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A heliocentric view of leptin

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Leptin is significantly broadening our understanding of the mechanisms underlying neuroendocrine function. Initially, based on a rather static view of the hormone, most investigations focused on the effects of leptin on food intake control and body-weight homeostasis, with attention primarily focused on the implications of leptin as a lipostatic factor and central satiety agent. However, the almost ubiquitous distribution of leptin receptors in peripheral tissues provided a fertile area for investigation and a more dynamic view of leptin started to unfold. This adipocyte-derived circulating peptidic hormone, with a tertiary structure resembling that of members of the long-chain helical cytokine family, has generated an enormous interest in the interaction as well as integration between brain targets and peripheral signals. Considerable evidence for systemic effects of leptin on specific tissues and metabolic pathways indicates that leptin operates both directly and indirectly to orchestrate complex pathophysiological processes. Disentangling the biochemical and molecular mechanisms in which leptin is involved represents one of the major challenges ahead.

OB protein: Obesity: Lipolysis: Reproduction: Angiogenesis: Hypertension

Overview

Animal models long available and commonly used in obesity research have included the genetically-obese, *ob/ob*, mouse and the diabetic, *db/db*, mouse. Both animal models develop obesity early in life, due to hyperphagia and reduced energy expenditure (Coleman, 1978). These mice are also hyperglycaemic, hyperinsulinaemic, hypothermic, stunted and infertile, an array of abnormalities not readily understandable in terms of a single gene defect. The two types of mutant are phenotypically identical when the mutant genes are expressed on the same genetic background. Observations from parabiosis experiments indicated many decades ago that *ob/ob* mice lack a factor in their blood that suppresses eating, whereas *db/db* mice lack the ability to decrease food intake in response to this factor (Hausberger, 1959; Hervey, 1959; Coleman & Hummel, 1969; Coleman, 1973, 1978). The cloning and characterization of the *ob* gene showed that it encodes a 16 kDa protein, which was called OB protein or leptin, a name derived from the Greek root leptos, meaning thin (Zhang *et al*. 1994). The identification that leptin is essential for body-weight homeostasis (Zhang *et al*. 1994; Campfield *et al*. 1995; Halaas *et al*. 1995; Pelleymounter *et al*. 1995) has permanently altered the field of metabolic physiology, with a substantial and rapidly changing body of knowledge being created since then.

Leptin structure

Interestingly, leptin presents striking structural similarities to members of the long-chain helical cytokine family (Madej *et al*. 1995; Zhang *et al*. 1997). The protein is an elongated molecule with approximate dimensions of 2·0 × 2·5 × 4·5 nm. It consists of four anti-parallel α-helices, connected by two long crossover links and one short loop arranged in a left-handed helical bundle, which forms a two-layer packing. The skew angle between the two layers is about 20°. The 167 amino acid sequence of leptin contains two cysteine residues, cysteine 96 and cysteine 146, that form a disulfide bond between the C-terminus of

Abbreviations: OB-R, leptin receptor; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; STAT, signal transducers and activators of transcription.
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the protein and the beginning of one of the loops. This bond appears to be important for structure folding and receptor binding, as mutations of either of the cysteine residues renders the protein biologically inactive. The other most conserved regions observed between OB proteins of different species are located within the four α-helices (Zhang *et al*. 1997). Furthermore, the synthesis and administration of fragment peptides based on the OB protein have shown that leptin activity is localized, at least in part, in the carboxy terminal region of the protein, in domains between residues 106 and 167 (Grasso *et al*. 1997; Frühbeck *et al*. 1998*b*).

Leptin is mainly produced by fat cells and is secreted into the bloodstream (Frühbeck *et al*. 1998*c*; Himms-Hagen, 1999; Ahima & Flier, 2000). However, other tissues such as placenta (Holness *et al*. 1999), mammary epithelium (Casabiell *et al*. 1997; Aoki *et al*. 1999), stomach (Bado *et al*. 1998), muscle (Wang J *et al*. 1998) and brain (Wiesner *et al*. 1999) are also able to produce leptin.

