

The Use of Animal Models to Dissect the Biology of Leptin

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ABSTRACT

Our understanding of the effects of leptin have stemmed mainly from animal studies, which continue to leave important clues of its roles in physiology, metabolism, neuroscience, and cell signaling. Since its discovery, leptin has been linked to various pathways, either directly at its primary site of action in the hypothalamus, or indirectly via downstream effector pathways such as in adipocytes and muscle. Leptin's importance is exemplified by the lack of redundant backup mechanisms, since leptin-deficient mice are obese, diabetic, and sterile. Investigations uncovering the pleiotropic actions of leptin were unfolded mainly from rodent models. Thus, this chapter focuses on these studies and, more specifically, on those findings recently brought forward by transgenic mice overexpressing leptin. The vast amount of biology that has been ascribed to leptin encompasses effects on food intake, insulin sensitivity, adiposity, thermogenesis, reproduction, immunity, and bone regulation. Mechanisms underlying leptin's action revolve essentially around neural pathways but also encompass to a lesser extent peripheral mechanisms. The roles of leptin along these axes are reviewed, with particular emphasis on pathways and phenotypes generated by transgenic hyperleptinemia. An evolutionary significance of hyperleptinemia in association with development of leptin resistance is suggested as a protective armament against some of the detrimental effects caused by hyperleptinemia in transgenic mice overexpressing leptin.

I. Introduction

The discovery of the adipocyte-derived hormone leptin (Zhang *et al.*, 1994) opened a new field of investigation, heightened interest in dissecting pathways regulating energy homeostasis, and a fresh viewpoint on treating obesity and associated disorders such as diabetes, syndrome X, polycystic ovary syndrome, and hypertension. It has long been known that obesity exacerbates these conditions; thus, the discovery of leptin replaced the interest brought earlier by adipisin (Flier *et al.*, 1987) and tumor necrosis factor (TNF) alpha (Hotamisligil *et al.*, 1993), by providing a new entry point to delineate mechanisms of energy homeostasis. Preparation of recombinant leptin allowed its exogenous effects to be tested on normal and leptin-deficient *ob/ob* mice (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995; Weigle *et al.*, 1995). The major outcomes from these early studies showed a significant decrease in food intake,

increase in energy expenditure, and selective attenuation of the adipose fat mass. Another major effect of leptin was on the reproductive axis (Chehab *et al.*, 1996), which is directly linked to an organism's nutritional status. It was shown that leptin treatment of *ob/ob* mice could rescue their lifelong sterility, while, in normal mice, it could suppress fasting-induced anestrus (Ahima *et al.*, 1996) and stimulate the onset of puberty (Ahima *et al.*, 1997; Chehab *et al.*, 1997; Yura *et al.*, 2000). Other functions of leptin were associated with immunity (Lord *et al.*, 1998) and a central regulation of bone formation (Ducy *et al.*, 2000; Takeda *et al.*, 2002). The failure of exogenous leptin treatment to attenuate the obesity in other animal models of obesity — such as the *db/db* mouse, the agouti yellow mouse, and the melanocortin 4 receptor (MC4-R) null mouse (Huszar *et al.*, 1997), which all are associated with elevated leptin levels — gave rise to the concept of leptin resistance and hyperleptinemia, a condition reflected in most forms of human obesity (Considine *et al.*, 1996). All these findings suggested that leptin has multiple roles beyond its body weight-thinning effects, as initially suggested by its name (Flier, 1998). Thus, to study leptin's systemic and chronic effects and to delineate mechanisms of action, transgenic animal models of leptin overexpression are warranted. This review is aimed mainly at pinpointing the most-relevant aspects of leptin biology that have and could emerge from overexpressing leptin in the mouse.

II. Animal Models of Leptin Overexpression

To date, there have been reports of four transgenic mouse models overexpressing leptin. One model resulted from leptin overexpression at very high levels under control of the liver-specific albumin promoter (Ogawa *et al.*, 1999). Two additional models used the fatty acid binding protein aP2 promoter to direct expression of leptin specifically to adipocytes, resulting in, in one case, moderately elevated leptin levels (Qiu *et al.*, 2001b) and in the other, very low leptin levels (Ioffe *et al.*, 1998). A fourth model directed leptin expression to the osteoblast using the alpha-1 collagen promoter (Takeda *et al.*, 2002). Although the low aP2-expressing line and the osteoblast line did not significantly alter circulating levels of leptin, they addressed specific questions regarding the sensitivity of *ob/ob* mice to small amounts of leptin and local effects on bone formation, respectively. Overexpression of leptin in a rat model also was generated using an adenoviral vector (Chen *et al.*, 1996). This review mainly addresses studies that emerged from the high and moderately elevated leptin-overexpressing lines (referred herein as LepTg) and the rat model, all of which investigated metabolic and broader effects of leptin.

III. Food Intake

Exogenous administration of leptin to *ob/ob* mice resulted in dose-dependent reductions in food intake (Pelleymounter *et al.*, 1995), with the greatest inhibition

