). The importance of glyceroneogenesis in pathophysiology was demonstrated by the fact that transgenic mice that overexpress PEPCK-C in adipose tissue become obese but not insulin resistant, whereas mice devoid of adipocyte PEPCK-C have a tendency to become insulin resistant (23,24). Several nutrients and hormones modulate glyceroneogenesis by means of alterations in Pck1 regulation, particularly at the transcriptional level, and PEPCK-C synthesis. One of the major transcriptional inducers of Pck1 is the γ isoform of PPAR, PPARγ, for which the hyperlipidemic and antiobidiatiazolidinediones are specific ligands (25,26). It was recently shown that chronic infusion of leptin into the mediobasal hypothalamus of rats inhibited Pck1 expression in adipose tissue (27). To our knowledge, the long-term action of leptin on adipocyte lipolysis and NEFA re-esterification has not been previously explored and this is the aim of the present study. The potential implication of NO in leptin effects is also investigated.

Materials and Methods

Materials. DMEM, penicillin, streptomycin, and TRizol Reagent (Total RNA Isolation Reagent) were from Invitrogen Life Technologies. SV total RNA Isolation System kit purchased from Promega, High Capacity cDNA Archive kit from Applied Biosystems, and Absolute qPCR SYBR green Rox mix from Thermo Scientific. Leptin was from Peprotech. [1-14C]Pyruvate acid, sodium salt (1 mCi/L), Hybond-N+ membranes, and Hyperfilm ECL were from Amersham Biosciences. Free Fatty Acids Half Micro test was from Roche and glycerol (glycerol UV-method) was from R-Biopharm. S-nitroso-N-acetyl-DL penicillamine (SNAP) was purchased from Cayman (SBI). Fetal bovine serum, essentially fatty acid-free bovine serum albumin (BSA), Nα-nitro-arginine methyl ester (L-NAME), dibutyryl-cAMP, sodium pyruvate, and all other products were purchased from Sigma. For Western-blot analysis, SDS-PAGE was performed using a SDS-MOPS running buffer and a Novex 4–12% Bis-Tris gel from Invitrogen Life Technologies. Rabbit anti-HSL antibody was purchased from Cell Signaling Technology, PEPCK-C antibody was a gift from Professor E. Beale (22). β-Actin antibody was from Santa-Cruz Biotechnology.

Animals. Male Sprague-Dawley rats (180–200 g) obtained from Centre d’Elevage de Rats Janvier at 6 wk of age were maintained at constant room temperature (24°C) on a 24-h light/dark cycle for 2 wk before being killed by CO2 asphyxiation at 1000 h. Rats consumed a standard balanced diet (60% carbohydrates, 16% proteins, 3% lipids, 5% vitamins and minerals, including retinol (1.98 mg/kg), cholecalciferol (506 µg/kg), d-α-tocopherol (20 mg/kg), and oligoelements such as calcium pantothenate, nicotinic acid, calcium iodate, ferrous carbonate, and copper sulfate from Safe and tap water ad libitum. The protocol for the animal studies was conducted according to the French Guidelines for the care and use of experimental animals.

Culture of explants from white adipose tissue and monitoring of fatty acid re-esterification. Epididymal fat pads were cut in fragments of ~200 mg and explants were incubated as previously described (22) in the absence or presence of leptin. At the end of incubation, the medium in each well was replaced with 1.5 mL of Krebs-Ringer bicarbonate buffer containing 2% fatty acid-free BSA, 5 mM/L pyruvate, and 20 µmol/L [1-14C]Pyruvate (1 mCi/L) as precursors of glycerol-3-phosphate. After 2 h, the incubation medium was discarded for the estimation of lipolytic NEFA and glycerol. Simultaneously, the corresponding tissue fragments were frozen in liquid nitrogen before lipid extraction according to the simplified method of Bligh and Dyer (28). The subsequent [1-14C] pyruvate incorporation into the lipid moiety was estimated as previously described (22). To assess NO implication, we used the NO donor SNAP (1 mM/L) and the NO synthase inhibitor L-NAME (1 mM/L).

RNA extraction and analysis. Total RNA was extracted from rat white adipose tissue (WAT) using TRizol and Dnase-treated with SV total RNA Isolation System kit as described (29). RNA was used for quantitative RT-PCR measurements using SYBR Green as described (22).

Results

Lipolytic and antilipolytic effects of leptin. We studied lipolytic effects of leptin in fat pad explants from epididymal WAT. A 2-h exposure of explants to 1–10 µg/L leptin resulted in a concentration-dependent increase in glycerol and NEFA release. Leptin (10 µg/L) induced significant increases in the concentrations of glycerol (70%) and NEFA (50%) above basal conditions (Fig. 1). Hence, a short-term treatment with leptin is lipolytic, as expected (9,11). Next, we incubated explants with 1–10 µg/L leptin for 18 h, then in lipolytic conditions with or without 1 mM/L db-cAMP for 2 h during which glycerol and NEFA concentrations were determined. Whereas leptin significantly reduced glycerol output (40–50%), NEFA release remained unchanged (Fig. 2). As expected, db-cAMP significantly stimulated glycerol and NEFA release, whereas leptin selectively inhibited db-cAMP induction of glycerol output (Fig. 2). Therefore, the long-term leptin treatment was antilipolytic for glycerol by counteracting the basal and CAMP-stimulated lipolysis but did not affect NEFA release.