Lipostatic factor

The original concept was that leptin's function was limited only to weight-gain control by reducing food intake as its concentration in blood rises with increasing adiposity. In fact, plasma leptin concentrations are correlated with total fat mass, percentage body fat and BMI, acting as a sensing hormone or 'lipostat' in a negative feedback control from adipose tissue to the hypothalamus, the brain centre responsible for satiety (Tritos & Mantzoros, 1997; Frühbeck *et al*. 1998*c*). Leptin informs the brain about the abundance of body fat, thereby allowing feeding behaviour, metabolism, and endocrine physiology to be coupled to the nutritional state of the organism. An increase in adiposity leads to an increase in circulating leptin concentrations, reducing the animal's appetite and increasing energy expenditure. Conversely, reduced fat stores lead to a decrease in leptin, which in turn leads to an increase in food intake together with a decrease in energy use, i.e. low leptin levels drive the organism to a state of energy sparing, of positive energy balance. In the absence of leptin, as is the case in *ob/ob* mice, animals fail to restrain their food intake, their energy expenditure is reduced and they become massively obese. Leptin-deficient *ob/ob* mice exogenously treated with leptin exhibit a marked body-weight loss with a distinct loss of discernible body fat (Campfield *et al*. 1995; Halaas *et al*. 1995; Pelleymounter *et al*. 1995). This effect is not only attributable to a decreased food intake, but also to an increased BMR, with selective promotion of fat metabolism (Pelleymounter *et al*. 1995; Chen *et al*. 1996; Levin *et al*. 1996; Hwa *et al*. 1997; Shimabukuro *et al*. 1997).

Since leptin was discovered in research directed at finding the cause of obesity, initially all efforts focused in this area. However, it now appears that leptin's major role is not to prevent obesity. Present views are that the signal generated by low leptin concentrations serves to initiate an array of adaptive changes aimed at conserving energy reserves and preventing reproduction during periods of food scarcity (Flier, 1998; Himms-Hagen, 1999). In this sense, reduced availability of leptin is thought to prevent excessive weight loss in order to combat extreme thinness.

Leptin receptors

Tartaglia *et al*. (1995) were the first researchers to isolate the leptin receptor (OB-R) from mouse choroid plexus by an expression cloning strategy. Since the sequence and expression of the initially cloned receptor are normal in *db*/*db* mice, it was suggested that the *db* mutation affected a different receptor or an alternatively-spliced isoform. Subsequent studies showed that the latter explanation proved to be correct. The OB-R belongs to the class I cytokine receptor family, which include receptors for interleukin 6, leukaemia inhibitory factor, granulocyte-colony stimulating factor and glycoprotein 130 (Tartaglia, 1997). The receptor is produced in several alternatively-spliced forms, designated OB-Ra, OB-Rb, OB-Rc, OB-Rd and OB-Re (Lee *et al*. 1996). The receptors have an extracellular domain of 840 amino acids, a domain of thirty-four amino acids and a variable intracellular domain, characteristic for each of the five receptor isoforms. Class I cytokine receptors are known to act through Janus kinases and signal transducers and activators of transcription (STAT). Only the full-length isoform, the OB-Rb, contains intracellular motifs required for activation of the Janus kinases–STAT signal transduction pathway (Chua *et al*. 1996; Ghilardi *et al*. 1996; Vaisse *et al*. 1996; Bjørbæck *et al*. 1997), and is considered to be the functional receptor. Janus kinase proteins are associated with membrane-proximal sequences of the receptor intracellular domain, which is phosphorylated on ligand binding. The phosphorylated intracellular domain then provides a binding site for a STAT protein, which is activated, translocates to the nucleus and stimulates transcription. The lack of the full-length OB-R has been shown to be responsible for the *db*/*db* mouse obesity phenotype and the fatty mutation (Baumann *et al*. 1996; Chua *et al*. 1996; Ghilardi *et al*. 1996; Vaisse *et al*. 1996; Bjørbæck *et al*. 1997; White *et al*. 1997). The OB-Re isoform, which lacks the transmembrane and intracellular domains, may encode a soluble receptor (Lee *et al*. 1996).

