Insulin and Leptin Acutely Regulate Cholesterol Ester Metabolism in Macrophages by Novel Signaling Pathways

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Leptin is produced in adipose tissue and acts in the hypothalamus to regulate food intake. However, recent evidence also indicates a potential for direct roles for leptin in peripheral tissues, including those of the immune system. In this study, we provide direct evidence that macrophages are a target tissue for leptin. We found that J774.2 macrophages express the functional long form of the leptin receptor (ObRb) and that this becomes tyrosine-phosphorylated after stimulation with low doses of leptin. Leptin also stimulates both phosphoinositide 3-kinase (PI 3-kinase) activity and tyrosine phosphorylation of JAK2 and STAT3 in these cells. We investigated the effects of leptin on hormone-sensitive lipase (HSL), which acts as a neutral cholesterol esterase in macrophages and is a rate-limiting step in cholesterol ester breakdown. Leptin significantly increased HSL activity in J774.2 macrophages, and these effects were additive with the effects of cAMP and were blocked by PI 3-kinase inhibitors. Conversely, insulin inhibited HSL in macrophages, but unlike adipocytes, this effect did not require PI 3-kinase. These results indicate that leptin and insulin regulate cholesterol-ester homeostasis in macrophages and, therefore, defects in this process caused by leptin and/or insulin resistance could contribute to the increased incidence of atherosclerosis found associated with obesity and type 2 diabetes. Diabetes 50:955–961, 2001

Obesity and insulin resistance are major contributory factors in premature death in the Western world, and these conditions are characterized by increased circulating levels of insulin (1) and leptin (2,3). Disturbances in lipid metabolism are characteristic features of insulin resistance and diabetes (4), and these are closely associated with the increased risk of atherosclerotic heart disease in individuals with these conditions (5). A key step in the development of atherosclerosis is the deposition of cholesterol ester–filled macrophage foam cells, which contribute to the formation of atherosclerotic plaques (6,7). Hormone-sensitive lipase (HSL) has neutral cholesterol esterase (nCE) activity, and this role of HSL in vivo is highlighted by the finding that targeted disruption of the HSL gene ablates nCE activity in most tissues (8). Although the knockout of the HSL gene does not remove all nCE activity from macrophages, there is now strong evidence that HSL is the enzyme responsible for a large proportion of nCE activity in these cells under normal conditions (6,9–14). Because HSL is involved in breakdown of cholesterol ester in macrophages, defects in the regulation of HSL could potentially promote formation of foam cells.

Regulation of HSL is best understood in adipocytes, where agents that increase cAMP activate lipolysis and where insulin counters this effect (15). The regulation of lipolysis in adipose tissue involves the coordinate regulation of HSL activity (15), subcellular localization of HSL, and subcellular localization of the lipid-coating protein perilipin (16). It also may involve interactions with associated proteins such as fatty acid–binding protein (17) and the docking protein lipotransin (18). More recently, there have been reports that leptin stimulates lipolysis in adipocytes (19–21), although it is not known whether this involves changes in HSL activity. The effects of leptin and insulin on HSL activity have not been investigated in macrophages; clearly, however, if leptin and insulin affect HSL activity in macrophages, they could play a role in regulating deposition of cholesterol ester and formation of foam cells. In the current study, we found that insulin acutely downregulates HSL activity in macrophages, whereas leptin stimulates the activity. We also found that the J774.2 macrophages possess the molecular machinery required to respond to leptin stimulation. They express high levels of the long form of the leptin receptor, and physiological concentrations of leptin stimulate intracellular signaling pathways known to be downstream of this receptor. Surprisingly, although both insulin and leptin activate phosphoinositide 3-kinase (PI 3-kinase) in macrophages, the attenuation of HSL by insulin was not blocked by wortmannin, but the stimulatory effects of leptin were blocked. These results identify new mechanisms by which leptin and insulin regulate HSL in macrophages and suggest that leptin and insulin may play direct roles in regulating formation of foam cells.
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RESEARCH DESIGN AND METHODS

