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Current Pharmaceutical Design, 2014, *20*, 000-000

Novel Superactive Leptin Antagonists and their Potential Therapeutic Applications

Arieh Gertler[1,]* and Eran Elinav[2,]*

[1]The Hebrew University, The Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot 76100, Israel; [2]Weizmann Institute of Science, Immunology Department; Rehovot 76100, Israel

Abstract: Random mutagenesis of mouse leptin antagonist (L39A/D40A/F41) followed by selection of high-affinity mutants by yeast-surface display indicated that replacing residue D23 with a non-negatively charged amino acid (most specifically with Leu) leads to dramatically enhanced affinity of leptin toward LEPR leading to development of superactive mouse, human, ovine and rat leptin antagonists (D23L/L39A/D40A/F41A). Superactive leptin antagonist mutants of mouse, human, rat or ovine leptins were developed in our laboratory, expressed in *E. coli,* refolded and purified to homogeneity as monomeric proteins. Pegylation of leptin antagonists resulted in potent and effective long-acting reagents suitable for *in vivo* studies or therapies. In the present review we explain the mechanism of leptin inhibition and summarize the possible use of leptin antagonists as possible leptin blockers in various human pathologies such as anti-inflammatory and anti-autoimmune diseases, uremic cachexia, and cancer. We also suggest the use of leptin antagonists as research reagents for creation of a novel, fast and reversible model of T2DM in mice.

Keywords: Leptin, antagonist, inflammatory disease, anti-autoimmune diseases, cancer, uremic cachexia, T2DM model.

DEVELOPMENT OF LEPTIN ANTAGONISTS

Though leptin and leptin receptor (LEPR) were cloned almost 20 years ago [1, 2] and leptin's 3D structure has been resolved [3], the mechanism of leptin:LEPR interaction and activation has not yet been elucidated. Nevertheless, several putative models have been proposed [4]. The extracellular domain (ECD) of LEPR, as depicted in (Fig. **1**), consists of several subdomains termed (from the N-terminus): cytokine homology region 1 (CHR 1), followed by an immunoglobulin-like domain (IGD), then by another CHR 2 and finally by two consecutive F3 domains. Fong and co-workers [5] localized the leptin-binding domain to the membrane-proximal CHR 2 (~ 200 amino acids) in the LEPR-ECD. This domain from human [6] and chicken LEPRs [7] was subcloned in Gertler's laboratory and expressed as a recombinant protein which showed a 1:1 molar interaction with leptin. A decade ago, Tavernier and his group [8] revealed that leptin binding to its receptor resembles the interaction between interleukin 6 (IL6) and IL6 receptor [9-11], and they formulated the existence of the putative leptin site III as a major site responsible for the formation of active 2:2 or 2:4 leptin:LEPR complex. The IGD located between the distal and proximal CK-F3 domains was clearly documented to be essential for productive dimerization (or even tetramerization) of the LEPR, as its removal attenuated activation, but not binding, of the ligand [8]. Schematic illustration of 2:2 leptin:LEPR complex showing interaction of two leptin molecules, each with the CHR 1 of one receptor (through binding site 1/2) and with the IGD of the second receptor (through binding site 3) is shown in (Fig. **1**). This model was further substantiated by the extensive mutagenesis of mouse and human leptins which led to the identification of Ser 120 and Thr 121, located in the N-terminal part of helix D, as contributors to site III [12]. Mutation of these amino acids to Ala led to the formation of leptin antagonist. In addition to the N-terminal part of helix D, we found that other parts of the leptin molecule also contribute to the interaction between leptin and LEPR's IGD. Carefully considering the known structures of IL6-receptor complexes [viral (v) IL6/gp130] [9] and IL6/IL6Rα/gp130 complex [10] in which site III was first identified, we examined the interface between the tips of the vIL6 bundle, comprising an A-B loop and the beginning of helix D, and the edge of one of the IGD β-sheets, involving strands F and G. We further analyzed the corresponding A-B loop in leptin and though its sequence differed greatly in length and in composition compared to IL6, we were able to identify, thanks to the use of the sensitive bidimensional Hydrophobic Cluster Analysis [13], a common short β-strand which interacts in the vIL6/gp130 complex (the only complex for which an ordered structure has been observed in this region) with a short β-strand in the receptor IGD. This strand was located before the first strand of the IGD core and is predicted to also be present in LEPR IGD. To verify this hypothesis and to test its generality, we prepared and purified to homogeneity several ovine and human recombinant leptin Ala muteins in the A-B loop (L39A/D40A, L39A/D40A/F41A or L39A/D40A/F41A/I41A) and documented their activity as potent competitive LEPR antagonists [14]. To verify the preservation and importance of this sequence for activation of LEPRs, we also prepared the corresponding muteins of mouse and rat leptin and documented their antagonistic activity [15]. In subsequent work, we increased the half life of the leptin antagonist by pegylation, resulting in significant extension of its bioavailability while retaining antagonistic activity [16]. In mice, administration of the pegylated antagonist produced rapid and dramatic weight gain, due to enhanced appetite and increased food consumption. The resulting fat was confined to the mesenteric region with no accumulation in the liver. Remarkably, weight changes were reversible upon cessation of leptin-antagonist treatment. The mechanism of severe central leptin deficiency was found to involve inhibition of leptin transport across the blood-brain barrier, as well as accumulation of pegylated antagonist within the central nervous system and direct inhibition of LEPRs in the hypothalamic region.

