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Review

Oral leptin administration for the management of body weight: current possibilities and future prospects

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ABSTRACT

Obesity is increasing at an alarming rate in humans, while very little pharmacological handling seems to be successful in preventing abnormal regulation of energy intake that exceeds energy expenditure. Leptin is a key hormone that regulates energy homeostasis. First identified as secreted by the adipose tissue, it is also released by gastric epithelial cells. While adipose tissue leptin acts in the long-term for energy expenditure, gastric leptin is active on a short-term, being released at time of meals. Leptin is secreted in an exocrine fashion into the gastric lumen associated to its soluble receptor that protects it from the harsh conditions of the gastric and intestinal lumina. Once transported across the duodenal wall, leptin reaches circulation as the leptin-leptin receptor complex. The presence of leptin in the gastric cavity prompted us to put forward its oral administration. Oral leptin protected by anti-proteases reaches the gastric cavity and is channelled towards the duodenum. Upon crossing the intestinal epithelium, it reaches circulation in its leptin-leptin receptor form to get to hypothalamic and other target cells. Oral leptin induces major decreases in food intake and consequently in body weight in mice. Changes correlate with amounts of oral leptin and are even more drastic in ob/ob leptin-deficient obese mice. Oral leptin administration to young rats led to significant losses of body weight gain. Once given to dogs, the effect on food intake was significant demonstrating that oral leptin is efficient in small as well as large mammals. Beside its role in food intake, oral leptin targets the brown adipose tissue to stimulate lipid oxidation and lipolysis, leading to reduction of adiposity. Long-term administration of oral leptin does not alter gastric, duodenal or liver tissues. Oral administration of leptin appears as a promising avenue for the management of food intake and control of body weight.

KEYWORDS: obesity, leptin, oral administration, gastric mucosa, adipose tissue

INTRODUCTION

By the end of the last millennium and ongoing, the Western society found itself confronted with a major health challenge. Obesity has increased at an alarming rate in the last 50 years, leading to several health problems [1-3]. It is of interest and also quite depressing to realize how human society has evolved during its history, finding itself nowadays split into two worlds; the Western one dealing with the issues of obesity while many developing countries remain at the edge of starvation. Along civilization, Man has always shown difficulties in establishing, sharing and staying in a comforting healthy condition. Many factors such as abundance and easy available food, media advertising creating false needs, a large variety of processed ready-made meals and fast food as well as sedentary social life, along with genetic susceptibilities triggered by psychological issues [4] are responsible for an imbalance between energy intake and energy expenditure. This imbalance contributes to the epidemic situation of obesity. Obesity is not anymore

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regarded as a simple cosmetic issue but rather as an illness in itself; a serious threat to the human well being [5, 6]. It is linked to diabetes and other endocrine-related disorders, to cardiovascular diseases, to some types of cancer, to pulmonary and gastrointestinal tract complications as well as to depression. Leading to increases in morbidity and mortality, it has become a major public health issue. Aside from some rather rare endocrine situations, obesity results from an abnormal regulation of energy homeostasis; energy intakes largely exceeding energy expenditure. In ideal situations, both are controlled and balanced by our endocrine regulatory system. However, appetite and feeding behavior are complex and depend on many factors including hunger but also on taste and olfactory sensations as well as visual and emotional issues. The need to consume food results from neurochemical events and involves various issues. Understanding the regulation of food intake has become increasingly complex not only because of the many factors, both orexigenic and anorexigenic that play important roles for the control of appetite, but also because of the intervention of social and emotional intricate aspects.

In spite of media awareness, long-term dietary intervention has failed in preventing the onset of obesity. We are facing today a quite urgent need for safe and efficient innovative therapies. Being a complex disease resulting from interactions between environment, intrinsic genetic and psychological factors, cure is rare having to turn ourselves toward palliative options [7-9]. Apart from bariatric surgery [10, 11], which has its own limitations and not without risks [12], there is currently little long-term treatment for obesity. Pharmacological handling of obesity has up to now, been quite chaotic largely due to intolerable side effects of different drugs that needed to be withdrawn from the market [13]. Unfortunately medical treatment as well as trying to modify life-style have largely failed to achieve success. Because of the severity of the situation, development of safe and efficient strategies is urgently needed for long-term prevention or treatment of obesity.

One of the key elements in the field of energy homeostasis is leptin [14-17], a hormone originally identified as one of the first peptides secreted by the adipose tissue [18-21]. It notifies the central nervous system of the nutritional state of the body [15] and acts on hypothalamic target cells to trigger the feeling of satiety [14]. First considered as a simple hormone that would prevent overeating conditions, we rapidly found that the hormone, its secretory mode and its way of action are by far, much more complex than expected [15, 16]. Indeed, first used in a leptin replacement therapy for the treatment of obesity in 1997 [22], it was followed by several attempts of daily leptin subcutaneous injections for very long periods of time all with rather poor results, far from those expected [23, 24].

Leptin

Early parasymbiosis experiments carried out by Jackson Laboratories on obese rodents, namely the ob/ob and the db/db mice, suggested the existence of lipostatic factors that act on the central nervous system to regulate food intake [25, 26]. This was validated much later about 25 years ago by J. M. Friedman who identified the hormone leptin (from the Greek *leptos* meaning *thin*) and its gene [14, 15]. Leptin was found to be secreted by the adipose tissue. Leptin is in fact the first adipokine to be described, establishing the adipose tissue as an endocrine gland [18-21]. Leptin is a small non-glycosylated peptide of 167 amino acids (146 without the signal peptide) displaying a disulfide bound and a structure with four anti-parallel α-helixes [17]. When analyzed by immunoblotting it is common to observe a 32kDa band demonstrating the ability for the peptide to form dimmers [27]. Leptin controls two related human actions, food intake and energy expenditure and acts as a music conductor to coordinate energy homeostasis to prevent excess accumulation of energy reserves, i.e. body fat [21, 28]. The role of leptin as a lipostatic factor was confirmed by chronic treatment of the leptin-deficient ob/ob mice. Indeed injection of leptin to these obese animals led to decreases in their hyperphagic behavior with reduction of their body weight [28, 29]. Leptin acts through its target cell membrane receptors that belong to the family of the gp130 receptors which are subdivided into six isoforms [30, 31]. Five of them are transmembrane proteins while one the OB-Re, the role of which is quite significant as we will see later, is a soluble isoform [32, 33]. This isoform corresponds to the extracellular domain of the complete transmembrane receptor. All receptors

possess the same extracellular domain and thus the same affinity for leptin [33]. They however differ in size and amino acid sequence of their transmembrane domains [34]. It is thus conceivable that each receptor isoform is related to and activates a different intracellular signaling pathway.

Leptin from adipose tissue

Immunolabelings at the light and electron microscope levels have demonstrated the presence of leptin in white adipocytes, being located in the small secreting granules of the narrow cytoplasmic ring that surrounds the large lipid droplets of fat cells [20]. Secretion of leptin by adipocytes follows the classical RER-Golgi-granule secretory pathway of endocrine cells and is discharged by exocytosis [20]. However, there is one particular feature in this leptin release by adipocytes; it is carried out by constitutive secretion, meaning that leptin is continuously synthesized and discharged by the fat cells [19, 20]. Cellular contents and amounts released, while differing from one adipose tissue to the other depending on abundance of adipocytes and location in the body, remain constant at all times. We found that once in culture, adipocytes while releasing leptin continuously are still reluctant to respond to stimulation [20]. Increases in leptin synthesis and release were only detected after one hour of steady stimulation [20]. Thus, since circulating levels of adipose tissue-secreted leptin are quite stable, it is difficult to conceive that they would intervene in the control of our food intake on a short-term basis. Indeed, more recently it has been established that the role of adipocyte-released leptin is oriented towards long-term regulation of energy expenditure.

Leptin involved in controlling food intake is not the one released by the adipose tissue but rather that from a different source, namely the gastric mucosa. Work performed in the last 10-15 years has established the leading role played by gastric leptin in regulating food intake [16]. Gastric leptin is secreted in an exocrine fashion by gastric chief cells at time of meals [16]. Upon secretion into the gastric cavity, leptin finds its way *via* the gastric juice to the intestinal lumen. It then interacts with transmembrane leptin receptors located on the luminal membrane of intestinal epithelial cells for its internalization. Upon crossing the duodenal mucosa it joins circulation to reach its target hypothalamic cells [16].