[1-14C]-Protein A and cholesterol 1-[14C]-oleate were obtained from Amersham-Pharma (Uppsala, Sweden). RPMI-1640 and newborn calf serum were obtained from Gibco-BRL (Gaithersburg, MD). Fetal calf serum, Dulbecco’s modified Eagle’s medium (DMEM), and 8-(4-chlorophenylthio)-adenosine 3’,5’-cyclic monophosphate (cPT-CAMP) were obtained from Sigma Chemical (Poole, Dorset, U.K.). Anti-phosphotyrosine antibody, PY99, was obtained from Santa Cruz Biochemicals (Santa Cruz, CA), anti-phosphoSTAT3 was obtained from New England Biolabs (Beverly, MA), and anti-HSL antibody raised against a segment of rat HSL corresponding to amino acids 399–608 was kindly supplied by Dr. Raj Beri, of Astra Zeneca (Macclesfield, U.K.). Rabbit polyclonal antibody raised to amino acids 541–840 of the human leptin receptor was obtained from Santa Cruz Biochemicals (sc8325) and recognized all forms of the leptin receptor. Polyclonal ObRb–specific antibodies raised to amino acids 1075–1174 of the murine leptin receptor and polyclonal JAK2 antibodies were supplied by Dr. Martin Myers of the Joslin Diabetes Center (Boston, MA) (22). Phosphodiesterase 3B (PDE3B) antibodies were supplied by Dr. Ulf Smith (Goteborg, Sweden). The leptin used in the experiments shown was obtained from Biogenesis (Poole, Dorset, U.K.), but because there is still some debate regarding the reliability of some commercially obtained leptin, we also obtained additional batches from Peprotech (London), Calbiochem (San Diego, CA), and R and D Systems (Minneapolis, MN). Leptin from all these sources gave very similar results.

Cell culture. The murine macrophage cell line J774.2 was grown in RPMI medium (2 g/l glucose) supplemented with 10% fetal calf serum (heat inactivated at 50°C for 30 min) and 1% antibiotic-antimycotic. The medium was removed and replaced with fresh medium every 48 h. 3T3-L1 fibroblasts were cultured and differentiated as we have described previously (23). Adipocytes were used on day 8.

Immunoprecipitation and Western blotting analysis. J774.2 macrophages were serum-starved overnight in serum-free RPMI medium containing 0.2% bovine serum albumin (BSA) (fatty acid–free). This was replaced with fresh RPMI/BSA before stimulation. 3T3-L1 adipocytes were washed twice with 1 ml DMEM (1 g/l glucose) containing 5 mg/ml BSA (fatty acid–free) before being incubated in DMEM/BSA for 2 h. This medium was replaced with DMEM/BSA containing 20 mmol/l HEPES before stimulation. After stimulation, cells were washed once with ice-cold phosphate-buffered saline (calcium- and magnesium-free) and lysed in lysis buffer containing 1% Triton X100, 10 mmol/l Tris phosphate, 1 mmol/l dithiothreitol, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 200 KIU aprotinin. Cell lysate was incubated at 4°C with primary antibody for 2 h before incubation with protein A-agarose (HSR) or anti-mouse IgG (Py99) for an additional hour. The immunoprecipitate was washed extensively before being resuspended in Western loading buffer (20% glycerol, 4% SDS, 200 mmol/l dithiothreitol, 0.2 mol/l Tris buffer pH 6.8) and subjected to SDS polyacrylamide gel electrophoresis. Samples were then transferred onto polyvinylidene difluoride. [1-14C]-protein A was used as secondary antibody, and reactive bands were detected using a Fuji FLA2000 phosphorimager (Tokyo).