In view of the potential pharmaceutical uses of leptin antagonists, the general question of how the biopotency of recombinant proteins can be enhanced *in vivo* needs to be explored. One possible approach is to increase the antagonist's affinity for the receptor by increasing k_{on}, or mainly by decreasing k_{off}, and thus prolonging receptor occupation. Theoretical thermodynamic considerations show that if antagonists and agonists exhibit the same affinity, at a 100-fold molar excess of antagonist, 99% of all occupied receptors will be occupied by antagonists. A 100-fold increase in antagonist affinity will lead to similar results at an approximate 1:1 molar

*Address correspondence to these authors at The Hebrew University, The Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot 76100, Israel; Tel:/Fax: +972 8 948 9006; E-mails: gertler@agri.huji.ac.il; eran.elinav@weizmann.ac.il
Immunology department, Weizmann Institute of Science, Rehovot 76100 Israel, Tel: +972 8 9344014

1381-6128/14 $58.00+.00 © 2014 Bentham Science Publishers

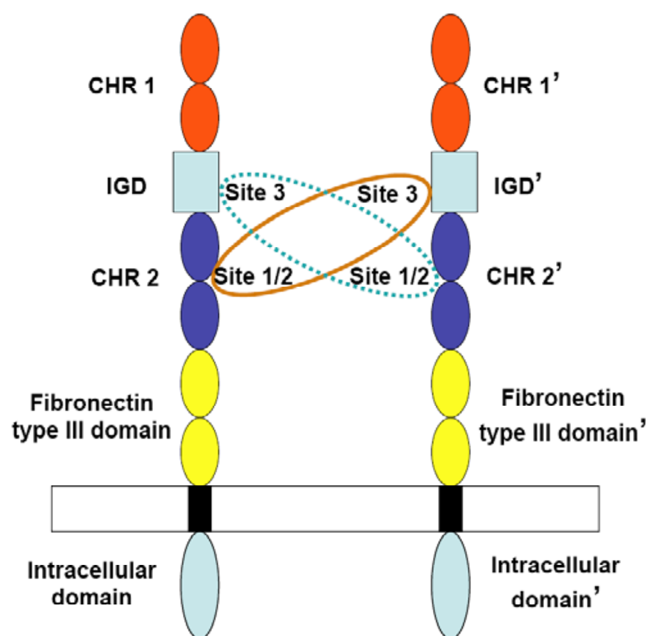

Fig. (1). Schematic illustration of the interaction of 2 leptin molecules with the extracellular domains of 2 leptin receptors. The first leptin molecule (designed as a brown-solid line oval) interacts through its 1/2 binding site with the cytokine homology domain 2 (CHR 2) of the left receptor and subsequently through its binding site 3 with the immunoglobulin-like domain 2' (IGD') of the second receptor. In parallel the second leptin molecule (designed as a green-pointed line oval) interacts through its 1/2 binding site with the CHR 2' of the right receptor and subsequently through its binding site 3 with the (IGD) of the left receptor. There is no known interaction between the two leptins. Mutations L39A/D40A/F41A abolish the interaction of leptins with the IGDs, preventing receptor activation but not the binding to CHR2 domains and just acts as antagonist. Mutation D23L increases the affinity of leptin (or leptin antagonist) toward CHR-2.

antagonist:agonist ratio. Pegylation of such muteins combines both approaches, resulting in a potent and effective long-acting competitive antagonist *in vivo*. To explore this approach, we employed random mutagenesis of leptin followed by selection of high-affinity mutants by yeast-surface display, and discovered that replacing residue D23 with a non-negatively charged amino acid leads to dramatically enhanced affinity of leptin for LEPR. Rational mutagenesis of D23 revealed the D23L substitution to be the most effective. Coupling the D23 mutation with Ala mutagenesis of three amino acids (L39A/D40A/F41A) previously reported to convert leptin into antagonist [14, 15] resulted in potent antagonistic activity [17]. Those novel superactive mouse and human leptin antagonists (D23L/L39A/D40A/F41A) termed, respectively, SMLA and SHLA, exhibited over 60-fold increased binding to LEPR and 14-fold higher antagonistic activity *in vitro* relative to the L39A/D40A/F41A mutants. To prolong and enhance the *in-vivo* activity, SMLA and SHLA were monopegylated mainly at the N terminus. Administration of the pegylated SMLA to mice resulted in a remarkably rapid, significant and reversible 27-fold more potent increase in body weight (as compared to pegylated L39A/ D40A/ F41A mutein) due to increased food consumption. To test whether the D23L mutations confer increased affinity in other leptin species, we have recently prepared D23L/L39A/D40A/F41A muteins of ovine [18] and rat leptins [L. Niv-Spector and A. Gertler, unpublished results] and found that they also act as potent leptin antagonists.

Thus recognition and mutagenesis of D23 enabled construction of novel compounds that induce potent and reversible central and peripheral leptin deficiency. In addition to enhancing our understanding of leptin interactions with its receptor, these antagonists have enabled *in-vivo* study of leptin's role in metabolic and immune processes, and hold potential for future therapeutic use in disease pathologies involving leptin.

LEPTIN ANTAGONISTS AS POTENTIAL ANTI-INFLAMMATORY AND ANTI-AUTOIMMUNE DISEASE TREATMENTS

For years, white adipose tissue has been regarded as an inert organ, with the exclusive function of long-term energy storage. With the cloning of leptin in 1994, that notion has drastically changed [1]. In subsequent years, a plethora of tissue-specific and nonspecific adipose-secreted factors were discovered and characterized and found to play fundamental roles in multiple aspects of metabolic processes, some of which take place in distant organs. Moreover, white adipose tissue has been found to dynamically integrate metabolic and immune signals through complex interactions between adipocytes and a complex network of innate and adaptive immune cells, including neutrophils, and mononuclear T and B cell subsets. Through this complex interaction it was suggested that immune components fundamentally affect metabolic processes such as insulin sensitivity, and conversely, adipocyte-secreted factors affect the innate and adaptive immune response through modulation of tissue-resident cells of hematopoietic origin [19 20].