The fact that leptin is present in the gastric cavity as part of the gastric juice, prompted us to put forward and design an approach for the oral administration of leptin in order to regulate food intake and control body weight. We have demonstrated that once oral exogenous leptin reaches the gastric cavity, it follows the same path as the endogenous one and reaches the hypothalamic cells to trigger the endocrine response for the feeling of satiety [35, 36].

Gastric leptin

The gastric mucosa has been recognized as a major source of leptin [16, 37, 38]. By immunocytochemistry, we have identified two types of secretory cells responsible for leptin secretion; endocrine cells and exocrine ones [16, 37] (Figure 1). The endocrine cells, few in number are located in the lamina propria of the gastric mucosa. These are pure classical hormone secreting cells with a large number of small leptin-containing granules that release leptin towards blood circulation by exocytosis (Figure 2). Secretion of leptin occurs along the classical RER-Golgi-granule secretory pathway and is regulated by food intake as well as hormonal triggering factors [16]. On the other hand, a large number of

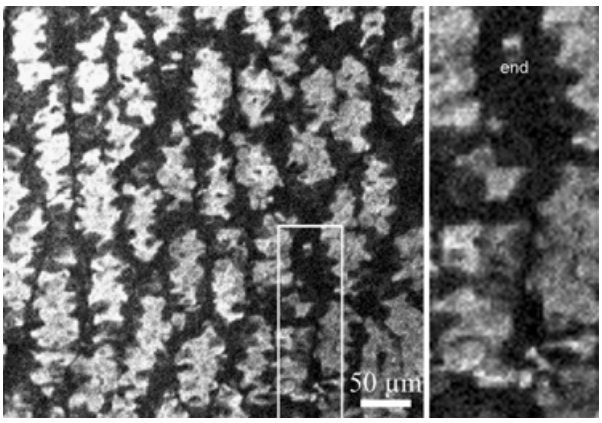
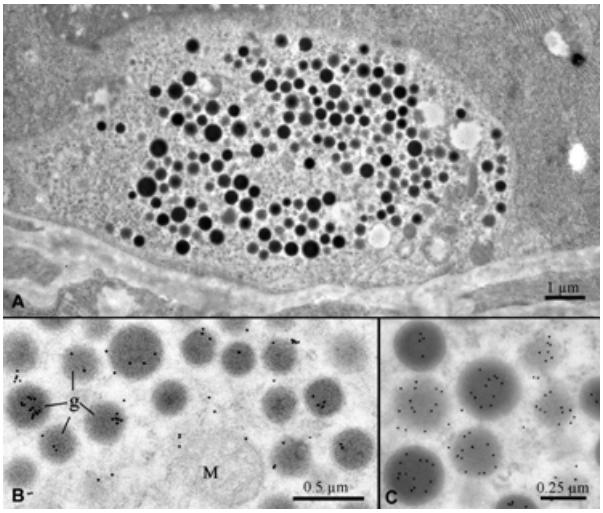


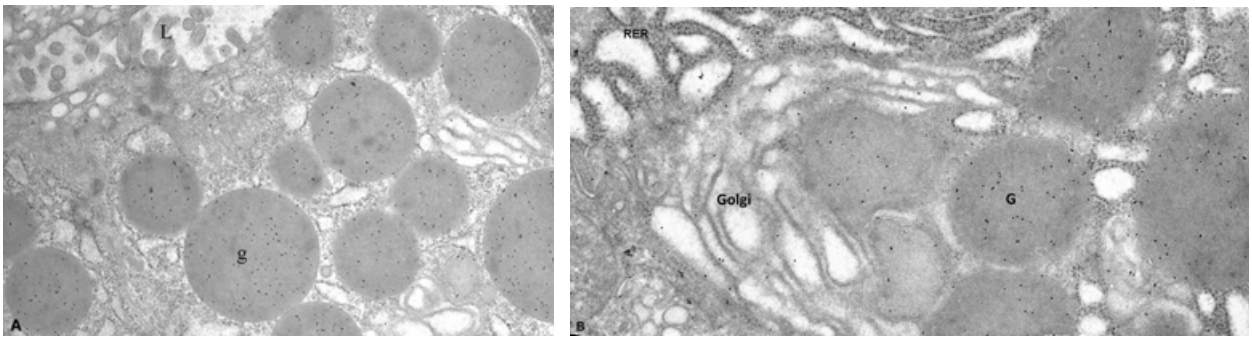
Figure 1. Light microscopy immunolocalization of leptin at the level of the gastric mucosa. The tissue sections of rat gastric mucosa were processed through immunofluorescence for the detection of leptin antigenic sites. Use of rabbit anti-leptin antibodies combined with FITC-conjugated anti-rabbit IgG. The immunolabeling reveals positive cells along the gastric epithelium; these are the gastric chief cells. Some isolated positive cells are present in the connective tissue; these correspond to endocrine cells (end).

gastric chief epithelial cells lining the epithelium of the lower half of the fundus secrete significant amounts of leptin (Figure 1). This is an exocrine secretion that takes place into the gastric cavity simultaneously with pepsinogen and lipase at time of food intake [16, 37].



Figures 2A, 2B & 2C. Electron microscopy immunolabeling of leptin in endocrine cells at the level of the gastric mucosa (Figures 2A & 2B). Use of anti-leptin antibodies combined to protein A-gold. Labeling by the 10 nm gold particles is present over the numerous secretory granules (g). The same cells also express the soluble leptin receptor at the level of the secretory granules (Figure 2C). This was revealed by the use of an anti-leptin receptor antibody combined to protein A-gold. M, mitochondria.

Release of leptin by chief cells is carried out through a regulated exocrine secretion into the gastric cavity along the typical RER-Golgi-granule secretory pathway [16, 37] (Figure 3). This regulated secretion occurs rapidly but only upon stimulation by food intake and/or action from some peptides such as secretin, cholecystokinin and insulin [39, 40, 41]. Being secreted in an exocrine manner, leptin finds itself in the gastric juice and as such is confronted to harsh conditions. Indeed, we have shown that exposing the leptin-free peptide to the gastric juice results in an immediate degradation [16]. Thus, leptin in the gastric lumen should be protected in order to carry out its physiological actions. Leptin is released by the gastric cells bound to a protecting chaperon that prevents its early degradation [27]. We have identified this chaperon as being the soluble isoform of the leptin receptor [27]. This soluble isoform is synthesized by the same gastric chief cells that secrete leptin. In fact the leptin receptor is processed along the same RER-Golgi-Granule secretory pathway as leptin. Molecular and cell biology studies have characterized the intricate secretory pathway of leptin and its receptor [27]. The chief epithelial cells synthesize the leptin-membrane-bound receptor molecule at the level of the RER and transfer it to the Golgi apparatus where it undergoes maturation. Furin and PC7, present in the same Golgi cisternae intervene in the maturation process of the leptin receptor. Furin activates PC7 which in turn cleaves the extracellular domain of the transmembrane leptin receptor releasing the soluble isoform [6, 27]. Indeed, the leptin membrane-bound receptor molecule possesses several PC7-



Figures 3A & 3B. Electron microscopy immunolabeling of leptin in gastric chief epithelial cells. Use of anti-leptin combined to protein A-gold. Labeling by the 10 nm gold particles is present over the rough endoplasmic reticulum (RER), the Golgi apparatus and the large exocrine secretory granules (g) as well as over the gastric lumen (L).

specifc cleavage sites making this transmembrane molecule a good target for the PC7 convertase [16, 27]. Once released from the membrane, the extracellular soluble portion of the receptor binds to the leptin molecules within the chief cell secretory granules (Figure 4). The binding of leptin to its soluble receptor generates a leptin-leptin receptor complex resistant to the harsh conditions of the gastric juice [27, 40]. Formation of the leptin-leptin receptor complex not only protects the leptin peptide from rapid degradation by gastric and pancreatic proteolytic enzymes but it also increases the half-life of the peptide. Indeed, free leptin in circulation has a half-life similar to that of other peptides, about 3 to 4 minutes. However, when bound to its soluble receptor its half-life increases up to 71 minutes allowing the peptide to reach its target tissues and act for long periods of time [42]. From the gastric cavity, the complex is channelled towards the duodenum. In the intestinal lumen, the leptin-leptin receptor complex gets dissociated and leptin interacts with its transmembrane receptor present on the luminal membrane (microvilli) of the enterocyte. The dissociation of leptin from its soluble receptor and its association to the duodenal transmembrane one remain elusive. It has been established that once internalized, leptin is transferred *via* the endosomal compartment of the enterocyte to the Golgi apparatus where it binds to a newly formed soluble isoform of its receptor [42, 43]. Indeed, we have demonstrated that the intestinal epithelial cells do synthesize the leptin receptor. The cells express the long-isoforms of the receptor at the levels of their plasma membranes, the apical as well as the baso-lateral ones [44]. But as for the gastric chief cells, they also convert the transmembrane leptin receptor into the soluble isoform at the level of the Golgi cisternae where it binds to the newly internalized leptin molecule. This recreates the leptin-leptin receptor complex that is released at the baso-lateral pole of the cell [54]. The leptin-leptin receptor complex joins the blood circulation and reaches target cells to regulate several processes [16, 43] (Figures 5, 6).