occurring during the early phases of leptin treatment. This demonstrated the exquisite sensitivity of *ob/ob* mice to leptin. In normal mice, large leptin doses were required to result in a 35% reduction of food intake, which eventually stabilized to match the food intake of nonleptin-treated normal mice (Halaas *et al.*, 1995). These studies demonstrate that normal mice respond to leptin treatment and adapt to its anorectic effects. In LepTg mice overexpressing 12-fold and 8-fold leptin, reductions in food intake were 25% and 15%, respectively (Ogawa *et al.*, 1999; Qiu *et al.*, 2001b). This once again demonstrated the dose-dependent effect but, interestingly, the lack of adaptation to anorectic effects of leptin. Adult rats infected with an adenoviral leptin construct resulted in a 10-fold hyperleptinemia and a 30–50% reduction in food intake (Chen *et al.*, 1998). These decreases in food intake are likely to represent near-maximal levels of tolerance that a mouse can sustain without suffering devastating physiological consequences of a caloric deficit. The implications of this functional hyperleptinemia (as opposed to hyperleptinemia associated with leptin resistance) suggests that while there may be a saturable transport of leptin to the hypothalamus (Banks *et al.*, 1996), normal circulating endogenous leptin levels are far below the threshold required to achieve a transport saturation. Because the 12-fold overexpressing LepTg line resulted in a larger decrease in food intake than the 8-fold overexpressing line, it is reasonable to assume that an 8-fold leptin overexpression still does not result in transport saturation. Mechanisms leading to chronic reductions of food intake in response to functional hyperleptinemia are likely to involve a continuous downregulation of the orexigenic pathway involving neuropeptide Y (NPY) neurons and activation of the anorectic pathway mediated by pro-opiomelanocortin (POMC) neurons and the melanocortin 4 receptor (MC4R) (Cowley *et al.*, 2001). Thus, steady-state reductions of food intake in normal mice suggest that pharmacological manipulations of MC4R could result in long-term reductions in food intake in individuals who might be responsive to leptin. Overall, the anorectic effects of leptin in an organism susceptible to hyperleptinemia appear to be dose related. While they are more pronounced when exogenously administered for short periods, they last longer when chronically secreted from a transgene. Interestingly, experiments by Coleman and Hummel (1969) involving the parabiotic union of *db/db* mice (overexpressing leptin) with normal mice led to wasting and death of the normal parabiont. In contrast, LepTg mice, while continuously responding to transgenic hyperleptinemia, do not suffer from energy deprivation or a wasting phenotype, suggesting that sensitivity to hyperleptinemia is not detrimental to energy homeostasis. It is to be noted that leptin-mediated anorexia is not associated with the detrimental effects of fasting, which unlike leptin treatment (Wang *et al.*, 1999), result in elevated circulating free fatty acids (FFAs), showing an unusual role for leptin in lipolysis.

IV. Regulation of Adiposity

The characteristic and most-prominent feature of exogenous leptin treatment by any means was a decrease in body weight, which was accounted for almost entirely by a reduced adipose tissue mass. In LepTg mice, the extent of this effect correlated with transgenic leptin levels and varied from a small fat mass (Qiu *et al.*, 2001b) to a virtual absence (Chen *et al.*, 1996; Ogawa *et al.*, 1999), reflecting quantitative effects of leptin on the fat mass. When adipose tissue could be recovered, histological analysis revealed that, compared to normal mice, LepTg adipocytes were 4-fold smaller and were either depleted of triacylglycerol stores or contained minute lipid droplets (Qiu *et al.*, 2001b; Ogus *et al.*, 2003). Whether these small cells represent preadipocytes, adipocytes, or a mixture of both remains to be determined. However, mRNA expression of the fatty acid binding protein aP2, which is expressed only in mature adipocytes, failed to reveal any quantitative differences between normal and leptin transgenic mice. This suggests that the majority of these cells consist of mature adipocytes, albeit without visible intracellular lipids. Recovery and growth of these adipocytes *in vitro* in a medium rich in glucose, insulin, and insulin-like growth factor-1 (IGF-1), which are all significantly decreased in LepTg mice, revealed a rapid accumulation of triacylglycerols, suggesting a heightened sensitivity to lipid accumulation (Ogus *et al.*, 2003).

The histological makeup of the adipose tissue in the leptin-overexpressing mice is not unique to leptin (Figure 1) but rather characteristic of lean mice with small fat masses, such as fasted mice, transgenic mice overexpressing a dominant-positive form of sterol regulatory element-binding protein-1c (SREBP1c) (Shimomura *et al.*, 1998), forkhead box subclass C member 2 (FOXO2) (Cederberg *et al.*, 2001), or knockout (KO) mice for either the type II regulatory subunit of protein kinase A (Cummings *et al.*, 1996), protein tyrosine phosphatase 1B (Elchebly *et al.*, 1999; Klamon *et al.*, 2000), or the lipid-coating protein perilipin (Martinez-Botas *et al.*, 2000; Tansey *et al.*, 2001). Thus, the convergence of different pathways to a common phenotype – namely, the depletion of triacylglycerols from adipocytes — demonstrates the various effectors that impact the adipose mass. The question of whether these molecules belong to a common pathway orchestrated by leptin remains to be answered.

In nonadipose tissues (e.g., liver, muscle), leptin prevents the lipotoxicity associated with triacylglycerol buildup (Lee *et al.*, 2001) by activating 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK), which stimulates fatty acid oxidation via inhibition of acetyl coenzyme A carboxylase. Leptin's action on stimulating AMPK activity is mediated chiefly via the central nervous system (CNS), with a moderate peripheral action (Minokoshi *et al.*, 2002). Since triacylglycerol buildup in muscle is associated with insulin resistance, it is conceivable that leptin treatment