Leptin, has been most widely studied for its effects on the immune response. Leptin secretion is inducible upon signaling from inflammatory mediators such as tumor necrosis factor alpha (TNF-α), IL1, and IL6. Indeed leptin levels have been shown to increase during infection and chronic inflammation [21]. Like leptin itself, LEPRs resemble cytokine receptors [22] and the long functional isoform of LEPR is found on T-cell subsets, monocytes [23], natural-killer (NK) lymphocytes [24], dendritic cells [25], hepatic stellate cells [26] and a variety of bone marrow progenitor cells. Functionally, leptin signaling promotes macrophage proliferation, phagocytosis and cytokine secretion [27], NK cell development, activation and survival [28], MAPK-mediated neutrophil chemotaxis [29], T-cell proliferation and IL2 secretion, especially when co-administered with classical T-cell mitogens [30]. Leptin has also been suggested to suppress regulatory T-cell proliferation and inflammation suppressive functions, further contributing to a general pro-inflammatory effect [31].

In vivo, leptin-deficient *ob/ob* mice are extremely vulnerable to the development of systemic infection and lipopolysaccharide (LPS)-induced organ damage, and resistant to several Th1 cells-mediated immune disorders, including experimental allergic encephalomyelitis, concanavalin A hepatitis, experimental arthritis, and autoimmune nephritis [32-34]. Leptin replenishment reverses these disorders [35]. These mice feature reduced cell density in their bone marrow and thymus, highlighting the importance of leptin signaling in the development of various subsets of the hematopoietic system. Similarly, humans with inherited or starvation-induced leptin deficiency feature thymic atrophy and severe functional alterations in the innate and adaptive immune response, rendering them susceptible to bacterial infection [36, 37].

In humans, the causative correlation between leptin levels and the propensity and severity of autoimmune disease remains controversial. Perhaps the strongest association between leptin levels and autoimmunity has been found in multiple sclerosis (MS), a T-cell-mediated autoimmune disorder of the central nervous system; *ob/ob* mice are protected from EAE, the most common small animal model of MS, possibly through leptin-deficiency-induced expansion of nTregs cells [38, 39]. In humans, leptin levels have been suggested to correlate with disease activity and to decrease following successful treatment with interferon beta (INF-β) [40]. In other systemic autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus, leptin levels have been suggested in some, but not all, reports to be elevated independently of gender or

weight and to possibly correlate with disease severity [41-46]. Interestingly, one study suggested that acute fasting promotes improvement in multiple immune parameters in rheumatoid arthritis patients, an effect that may be linked to the reduced leptin levels in these patients [47]. Similarly, leptin was suggested to contribute to the pathogenesis of diabetes mellitus in mice, while a mutation in the LEPR of mice with a NOD background ameliorated diabetes severity in these mice [48, 49]. Yet another example of leptin's effect on the immune response was recently demonstrated by our groups [50], whereby administration of competitive leptin antagonists induced significant amelioration in a model of chronic liver inflammation and fibrosis.

A role for leptin was suggested in the pathogenesis of intestinal autoinflammation. Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting 0.3% of the Western population; its pathogenesis is thought to result from loss of tolerance of the intestinal immune system in the presence of constant antigenic stimuli mediated by resident microflora. In recent years, mounting evidence has suggested that IBD pathogenesis is closely related to a combination of abnormal challenges by normal gut microflora, coupled with inherent or acquired aberrations in the intestinal innate immune response. Leptin's central role as a mediator of colonic autoinflammation has been suggested in both animal models and human studies. Leptin-deficient *ob/ob* mice are resistant to acute and chronic experimental colitis [51, 52]. In those studies, leptin was suggested to augment pro-inflammatory cytokine secretion, including IFN-γ, TNF-α, IL18, IL1b, IL6 and IL10, and to inhibit inflammation-associated apoptosis of colonic mononuclear cells. Inflamed colonic epithelial cells were also found to express and release leptin apically into the intestinal lumen. Intrarectal administration of leptin induced activation of NF-κB and epithelial wall damage associated with neutrophil infiltration [53]. The source of local and/or systemic leptin has not been elucidated, but it has been suggested not to be the lamina propria T cells themselves. Similarly, the leptin-responsive cells have not yet been identified. Collateral evidence for a possible pro-inflammatory interaction between leptin and lamina propria dendritic cells (lpDCs) comes from studies showing that LEPRb is expressed on immature and mature human DCs, and that leptin induces STAT3 and NF-κB activation, production of IL1, IL6, IL12, TNF-α and MIP-1α, an anti-apoptotic effect through Bcl-2 and Bcl-XL, downregulation of IL10, polarization into Th-1, and increased CD40 expression [54, 55]. In human IBD patients, leptin levels have been suggested to correlate with the severity of the disease [56-58].

Taken together, a large body of evidence suggests an association between leptin levels and responsiveness in terms of propensity for autoimmunity, autoinflammation and infection. While much of the current data, particularly in humans, is observational and associative, they suggest that leptin may play direct or indirect roles in the modulation and regulation of innate and adoptive immune responses and that excessive leptin signaling might result in a deleterious tendency toward autoinflammation in susceptible populations. As such, inhibition of leptin signaling, as has been recently suggested by Gertler's and Elinav's groups [16, 17, 50], may offer a unique therapeutic modality targeting leptin signaling in the autoinflammatory setup.

LEPTIN ANTAGONISTS AS ANTICANCER AGENTS

The epidemiological evidence linking obesity and breast, cervical, colon, rectal, esophageal, gall bladder, kidney, liver, ovarian, pancreatic, stomach and uterine cancers is well-established [59-64]; the high level of leptin in obese subjects thus raises the notion of a possible link between leptin and cancer. Leptin is an anti-apoptotic molecule in many cell types and its possible role in obesity-linked cancers originates from its known pro-angiogenic, pro-inflammatory and mitogenic activities. Furthermore, leptin was defined as a growth factor not only because of its proliferative activity but also due to its effect on cell motility and migration [65]. Leptin is overexpressed in at least 80% of cases of human skin melanoma [66], breast [67], ovarian [68] and prostate [69] cancers. In most of those cases, LEPR is overexpressed as well [70]. Interestingly, cancer stem cells, and particularly breast cancer stem cells, also overexpress LEPR, thus sensitizing those cells to leptin action [71]. In one such cell type, termed triple-negative breast cancer cell, leptin attenuated the inhibitory effect of cisplatin on cell proliferation and viability [72].