Effect of leptin on gene expression and glyceroneogenesis. To explore this unexpected result further, we evaluated the level of expression of genes encoding proteins involved in lipid metabolism. As expected, the treatment with 10 µg/L leptin for 2–18 h resulted in a drastically reduced FAS mRNA, reaching 55–40% of control, respectively (11) (Fig. 3A). Whereas leptin treatment did not affect ATGL mRNA, the HSL transcript decreased similarly to that of FAS (Fig. 3A). The amount of HSL protein was also reduced (Fig. 3B). These results can explain the diminished glycerol release but not the unchanged NEFA concentration. In addition, the expression of glycerol kinase

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(GyK) was not affected by leptin (Niang et al., data not shown). A 6-h exposure of leptin induced an increase of ~3-fold in the expression of a gene involved in the first step of mitochondrial NEF A oxidation, the very long-chain acyl dehydrogenase gene (F. Niang and A. M. Jaubert, unpublished data). Leptin also reduced PEPCk-C mRNA by ~40%, starting at 6 h, and PEPCk-C protein (Fig. 3). It induced a sharp decrease in PPARγ mRNA of ~60% at 2 h and thereafter (Fig. 3A). Thus, the leptin-induced decrease in PPARγ gene expression preceded that of PEPCk-C mRNA. To explore the resulting effects on glyceroneogenesis, we measured [1-14C] pyruvate incorporation into neutral lipids in explants pretreated for 18 h with 10 μg/L leptin. A 30% decrease in radioactive labeling of neutral lipids, therefore in glyceroneogenesis, was observed (Fig. 4).

**FIGURE 1** Leptin stimulates glycerol and NEFA release in rat epididymal fat pads. WAT explants were treated or not with leptin (1 μg/L; 10 μg/L) for 2 h in KRB medium containing 2% BSA and 5 mmol/L pyruvate (lipolysis medium). Results are expressed as percent of glycerol or NEFA release in the culture medium relative to the corresponding untreated control set as 100%. Crude values for control were 4.19 nmol-mg tissue^{-1}·h^{-1} for glycerol and 4.05 nmol-mg^{-1} tissue^{-1}·2 h^{-1} for NEFA. Values are mean ± SEM, n = 4 (means of 2 samples/rat). Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001.

Involvement of NO on leptin effects. Because leptin induces NO production in isolated adipocytes (16), we tested whether NO was implicated in leptin’s effects on glyceroneogenesis and on lipolysis. When explants were pretreated for 30 min with the NOS inhibitor L-NAME and then with leptin for 18 h, the effect of leptin on glyceroneogenesis was completely abolished (Fig. 4). Conversely, the NO donor SNAP significantly reduced the glyceroneogenic flux by 60% (Fig. 4). Additionally, L-NAME completely abolished leptin reduction in glycerol release, whereas NEFA was unaffected (Fig. 5). Furthermore, SNAP dramatically induced a 70% decrease in glycerol concentration and a 70% increase in NEFA release (Fig. 5) while decreasing the expression of Pck1 and of the HSL gene by >90% (P < 0.001; data not shown). Pretreatment of explants with L-NAME prevented leptin’s repressive action on HSL, PEPCk-C, and PPARγ mRNA (Fig. 6).

**FIGURE 2** Leptin induces a long-term inhibition on glycerol and NEFA release in rat epididymal fat pads. WAT explants were treated or not with leptin (1 μg/L; 10 μg/L) for 18 h in DMEM. Incubation medium was then removed and changed for lipolysis medium in the absence or presence of db-cAMP (1 mmol/L) for 2 h. Results are expressed as percent of glycerol or NEFA relative to the corresponding untreated control. Crude values for control were 11.9 nmol-mg tissue^{-1}·2 h^{-1} for glycerol and 2.92 nmol-mg tissue^{-1}·2 h^{-1} for NEFA. Values are mean ± SEM, n = 4 (means of 2 samples/rat). Asterisks indicate different from control: ***P < 0.01; ****P < 0.001; + P < 0.01 vs. db-cAMP (1 mmol/L)-treated explants.