HSL/nCE assay. To measure the nCE activity in macrophage lysates, the cell monolayers were washed with phosphate-buffered saline before being scraped into 200 μl of assay buffer (5 mmol/l imidazole pH 7.0, 30% glycerol, 50 mmol/l NaCl, 20 mmol/l EDTA, 0.1 mmol/l sodium orthovanadate, 0.5 μmol/l sodium pyrophosphate, 1 mmol/l dithiothreitol, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 200 KIU aprotinin). Cells were lysed with four rounds of freeze-thawing followed by centrifugation at 14,000 rpm at 4°C, and 25-μl aliquots of the cell lysate (∼0.15 mg protein) were assayed. nCE activity was measured either directly in lysates or in anti-HSL immunoprecipitates.

The assay was performed as described previously (23). Briefly, the substrate was prepared by drying down a mixture of cholesterol [1-14C]-oleate (2.5 μCi, 30 μg) and cholesterol oleate (400 μg) under nitrogen. The lipid was then resuspended in 100 μl ethanol and added to a mixture of 100 μg BSA, 1 ml 0.2 mol/l sodium phosphate buffer pH 7, and 2.4 ml distilled water. A total of 175 μl substrate was added to a 25-μl sample and incubated for 50 min at 37°C. To stop the reaction, 0.5 ml of stopping buffer was added (0.1 mol/l potassium tetraborate, 0.1 mol/l potassium carbonate, pH 10.5), followed by 1.6 ml extraction buffer (methanol [1:41]chloroform [1:25]:heptane [1]) containing 0.3% (w/v) of unlabeled oleic acid as a carrier. The mixture was vortexed vigorously and then centrifuged at 3,000 rpm for 10 min at 10°C, and 0.5 ml of the upper phase containing [1-14C]-oleic acid was transferred to vials containing 5 ml scintillation fluid. We found that 1 unit of enzyme activity catalyzes the release of 1 μmol oleate per minute.

RESULTS

Acute regulation of nCE activity of HSL by insulin. In the current study, we investigated the acute effects of insulin and leptin on HSL activity in adipocytes and macrophages. The 3T3-L1 system was used as an adipocyte model because the effects of insulin on HSL have been partially characterized in these cells (18,24). The murine J774.2 macrophage cell line was used because it has been demonstrated to be a good model of mouse peritoneal macrophages (25) and some characterization of HSL activity has been performed in these cells (13,24,26). We find that both cell types express the 85-kDa active form of HSL, whereas in 3T3-L1 adipocytes, the HSL antibodies also react with a band of 80 kDa (Fig. 1). The identity of the 80-kDa band is not clear, but alternative splicing of the HSL gene has been reported previously (27). PDE3B, which is important in regulation of HSL by insulin in adipocytes, is expressed in 3T3-L1 adipocytes but is not found in J774.2 macrophages (Fig. 1).

Short-term incubation of serum-starved cells with insulin caused a ∼30% reduction in HSL nCE activity in 3T3-L1 adipocytes (Fig. 2). Insulin causes a similar reduction in activity in HSL immunoprecipitates from J774.2 macrophages, and this was paralleled by a very similar decrease in nCE activity in the cell lysates (Fig. 3).

Insoprenaline acutely stimulates HSL nCE activity in 3T3-L1 adipocytes, presumably via an increase in cAMP levels and activation of protein kinase A (PKA) (Fig. 4A). The magnitude of the effects on HSL are very similar to the level of activation induced by directly phosphorylating HSL with PKA in vitro (28). Insulin counters the isoprenaline stimulation of HSL in adipocytes (Fig. 4A). This might be expected, given that these effects parallel the effects that insulin and adrenergic stimuli have on HSL activity in these cells (24), although this has not previously been demonstrated in anti-HSL immunoprecipitates.

Activation of HSL by cAMP has been reported previ-
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FIG. 3. Insulin causes similar downregulation of the nCE activity in lysates and in HSL immunoprecipitates in J774.2 macrophages. nCE activity was measured in aliquots of lysate containing 100 μg protein (A) and HSL immunoprecipitates (B) from J774.2 macrophages after stimulation with 1 μmol/l insulin for varying times. Data represent the mean ± SE of four independent determinations. **P < 0.01 vs. basal.