Leptin-induced modulation of colorectal cancer (CRC) has been reported in several *in-vitro* and *in-vivo* studies (for most recent report see ref. [79]). Leptin's stimulation of proliferation and inhibition of apoptosis have been shown in several human epithelial colon cancer cells, such as HT-29, CACO-2, DDL-1, SW480, HCT116, LS174-T and Lovo [73-75]. However the *in vivo* role of leptin in CRC remains unclear. In spontaneous CRC models, such as in ApcMin/+ mice, leptin did not increase and even decreased intestinal tumorigenesis in ApcMin/+ mice, a mutation that predisposes the animal to tumor development in the intestine and colon [74, 76]. In contrast, inflammation-induced CRC induced by Dextrane Sodium Sulphate - axoxymethane was attenuated in *ob/ob* and *db/db* mice as compared to wild-type mice [77]. Note that leptin's effect may be indirect as leptin is known to induce inflammatory cytokines in colonic tissue that are implicated in colon carcinogenesis, such as IL6, IL1β and CXC [78]. Likewise the impact of leptin on colon cancer in humans remains unclear. While some reports show increased leptin expression with progressing tumorigenesis, other have failed to confirm such observations [79]. However, it should be noted that serum leptin levels may not be indicative of local leptin levels. Putatively, blocking localized leptin secreted by leptin antagonists endoscopically applied to colon cancer cells may be considered as a possible future treatment option.

The role of leptin in hepatocellular carcinoma (HCC), the third leading cause of cancer death in the world, is also not clear. Patients with cirrhosis resulting from hepatitis B or C infection were found to exhibit increased leptin levels which were suggested to represent a negative prognostic factor [80], but again, studies have reported contradictory findings [81]. Another major factor leading to HCC is non-alcoholic fatty liver disease (NAFLD) ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). In an animal model of NAFLD, leptin was suggested to contribute to insulin resistance and steatosis, and leptin injections increased the expression of procollagen-I, TGF-β1 and smooth muscle actin and led to increased liver fibrosis [82]. In another study without leptin signaling, neither fibrosis nor HCC developed in the rat NASH model [83]. However, exogenous leptin significantly decreased tumor size and increased survival rate in an HCC mouse model [84]. In human studies, leptin levels were significantly higher in NASH patients and correlated with severity of hepatic steatosis [85], but in another study no differences were found [86]. Furthermore, high leptin expression correlated with better survival in HCC patients [87].

More recently, attention has been given to the role of leptin in glioblastoma and is reviewed elsewhere (see ref. [65]). Though extensive *in-vivo* studies are still lacking and the information linking leptin to glioblastoma stems mainly from studies with glioma cell line C6 [88, 89], the authors strongly suggest leptin's role not only in cell proliferation and inhibition of apoptosis but also in leptin-enhanced cell migration [90]. Moreover, indirect leptin actions such as promotion of angiogenesis and augmentation of VEGF levels may also play an important role in leptin promotion of glioblastoma and other cancers [91-94].

One of the most interesting findings connecting leptin to susceptibility to cancer in mouse models of melanoma and colon cancer, related to environmental enrichment, showed that mice kept in an enriched environment express higher levels of brain-derived nerve factor (BDNF), which in turn activates sympathetic nerve fiber innervation of white adipose tissue, resulting in decreased

leptin secretion, cancer inhibition and remission [95]. Thus, although the role of leptin in tumor promotion is controversial and may depend on tumor type, at least in those cases in which leptin plays a negative role, leptin antagonists may be potentially used as both important research tools and potential therapeutic modalities.

LEPTIN ANTAGONISTS IN UREMIC CACHEXIA

Most recently, the negative role of leptin in uremic patients has been reviewed [96]. Leptin has been defined as a true uremic toxin, and reducing leptin levels in uremic patients, particularly those suffering from uremia-related cachexia, may have potential beneficial effects. This suggestion is based on the finding that plasma leptin is associated with reduced energy intake and protein-wasting in uremic patients [97]. In a murine animal model of uremic cachexia, application of pegylated superactive leptin antagonist prevented weight gain, protein and fat losses and normalized muscle function (Michal Ayalon-Soffer and Robert Mak, personal communication). Use of leptin antagonists in the treatment of uremic patients seems at present as a most feasible therapeutic application.

LEPTIN ANTAGONISTS IN OTHER PATHOLOGIES

The role of leptin signaling in myocardial hypertrophy, heart diseases associated with metabolic syndrome, endothelial dysfunction, arterial hypertension are reviewed in depth in the present issue. In all of these conditions, leptin's negative effects can be potentially antagonized by leptin antagonists.

LEPTIN ANTAGONIST-INDUCED RAPID AND REVERSIBLE MOUSE MODEL OF T2DM

Obesity and its major consequence, type II diabetes mellitus (T2DM), has become an epidemic in Western society. T2DM accounts for 95% of the diabetes worldwide. One limitation to the development of new T2DM treatments has been a lack of effective animal models to use in research. There are no rodent models that recapitulate the pancreatic β-cell lesions of humans with T2DM. Moreover, animal models of obesity require either overfeeding, which is expensive and takes weeks to months to establish, or specific genetic mutations that cause lifelong metabolic dysfunction. Thus the ability to rapidly induce obesity in healthy rat and mouse strains would constitute a major advance in diabetes and obesity research leading to the development of novel therapies. In its monopegylated form, the compound PEG-SMLA has strong orexigenic properties, and when given to mice every 24 to 48 h leads to remarkable weight gain. So far, over 15 experiments have been performed over the course of 2 to 12 weeks to test the effects of PEG-SMLA on weight gain in mice, achieving a uniform 25 to 45% weight gain in 14 to 21 days. Some representative data from these studies are presented in Table **1** and show that weight gain is accompanied by elevated glucose, cholesterol and triglyceride levels, and an even more dramatic increase in insulin levels and insulin resistance (HOMA-IR). Interestingly no hepatic damage was observed, even after 12 weeks of treatment, although morphologically the livers of PEG-SMLA-treated animals seem fatty. PEG-SMLA treatment did not affect other blood parameters such as albumin, globulin, creatinine, urea, calcium, potassium, phosphorus or bilirubin (not shown). An additional experiment was carried out to test the effects of PEG-SMLA, and showed abnormal glucose tolerance (by oral glucose tolerance test) after 3 weeks of treatment. This change, along with others such as weight gain, increased fat content, hyperinsulinemia and hypertriglyceridemia, were fully reversible with cessation of PEG-SMLA injections, disappearing within 10 to 14 days.