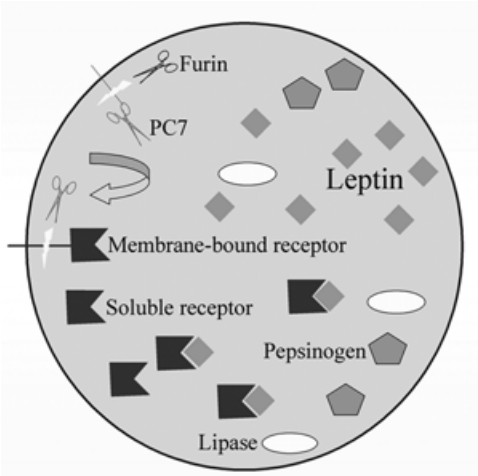


Figure 4. Diagram illustrating the different steps in the maturation of the leptin receptor and the formation of the leptin-leptin receptor complex within a secretory granule of the gastric chief cell. Furin activates PC7 which in turn cleaves the transmembrane leptin receptor, generating the soluble leptin receptor isoform. This isoform binds leptin within the secretory granule generating the leptin-leptin receptor complex. Pepsinogen and lipase are also present in the same secretory granule. All will be released be exocytosis into the gastric lumen.

Being a regulated secretion, gastric leptin is released only upon stimulation by specific triggers such as food intake and takes place for a short period of time [38, 45, 46]. Chief cells return to their basal state quite rapidly. This pattern differs drastically from the leptin secretion by adipose tissue which being constitutive, releases leptin at a set concentration and at a constant rate. Differences in time frame of secretion between white adipose tissue and gastric mucosa must reflect differences in roles. Indeed, leptin from adipose tissue acts on a long-term targeting the central nervous system for energy expenditure and glucose metabolism [28, 29, 47] while gastric leptin is active on a short-term basis, being secreted only at time of meals. It exerts a transient regulatory function on the digestive tract [37, 48] and triggers the feeling of satiety at the level of the hypothalamus. Action on the digestive tract takes place through paracrine action upon binding to leptin receptors located on the baso-lateral membrane of gastric as well as intestinal epithelial cells. Indeed, these cells express leptin receptors on both their apical and baso-lateral membranes. Leptin potentiates the effects of cholecystokinin slowing down gastric emptying and promoting gastric distension [49]. Leptin also stimulates GLP1 and GLP2 [50-52]. The feeling of satiety triggered by leptin occurs through its target hypothalamic cells.

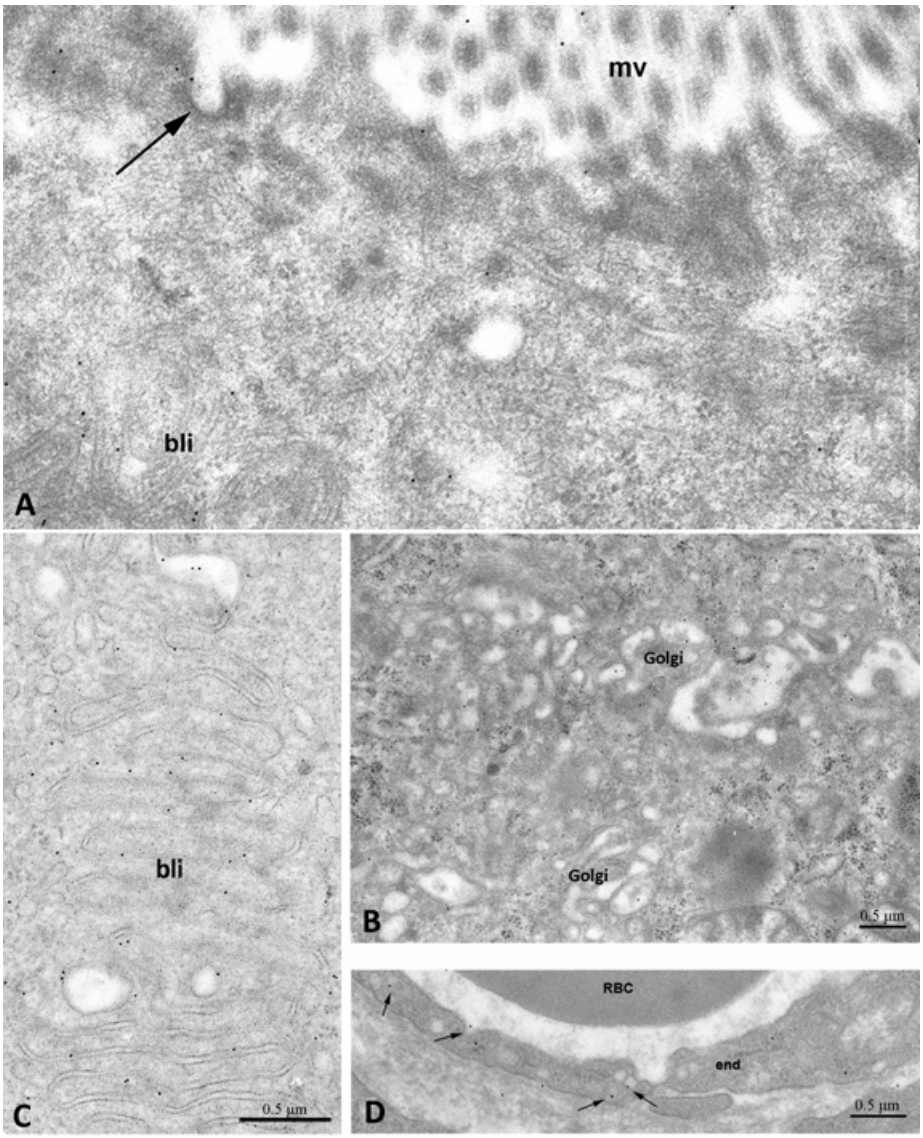


Figure 5. Electron microscopy immunolabeling of leptin at the level of duodenal epithelial cells. Use of anti-leptin combined to protein A-gold. Tissue was sampled 30 minutes after introduction of leptin into the duodenal lumen. Labelings by gold particles are seen at the level of the microvilli (mv), endocytotic vesicles (large arrow), the Golgi apparatus and the basolateral interdigitations (bli). Labeling by gold particles is also detected at the level of the endothelial cells (end) of the blood capillary, particularly associated with the plasmalemmal vesicles (arrows). RBC, red blood cell.

While leptin secretion by the gastric mucosa is encountered in all mammals, differences exist between humans and rodents. When food is easily available, circulating levels of leptin reflect amounts of body fat. However, when food becomes short to the point of decreasing body weight, levels of circulating leptin drop, reflecting an energy imbalance [53]. In rodents this occurs rapidly only in a matter of hours while it takes at least a couple of days for humans to register a leptin decrease. This must be due to differences in body mass. While rodents eat about half of their weight every day, humans ingest only 2% of their weight per day. Changes in circulating leptin levels in humans are therefore less sensitive to weight loss than in small animals [15]. On the other hand, in Western countries, circulating levels of leptin in men follow the nycthemeral cycle, independent of food intake.