FIG. 2. Effect of insulin on basal HSL activity in adipocytes. 3T3-L1 adipocytes were serum-starved for 2 h in DMEM containing 5 mg/ml BSA. Cells were stimulated with 1 μmol/l insulin. At various time points, cells were lysed, and HSL was immunoprecipitated and assayed for nCE activity. Data represent the mean ± SE of two independent experiments, each performed in triplicate.

Effects of leptin on nCE activity. There is some debate regarding whether leptin has direct effects in peripheral tissues. Therefore, we sought to determine whether leptin signaling systems existed in J774.2 macrophages before conducting experiments on the effects of leptin on HSL. We used two antibodies to detect the leptin receptor: one recognizing the extracellular domains, and thus recognizing all forms of the receptor (Fig. 5A), and another specific for the intracellular domain of the long (ObRb) form of the receptor (Fig. 5B). It has been established previously that ObRb runs at ~210 kDa under the gel conditions used (31,32) and that both leptin-receptor antibodies recognize a band of this molecular weight in J774.2 cells. ObRb was not detected in 3T3-L1 adipocytes, although several short forms of the leptin receptor are expressed in these cells (data not shown). In J774.2 macrophages, the level of the ObRb is much greater than the level of the ObRa form (Fig. 5A), and the ratio of these isoforms is similar to that seen in hypothalamus (33). Evidence that the ObRb is capable of mediating leptin signaling in these cells comes from the finding that the ObRb becomes rapidly tyrosine-phosphorylated after stimulation with low doses of leptin (Fig. 5C). This stimulation was 20-fold higher than basal levels after 15 min. Furthermore, leptin acutely stimulates tyrosine phosphorylation of JAK2 (Fig. 5D) and STAT3 (Fig. 5E) more than fourfold in J774.2 cells. It has been found previously that leptin stimulation of phosphorylation of both STAT3 and JAK2 requires the ObRb form of the leptin receptor (22,34). An important finding is that leptin can increase the nCE activity in anti-HSL immunoprecipitates

FIG. 4. Insulin counteracts cAMP-induced activation of HSL in both adipocytes and macrophages. 3T3-L1 adipocytes were treated with 10 μmol/l isoprenaline (A), or J774.2 macrophages were treated with 1 mmol/l CPT-cAMP (B). Treatment was for 15 min in each case. Where indicated, 10 mmol/l insulin was added for the last 5 min. HSL was then immunoprecipitated and nCE activity was measured in these immunoprecipitates. Data represent the mean ± SE of five independent determinations.
in J774.2 macrophages (Fig. 6A). This effect was maximal at 2 nmol/l leptin. The effect was seen at concentrations of leptin as low as 0.5 nmol/l, which is within the range of concentrations found in obese humans (2,3); surprisingly, however, this effect diminishes greatly at concentrations of leptin higher than 10 nmol/l (not shown). The effects of leptin were rapid, reaching maximum within 15 min (Fig. 6B). Furthermore, the effects of leptin were additive with those of CPT-cAMP (Fig. 7).

**Role of PI 3-kinase in signaling pathways regulating the nCE activity of HSL.** There is strong evidence that the regulation of HSL activity by insulin in adipocytes is mediated by PI 3-kinase, because the PI 3-kinase inhibitor wortmannin blocks the inhibition of lipolysis by insulin (35). We found that the inhibition of HSL by insulin in 3T3-L1 adipocytes was also blocked using those concentrations of wortmannin at which the drug specifically inhibits PI 3-kinase activity (Fig. 8A). However, similar concentrations of wortmannin had no effect on the attenuation of HSL activity by insulin in J774 macrophages (Fig. 8B). Similar results were obtained using LY294002, an alternative PI 3-kinase inhibitor (data not shown). Surprisingly, 100 nmol/l wortmannin completely blocked the leptin-induced stimulation of HSL nCE activity, whereas wortmannin alone had no significant effect (Fig. 8C).

