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Commentary

Leptin expression and action: New experimental paradigms

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The identification of the *ob* gene through positional cloning (1) and the demonstration that its protein product leptin reverses the obesity–diabetes syndrome in *ob*/*ob* mice (2–4) have ushered in an exciting new era in nutritional physiology and obesity research. Preeminent among a rapidly expanding list of molecules whose discovery has fundamentally advanced our understanding of the regulation of appetite and energy expenditure, leptin deserves special status. With the realization that adipocytes produce a hormone that acts through discrete receptors (5, 6) on distant targets to create a feedback loop for body weight regulation, our understanding of the pathophysiology of obesity has entered the "endocrine era." Although numerous endocrine abnormalities have been identified in association with obesity, endocrine insights into the etiology and therapy of obesity were lacking until leptin appeared on the scene. Now, laboratories race to measure leptin secretion and levels in disorders of body weight, and to characterize the sites and mechanisms of leptin action, much as insulin secretion and action have been studied for many years in obesity and obesity-linked non-insulin-dependent diabetes mellitus. Indeed, interesting parallels between research on insulin and leptin continue to emerge.

As is the case with insulin and its receptor in diabetes, mutations of leptin and its receptor are rare in human obesity (7, 8) (none have yet been described), and most obese individuals have higher levels of serum-immunoreactive leptin than do nonobese individuals, raising the specter of "leptin resistance" (9, 10). Although absolutely increased compared with lean individuals, leptin levels may be relatively lower as a function of body fat content in some obese individuals than in others, and individuals with relatively "low leptin" may be more likely to gain weight over subsequent years (11). It will be interesting to determine whether these lower levels are a fixed attribute of certain individuals or, analagous to the pathophysiology of insulin secretion in developing non-insulin-dependent diabetes, leptin production diminishes over time. These findings increase the importance of elucidating the factors that determine leptin expression in adipocytes, and the mechanism of leptin action in target cells. This issue of the Proceedings has two papers that address these opposite poles of leptin biology. The paper by Mandrup *et al.* (12) establishes a new system for defining the regulation of leptin expression by adipocytes, and the paper by Shimabukuro *et al.* (13) raises a provocative new hypothesis regarding the nature of leptin actions on metabolic pathways. In this commentary, I will attempt to place these findings in the broader perspective of leptin biology two and one-half years after the initial discovery of leptin.

Several aspects of regulated expression of leptin are worthy of note. The first involves the tissue-specific expression of leptin. Leptin is strongly expressed in white adipose tissue, and is absent or expressed at extremely low levels in other tissues. Although expression of leptin mRNA has been described in the closely related brown adipose tissue (BAT; refs. 14–16), most of this expression may be due to contaminating white adipocytes (17). Because white adipocytes are primarily involved in energy storage, for which leptin expression is the measure, whereas brown adipocytes subserve regulated energy dissipation, it is not surprising that these two tissues have divergent capacities for leptin expression. Similarly, uncoupling protein (UCP; ref. 18), the gene most fundamental to brown adipocyte thermogenic function (19), is expressed exclusively in BAT. On the other hand, leptin may be the first gene described to be expressed in white adipose tissue (WAT) but to a limited extent or not at all in BAT, a fact that creates new opportunities to evaluate the molecular basis for tissue-specific gene expression in these tissues.