Determining insulin and glucose levels, and HOMA scores can indicate whether mice are normal (euglycemic with normal insulin levels), insulin-resistant, or have developed T2DM according to the following criteria:

Normal: Glucose and insulin are less than 2.5 SD above control average

Insulin-resistant: Insulin more than 2.5 SD above control average but glucose less than

Table 1. Weight gain and selected biochemical and hormonal parameters in 8-week-old male and 4-week-old female mice after long-term 4 or 12 weeks IP administration of PEG-SMLA at 12 (male) or 5 (female) mg/kg daily.

	Males[1]		Females[2]			
	after 4 weeks		after 4 weeks		after 12 weeks	
	Control	PEG-SMLA	Control	PEG-SMLA	Control	PEG-SMLA
Weight gain (%)	15 ± 0.3	65 ±1.2**	20 ± 1	69 ± 7***	39 ± 4	124 ± 6***
Glucose (mg/dl)	97 ± 6	151 ± 8**	178 ± 15	246 ± 21*	131 ± 8	183 ± 10*
Chol (mg/dl)	133 ± 4	173 ± 10**	96 ± 8	147 ± 9**	105 ± 4	153 ± 4**
TG (mg/dl)	63 ± 3	86 ± 4**	63 ± 3	87 ± 4*	79 ± 5	106 ± 8**
Insulin (ng/ml)	Not tested	0.80 ± 0.1	4.1 ± 0.7**	0.31 ± 0.07		1.84 ± 0.08***
HOMA (arbit U)	Not tested	57 ± 9	380 ± 80***	18 ± 4		146 ± 28***
SGOT (IU/ml)	150 ± 19	165 ± 20	120 ± 23	249 ± 54	187 ± 13	173 ± 16
SGPT (IU/ml)	158 ± 37	136 ± 16	74 ± 41	203 ± 67	129 ± 41	163 ± 45
AP (IU/ml)	122 ± 10	136 ± 18	192 ± 6	166 ± 19	124 ± 5	115 ± 6

[1]Initial weight 20.8 g at 8 weeks of age.
[2]Initial weight 11.8 g at 4 weeks of age.
Values are mean ± SEM, *P > 0.05, **P > 0.01, ***P > 0.001; male: 4 h post-fasting, females: not fasted.
Chol – cholesterol, TG – triglycerides, HOMA - homeostatic model assessment, SGOT - serum glutamic oxaloacetic transaminase, SGPT - serum glutamic pyruvic transaminase, AP – alkaline phosphatase

2.5 SD above control average

T2DM: Both insulin and glucose more than 2.5 SD above control average.

Using these criteria, we have found in various experiments that 36 to 100% of PEG-SMLA-treated animals develop insulin resistance or T2DM in a time-dependent manner within 3 to 6 weeks of PEG-SMLA treatment. In view of these results, we conclude that PEG-SMLA treatment leads to the appearance of metabolic syndrome and even to a reversible T2DM phenotype.

In conclusion, administration of PEG-SMLA or even PEG-MLA to mice causes reversible obesity, hyperglycemia and hyperinsulinemia. These findings raise questions as to whether islet β-cells are specifically affected by a LEPR blockade preventing their full compensation for insulin resistance. A second issue raised by these findings is the role of hepatic glucose production and insulin sensitivity in the rapid development of hyperglycemia. Hypothalamic LEPRs have been implicated in the regulation of hepatic glucose production, and direct effects of leptin on hepatocytes have also been demonstrated. The ability to rapidly and directly affect systemic and CNS leptin signaling with high potency LEPR antagonists provides a powerful means of addressing the breadth of leptin action.

One-month exposure to PEG-SMLA altered the expression of key regulatory genes in adipose tissue, muscle and brain. These findings indicate that leptin antagonism induces systemic dysmetabolism in a rapid and practical manner, and provides a valuable tool for research in obesity and diabetes.

CONFLICT OF INTEREST

Both authors are employed as consultants to BiolineRx, a R&D company exploring the therapeutic potential of leptin antagonists.

ACKNOWLEDGEMENTS

This work was supported by the Israel Science Foundation, grant no. 521/07 to AG and EE. The authors thank the members of their teams for their valuable contribution to this work: Dr. Leonora Niv-Spector, Dr. Michal Shpilman, Ms. Gili Solomon from the Hebrew University of Jerusalem, and Ms. Meirav Katz and Dr. Chen Varol from Tel-Aviv Sourasky Medical Center.

REFERENCES

[1] Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372: 425-32.

[2] Tartaglia LA, Dembski M, Weng X, *et al*. Identification and expression cloning of a leptin receptor, OB-R. Cell 1995; 83: 1263-71.

[3] Zhang F, Basinski MB, Beals JM, *et al*. Crystal structure of the obese protein leptin-E100. Nature 1997; 387: 206-9.

[4] Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, Tavernier J. The ins and outs of leptin receptor activation. FEBS Lett 2003; 546: 45-50.

[5] Fong TM, Huang RR, Tota MR, *et al*. Localization of leptin binding domain in the leptin receptor. Mol Pharmacol 1998; 53: 234-40.

[6] Sandowski Y, Raver N, Gussakovsky EE, *et al*. Subcloning, expression, purification, and characterization of recombinant human leptin-binding domain. J Biol Chem 2002; 277: 46304-9.