Because wortmannin blocked the effects of leptin but not those of insulin, we investigated whether these stimuli were able to activate PI 3-kinase in the J774.2 cells. We found that both insulin and leptin rapidly stimulate recruitment of the class Ia PI 3-kinase adapter subunit (p85α) to tyrosine-phosphorylated signaling complexes in J774.2 macrophages (Fig. 9); however, the stimulation by insulin is only 70% above basal, whereas leptin stimulates p85 recruitment 400% above basal. This indicates that they are activating PI 3-kinase, and in support of this, we found that both of these stimuli also cause an increase in PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates that parallel their ability to recruit p85α (data not shown).

**DISCUSSION**

Coronary artery disease is a major cause of premature death in obese and diabetic individuals (5,36), and these conditions are characterized by increased levels of insulin (1) and leptin (2). There is accumulating evidence that hyperleptinemia (37) and the combination of hyperinsulinemia and hyperglycemia (5) play direct roles in the development of atherosclerosis. Much attention has focused on understanding the mechanisms and regulation of deposition of esterified cholesterol in macrophage foam cells, although until recently, not much attention has been given to the role of nCE in this process. Evidence from mice with targeted disruption of the HSL gene strongly suggest that HSL is the enzyme responsible for nCE activity in a wide...
Evidence from several experimental approaches also indicates that HSL is a rate-limiting enzyme in cholesterol ester efflux from macrophages (9–14). It is possible that regulation of HSL by leptin and insulin could, at least in part, explain deposition of cholesterol ester and formation of foam cells. Such a scenario is best established for insulin, which is known to counter cAMP-induced increases in HSL activity in adipocytes; this is believed to be a key step in mediating the antilipolytic effects of insulin (15). It is known that HSL in macrophages is activated by agents that increase cAMP levels (26,29,30), but the effect of insulin on HSL in macrophages has not been investigated previously. Our finding that insulin cannot only counter the cAMP-induced activation of HSL but also acutely downregulate basal activity of HSL thus provides the first direct evidence that insulin could contribute to formation of macrophage foam cells via effects on HSL.

The finding that leptin stimulates HSL in macrophages is novel and suggests that leptin has an acute protective effect against accumulation of cholesterol ester in these cells. There is currently a great deal of controversy regarding whether leptin directly regulates glucose and lipid metabolism in peripheral tissues. A number of studies have reported that leptin has direct effects (19–22,33,34,38,39), whereas an almost equal number of studies in similar tissue types have found that leptin alone has no direct effects (32,40–42). One explanation for this could be variability in the source of leptin; for this reason, we obtained leptin from four different sources, and all gave essentially similar results. Another source of variation in the experimental observations could lie in differences in leptin signaling systems present in the different cell types used. Evidence indicates that leptin only signals inside the cells when the long form (ObRb) of the leptin receptor is expressed (22,31,34,38,39), whereas an almost equal number of studies in similar tissue types have found that leptin alone has no direct effects (32,40–42). The apparent absence of ObRb in peripheral tissues is cited as evidence that the main site of action for leptin is the hypothalamus. However, there are reports that T-cells express ObRb, at least
as determined by reverse transcriptase–polymerase chain reaction (45–49), although these studies have not explored expression at the protein level or leptin stimulation of signaling pathways. Our studies clearly establish that ObRb is the major form of leptin receptor present in J774.2 macrophages and that signaling pathways downstream of the leptin receptor are activated in these cells. These findings, along with the finding that leptin activates HSL, provide strong evidence that macrophages are a functionally relevant target tissue for leptin. Leptin has been shown previously to modulate immune cell functions, such as proliferation in T-cells (45–49) and cytokine production and phagocytosis in macrophages (45). Currently, adipocytes are the only known site of leptin production, and it is not clear why a cytokine secreted from adipose tissue would be useful in controlling these immune cell functions. However, the link between nutritional status and effects of leptin on HSL in macrophages is more easily rationalized because leptin has been reported previously to stimulate lipolysis in several other tissues (19–21). This has led to the proposal that, in addition to its role in regulating food intake, leptin has a protective effect on peripheral tissues in times of energy excess by limiting accumulation of lipid in nonadipose tissues via its ability to regulate lipolysis (50). Such a role would certainly be valuable in preventing formation of foam cells in macrophages.