A second issue relates to the mechanism for regulated expression of leptin within WAT in physiology and disease. In experimental animals and man that were studied in the fed state, leptin expression and levels generally increase in parallel to adipose stores (9, 20), in agreement with the proposed role of leptin as a readout signal of adipocyte triglyceride stores. The mechanism for this tight coupling between triglyceride stores and leptin expression and secretion remains obscure. Many studies have observed a correlation between insulin and leptin levels (21), but this is most often explained by leptin and insulin each covarying with obesity. Insulin however has been found to be capable of increasing leptin expression (22) and levels (23) under some circumstances, and the idea that insulin may be a controlling factor over leptin expression has been suggested. Although possible, dominant control of leptin expression by an exogenous factor such as insulin would seem to diminish the rationale for the adipocyte being in the feedback loop in the first place. On the other hand, leptin expression and levels fall rapidly with starvation (14, 24), and this suppression is disproportionate to the fall in adipocyte energy stores. The fall in leptin appears to be central to the neuroendocrine adaptation to starvation (25), and could be the primary purpose for which leptin evolved. Falling insulin may be a key regulatory signal for the suppression of leptin expression with starvation (24). Other positive regulators include glucocorticoids at high doses (26, 27) and certain cytokines (28, 29), and negative regulators include beta adrenergic agonists or cAMP (27, 30). Despite these external influences, it is likely that cell-autonomous factors are the major links between adipocyte size and leptin gene expression. Which intracellular metabolites, signaling molecules, or transcription factors provide the necessary link is as yet unclear. Like many other adipocyte genes, the *ob* gene promoter is positively regulated through a functional binding site for CEBPα (31–34). In contrast, thiazoladinedione agonists for PPARγ transcription factors (35, 36) suppress leptin expression *in vitro* and *in vivo* in rodents (37–40), and this may involve, at least in part, a functional antagonism between CEBPα and PPARγ on the leptin promoter (41).

The study of leptin gene expression has been hampered by the absence of a suitable *in vitro* test system. Preadipocyte cell lines that differentiate in culture have been used extensively to characterize cis and trans factors that regulate adipocyte gene

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Abbreviations: CNS, central nervous system; FFA, free fatty acids.

expression (42, 43). These cells typically display robust expression of adipocyte genes, often to levels seen in adipose cells *in situ*. Leptin is a notable exception, however, as no mixture of ligands has so far been able to induce expression beyond 1–2% of that seen in adipose cells (44). Mandrup *et al.* (12) have made clever use of the observation of Greene and Kehinde (45) that cultured adipocytes can grow into fat pads after being placed subcutaneously into athymic mice. Using this technique, they have shown a 10-fold increase in leptin expression when such cells reside *in vivo*, to a level 10–15% of that seen in adipocytes *in situ*, and the cells acquire a brisk response to glucocorticoids (12). These results suggest the existence of a key, unidentified *in vivo* regulatory factor, either a soluble mediator or a cell–cell interaction. Alternatively, adipocytes may simply enlarge to a greater extent *in vivo* than *in vitro*, with leptin expression increasing on that basis. Whether injection into mice of modified cell lines will be better than a transgenic approach for studying leptin expression will depend on the time required to create multiple stable cell lines vs. the time and expense of transgenesis.

While the basis for regulated leptin expression is being studied, the mechanism of leptin action is being addressed using genetic, biochemical, and physiologic approaches. The cloning of the leptin receptor gene (6, 46) has revealed unexpected complexity, with five or more isoforms encoded by a single gene. Among these are a form predicted to be soluble, several short forms with minimal intracellular domains, and one long form with a substantial homology to the signaling domain of the type I cytokine receptor family that employs the JAK-STAT pathway for signal transduction. Genetic obesity syndromes have been employed to reveal important insights into the function of these forms. The *db/db* mouse has a missense mutation that, through an effect on mRNA splicing, prevents expression solely of the long receptor form (6, 46), resulting in a short isoform wherever the long form would normally be expressed. Since these mice have a florid syndrome of obesity that appears to be totally resistant to leptin (2–4), it is clear that the long receptor form is essential for avoidance of obesity. Consistent with this genetic evidence is biochemical confirmation that the long form signals through the JAK-STAT pathway to regulate gene expression *in vitro* (47–49) and *in vivo* (50), whereas the short form has heretofore been found to be devoid of such activity (47, 48, 50). The fact that mRNA encoding the long receptor form is most strongly expressed in the hypothalamus (6) is also consistent with the anti-obesity effect of leptin being exerted primarily through regulated gene expression by the long receptor isoform in the brain (50). On the other hand, since the long-form mRNA is present at lower amounts in several peripheral tissues, at least as assessed by PCR (6, 46), it is possible that peripheral actions are exerted through this receptor isoform as well. The mutations responsible for the fa/fa and Koletsky rats also map to the leptin receptor gene (51–54). Unlike the *db* mutation that selectively affects the long receptor, these two mutations affect all receptor isoforms through a missense mutation in the common extracellular domain in fa/fa rats (51–53) and a nonsense mutation that is predicted to cause a total absence of receptors in Koletsky rats (54). It is not known whether the absence or dysfunction of the receptor short forms in these two models has any phenotypic consequence beyond that seen with selective deletion of the long form in *db/db* mice.