[7] Niv-Spector L, Raver N, Friedman-Einat M, *et al*. Mapping leptin-interacting sites in recombinant leptin-binding domain (LBD) subcloned from chicken leptin receptor. Biochem J 2005; 390: 475-84.

[8] Zabeau L, Defeau D, Van der Heyden J, Iserentant H, Vandekerckhove J, Tavernier J. Functional analysis of leptin receptor activation using a Janus kinase/signal transducer and activator of transcription complementation assay. Mol Endocrinol 2004; 18: 150-61.

[9] Chow D, He X, Snow AL, Rose-John S, Garcia KC. Structure of an extracellular gp130 cytokine receptor signaling complex. Science 2001; 291: 2150-5.

[10] Boulanger MJ, Chow DC, Brevnova EE, Garcia KC. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. Science 2003; 300: 2101-4.

[11] Muller-Newen G. The cytokine receptor gp130: faithfully promiscuous. Sci STKE 2003; 2003: PE40.

[12] Peelman F, Van Beneden K, Zabeau L, *et al*. Mapping of the leptin binding sites and design of a leptin antagonist. J Biol Chem 2004; 279: 41038-46.

[13] Callebaut I, Labesse G, Durand P, *et al*. Deciphering protein sequence information through hydrophobic cluster analysis (HCA): current status and perspectives. Cell Mol Life Sci 1997; 53: 621-45.

[14] Niv-Spector L, Gonen-Berger D, Gourdou I, *et al*. Identification of the hydrophobic strand in the A-B loop of leptin as major binding site III: implications for large-scale preparation of potent recombinant human and ovine leptin antagonists. Biochem J 2005; 391: 221-30.

[15] Salomon G, Niv-Spector L, Gussakovsky EE, Gertler A. Large-scale preparation of biologically active mouse and rat leptins and their L39A/D40A/F41A muteins which act as potent antagonists. Protein Expr Purif 2006; 47: 128-36.

[16] Elinav E, Niv-Spector L, Katz M, *et al*. Pegylated leptin antagonist is a potent orexigenic agent: preparation and mechanism of activity. Endocrinology 2009; 150: 3083-91.

[17] Shpilman M, Niv-Spector L, Katz M, *et al*. Development and characterization of high affinity leptins and leptin antagonists. J Biol Chem 2011; 286: 4429-42.

[18] Niv-Spector L, Shpilman M, Boisclair Y, Gertler A. Large-scale preparation and characterization of non-pegylated and pegylated superactive ovine leptin antagonist. Protein Expr Purif 2012; 81: 186-92.

[19] Cipolletta D, Kolodin D, Benoist C, Mathis D. Tissular T(regs): a unique population of adipose-tissue-resident Foxp3+CD4+ T cells that impacts organismal metabolism. Semin Immunol 2011; 23: 431-7.

[20] Schaffler A, Scholmerich J. Innate immunity and adipose tissue biology. Trends Immunol 2010; 31: 228-35.

[21] Sarraf P, Frederich RC, Turner EM, *et al*. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. J Exp Med 1997; 185: 171-5.

[22] Gimble JM, Robinson CE, Wu X, Kelly KA. The function of adipocytes in the bone marrow stroma: an update. Bone 1996; 19: 421-8.

[23] Sanchez-Margalet V, Martin-Romero C, Gonzalez-Yanes C, Goberna R, Rodriguez-Bano J, Muniain MA. Leptin receptor (Ob-R) expression is induced in peripheral blood mononuclear cells by *in vitro* activation and *in vivo* in HIV-infected patients. Clin Exp Immunol 2002; 129: 119-24.

[24] Motivala SJ, Dang J, Obradovic T, Meadows GG, Butch AW, Irwin MR. Leptin and cellular and innate immunity in abstinent alcoholics and controls. Alcohol Clin Exp Res 2003; 27: 1819-24.

[25] Lam QL, Liu S, Cao X, Lu L. Involvement of leptin signaling in the survival and maturation of bone marrow-derived dendritic cells. Eur J Immunol 2006; 36: 3118-30.

[26] Saxena NK, Ikeda K, Rockey DC, Friedman SL, Anania FA. Leptin in hepatic fibrosis: evidence for increased collagen production in stellate cells and lean littermates of ob/ob mice. Hepatology 2002; 35: 762-71.

[27] Sanchez-Margalet V, Martin-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. Clin Exp Immunol 2003; 133: 11-9.

[28] Tian Z, Sun R, Wei H, Gao B. Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation. Biochem Biophys Res Commun 2002; 298: 297-302.

[29] Montecucco F, Bianchi G, Gnerre P, Bertolotto M, Dallegri F, Ottonello L. Induction of neutrophil chemotaxis by leptin: crucial role for p38 and Src kinases. Ann N Y Acad Sci 2006; 1069: 463-71.

[30] Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 1998; 394: 897-901.

[31] De Rosa V, Procaccini C, Cali G, *et al*. A key role of leptin in the control of regulatory T cell proliferation. Immunity 2007; 26: 241-55.

[32] Matarese G, Di Giacomo A, Sanna V, *et al*. Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. J Immunol 2001; 166: 5909-16.

[33] Elinav E, Pappo O, Sklair-Levy M, *et al*. Adoptive transfer of regulatory NKT lymphocytes ameliorates non-alcoholic steatohepatitis and glucose intolerance in ob/ob mice and is associated with intrahepatic CD8 trapping. J Pathol 2006; 209: 121-8.

[34] Elinav E, Pappo O, Sklair-Levy M, *et al*. Amelioration of non-alcoholic steatohepatitis and glucose intolerance in ob/ob mice by oral immune regulation towards liver-extracted proteins is associated with elevated intrahepatic NKT lymphocytes and serum IL-10 levels. J Pathol 2006; 208: 74-81.

[35] Ahima RS, Osei SY. Leptin signaling. Physiol Behav 2004; 81: 223-41.

[36] Palmer G, Gabay C. A role for leptin in rheumatic diseases? Ann Rheum Dis 2003; 62: 913-5.