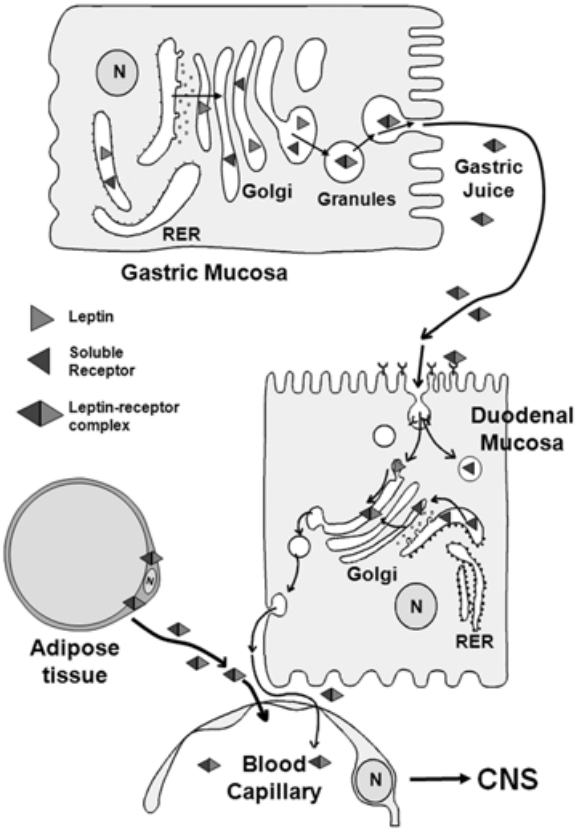


Figure 6. Diagram illustrating the path taken by gastric and adipose tissue leptins to reach circulation. Synthesis of gastric leptin takes place at the level of the RER of the gastric chief cell. It is transferred to the Golgi apparatus where it binds to the soluble leptin receptor for the formation of the leptin-leptin receptor complex. Upon transfer to the apical membrane by secretory granules, the complex is discharged into the gastric lumen by exocytosis. The gastric juice carries the complex towards the duodenal lumen where gastric leptin binds to the transmembrane leptin receptor present at the apical microvilli of the duodenal cell. Upon internalization leptin reaches the Golgi apparatus where it binds to a newly synthesised soluble leptin receptor and this newly formed complex is then secreted on the baso-lateral pole of the cell to reach blood circulation. In parallel, the adipocyte secretes a leptin-leptin receptor complex that also reaches blood circulation.

Under those conditions, insulin which also interacts with leptin [54, 55] has little effect on leptin levels [21]. Indeed, insulin also participates in the process of inhibiting food intake [56] and there is a cross-talk between insulin and leptin at the level of the hypothalamic target cells [19, 40, 56-58]. In fact, signals of hunger and satiety are orchestrated by several orexigenic and anorexigenic factors, many of them being gastro-intestinal peptides [39, 40, 59, 60] and/or adipokines [59, 61, 62]. While secretion of ghrelin that stimulates hunger is reduced by leptin [46], cholecystokinin has a satiety effect [61, 62] potentiating leptin and creating a positive feed-back loop [62].

Leptin replacement therapy

The fact that leptin is secreted by the gastric mucosa into the gastric lumen, is vehiculated towards the duodenal lumen and transferred to blood circulation, triggered the proposition that exogenous leptin could be administered orally. This exogenous oral leptin by reaching the gastric juice should follow the physiological path towards the duodenal lumen, cross the duodenal mucosa to reach systemic circulation and its hypothalamic target cells.

The proposal of an oral leptin opens a new approach and a powerful alternative to the leptin replacement therapy carried out by injection. Indeed, up to now the leptin replacement therapy has not been as successful as expected or predicted mainly because free leptin has been injected directly or indirectly into circulation. We are now well aware that the circulating hormone is not free leptin but rather the leptin-leptin receptor complex which displays a much higher half-life reaching efficiently the targeted hypothalamic cells. The results obtained in ours studies on the exocrine secretion of gastric leptin with its efficient although complicated transport across the intestinal mucosa and its release in its leptin-leptin receptor complex form towards circulation, have opened new perspectives for leptin therapy. In fact different avenues can now be put forward for successful leptin replacement therapy: 1- We could engineer the leptin-leptin receptor complex to be administered orally. This complex will resist the gastric conditions and follow the physiological path already existing in our digestive system, to reach circulation; 2- a second proposition would consist in an oral administration of free leptin taking however the precaution to protect it from early degradation by gastric and pancreatic juices; 3- the third alternative that can also be put forward would be to use the gut microbiota with particular leptin-expressing bacteria that would secrete leptin in the intestinal lumen.

Concerning the last proposition, recent studies have introduced this new avenue using gut microbiota

as therapeutic agents. Genetically modified bacteria are being envisioned as drug delivery systems [63]. In this particular case, probiotic bacteria engineered to express and secrete human leptin could be incorporated into the gut microbiota. Inserting the leptin-secreting bacteria into our gut microbiota can easily be achieved and once implanted, these bacteria should release significant amounts of leptin in the intestinal lumen. From there, the bacteria-secreted leptin should interact with the transmembrane leptin receptors on the luminal membrane of intestinal cells and be internalized. Once in the endosomal compartment of the intestinal cells, it should bind to its soluble receptor in the Golgi apparatus and then released towards circulation in its usual very efficient leptin-leptin complex form. Thus inserting leptin-secreting bacteria into the intestinal lumen could provide and sustain treatment for obesity.

From the three alternative propositions mentioned above, we report herein results obtained by the oral administration of free leptin.

Oral administration of leptin

Leptin levels rise within 15 minutes after the onset of food intake in mice [38]. We have demonstrated that this postprandial increase in circulating leptin results from gastric secretion and not from adipose tissue, with a very efficient transport towards blood across the intestinal mucosa [16]. In view of this, we put forward the proposal that leptin given orally should reach blood circulation using the same efficient transfer system. This hypothesis was first tested on normal and genetically obese mice.

One of the main issues in the oral proposition consists in protecting the leptin peptide from the harsh conditions existing in the gastric and intestinal lumina. Indeed, gastric as well pancreatic juices contain proteolytic enzymes that degrade the free leptin peptide very rapidly [16]. As mentioned above, nature has provided a protecting chaperon to the endogenous leptin in the form of the soluble leptin receptor isoform. Since we are working with free leptin, this constitutes a main issue and the peptide given orally should be protected from early degradation. Thus, we force-fed free leptin to mice in a vehicle [35] that contains anti-proteases to provide protection against proteolysis and bile salts to promote internalisation of the peptide by the intestinal mucosa [64]. Upon several essays and tests carried out on the vehicle [65], the optimal one was composed by mouse recombinant leptin (R&D System Inc. Minneapolis MN, USA) combined to 2000 Kallikrein inhibitor units of aprotinin (Trasylol, Bayer, Leverkusen FRG), 5 mg of sodium deoxycholate (22 mmol/l) (Sigma St-Louis, MO USA) dissolved in 0.5 ml of bicarbonate buffer at pH 9.0. When 0.5 ml of the vehicle containing 50 µg of mouse recombinant leptin were force-fed to normal mice, significant amounts of this exogenous leptin were detected in plasma very rapidly after its oral administration [35, 65] reaching a peak at 5 minutes (Figure 7). This was followed by a slow decrease with levels returning to base line by sixty minutes (Figure 7), which indicates that the vehicle is able to protect the exogenous leptin from being degraded and to promote its transfer to blood quite efficiently. Once in circulation, oral leptin reached the hypothalamic target cells and triggered the feelings of satiety. Indeed, oral leptin was able to significantly decrease the amounts of food intake in normal mice and upon steady oral administration for several days, it did influence gain in body weight (Figure 8). The effects of oral leptin in terms of food intake and loss of body weight are proportional to the amounts of leptin administered orally (Figure 9).

Upon oral administration of leptin to ob/ob obese mice that lack secretion of the leptin peptide [66], the effects were even more striking. Indeed the ob/ob mice are leptin-deficient animals and as

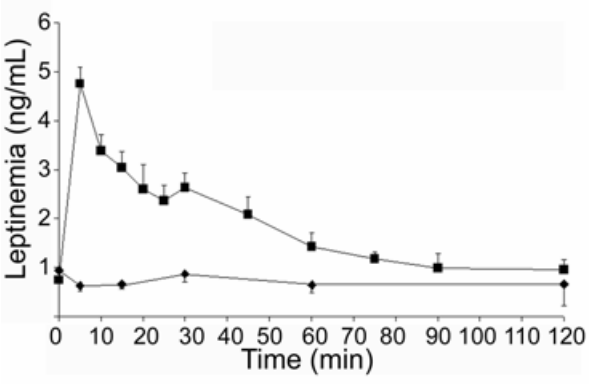


Figure 7. Plasma leptin levels upon oral administration of 10 µg of leptin to normal mice. Oral leptin reaches circulation quite rapidly and reaches a maximum within the first 5 minutes. Levels return to base line after 90 minutes (N = 8).