What are the functions of the receptor short forms, the mRNAs for which are surprisingly abundant in peripheral tissues, including lung and kidney, as well as the choroid plexus in the brain, from which it was originally cloned (5)? The most obvious suggestion is that one or more of these receptors mediate transport from the plasma into the central nervous system (CNS), through either the blood–cerebrospinal fluid or blood–brain barriers (55). The observation that leptin is in part cleared by a renal mechanism (56) might suggest a clearance function for receptors in the kidney, whereas a function in the lung remains obscure. Because transport into the CNS may be a rate-limiting step in leptin action in both animals with diet-induced obesity (57) and obese humans (58), it will be critical to determine the specific receptor through which transport and/or clearance occur, and to elucidate the biochemical mechanisms involved.

From a physiologic perspective, the action of leptin that has received the most experimental attention is its ability to influence food intake. This involves the regulated expression of hypothalamic neuropeptides, which include (59) but cannot be limited to neuropeptide Y, because neuropeptide Y knockout mice respond at least as well as wild-type mice to leptin injection (60). The existence of a mechanism for rapid leptin uptake from blood into the brain (61), the presence of leptin receptors on cells within key hypothalamic nuclei (62, 63), the rapid activation of *fos* expression in a subset of these nuclei after peripheral injection of leptin (64–66), and the potent peripheral metabolic response that follows administration of small amounts of leptin into the cerebrospinal fluid (3) combine to support the idea that leptin action is initiated within the CNS. If so, then the powerful actions of leptin to reverse not only hyperphagia, but the major metabolic defects of *ob/ob* mice, including diabetes, insulin resistance, and altered thermogenesis, must arise within the CNS. There are venerable precedents for central lesions, such as those in the ventromedial hypothalamic nucleus, causing peripheral metabolic defects (67, 68) that are mediated by changes in activity of the autonomic nervous system, which exerts profound effects on insulin secretion and the thermogenic state of brown adipose tissue.

On the other hand, since several leptin receptor isoforms are expressed in peripheral sites, direct effects on peripheral tissues could underlie some of leptin's biological actions. This issue is addressed in the paper of Shimabukuro *et al.* (13) in this issue of the Proceedings. Using recombinant adenoviral vectors to create continuously high leptin levels in normal rats, these researchers previously reported severe depletion of adipose stores, far exceeding the consequences of restricted food intake alone (69). Because leptin has been shown in a preliminary report to activate nerve activity in highly thermogenic brown adipose tissue (70), it is not surprising that leptin-induced weight loss exceeded that from food restriction. Indeed, this was previously reported in mice receiving leptin injections (71). However, the lipid depletion they observed (69) was so extreme that it raised questions about the biochemical and physiologic mechanisms involved, and the current paper (13) raises two important points in this regard. Their work builds upon earlier studies of the Unger group, and reports that fa/fa rats have markedly increased intracellular triglyceride content in a number of tissues, including islets of Langerhans, where a role for the lipid as a "lipotoxic" mediator was proposed (72). Here, they suggest that leptin may reverse this lipid accumulation through a direct peripheral action. This surprising conclusion is based on the observation that leptin reduced triglyceride synthesis and increased intracellular lipid oxidation upon direct addition to normal islets in short-term culture (13). Peripheral hyperleptinemia induced by adenovirus reduced intracellular triglyceride in multiple other tissues, but whether this occurred directly or indirectly through a CNS signal was not addressed by this study. The inability of leptin to induce these effects in fa/fa rats proves that leptin receptors are involved, but cannot clarify whether the actions are exerted centrally or peripherally, since all receptor forms are defective in the fa/fa rat. On the basis of their data, the authors propose that leptin-mediated lipid oxidation may occur through a novel pathway that could be critical to the peripheral actions of this hormone.

This raises two major questions. First, which of leptin's actions are exerted in the CNS, and which are exerted directly

via leptin receptors (presumably long form) in the peripheral tissues? Second, whatever the anatomical site at which the signal is initiated, by what intracellular mechanism does leptin cause the observed profound metabolic changes in carbohydrate and lipid metabolism? Shimabukuro *et al.* (13) suggest that leptin inhibits triglyceride synthesis and increases triglyceride oxidation within cells. This contrasts with the mechanism through which triglycerides are lost when adipocytes are deprived of insulin, which involves triglyceride breakdown and release (i.e., lipolysis). To determine the biochemical pathways involved in this action of leptin will require examination of the pathways that determine switching between fatty acid and carbohydrate use within cells.

The latter question brings us full circle from the hypothesis and mechanism-free world of positional cloning, to the world of intermediary metabolism and mitochondrial energetics, where the rubber of energy homeostasis meets the road. A fundamental issue in metabolic physiology is the mechanism by which organisms regulate the choice of fuels that they will utilize under varying circumstances of nutrition and exercise. In the transition from the fed to the starved state, for example, there is a switch from the predominant use of carbohydrate to fat as an energy source, and this is orchestrated by the fall in levels of insulin and the rise in levels of glucagon and cortisol. Under these conditions, free fatty acids (FFAs) are released from adipose stores, taken up into liver and muscle cells (among others), and transported into mitochondria, where they are oxidized for the provision of energy, as well as used by hepatocytes to synthesize ketone bodies that are exported for use elsewhere in the body. The study by Shimabukuro *et al.* (13) suggests the existence of a parallel but presumably distinct system through which leptin may regulate triglyceride synthesis and oxidation in tissues. That is, a system by which leptin, perhaps acting in part directly on peripheral cells, simultaneously inhibits triglyceride synthesis and stimulates oxidation within the cell. How might this occur? One hint is the observation that leptin may be able to inhibit the activity of acetyl CoA carboxylase in a cultured adipocyte cell line (73). Acetyl CoA carboxylase is the rate-limiting step in fatty acid synthesis and has also been proposed to serve as a metabolic switch for fatty acid oxidation. Lower activity of the enzyme would reduce levels of malonyl CoA (74), disinhibiting carnitylacyltransferase 1 (CPT1; refs. 75 and 76), and thereby increasing uptake of FFA into the mitochondria. Further evidence that leptin actually inhibits acetyl CoA carboxylase activity in various tissues must be sought, and the mechanism through which leptin might accomplish this must be determined.

Is this ability of leptin to increase fuel oxidation likely to involve additional mechanisms, apart from provision of FFA substrate to mitochondria? Under conditions of tight coupling, fuel is oxidized only to the extent that energy is needed, as assessed by the ATP/ADP ratio within the mitochondria. Can leptin, in addition to increasing FFA availability to the mitochondrial oxidative machinery through inhibition of acetyl CoA carboxylase or some other mechanism, cause uncoupling of mitochondria to permit oxidation of FFA that is not obligatorily linked to ATP synthesis, with the consequence being increased thermogenesis? Such a mechanism is clearly involved in the mitochondria of brown adipocytes, through the function of uncoupling protein. The recent discovery of a novel uncoupling protein (UCP-2) (77) that is homologous to that found in brown adipocytes but widely expressed in peripheral tissues will permit this idea to be readily tested.

In summary, it is clear that our understanding of metabolism and nutritional homeostasis has been profoundly affected by the discovery of leptin and the proof that its replacement cures the obesity syndrome of *ob/ob* mice. As we make the transition from the stunningly successful studies of this severe monogenic model to the question of how leptin's expression is regulated, how leptin brings about its complex effects, and why it so often seems unable to prevent human obesity, it is apparent that much hard work lies ahead.

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