[37] Lago F, Dieguez C, Gomez-Reino J, Gualillo O. The emerging role of adipokines as mediators of inflammation and immune responses. Cytokine Growth Factor Rev 2007; 18: 313-25.

[38] Sanna V, Di Giacomo A, La Cava A, *et al*. Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. J Clin Invest 2003; 111: 241-50.

[39] Matarese G, Carrieri PB, La Cava A, *et al*. Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells. Proc Natl Acad Sci U S A 2005; 102: 5150-5.

[40] Batocchi AP, Rotondi M, Caggiula M, *et al*. Leptin as a marker of multiple sclerosis activity in patients treated with interferon-beta. J Neuroimmunol 2003; 139: 150-4.

[41] Bokarewa M, Tarkowski A. Local infusion of infliximab for the treatment of acute joint inflammation. Ann Rheum Dis 2003; 62: 783-4.

[42] Anders HJ, Rihl M, Heufelder A, Loch O, Schattenkirchner M. Leptin serum levels are not correlated with disease activity in patients with rheumatoid arthritis. Metabolism 1999; 48: 745-8.

[43] Vadacca M, Margiotta D, Rigon A, *et al*. Adipokines and systemic lupus erythematosus: relationship with metabolic syndrome and cardiovascular disease risk factors. J Rheumatol 2009; 36: 295-7.

[44] Chung CP, Long AG, Solus JF, *et al*. Adipocytokines in systemic lupus erythematosus: relationship to inflammation, insulin resistance and coronary atherosclerosis. Lupus 2009; 18: 799-806.

[45] De Sanctis JB, Zabaleta M, Bianco NE, Garmendia JV, Rivas L. Serum adipokine levels in patients with systemic lupus erythematosus. Autoimmunity 2009; 42: 272-4.

[46] Rho YH, Solus J, Sokka T, *et al*. Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis. Arthritis Rheum 2009; 60: 1906-14.

[47] Fraser DA, Thoen J, Reseland JE, Forre O, Kjeldsen-Kragh J. Decreased CD4+ lymphocyte activation and increased interleukin-4 production in peripheral blood of rheumatoid arthritis patients after acute starvation. Clin Rheumatol 1999; 18: 394-401.

[48] Matarese G, Sanna V, Lechler RI, *et al*. Leptin accelerates autoimmune diabetes in female NOD mice. Diabetes 2002; 51: 1356-61.

[49] Lee CH, Chen YG, Chen J, *et al*. Novel leptin receptor mutation in NOD/LtJ mice suppresses type 1 diabetes progression: II. Immunologic analysis. Diabetes 2006; 55: 171-8.

[50] Elinav E, Ali M, Bruck R, *et al*. Competitive inhibition of leptin signaling results in amelioration of liver fibrosis through modulation of stellate cell function. Hepatology 2009; 49: 278-86.

[51] Siegmund B, Lehr HA, Fantuzzi G. Leptin: a pivotal mediator of intestinal inflammation in mice. Gastroenterology 2002; 122: 2011-25.

[52] Siegmund B, Sennello JA, Jones-Carson J, *et al*. Leptin receptor expression on T lymphocytes modulates chronic intestinal inflammation in mice. Gut 2004; 53: 965-72.

[53] Sitaraman S, Liu X, Charrier L, *et al*. Colonic leptin: source of a novel proinflammatory cytokine involved in IBD. Faseb J 2004; 18: 696-8.

[54] Fantuzzi G, Sennello JA, Batra A, *et al*. Defining the role of T cell-derived leptin in the modulation of hepatic or intestinal inflammation in mice. Clin Exp Immunol 2005; 142: 31-8.

[55] Mattioli B, Straface E, Quaranta MG, Giordani L, Viora M. Leptin promotes differentiation and survival of human dendritic cells and licenses them for Th1 priming. J Immunol 2005; 174: 6820-8.

[56] Karmiris K, Koutroubakis IE, Kouroumalis EA. The emerging role of adipocytokines as inflammatory mediators in inflammatory bowel disease. Inflamm Bowel Dis 2005; 11: 847-55.

[57] Ballinger A, Kelly P, Hallyburton E, Besser R, Farthing M. Plasma leptin in chronic inflammatory bowel disease and HIV: implications for the pathogenesis of anorexia and weight loss. Clin Sci (Lond) 1998; 94: 479-83.

[58] Karmiris K, Koutroubakis IE, Xidakis C, Polychronaki M, Kouroumalis EA. The effect of infliximab on circulating levels of leptin, adiponectin and resistin in patients with inflammatory bowel disease. Eur J Gastroenterol Hepatol 2007; 19: 789-94.

[59] Basen-Engquist K, Chang M. Obesity and cancer risk: recent review and evidence. Curr Oncol Rep 2011; 13: 71-6.

[60] Paz-Filho G, Lim EL, Wong ML, Licinio J. Associations between adipokines and obesity-related cancer. Front Biosci 2011; 16: 1634-50.

[61] Teucher B, Rohrmann S, Kaaks R. Obesity: focus on all-cause mortality and cancer. Maturitas 2010; 65: 112-6.

[62] Wolin KY, Carson K, Colditz GA. Obesity and cancer. Oncologist 2010; 15: 556-65.

[63] Food Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. World Cancer Research Fund/American Institute for Cancer Research: Washington, DC 2007.

[64] Systematic Literature Review Continuous Update Project Report. The Associations between Food, Nutrition and Physical Activity and the Risk of Colorectal Cancer. World Cancer Research Fund/American Institute for Cancer Research 2010.

[65] Lawrence JE, Cook NJ, Rovin RA, Winn RJ. Leptin promotes glioblastoma. Neurol Res Int 2012; 2012: 870807.

[66] Ellerhorst JA, Diwan AH, Dang SM, *et al*. Promotion of melanoma growth by the metabolic hormone leptin. Oncol Rep 2010; 23: 901-7.

[67] Ishikawa M, Kitayama J, Nagawa H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. Clin Cancer Res 2004; 10: 4325-31.