**FIGURE 3** Leptin regulates FAS, ATGL, HSL, PEPCk-C, and PPARγ mRNA (A) and HSL and PEPCk-C proteins (B) in rat epididymal fat pads. WAT explants were treated in the absence or presence of leptin (10 μg/L) for 2, 6, or 18 h. (A) FAS, ATGL, HSL, PEPCk-C, and PPARγ mRNA levels were analyzed by RT-qPCR. Results are normalized using 18S mRNA. Values are mean ± SEM, n = 4 (means of 2 samples/rat). Asterisks indicate different from control: *P < 0.05; **P < 0.01; ***P < 0.001. (B) HSL, PEPCk-C, and β-actin proteins were revealed by Western blotting performed on fat cake-linked proteins for HSL and cytosolic proteins for PEPCk-C and β-actin at 18 h (HSL) or 6 h (PEPCk-C and β-actin) of leptin treatment. Ponceau red staining of the gel was used as a marker of equal loading of fat cake-linked proteins.

**Discussion**

The main findings of the present study are that a long-term treatment (18 h) with leptin significantly decreased lipolysis and glyceroneogenesis and the expression of Pck1, HSL, and PPARγ genes in WAT and that NO is implicated in the effects of leptin. Lipolysis is usually monitored by the release of glycerol in the incubation medium, because NEFA output strongly depends on intracellular utilization. Several studies demonstrated that short-term exposure of WAT explants to leptin resulted in lipolytic activity (9,12,31). Therefore, leptin can be both lipolytic and antilipolytic depending on the length of treatment. One of the
main consequences of long-term effects of leptin is the decrease in HSL expression and glycerol release. Although one would have expected a concomitant reduction in medium NEFA, this did not occur. However, the net fatty acid output is mainly controlled by re-esterification. We observed that the incorporation of radiolabeled pyruvate into triglycerides was reduced upon leptin treatment, demonstrating that glyceroneogenesis and NEFA re-esterification were decreased. Additionally, Pck1 expression was diminished, a result reminiscent to what we observed previously with interferon-γ (32). GyK-induced glycerol phosphorylation is not a major pathway in adipocytes and GyK expression remained unchanged under leptin treatment.

Hence, this hormone reduces glyceroneogenesis but probably not glycerol phosphorylation. Such a reduction in re-esterification could, by itself, result in increased NEFA release even with a diminished lipolysis. The observation that fatty acid output is actually stable under leptin treatment could be due to induced β-oxidation. It was previously shown that leptin stimulated NEFA oxidation in adipocytes (9,11,33). We observed an increase in very long-chain acyl dehydrogenase gene expression, suggesting that this hormone is probably also implicated in NEFA oxidation under our experimental conditions. It thus can be concluded that such a catabolism is coherent with the absence of long-term leptin effect on NEFA release because HSL and PEPCK-C are downregulated, leading simultaneously to reduced lipolysis and re-esterification, in addition to increased oxidation. Because PPARγ transcriptionally regulates both HSL (34,35) and Pck1 (25,26), we explored PPARγ gene expression. Of interest was the fact that PPARγ mRNA was drastically reduced under leptin, ahead of the decrease in HSL and Pck1 gene expression. Because PPARγ maintains a positive transcriptional tone on these genes, it can be assumed that decreasing expression of this transcription factor would result in reduced transcription of HSL and Pck1 genes and ultimately downregulated lipolysis and glyceroneogenesis. The possible role of NO in lipolysis has been discussed by Gaudiot (14) and Frühbeck (15). More recently, we showed that leptin induced NOS III phosphorylation and increased NO production in adipocytes (16). Our present results demonstrate that NO is an important mediator in the long-term leptin-induced downregulation of lipolysis, glyceroneogenesis, the key genes in these pathways, and their major transcriptional regulator, PPARγ. Such results are in agreement with other studies carried out on 3T3-L1 adipocytes or with iNOS−/− transgenic mice that concluded that NO reduced PPARγ expression in adipocytes (36,37).

What could be the metabolic consequences of our observations? An explanation could be that such a leptin-induced decrease in lipolysis might represent a counter-regulatory adaptive process that cells develop to offset the action of lipolytic agents on a long-term basis. Examples of counter-regulatory processes are frequently observed in physiology. However, in our case, glycerol release, but not NEFA output, is downregulated. WAT-derived glycerol is thought to be used by the liver for VLDL synthesis and a decrease in this process could contribute to limiting hypertriglycerid-
eridemia. Such an effect by leptin was previously reported both in humans and rats (38–41). The stable NEFA output whether leptin is administered or not is the consequence of both reduced re-esterification (our results) and upregulated oxidation (33,42). The lipooxidative action of leptin in nonadipose cells, particularly in hepatocytes, could accompany the limited hepatic VLDL synthesis (42,43). This peripheral mechanism could contribute to the putative physiological role of leptin in normal animals preventing the development of an obese phenotype. This new metabolic state is a favorable situation in which energy storage is limited and expenditure is augmented. The elucidation of leptin action on these WAT metabolic pathways and genes in obese states is an open issue that remains to be investigated.

Acknowledgments

C.R., C.F., and A-M.J. designed research; F.N., A-M.J., M.C., N.M-M., and G.P. conducted research; F.N., C.B., C.R., C.F., and A-M.J. analyzed data; C.B., C.R., C.F., and A-M.J. wrote the paper; and C.F. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited