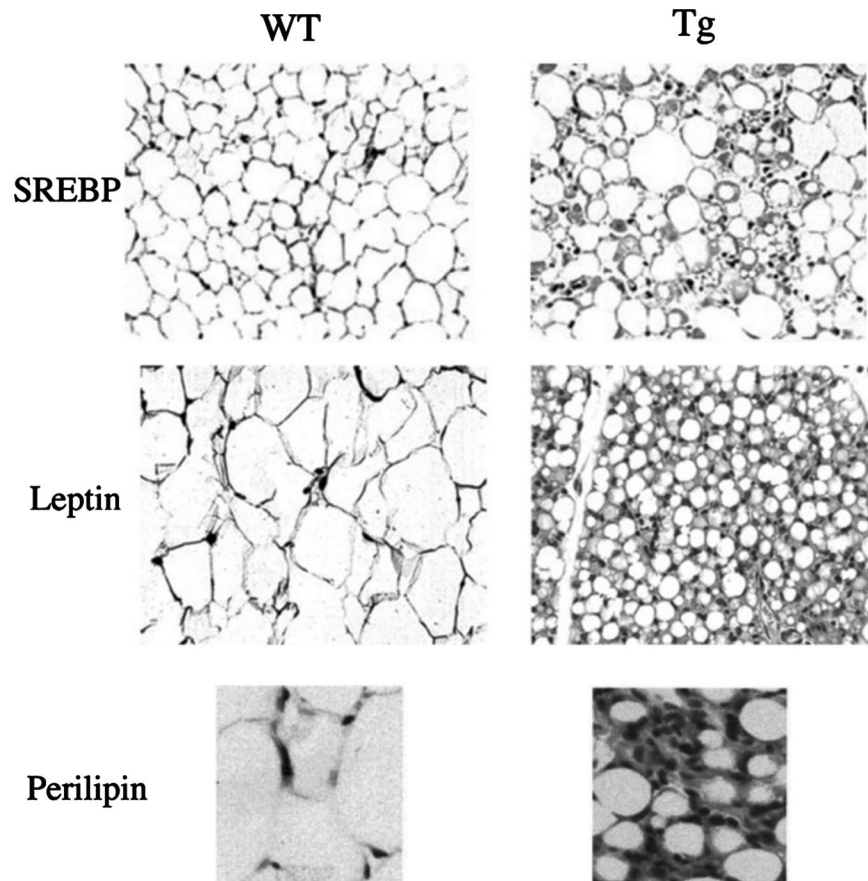


FIG. 1. Histology of white adipose tissue, stained with hematoxylin/eosin (H/E) from normal and lipodystrophic mice overexpressing either sterol regulatory element-binding protein-1c (SREBP-1c), leptin, or homozygous for a targeted deletion in perilipin. The extent of lipid accumulation and adipocyte sizes of the transgenic (Tg) mice are greatly reduced, compared to those of wild-type (WT) mice. [Reprinted with permission from Shimomura S, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS 1998 Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 12:3182–3194; Qiu J, Ogus S, Lu R, Chehab FF 2001 Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. *Endocrinology* 142:348–358. Copyright The Endocrine Society; Tamsey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, Londos C 2001 Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production and resistance to diet induced obesity. *Proc Natl Acad Sci USA* 98:6494–6499. Copyright 2001 National Academy of Sciences, U.S.A.]

might alleviate this effect by stimulating intramuscular lipolysis, thereby improving the diabetes phenotype.

V. Adaptation to Cold and Diet-induced Thermogenesis

Brown adipose tissue (BAT) is greatly affected in LepTg mice. However, in contrast to white adipose tissue, brown adipocytes appeared entirely devoid of triacylglycerol stores, at least as seen by light microscopic analysis (Qiu *et al.*, 2001b), suggesting a dysfunctional hypothalamic-BAT axis. A prominent characteristic of rodents is their adaptation to cold temperatures, a mechanism that ensures their survival in nature. The underlying mechanism for adaptive thermogenesis involves the uncoupling of oxidative phosphorylation to generate heat via increased expression and activity of mitochondrial uncoupling protein 1 (UCP-1) in BAT. Moreover, UCP-1 expression and activity can be stimulated via diet-induced thermogenesis, converting excess calories into heat (Himms-Hagen, 1984) and protecting the animal against obesity to some extent. Leptin's role in thermogenesis is evidenced by the cold intolerance of leptin-deficient and leptin receptor-deficient obese mice and, paradoxically, by leptin-overexpressing lean mice. Indeed, LepTg mice, unlike normal mice, were sensitive to a cold challenge and quickly succumbed to hypothermia if not rescued from the cold. This effect was independent of UCP-1 expression in BAT, since restoration of triacylglycerols in adipose tissue via high-fat diet feeding resulted in normal cold tolerance of the leptin transgenic mice (Qiu *et al.*, 2001b). Therefore, the absence of triacylglycerols in BAT failed to provide substrates for the ability of UCP-1 to uncouple oxidative phosphorylation. On the other end of the spectrum, obese mice deficient in either leptin or leptin signaling are also cold intolerant, despite the massive accumulation of triacylglycerols in BAT (Trayhurn and James, 1978; Trayhurn, 1979). Therefore, leptin plays a primordial role in regulating the hypothalamic-BAT axis. Any interference with its regulation, whether manifested in an excessively lean or obese state, is reflected by a perturbation of thermogenesis. This effect appears to be quantitative and subject to threshold levels of triacylglycerols. For example, unlike LepTg mice, perilipin KO mice could adapt to the cold (Figure 2), despite their reduced fat mass and significantly decreased intracellular triacylglycerols. However, when further depleted of their residual intracellular triacylglycerols by fasting, they could not withstand a cold challenge (Martinez-Botas *et al.*, 2000). Lipoatrophy of the perilipin KO mice is not as severe as that of LepTg mice, which quickly succumb to a cold challenge in the fed state. Thus, regulatory thermogenesis requires the presence of leptin and of a critical intracellular level of triacylglycerols in adipocytes, to elicit the induction of UCP-1 expression and activity, thereby ensuring survival of the animal at low temperatures.

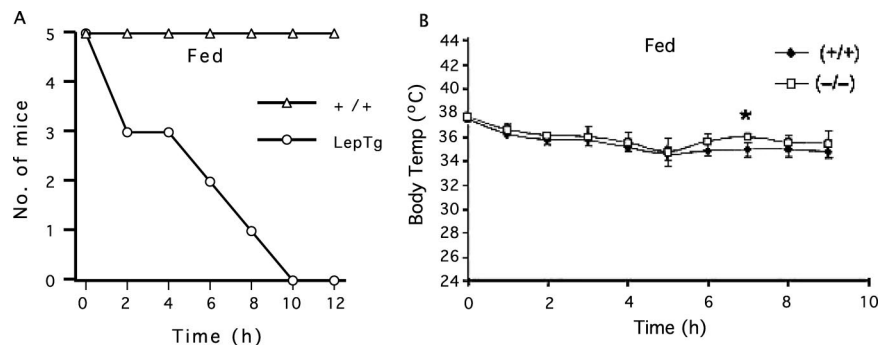


FIG. 2. Differential effects of thermogenesis in lipodystrophy in *ad libitum*-fed LepTg and perilipin knockout (KO) mice. (A) Number of LepTg mice (○) that did not tolerate the cold, resulting in a significant drop of their body temperature to 17.4°C–25°C, compared to the stable body temperature of normal mice (△). (B) Perilipin KO (–/–) were insensitive to the cold and adjusted appropriately, similar to WT controls (+/+). [(B) Reprinted with permission from Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, Chan L 2000 Absence of perilipin results in leanness and reverse obesity in *Lepr* (*db/db*) mice. *Nature Genet* 26:474–479. Copyright Nature Publishing Group.]

The mechanism by which leptin induces UCP-1 is mainly through the sympathetic nervous system (SNS) (Collins *et al.*, 1996), since central administrations of leptin resulted in increased UCP-1 expression. In addition, the MC4R, a downstream target of leptin, appears to mediate this effect because the leptin-mediated induction of UCP-1 expression could be blocked with SHU9119, a potent antagonist of alpha-melanocyte stimulating hormone (α -MSH) (Satoh *et al.*, 1998). Since α -MSH acts as an agonist at the MC4R, one would expect that MC4R KO mice would not tolerate the cold. However, this is not the case. Adult — and therefore obese — MC4R null animals have normal body temperature and tolerate cold well (Ste Marie *et al.*, 2000), suggesting that additional α -MSH-mediated mechanisms are involved in the SNS response to cold.