[68] Uddin S, Bu R, Ahmed M, *et al*. Overexpression of leptin receptor predicts an unfavorable outcome in Middle Eastern ovarian cancer. Mol Cancer 2009; 8: 74.

[69] Hoon Kim J, Lee SY, Myung SC, Kim YS, Kim TH, Kim MK. Clinical significance of the leptin and leptin receptor expressions in prostate tissues. Asian J Androl 2008; 10: 923-8.

[70] Guo S, Liu M, Wang G, Torroella-Kouri M, Gonzalez-Perez RR. Oncogenic role and therapeutic target of leptin signaling in breast cancer and cancer stem cells. Biochim Biophys Acta 2012.

[71] Feldman DE, Chen C, Punj V, Tsukamoto H, Machida K. Pluripotency factor-mediated expression of the leptin receptor (OB-R) links obesity to oncogenesis through tumor-initiating stem cells. Proc Natl Acad Sci U S A 2011; 109: 829-34.

[72] McGlothen T, Gillespie C, Colbert L, Guo S, Gonzalez-Perez R. Leptin signaling impacts on notch and Wnt crosstalk in breast cancer. CTRC-AACR San Antonio Breast Cancer Symposium: San Antonio 2011.

[73] Hardwick JC, Van Den Brink GR, Offerhaus GJ, Van Deventer SJ, Peppelenbosch MP. Leptin is a growth factor for colonic epithelial cells. Gastroenterology 2001; 121: 79-90.

[74] Aparicio T, Kotelevets L, Tsocas A, *et al*. Leptin stimulates the proliferation of human colon cancer cells *in vitro* but does not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in Apc(Min/+) mice. Gut 2005; 54: 1136-45.

[75] Hoda MR, Keely SJ, Bertelsen LS, Junger WG, Dharmasena D, Barrett KE. Leptin acts as a mitogenic and antiapoptotic factor for colonic cancer cells. Br J Surg 2007; 94: 346-54.

[76] Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res 1994; 54: 5947-52.

[77] Endo H, Hosono K, Uchiyama T, *et al*. Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. Gut 2011; 60: 1363-71.

[78] Padidar S, Farquharson AJ, Williams LM, *et al*. Leptin up-regulates pro-inflammatory cytokines in discrete cells within mouse colon. J Cell Physiol 2011; 226: 2123-30.

[79] Drew JE. Molecular mechanisms linking adipokines to obesity-related colon cancer: focus on leptin. Proc Nutr Soc 2012; 71: 175-80.

[80] Manolakopoulos S, Bethanis S, Liapi C, *et al*. An assessment of serum leptin levels in patients with chronic viral hepatitis: a prospective study. BMC Gastroenterol 2007; 7: 17.

[81] Stefanou N, Satra M, Papanikolaou V, *et al*. Leptin receptor isoforms mRNA expression in peripheral blood mononuclear cells from patients with chronic viral hepatitis. Exp Biol Med (Maywood) 2006; 231: 1653-63.

[82] Wang SN, Lee KT, Ker CG. Leptin in hepatocellular carcinoma. World J Gastroenterol 2010; 16: 5801-9.

[83] Kitade M, Yoshiji H, Kojima H, *et al*. Leptin-mediated neovascularization is a prerequisite for progression of nonalcoholic steatohepatitis in rats. Hepatology 2006; 44: 983-91.

[84] Elinav E, Abd-Elnabi A, Pappo O, *et al*. Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation. J Hepatol 2006; 44: 529-36.

[85] Chitturi S, Farrell G, Frost L, *et al*. Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity? Hepatology 2002; 36: 403-9.

[86] Angulo P, Alba LM, Petrovic LM, Adams LA, Lindor KD, Jensen MD. Leptin, insulin resistance, and liver fibrosis in human nonalcoholic fatty liver disease. J Hepatol 2004; 41: 943-9.

[87] Wang SN, Chuang SC, Yeh YT, *et al*. Potential prognostic value of leptin receptor in hepatocellular carcinoma. J Clin Pathol 2006; 59: 1267-71.

[88] Morash B, Li A, Murphy PR, Wilkinson M, Ur E. Leptin gene expression in the brain and pituitary gland. Endocrinology 1999; 140: 5995-8.

Received: April 3, 2013 **Accepted: May 13, 2013**

[89] Riolfi M, Ferla R, Del Valle L, *et al*. Leptin and its receptor are overexpressed in brain tumors and correlate with the degree of malignancy. Brain Pathol 2010; 20: 481-9.

[90] Yeh WL, Lu DY, Lee MJ, Fu WM. Leptin induces migration and invasion of glioma cells through MMP-13 production. Glia 2009; 57: 454-64.

[91] Frankenberry KA, Somasundar P, McFadden DW, Vona-Davis LC. Leptin induces cell migration and the expression of growth factors in human prostate cancer cells. Am J Surg 2004; 188: 560-5.

[92] Zhou W, Guo S, Gonzalez-Perez RR. Leptin pro-angiogenic signature in breast cancer is linked to IL-1 signalling. Br J Cancer 2011; 104: 128-37.

[93] Liu H, Wan D, Pan Z, *et al*. Expression and biological significance of leptin, leptin receptor, VEGF, and CD34 in colorectal carcinoma. Cell Biochem Biophys 2011; 60: 241-4.

[94] Knight BB, Oprea-Ilies GM, Nagalingam A, *et al*. Survivin upregulation, dependent on leptin-EGFR-Notch1 axis, is essential for leptin-induced migration of breast carcinoma cells. Endocr Relat Cancer 2011; 18: 413-28.

[95] Cao L, Liu X, Lin EJ, *et al*. Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. Cell 2010; 142: 52-64.

[96] Teta D. Adipokines as uremic toxins. J Ren Nutr 2012; 22: 81-5.

[97] Odamaki M, Furuya R, Yoneyama T, *et al*. Association of the serum leptin concentration with weight loss in chronic hemodialysis patients. Am J Kidney Dis 1999; 33: 361-8.