The current study also established that insulin and leptin regulate HSL activity in macrophages using signaling mechanisms distinct from those in adipocytes described previously. In particular, it is surprising that the effects of insulin on HSL in J774.2 macrophages are not blocked by wortmannin and thus presumably do not require activation of PI 3-kinase. This suggests that there are pathways regulating HSL other than the classical PI 3-kinase–protein kinase B (PKB)–PDE3B pathway. Indeed, there is previous evidence to support this. When there are relatively low levels of cAMP in adipocytes, the insulin-induced inhibition of lipolysis acts via phosphodiesterases (51). It is believed that this is achieved via activation of PI 3-kinase, which causes activation of PKB, which phosphorylates and activates PDE3B (52). This, in turn, decreases the cAMP levels produced in response to lipolytic stimuli and thus reduces the phosphorylation of HSL by PKA (15). However, at high cellular concentrations of cAMP, the inhibition of lipolysis by insulin is not mediated by phosphodiesterases (51), indicating that there are several mechanisms contributing to the regulation of lipolysis by insulin in adipocytes. Our evidence suggests that insulin regulates HSL by non–PI 3-kinase–dependent pathways in the J774.2 macrophage line. In support of this, we found that although insulin activates PI 3-kinase in the J774.2 cells, the effect is small. However, even if PI 3-kinase was highly stimulated in these cells, it is unlikely that this would be sufficient to regulate cAMP via PKB because PDE3B levels are so low in these cells. It could be that insulin is regulating other PDE isoforms in macrophages. There is evidence that PDE4 can take part in non–PI 3-kinase–dependent pathways regulating fatty acid release (53) and that PDE4 is present in macrophages (54). It is known that PDE4 can be regulated by interleukin-4 in myeloid cells (55) and that insulin and interleukin-4 share some overlap in their intracellular signaling pathways in hematopoietic cells (56). Alternatively, it is possible that the regulation of HSL by insulin does not involve cAMP-dependent pathways but instead acts via regulation of the protein phosphatases that have been demonstrated previously to act on HSL (57).

The mechanism by which leptin acts to increase HSL activity in macrophages is unclear. Increasing cAMP is the most well-established mechanism for activating HSL, but there are conflicting reports regarding the effect of leptin on cellular levels of cAMP. In chromaffin cells, leptin causes a very modest increase in cAMP levels (58), whereas in pancreatic β-cells, leptin causes a decrease in cAMP levels (39). However, in the β-cells, the decrease in cAMP is apparently caused by a leptin-induced activation of PDE3B (39). Because the level of PDE3B is very low in the J774.2 macrophages, it is possible that in the absence of this negative regulator of cAMP, leptin is actually increasing cAMP levels, as seen in chromaffin cells. However, it is unclear how leptin, but not insulin, would achieve this in a PI 3-kinase–dependent manner.

In conclusion, we have identified macrophages as a novel target for leptin-mediated effects on lipid metabolism. Furthermore, we found that leptin- and insulin-mediated pathways acutely regulating HSL activity in macrophages differ from those defined previously in other tissues. Based on these findings, we suggest that leptin and insulin could play counteracting roles in regulating cholesterol ester homeostasis in macrophages. Therefore, either leptin resistance or increased insulin action could contribute to the increased formation of foam cells and atherosclerosis in obese and diabetic individuals.

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