VI. Glucose Homeostasis and Insulin Sensitivity

A hallmark of leptin treatment of *ob/ob* mice was their improved insulin sensitivity and amelioration of their hyperglycemia independently of effects on food intake (Halaas *et al.*, 1995). Thus, the link between insulin sensitivity, glucose metabolism, and leptin became the focus of intense investigation. Although circulating glucose levels in LepTg mice ranged from normal (Ogawa *et al.*, 1999) to low (Qiu *et al.*, 2001b), plasma levels of insulin, FFAs, and triglycerides were depressed in both transgenic lines. The antidiabetic properties of leptin also were demonstrated in lipoatrophic diabetic mice treated with leptin,

either exogenously, as with the aP2-nSREBP1c transgenic mice (Shimomura *et al.*, 1998), or by crossing the LepTg mice with the A-ZIP-F1 KO mice (Ebihara *et al.*, 2001) that are lipoatrophic and diabetic (Moitra *et al.*, 1998). Although the mechanisms underlying this leptin-mediated improvement in insulin sensitivity remain to be determined, it is likely that the steatosis associated with lipodystrophy could be ameliorated by leptin. This makes it as a prime candidate for the treatment of lipodystrophies such as in the Berardinelli-Seip syndrome (Berardinelli, 1954; Seip and Trygstad, 1963; Savage and O'Rahilly, 2002).

VII. Reproduction

While the role of leptin in energy homeostasis was the center of investigation, due mainly to the morbid obesity of leptin-deficient *ob/ob* mice, their infertility had not caught up with the excitement of leptin's discovery, even though it had been investigated in parabiosis experiments (Lane, 1959) prior to those by Coleman (Coleman and Hummel, 1969). The finding that leptin rescued the sterility of *ob/ob* mice (Chehab *et al.*, 1996) and advanced the puberty of normal mice (Ahima *et al.*, 1997; Chehab *et al.*, 1997; Yura *et al.*, 2000) carved a place for it in reproductive biology.

It is often assumed — erroneously — that obesity is always associated with hypogonadism and infertility. While this may be correct for the Prader-Willi syndrome (Hamilton *et al.*, 1972) and the rare occurrences of leptin and leptin receptor mutations (Montague *et al.*, 1997; Clement *et al.*, 1998), only 60% of obese patients with the Bardet-Biedl syndrome exhibit hypogonadism (Jones, 1988). Association studies between nonsyndromic obesity and hypogonadism remain scarce and worthy of investigation.

In animal models, obesity caused by leptin or leptin receptor mutations results in infertility, whereas KO mice at the MC4-R (which is downstream of leptin) are obese and fertile. So what is the role of leptin in reproduction and what is its mechanism of action on the reproductive system? Exogenous leptin treatment of normal mice advanced vaginal opening (Figure 3) by a few days (Ahima *et al.*, 1997; Chehab *et al.*, 1997). However, in *ad libitum*-fed rats, leptin did not significantly alter the normal course of puberty, although it was able to partially reverse the delay in sexual maturation caused by reduced feeding (Cheung *et al.*, 1997). Studies have shown that intracerebroventricular (ICV) injections of leptin induce prepubertal secretion of luteinizing hormone (LH) (Dearth *et al.*, 2000), whereas leptin antibodies inhibit the leptin-mediated stimulation of LH release (Carro *et al.*, 1997). Transgenic mice overexpressing 12-fold excess leptin showed early vaginal opening (Yura *et al.*, 2000) but those with 8-fold overexpression did not (F.F. Chehab, unpublished observations). Thus, for leptin to significantly impact the onset of puberty and the firing of the reproductive system, elevated leptin levels analogous to the prepubertal leptin

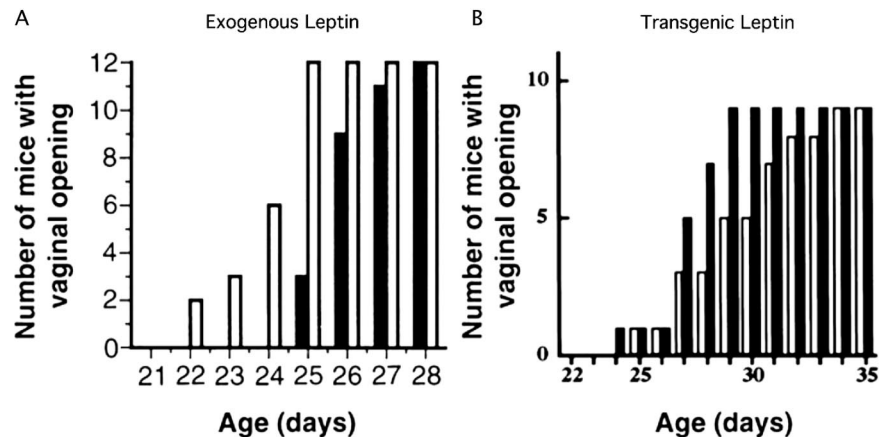


FIG. 3. Stimulation of puberty by exogenous leptin treatment or transgenic hyperleptinemia. Leptin treatment of prepubertal normal mice resulted in earlier vaginal opening (A), similar to LepTg mice (B), demonstrating a stimulatory effect on the reproductive system. [(A) Reprinted with permission from Chehab FF, Mounzih K, Lu R, Lim ME 1997 Early onset of reproductive function in normal female mice treated with leptin. *Science* 275:88–90. Copyright The American Association for the Advancement of Science; (B) from Yura S, Ogawa Y, Sagawa N, Masuzaki H, Itoh H, Ebihara K, Aizawa-Abe M, Fujii S, Nakao K 2000 Accelerated puberty and late-onset hypothalamic hypogonadism in female transgenic skinny mice overexpressing leptin. *J Clin Invest* 105:749–755.]

spike found in mice (Ahima *et al.*, 1998) and rats (Nagatani *et al.*, 2000) may be required. In the former case, this preceded the appearance of estradiol but coincided with that of insulin (Figure 4), suggesting an indirect stimulation of estradiol secretion and a concomitant action with insulin. Furthermore, the presence of a transgene overexpressing small amounts of leptin onto the *ob/ob* background rescued only the infertility of the phenotype, without affecting obesity (Ioffe *et al.*, 1998), demonstrating the exquisite sensitivity of the reproductive system to leptin. Similarly, human studies have shown that leptin-deficient patients who fail to enter puberty naturally progress through puberty with leptin treatment (Farooqi *et al.*, 1999). Paradoxically, in rhesus monkeys, associations between prepubertal leptin rise and the onset of puberty were lacking in one study (Plant and Durrant, 1997) but were evident in another using a different leptin-sampling paradigm (Suter *et al.*, 2000). Nevertheless, exogenous leptin treatment of rhesus monkeys did not appear to elicit precocious puberty, which could be attributed to the fact that the monkeys had developed leptin antibodies (Barker-Gibb *et al.*, 2002).

The role of leptin as a conveyor of energy stores to the reproductive system is logical. While studies have concentrated on the fact that leptin may perform this function single-handedly, the complexity of the mammalian system and the

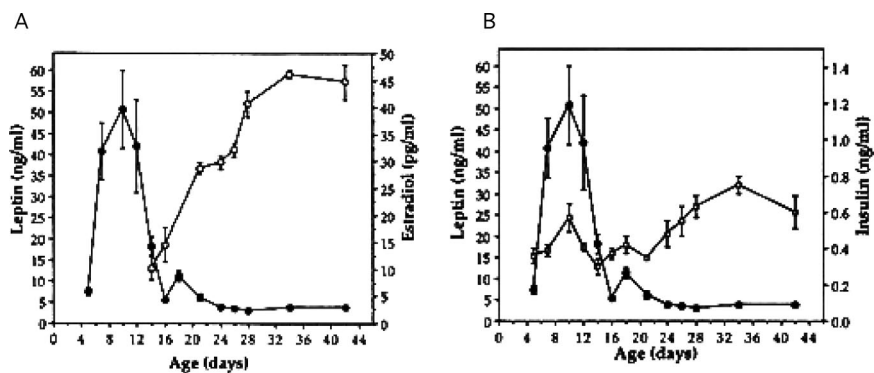


FIG. 4. Circulatory levels of leptin, estradiol, and insulin in neonatal female mice. (A) The leptin spike at 10 days of age preceding that of estradiol is consistent with a stimulatory role of leptin on the reproductive axis around the time of puberty. (B) The coincident rise in leptin and insulin at 10 days of age suggests that leptin and insulin may act together to stimulate the reproductive system. [Reprinted with permission from Ahima RS, Prakashakaran D, Flier JS 1998 Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. *J Clin Invest* 101:1020–1027.]

redundancy of supportive mechanisms ensuring the survival of the reproductive system — and therefore of the species — suggest that leptin might recruit other factors (e.g., insulin, IGF-1) to stimulate the release of gonadotropin-releasing hormone (GnRH) and open the gate of the reproductive system. Variations in the expression of leptin's cofactors at critical times during the progression to puberty may be detrimental to the reproductive system. For example, disruption of insulin signaling in the brain has revealed disturbances of the reproductive system (Burks *et al.*, 2000). ICV injections of IGF-1 have been known for some time to induce early puberty (Hiney *et al.*, 1991,1996). Thus, the actions of leptin, insulin, and IGF-1 in the brain could constitute a driving force aimed at the stimulation and firing of the reproductive system.

A mechanism of action of leptin on the reproductive system remains elusive. However, two critical components of the leptin energy homeostasis pathway have been ruled out as playing a role in reproduction and, in fact, have dissociated the obesity from the sterility phenotypes. As previously stated, MC4R KO mice are obese but fertile, ruling out that the MC4R and its downstream targets are critical for reproduction. Thus, bifurcation from the leptin-energy homeostasis pathway could occur at the MC4-R or upstream from it. Furthermore, the role of orexigenic NPY appears to be central for the leptin-reproductive pathway. NPY inhibits reproductive function in male and female rats (Clark *et al.*, 1985). Its overexpression in obese-sterile, leptin-deficient *ob/ob* mice is reduced by leptin treatment (Stephens *et al.*, 1995), which is accompanied by a rescuing of the sterility phenotype (Chehab *et al.*, 1996).

NPY's inhibitory effect on reproductive function appears to be mediated by the Y4 receptor, since *ob/ob* mice homozygous for an Y4 receptor deletion remain obese but are fertile (Sainsbury *et al.*, 2002). The exclusion of a major leptin signaling event in the hypothalamus stemmed from the finding that mutation of the critical tyrosine 1138 phosphorylation site in the long form of the leptin receptor, largely responsible for signal transducer and activator of transcription (STAT)-3 activation, resulted in an obese but fertile mouse (Bates *et al.*, 2003). Thus, the pathway elicited by tyrosine 1138 phosphorylation is not critical for reproduction. These observations suggest that the leptin-reproductive pathway bifurcates from the leptin-energy homeostasis pathway prior to the MC4-R and may involve a separate signaling pathway at other leptin receptor sites. The complexity of this pathway is demonstrated by the fact that modifier genes rescued the sterility phenotype of leptin-deficient mice, demonstrating the multifactorial nature of the leptin-reproductive pathway (Ewart-Toland *et al.*, 1999; Qiu *et al.*, 2001a).

VIII. Leptin and the Melanocortin System

It has been demonstrated that a major downstream target of leptin-binding neurons is a downregulation of NPY neurons and a stimulation of MC4R neurons, which act to stimulate and inhibit food intake, respectively (Cowley *et al.*, 2001). While this topic has been extensively reviewed, the issue of adult-onset obesity, as in agouti yellow obese mice (yA), has received little attention. The molecular defect in yA mice has been elucidated and consists of a blockade at the MC4R by the agouti protein, which normally is expressed only in melanocytes, but becomes ubiquitously expressed in the agouti mouse. As a result, it competes with α -MSH binding at the MC4R (Bultman *et al.*, 1992; Miller *et al.*, 1993; Lu *et al.*, 1994). These studies led to the discovery of agouti-related protein (AgRP) (Ollmann *et al.*, 1997), which is the endogenous competitor of α -MSH. AgRP and α -MSH act together to regulate energy homeostasis (Ebihara *et al.*, 1999). Interestingly, AgRP overexpression in transgenic mice leads to obesity (Ollman *et al.*, 1997), whereas its deletion by gene targeting did not result in any obvious phenotype, even in the absence of NPY (Qian *et al.*, 2002). This suggests that other factors may regulate energy homeostasis via the MC4R, either in the presence or absence of AgRP and NPY.

The issue of adult-onset obesity is central and has been neglected. For example, in the yA mouse, despite the ubiquitous expression of agouti protein and its antagonism of melanocyte signaling and pigmentation from an early age, it is not clear why antagonism at the MC4R is delayed and not evident from a young age. To address whether leptin sensitivity is reduced from a young age in agouti mice, we crossed LepTg mice with yA mice and generated double heterozygotes for yA and the leptin transgene. The resulting mice were largely devoid of

adipose mass, similar to the transgenic mice, but showed the characteristic agouti color of y^A mice (Figure 5). The statistical differences in body weight, reflecting the lean phenotype of the double heterozygotes, did not last beyond 6 weeks in females and 8 weeks in males (Figure 5), when obesity started to become evident and the lean phenotype caused by transgenic hyperleptinemia started to wane. The color coat of the mutant mice demonstrated expression of the agouti protein from a young age; studies have shown that the MC4R also is expressed from a young age (Mountjoy and Wild, 1998). Then why is the obesity associated with the lack of neuronal MC4R signaling not manifested from a young age, as in *ob/ob* and *db/db* mice, but manifested at a later age, as in y^A and MC4R KO mice? One would have to speculate that neuronal projections downstream of the MC4R conceivably either do not develop or fail to be activated until a later age. Thus, these animal models will be valuable in elucidating pathways downstream of MC4R neurons that may develop to counteract leptin sensitivity.

IX. Leptin and the Sympathetic Nervous System

The binding of leptin to its receptor in the arcuate nucleus eventually is reflected by the adrenergic stimulation of lipolysis (Collins *et al.*, 1996) on adipocytes via beta3 adrenergic receptors (β 3AR). Deletion of the β 3AR by gene targeting did not yield to overt obesity, due to an upregulation and activation of β 1AR and β 2ARs (Susulic *et al.*, 1995). In addition, deletion of β 1, -2, and -3

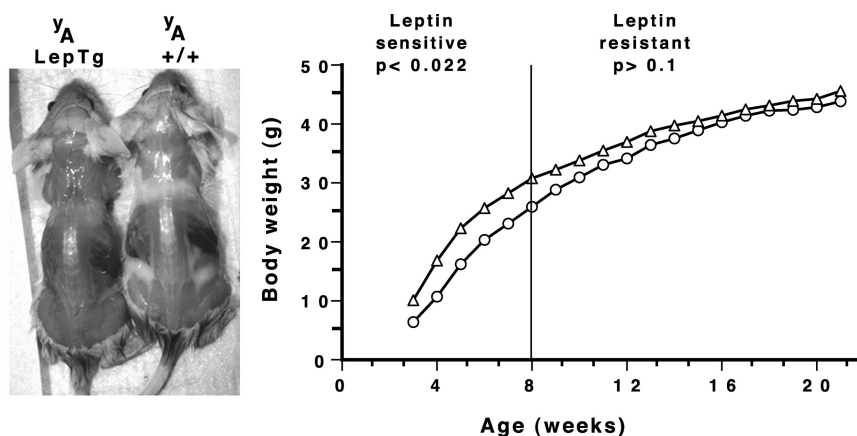


FIG. 5. Sensitivity and resistance of agouti yellow (y^A) mice to transgenic leptin. The photograph shows the lack of subcutaneous adipose mass in 6-week-old agouti LepTg males (y^A LepTg), compared to yellow agouti (y^A +/+) controls without transgenic hyperleptinemia. The growth curve shows the biphasic states of the y^A /LepTg mice (○) between 3–8 weeks of age, when their body weights are significantly less than those of y^A +/+) mice (△).

ARs yielded mice that were slightly obese on a chow diet but grossly obese on a high-fat diet (Bachman *et al.*, 2002). This suggested that β ARs are necessary to counteract diet-induced obesity (DIO). We asked whether the leptin-mediated stimulation of the SNS acting on adipocytes would be attenuated by the absence of β 3AR on adipocytes. This effect would be most noticeable in animals with no or little fat (e.g., LepTg mice). Thus, generation of LepTg mice with no β 3AR resulted in a lean phenotype similar to that of LepTg mice with β 3AR, as judged by their growth curves (Ke *et al.*, 2003) (Figure 6). This infers a normal response of adipocytes to leptin-mediated SNS activation of lipolysis. Therefore, leptin-mediated activation of the SNS is indifferent to the absence of β 3AR, which are chiefly found on adipocytes.

Another axis that has been investigated is the renal axis, with possible connections to hypertension. Leptin treatment caused a dose-dependent increase in sympathetic nerve activity to the kidney (Haynes *et al.*, 1997). This increase could be blocked by the MC4-R antagonist SHU9119, thereby involving the melanocortin system in the sympathoexcitatory effects of leptin on the kidney (Haynes *et al.*, 1999). LepTg mice consistently had elevated blood pressure, whereas the low blood pressure characteristic of *ob/ob* mice was reversed with leptin treatment (Aizawa-Abe *et al.*, 2000). However, ICV administrations of SHU9119 increased food intake in the LepTg mice but did not affect their blood pressure, suggesting that the leptin-mediated effect on blood pressure involves a

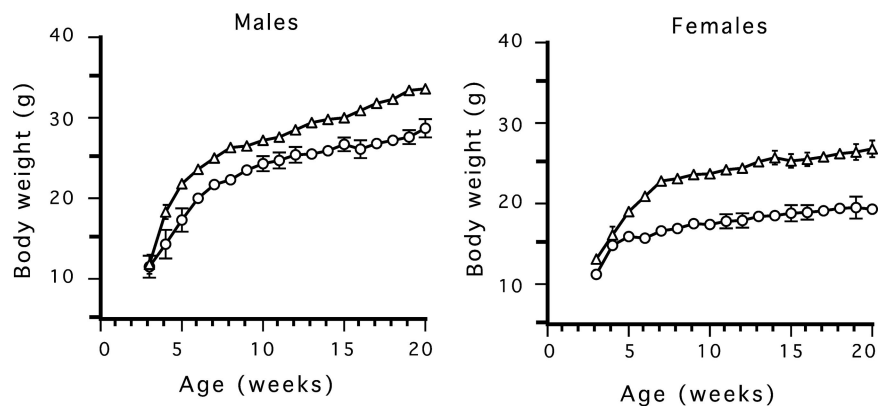


FIG. 6. Transgenic hyperleptinemia exerts its effects independently of beta3-adrenergic receptors (β 3ARs). Growth curves of β 3AR KO mice (β 3ARKO) with (O) and without (Δ) transgenic hyperleptinemia. The body weights of the LepTg mice on a null β 3AR background remain smaller than those of β 3ARKO mice without hyperleptinemia, demonstrating the lack of specificity of the leptin-mediated response to β 3ARs. [Reprinted from Ke Y, Qiu J, Ogus S, Shen WJ, Kraemer PB, Chehab FF 2003 Overexpression of leptin in transgenic mice leads to decreased basal lipolysis, PKA activity and perilipin levels. BBRC 312:1165–1170.]