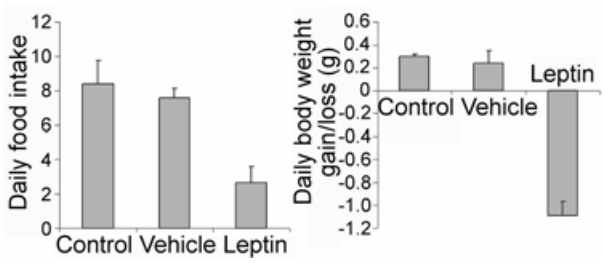


Figure 8. Daily food intake (in g) and changes in body weight (in g) of ob/ob mice upon receiving 50 µg of oral leptin twice a day (N = 8).

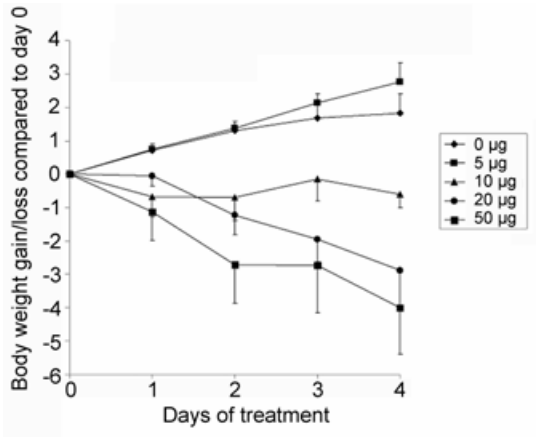


Figure 9. Effect of oral administration of various concentrations of leptin on body weight. The effect is directly related to amounts of oral leptin (N = 5 for each group).

such have no feelings of satiety; they ingest food non-stop and become rapidly obese. Upon oral administration of leptin, these animals reacted immediately by decreasing their daily food intake by 60% (Figure 10), loosing body-weight of about 1 g a day (Figure 10). In fact the changes in amounts of food intake and in loss of body weight were also proportional to the daily amounts of leptin administered [35, 36]. We carried out experiments on these ob/ob mice for long periods of time. We started with young not yet obese animals and followed with a second experiment performed on older obese ones (Figure 10). Results from these experiments, reported in figure 10, demonstrate that upon oral administration of leptin, the response in lowering food intake and reducing body weight is immediate starting on the first days of the experiment. Also upon stopping the oral treatment the animals return to their over-eating status and start regaining weight (Figure 10). When given to young animals it mainly led to reduction of food intake with slow decreases in body weight. On the other hand, when given to older obese animals, it triggered reduction in food intake and lowered the animal body weight very drastically (Figure 10).

Interesting results were obtained when oral leptin was administered to the db/db mice. The db/db animals do secrete their own leptin but don't

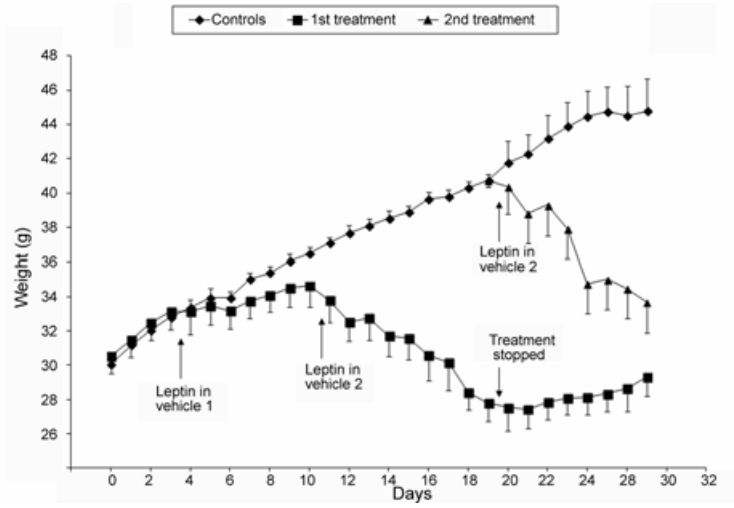


Figure 10. Changes in body weight in ob/ob mice upon oral administration of 50 µg of leptin twice a day. Experiments were done on young non-obese as well as on adult obese animals. Effect of oral leptin on adult animals is immediate and very significant, while young animals respond more gradually (N = 15).

express the corresponding transmembrane leptin receptors at the level of the hypothalamic cells [66]. Thus, in spite of high levels of circulating endogenous leptin, these are not efficient [35]. As for the ob/ob animals, the db/db ones have no satiety feelings and thus demonstrate hyperphagia leading to morbid obesity. Oral administration of leptin to these animals was found not to be efficient; indeed even if the exogenous oral leptin was able to reach circulation, it did not trigger any hypothalamic response [35]. These results are important since they indicate that oral leptin follows the same path as the endogenous gastric one reaching circulation and does require the presence of intact and functional leptin receptors at the level of the hypothalamic cells to trigger a response. Thus, oral administration of leptin appears to be very efficient provided that the leptin receptor system at the level of the hypothalamic cell membrane is present and functional.

In the next step of the investigation, we evaluated the efficiency of the oral administration on a long term basis. We orally administered leptin at a concentration of 10 µg per day to ob/ob mice for periods of 10, 20 and 30 days. By the second day of the treatment the animal body weight stabilized and remained steady during the period of the treatment [35]. At the end of the experiment, tissues were sampled and analyzed by light and electron microscopy to evaluate the presence of pathological changes. As illustrated in figure 11 and reported previously [35], after 30 days of treatment, none of the examined tissues show signs of alteration. Gastric and intestinal mucosa displayed their classical brush border at their luminal membranes and their junctional complexes on the lateral membranes remained tight (Figure 11). Intracellular cytoplasmic components displayed their normal morphology. Similarly, liver tissue did not show any sign of alteration [35].

Following the work carried out on mice, we switch to other rodents in order to confirm the anorexic properties of orally administered leptin. Studies were thus carried out on rats and the gerbils (see below). Concerning the rat, we choose to work with young animals. Wistar rats were force-fed with 200 µg of rat leptin in the appropriate vehicle for four days. This was followed by a four-day recovery. Body weight

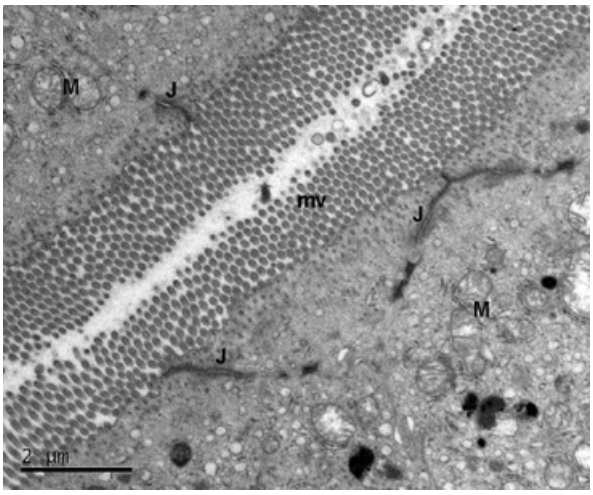
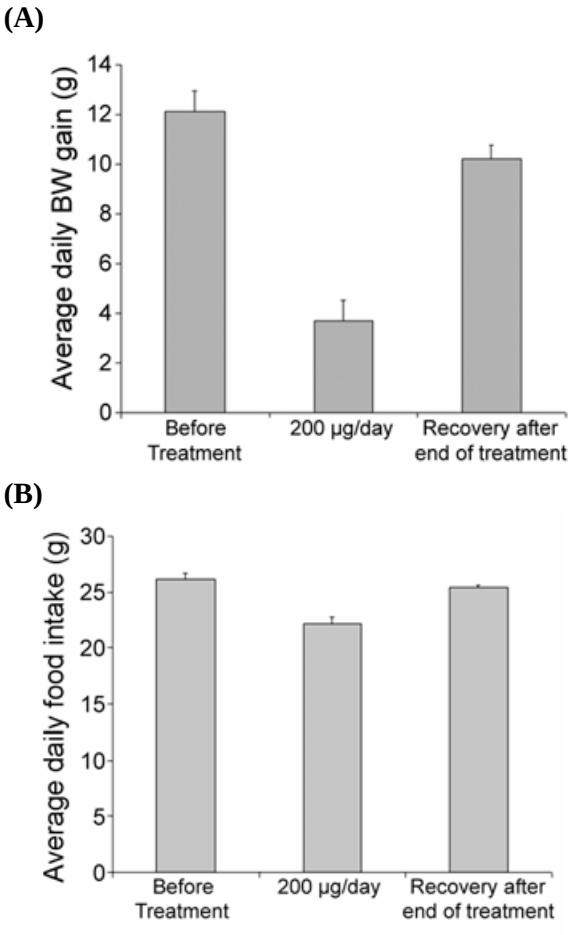


Figure 11. Electron micrograph of the mouse duodenal mucosa sampled after 30 days of daily oral administration of leptin. The duodenal epithelial cells display their normal characteristics with apical microvilli (mv) and junctional complexes (J). M, mitochondria.

and daily food intake were monitored each day. The results obtained, illustrated in figures 12 and 13, demonstrate that rat leptin administered orally to rats is able to reduce their body weight gain. Data clearly demonstrate that while food intake barely declined (from an average of 26.1 ± 0.52 g/day to 22.16 ± 0.62 g/day), increases in body weight were reduced by 70% (from an average of 11.66 ± 1.08 g/day to 3.71 ± 0.79 g/day). Thus upon administration of leptin, these animals slightly reduced their food intake but slowed down their growth. As previously observed with mice, upon ending the treatment, changes were restored to normal. To complete the experiments with the rat, we evaluated the rise in circulating leptin upon oral administration of rat leptin which reached a maximum at 30 minutes. Levels were restored towards their normal values by the third hour after administration. As for the mice, oral leptin reaches blood circulation and target tissues quite rapidly influencing growth of the animals. However differences between these results and those obtained with mice were noticed. Indeed, even in normal mice leptin was able to significantly reduce food intake with decreases in their body weight. In the case of the rat, growth of the animal was the main target with little effects on food intake. Differences exist between the two



Figures 12. Daily oral administration of 200µg of leptin to rats (N=5). Figure 12A. Body weight gain by 200 g rats. Upon daily oral administration of leptin the animals gained only 30% of the weight normally gained by the control animals. This was recovered rapidly upon ending the treatment. Figure 12B. Daily food intake by the rats upon receiving the oral leptin. In contrast to the mice, leptin has little influence on the amount of food ingested by the animals.

experiments, the main ones being the stage of the animals. Mice were adult animals, while rats were still in their growing stage. As we will show and discuss later, leptin does not solely influence food intake but has other target tissues such as the brown adipose tissue that do manage body weight.

A further experiment was performed on rats in order to test the efficiency of human leptin. Rat or human leptin was administered in a comparative way to rat for a period of two days. Gains in body weight as well as food intake were monitored and

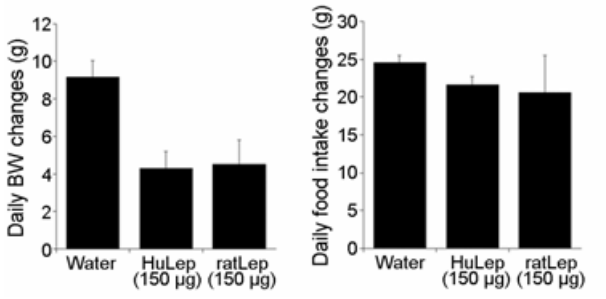


Figure 13. Rat or human leptin was administered orally to rats (N = 3) in order to evaluate the efficiency of both molecules. Both rat leptin as well as human leptin yield exactly the same results in terms of body weight and food intake changes.

demonstrate that both rat as well a human leptins induce the same changes (Figure 13). This indicates that human leptin is physiologically as active as rat leptin in rodents.

In view of the efficiency demonstrated by oral leptin in reaching the hypothalamus and triggering a physiological response in rodents, we pursued the work and carried out further experiments on larger mammals, namely the dog. For such experiments we designed a tablet containing the different elements that allow oral leptin to reach blood circulation. Composition of the tablet was as follows: 0.8 g of sodium bicarbonate, 0.5 g of sodium deoxycholate, 100 mg of trypsin inhibitor, 500 mg of Aprotinin and 1 mg of human leptin (about 100 µg/kg) [36]. These elements were inserted into gelatin capsules and given orally to the animals. Dogs were trained to swallow the pills with 20 ml of water. They were also trained to be fed twice a day at precise times in the morning and afternoon and allowed to eat for a period of 30 minutes, upon which food was removed, weighted and exact amounts of food intake measured. Results have shown that upon oral administration of the leptin pill to young dogs, beagles about one year-old weighting between 8 and 10 kg, leptin appeared in circulation within the first 15 minutes and reached a peak at 60 minutes (Figure 14), which prompted us to administer the pill 60 minutes prior to feeding the animals. Upon reaching its peak, blood levels of exogenous leptin returned to baseline by the third hour (Figure 14).

As expected, upon receiving the oral leptin, the dogs significantly reduced their food intake [36].

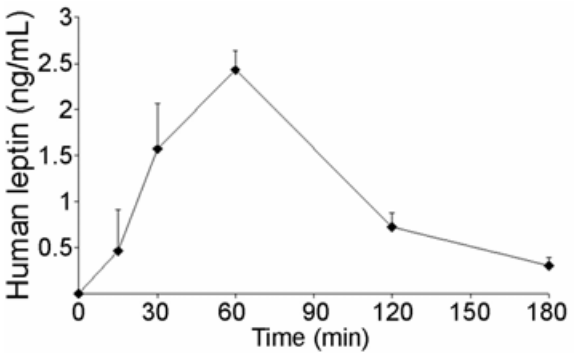


Figure 14. Experiments on dogs. Circulating levels of human leptin upon oral administration of 1 mg of human leptin to dogs (N = 5). Use of an Elisa test specific for human leptin not interfering with dog leptin. High levels of circulating human leptin were reached 60 minutes after administration of the leptin pill. Return to base lane occurs 2 hours later.

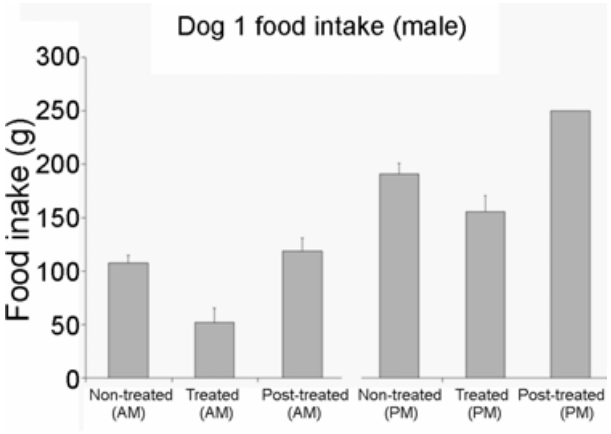


Figure 15. Example of one experiment. Food intake by one male dog upon swallowing a leptin pill containing 1 mg of human leptin. Food intake is notably reduced upon receiving the leptin pill. The effect is more significant in the morning; amounts of food ingested in the morning are less than in the afternoon. Recovery occurs quite rapidly.

Some experiments were performed in early mornings while others in early afternoons, at normal feeding times. As found in rodents, oral administration of leptin influenced food intake (Figure 15). However, the reductions in food intake differed significantly between mornings and afternoons. Indeed, the animals were much more sensitive to the treatment in the mornings (Figure 15). Decreases in food intake in the morning reached an average of 54% while in the afternoon it was only 19%. No major differences were noted between male and female dogs. The experiments also demonstrated that the effect of oral leptin was of short duration influencing food intake within the first hours after administration. Upon discontinuing the leptin treatment, the eating behavior returned immediately even in the same day, back to normal (Figure 15). Blood levels of oral leptin were measured and correlated to amounts of food intake in the same experiment. Figure 16 illustrates such an evaluation and demonstrates that the amount of food intake is directly related to circulating levels of oral leptin with a correlation factor of $R^2 = 0.75$ (Figure 16). This indicates that the effectiveness of the experiment relates on the efficiency of leptin in crossing the intestinal barrier. One possibility that would explain the differences between mornings and afternoons may lie in avoiding leptin degradation at the level of the gastric and pancreatic juices and/or on the efficiency of leptin

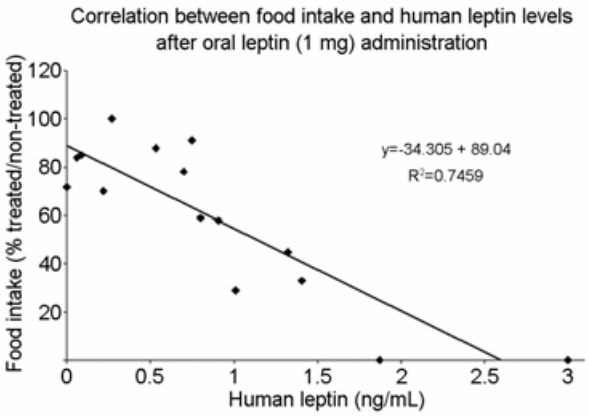


Figure 16. Correlation between food intake and circulating levels of human leptin in dogs. Amounts of food intake are directly proportional to the levels of circulating oral human leptin (N = 15).

to cross the intestinal barrier. Morning leptin administration may well be confronted to smaller amounts of digestive enzymes [67]. Results from these experiments carried out on large animals do confirm those obtained previously on small rodents. However, work still remains to be carried out to design the best protocol for leptin administration [65]. Thus overall, oral leptin appears as a powerful and promising approach for the management of food intake in small rodents as well as in large mammals.

Work was further pursued to assess some physiological actions of oral leptin. We know that leptin regulates adiposity and body weight by controlling food intake and energy homeostasis through its membrane receptors at the level of hypothalamic target cells but it also acts through receptors present at the plasma membrane of some peripheral tissues such as the brown adipose tissue (BAT) [68]. BAT plays crucial roles in thermogenesis, being particularly abundant in hibernating mammals, providing non-shivering thermogenesis for cold acclimation [69]. In humans the existence of BAT has been debated for a long time [70]. It was first found in newborn babies, located under the epithelial layer of the skin [71-73]. BAT allows babies to overcome the drastic temperature changes to which they are confronted at the time of birth. Indeed, upon spending nine months in a controlled environment at 37 °C, newborns are exposed in a matter of minutes to much colder surroundings; they must be able to overcome those changes. The presence of BAT allows them to generate energy and maintain their internal temperature at the suitable 37 °C. Recently the presence of some BAT in adult men has been confirmed particularly in woodcutters working in Nordic areas [74, 75]. Once activated, BAT generates heat. Leptin appears to be one of the hormones that activate BAT and stimulate thermogenesis [68]. A key protein in the activation of BAT is a mitochondrial membrane protein UCP1, a leptin-target protein [76, 77]. Leptin activates UCP1 which in turn increases lipid oxidation, increases lipolysis and decreases fat synthesis leading to a rapid reduction of body weight and adiposity [78].

To evaluate the action of oral leptin on BAT, we administered leptin orally to gerbils for a period of ten days [78]. Their food intake and loss of body weight were monitored daily. Results obtained mimic those on mice with a 55% decrease in food intake and steady decreases in body weight that persisted as long as the leptin treatment was maintained (Figure 17). Morphological examination of BAT was carried out by light and electron microscopy revealing that brown adipocytes which normally display one or two large lipid droplets surrounded by a cytoplasmic layer containing mitochondria and a peripheral nucleus (Figure 18A) underwent major morphological alterations (Figure 18B). The adipocyte peripheral nucleus moved towards the center of the cell; its large lipid droplets broke down into numerous smaller ones (Figure 18B) that underwent lipolysis through autophago-lysosomal activity (Figure 18C); mitochondria increased in number and display abundant cristea and a denser matrix (Figure 18C); blood circulation in the BAT increased with capillaries moving closer to the adipocytes (Figures 18B & 18C). By biochemical evaluation, UCP1, the main protein involved in thermogenesis and present exclusively in BAT mitochondria, was found to increase significantly [78]. Other BAT enzymes involved in lipogenesis were also increased [78]. By the tenth day of oral leptin treatment BAT showed major signs of disappearance. Not interested in losing our leptin-treated animals the experiments were suspended after ten days [78].

These results indicate that leptin once given orally, is able to reach blood circulation and its target cells in different locations of the body such as the hypothalamus and peripheral BAT. Besides acting as a satiety hormone reducing appetite and decreasing food intake, oral leptin stimulates brown adipocytes triggering lipolysis and generating large amounts of heat, thus contributing to major losses of body weight.

Finally it was important for pharmaceutical purposes to evaluate the segment of the digestive track that internalizes leptin most efficiently and transfers it toward blood circulation [65]. Results have shown

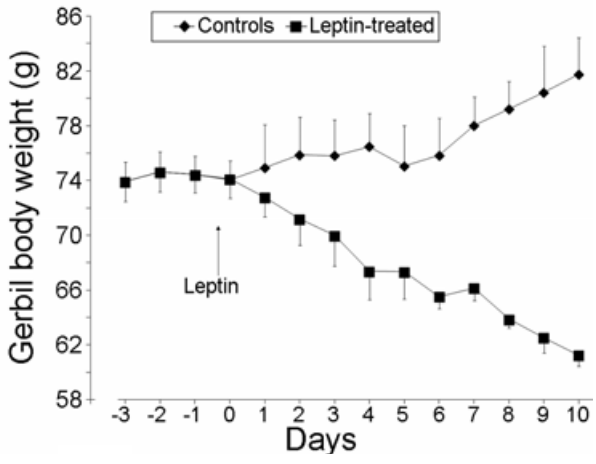
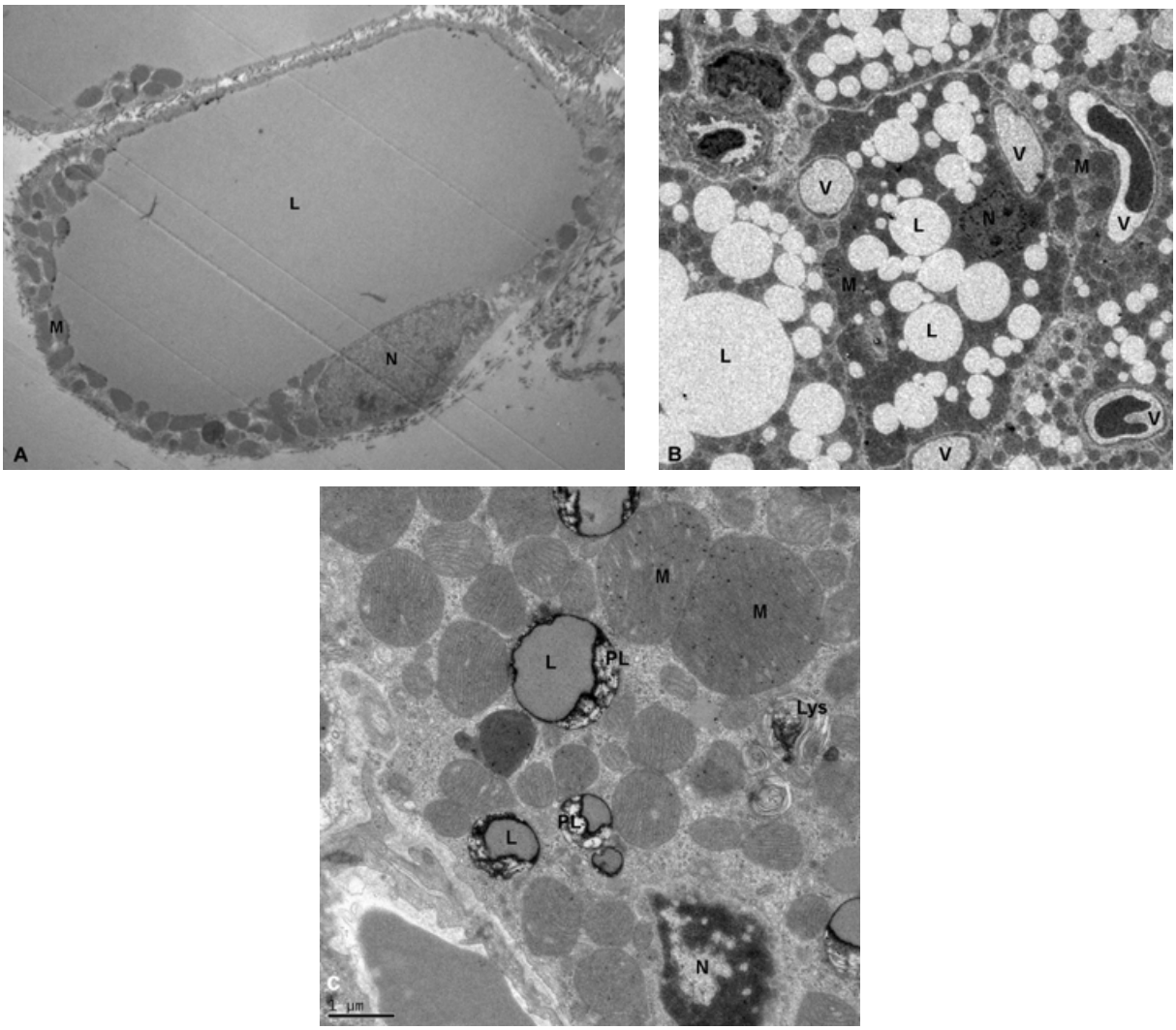


Figure 17. Experiment on gerbils. Changes in body weight upon oral administration of leptin to gerbils (N = 8 for leptin-treated animals vs N = 5 for non-treated animals). Daily oral administration of 100 µg of leptin induced major loss in body weight.



Figures 18. Electron micrographs of adipocytes from the gerbil interscapular brown adipose tissue. Figure 18A. Brown adipose tissue from a control animal not receiving leptin. The adipocyte displays a large lipid droplet (L) surrounded by a wide ring of cytoplasm containing numerous mitochondria (M) and a peripheral nucleus (N). Figure 18B. Brown adipose tissue from an animal receiving 100 µg of oral leptin daily for a period of 3 days. The large lipid droplet has fragmented into numerous smaller ones (L). The nuclei (N) have moved towards the center of the cells. Mitochondria (M) remain abundant. Numerous blood vessels (V) are closely related to the adipocytes. Figure 18C. Brown adipose tissue from an animal receiving 100 µg of oral leptin daily for a period of 8 days. Lipid droplets (L) surrounded by phagolysosomal structures (PL) undergo degradation. Mitochondria (M) are numerous and display a large number of cristea.

that enterocytes in all intestinal segments display leptin receptors on their luminal membrane and have the ability to internalize leptin and transfer it to circulation. However, the ileum appeared as being the most efficient segment [65]. One explanation we could put forward is the fact that being far from the duodenum, it displays mush lower concentrations of pancreatic enzymes and thus leptin could be less degraded prior to internalization.

The second issue tested was the efficiency in the methods of administration [65]. We compared the action of leptin upon its delivery through different paths. We evaluated the functional efficiency of leptin administered either orally, by subcutaneous injection or by intra-peritoneal injection. As can be seen in figure 19, there are major differences in the biological efficiency of the delivered leptin. Orally-administered leptin appears to be by far,

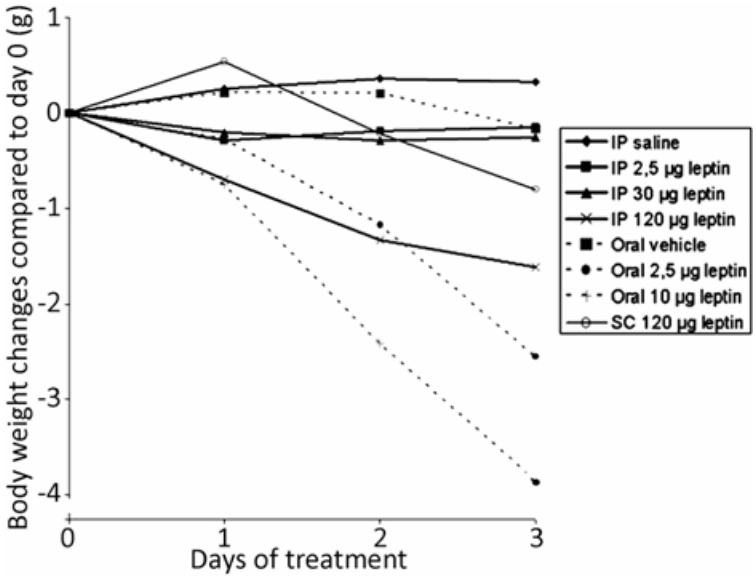


Figure 19. Comparative evaluation of leptin efficiency depending on its method of administration; intraperitoneal (ip), subcutaneous (sc) or oral. The experiment was performed on normal mice. Experiment was carried out for a period of three days. Loss of body weight is particularly significant when leptin is administered orally even in very small amounts (N = 5 in each group).

the one that carries the highest physiological activity. This is due to the fact that as demonstrated before, the circulating hormone is not the free leptin but rather the leptin-leptin receptor complex. Intraperitoneal or subcutaneous injections of leptin lead to peptide reaching blood circulation rapidly in its free form which has a very short half-life. When free leptin is administered orally, it gets first to the gastric cavity and then to the duodenal lumen prior being internalized. While crossing the duodenal cells, in its transit along the endosomal compartment, Golgi apparatus and secretory vesicles, leptin gets associated to its soluble receptor prior to secretion and thus reaches circulation in its complexed form bound to its soluble receptor. This leptin-leptin receptor complex with a much longer half-life interacts with its target cells through normal physiological pathways with optimal efficiency.

Taken together the results presented herein demonstrate that oral administration of leptin is a promising avenue for the leptin replacement therapy and should be considered for regulating food intake in order to control and maintain body weight. Several points should be kept in mind when administering leptin orally: 1- it should be protected from early degradation by gastric and pancreatic juices; 2- the physiologically active hormone is the one bound to its soluble receptor i.e. the leptin-leptin receptor complex; and 3- leptin is not only a satiety factor but it also acts on different systems particularly activating the brown adipose tissue contributing to major losses of body weight.

CONCLUSION

Thus perspectives for an endocrine approach in the treatment of obesity are quite positive and convincing. Earlier we introduced three propositions for dealing with obesity. While exposing at length the results and high potential of oral administration of free leptin, we should not rebuff the two other approaches.

The first one, the oral administration of the leptin-leptin receptor complex will certainly lead to very efficient results since it mimics exactly the physiological condition. One major drawback however would be the preparation of the leptin-leptin receptor complex. Synthesis of the receptor, a quite large protein, will require sophisticated technology. Once prepared and purified, the receptor should be bound to leptin *in vitro* prior to

insertion into a capsule for oral administration. While the technology for preparing such a complex exists, it will certainly require large financial investments and major pharmaceutical labour to generate a functional pill. The required funds and labour will end up being rather expensive and will be reflected in the cost to the patient. The alternative approach, the oral administration of free leptin, exposed in the present study, has demonstrated that anti-proteases carry the work of protecting leptin in the gastric and pancreatic juices quite efficiently. This proposition appears simple, efficient and straightforward. The third approach consisted in the implantation of particular microbiota into the digestive system of obese patients. The genetically modified bacteria will secrete leptin directly in the intestinal lumen and the secreted leptin will follow the physiological path already present in the intestinal system by binding to the leptin receptors present on the apical membrane of the intestinal cells to cross the intestinal barrier and reach circulation. Chen *et al*. [63] have tested with success this approach. However, they did not implant leptin-secreting bacterial. Their study focused on a compound, the NAPE (N-acylphosphatidylethanolamines), synthesized in the small intestine in response to feeding, which reduces food intake with loss of body weight. They have shown that implanting the engineered NAPEs expressing bacteria in the obese mice intestine led in reducing their level of obesity [63]. Thus, appropriate alteration of the gut microbiota could provide sustained treatment for a chronic condition such as obesity and will relieve the need for continuing daily administration of therapeutic compounds. In the case of obesity the strategy would be to alter the gut microbiota incorporating genetically modified bacteria that express and secrete leptin. What remains to be evaluated in this strategy is the amount of leptin secreted by such bacteria and the length of time that the treatment will be efficient. Not being a natural component of the intestinal flora, it is conceivable that the engineered bacteria will not reside for long periods of time in the gut and will need periodical booster administrations. In a certain sense these boosters will allow for roughly controlling amounts of bacteria-secreted-leptin and thus amounts of secreted leptin. Once established this approach would be a very efficient and economic one to deal with the problem of obesity.

This report has put forward options for the efficient delivery of leptin into circulation. Now that we are better aware of the cells that secrete leptin, how it is secreted, and how it circulates and acts at the level of targets tissues, all leading to major control of appetite and adipose tissue mass, the chances of success for the leptin therapy replacement approach in dealing with obesity are markedly improved.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest.

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