different pathway than the melanocortin system. Thus, to reconcile the differences between the SHU9119 blockade of the leptin-mediated sympathetic activation of the kidney and its lack of effect on the blood pressure of the LepTg mice, one would have to assume that the two pathways are centrally divergent at the MC4-R.

X. Leptin and the Bone Axis

Increased weight bearing caused by obesity results in increased bone remodeling and decreased bone removal. This observation is significant in obese, postmenopausal women, who show diminished rates of hip fractures caused by osteoporosis. Thus, a mechanism linking obesity and osteoporosis would be of great interest in the treatment of osteoporosis in nonobese women. The observation that ICV leptin treatment of normal and leptin-deficient *ob/ob* mice resulted in bone loss led to the hypothesis that leptin participates in the modulation of bone formation via its action on the hypothalamus, acting as an inhibitor of this process (Ducy *et al.*, 2000) independently of its anorexigenic function (Takeda *et al.*, 2002). Based on an increased bone mass in leptin-deficient mice, we asked whether an excess of leptin, as found in the LepTg mice, would result in decreased bone mass. Histomorphometric studies showed that bone formation was not affected by transgenic hyperleptinemia (F.F. Chehab and M. Horowitz, unpublished observations), despite a modest and significant decrease in bone weight, which could be attributed to the smaller weight and lean phenotype of the LepTg mice. Thus, leptin deficiency strengthens bone formation, while its abundance in organisms that respond to hyperleptinemia does not appear to cause a detrimental effect on bone remodeling.

XI. Resistance to Transgenic Hyperleptinemia

It is clear from the phenotype of LepTg mice that they chronically respond to transgenic hyperleptinemia, as evidenced by reductions in food intake and fat mass, two parameters that are characteristic of a leptin response. We found that even though the LepTg mice continue to secrete transgenic leptin throughout their life, there are at least three situations that make them resistant to transgenic hyperleptinemia: aging, a high-fat diet, and genetics (Figure 7).

When maintained on a C57BL/6J/DBA heterogeneous genetic background, the LepTg mice exhibit a lean phenotype characterized by very little fat mass, which gradually builds up to approximate that of normal mice around 33–36 weeks of age (Qiu *et al.*, 2001b). On a pure C57BL/6J genetic background, the LepTg mice remain lean for a longer period of time than on a mixed genetic background but eventually also gain large amounts of fat that remain, however, significantly less than those of nontransgenic littermates. Our observation that the LepTg mice fail to remain lean over time, an effect more pronounced in males than females, is puzzling and interesting. It has been reported that aging results

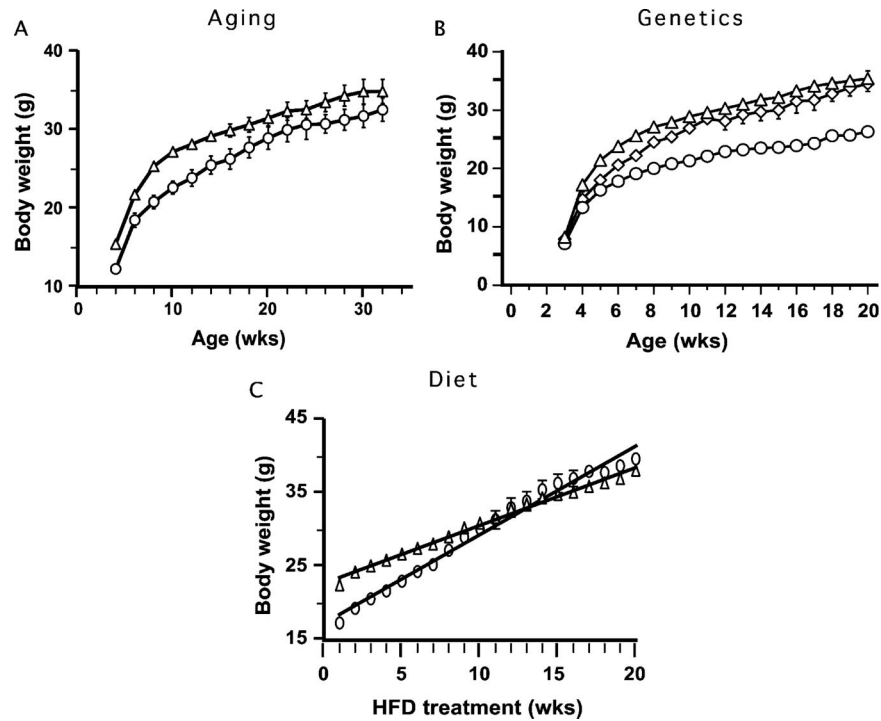


FIG. 7. Resistance to transgenic hyperleptinemia as a result of aging, genetic background, and diet-induced obesity (DIO). (A) At a young age, the body weights of LepTg mice on a mixed genetic background (○) are lower than those of normal mice (△), reflecting lower adiposity, but then start to approach the body weights of normal mice at a later age. (B) Genetic resistance to transgenic hyperleptinemia in LepTg males bred on a mixed genetic background (◇) is demonstrated by their normal body weight, compared to WT (△) and LepTg (○) mice on the same genetic background. (C) Body weights of high-fat diet-fed C57BL/6J normal (△) and LepTg (○) mice show the precipitous DIO of LepTg mice. [(A) Reprinted with permission from Qiu J, Ogus S, Lu R, Chehab FF 2001 Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. *Endocrinology* 142:348–358. Copyright The Endocrine Society; (C) Modified with permission from Ogus S, Ke Y, Qiu J, Wang B, Chehab FF 2003 Hyperleptinemia precipitates diet-induced obesity in transgenic mice overexpressing leptin. *Endocrinology* 144:2865–2869. Copyright The Endocrine Society.]

in a leptin resistance state (Scarpace *et al.*, 2000a,b). Thus, it would make sense to assume the same for the LepTg mice. Whether the effects of functional transgenic hyperleptinemia become blunted with aging or whether a mechanism that supercedes the transgenic leptin effect becomes established with age remains to be determined. However, our studies tend to favor the latter hypothesis because transgenic mice that have accumulated adipose mass with age remain sensitive to peripheral administrations of large doses of leptin (Qiu *et al.*, 2001b).

An unexpected finding underlying the loss of effect associated with functional transgenic hyperleptinemia was a high-fat diet. Since the young LepTg mice are sensitive to transgenic hyperleptinemia — as indicated by a reduction in food intake and adipose tissue mass — we reasoned that they actually would be resistant or show at least an attenuated obesity on a high-fat diet treatment. This was clearly not the case when they were fed a diet consisting of 42% milk fat, as they manifested a more-precipitous obesity than that of normal mice fed the same diet (Ogus *et al.*, 2003). This interesting finding raises a number of issues as to the cause of their resistance to transgenic hyperleptinemia. In the absence of significant differences in high-fat food intake, one should look for differences in energy expenditure, metabolism, or diet composition. It is possible that the levels of hyperleptinemia were not sufficient to counteract the rapid buildup of adipose mass, indicating that the ratio of leptin to fat accumulation would be the culprit of the resulting phenotype. Moreover, the composition of the high-fat diet, which consisted of 61% saturated and 39% unsaturated fats, may be critical. Thus, feeding the LepTg mice different amounts and types of fat might provide clues about the interactions between their hyperleptinemia and their diet, possibly extending the resulting observations to the broader problem of hyperleptinemia in DIO in animals and humans.

Another interesting finding in our LepTg mice is the fact that some on the mixed genetic background exhibit a transgenic hyperleptinemia that is not associated with a lean phenotype, even from a young age (Figure 7). When backcrossed to the C57BL/6J genetic background for 6–10 generations, this effect is lost, pointing to a powerful effect of modifier genes from the C57BL/6J and DBA/2J genetic backgrounds. Backcrossing the LepTg mice to the DBA/2J background revealed that, at the N5 generation, the LepTg mice remain lean, similar to C57BL/6J LepTg mice. Thus, genetic leptin resistance must have arisen from a polygenic interaction of the two genomes. Furthermore, crosses of genetically resistant LepTg mice did not result in recapitulation of the phenotype according to Mendel's laws for single-gene disorders. Thus, it is unlikely that dominant or recessive genes accounted for this early leptin resistance phenotype. Instead, it suggests that modifier genes had accumulated in these mice but that genetic crosses allowed their segregation. Although these findings reinforce the effect of genetics on the phenotype of leptin resistance, along with the data mentioned earlier, they pinpoint what has commonly been suspected: that complex genetics account for the common DIO phenotype.

XII. Perspectives on Mechanisms of Leptin Resistance

Leptin resistance refers to a state of elevated leptin levels without a lean phenotype but, paradoxically, with an obese state. It has been suggested that the hypothalamus may be insensitive to elevated leptin levels but would, on the

contrary, sense low leptin levels, such as in fasting states. This would activate a survival response that would conserve energy resources by shutting down, for example, the reproductive system. Remarkably, the response to fasting can be rescued by leptin injections, demonstrating that leptin mediates the starvation-induced response (Ahima *et al.*, 1996).

What distinguishes LepTg mice with elevated leptin levels and a clear response to hyperleptinemia from hyperleptinemia in common obesity remains for the most part unknown. It is unlikely, but still not completely ruled out, that the primary defect could lie in the saturation transport of leptin to the hypothalamus. There appears to be enough leeway to allow large quantities of leptin (such as in LepTg mice) to elicit a physiological response (Ogawa *et al.*, 1999; Qiu *et al.*, 2001b). The culprit may, however, be in the diet, which could block leptin's access to the hypothalamus or its central action. It has been shown that prolonged, high-fat diet feeding of normal mice fails and reduces STAT-3 activation in the hypothalamus following peripheral and central administrations of leptin, respectively (El-Haschimi *et al.*, 2000). The contribution of the diet and its mechanisms of action to the establishment of a leptin-resistant state in LepTg mice will be invaluable in deciphering pathways that result in dysfunctional hyperleptinemia. One possibility is that components of a high-fat diet might alter the permeability of leptin to the brain and reduce leptin signaling in the hypothalamus.

XIII. Evolutionary Significance of Hyperleptinemia

The possibility that resistance to hyperleptinemia might have evolved as a protective mechanism to ensure the survival of an organism is a worthwhile hypothesis. As previously noted, LepTg mice are cold sensitive and succumb rapidly to a low temperature. Should a similar situation arise in wild habitats, such mice would be quickly eliminated by natural selection, unless they are capable of developing leptin resistance. This is particularly significant for hibernating animals, which stock up on energy stores prior to hibernation, increasing their adiposity and leptin levels. Should they fail to develop leptin resistance, the process of adipose mass deposition would fail. Another example is pregnancy. We have shown that leptin resistance is established in midpregnancy in normal mice (Mounzih *et al.*, 1998) as a mechanism to increase energy intake to build up enough adipose tissue in anticipation of the large energy requirements of lactation. If the mother fails to build up adipose tissue, lactation and survival of the pups would be seriously jeopardized. Therefore, the need to develop a resistance to hyperleptinemia is teologically warranted and would protect the organism against the detrimental effects of functional hyperleptinemia to ensure its survival. Thus, although leptin resistance in pregnancy makes sense and is essential, it occasionally extends beyond gestation, resulting in postpartum

weight retention. This again demonstrates a close interaction between the reproductive system, leptin signaling, and obesity.

XIV. Concluding Remarks

Much remains to be learned about the biology of leptin and its direct and indirect effects on various systems. The use of leptin-deficient, leptin-resistant, and leptin-overexpressing mice has demonstrated the detrimental consequences of leptin resistance and sensitivity, albeit in different ways. Animal models will continue to unravel the biology of leptin and are destined to unlock leptin resistance and provide a treatment for obesity. However, timed leptin resistance, as in pregnancy, is essential and would need to be maintained to ensure the survival of the next generation.

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