Consistent with leptin's role in controlling appetite and energy metabolism, OB-R have been found in the hypothalamus and adjacent brain regions (Tartaglia, 1997; Trayhurn *et al*. 1999). Initially, direct leptin actions were thought to be exclusively restricted to the central nervous system. However, the almost universal distribution of functional OB-R (Cioffi *et al*. 1996; Lee *et al*. 1996; Hoggard *et al*. 1997, 2000; Zamorano *et al*. 1997; Frühbeck *et al*. 1999), which reflects the multiplicity of biological effects in extraneural tissues, is a good example of the extreme functional pleiotropy of leptin (Fig. 1). OB-R are present in organs involved in energy storage, metabolism and digestion, such as skeletal muscle, adipose tissue, pancreas, stomach, small intestine, colon and liver. Functional OB-R are expressed in reproductive organs such as ovaries, uterus and testes. Interestingly, OB-R can be also found in tissues related to immunity, such as spleen, thymus, lymph nodes, haematopoietic cells and T-cells. Other localizations include the endothelium, kidneys, adrenals and heart,

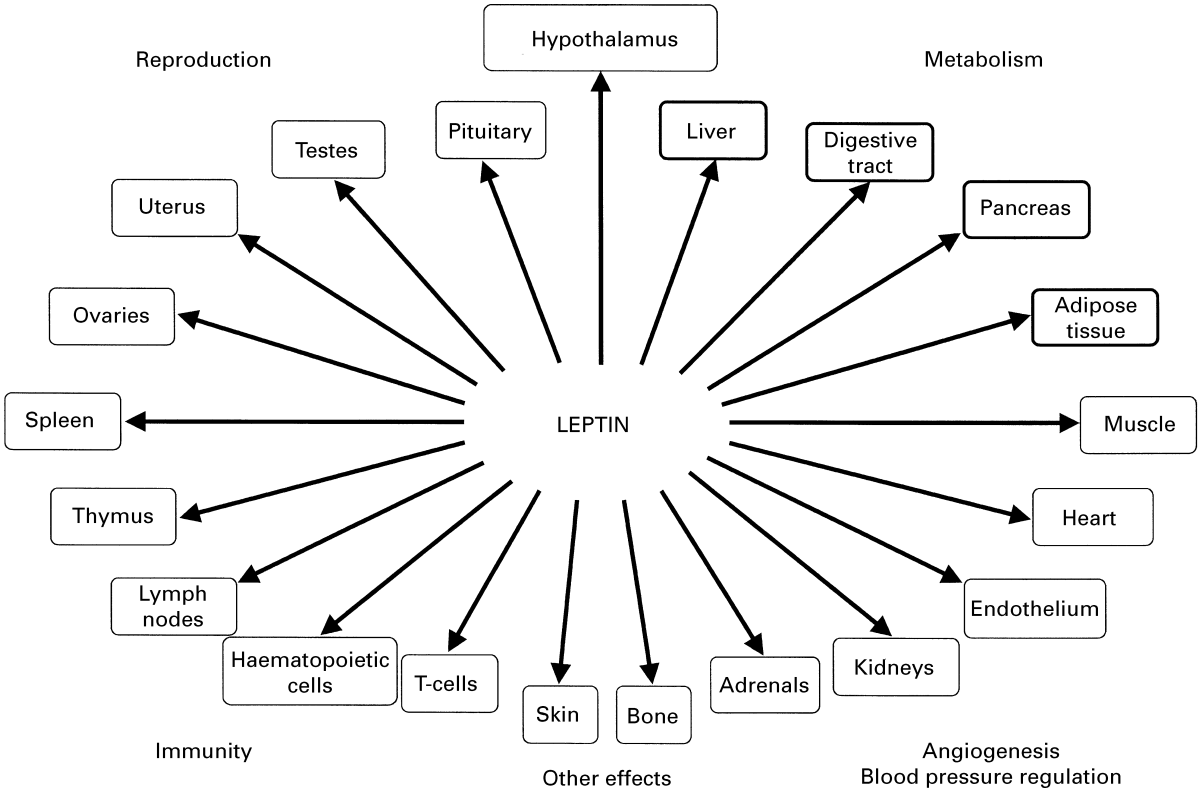

Fig. 1. Localization of functional leptin receptors showing the involvement of leptin in peripheral effects.

tissues involved in angiogenesis and blood pressure regulation.

Relevance of leptin to human obesity

To date, only a few cases of congenital leptin deficiency or OB-R mutation associated with severe early-onset obesity have been reported (Montague *et al*. 1997*a*; Clement *et al*. 1998; Strobel *et al*. 1998). Human *ob* mutations were first reported in two children from a highly-consanguineous Pakistani family (Montague *et al*. 1997*a*). In these cousins, deletion of a single guanine nucleotide in codon 133 led to a frameshift mutation and synthesis of a truncated OB protein that undergoes proteosomal degradation (Rau *et al*. 1999). Treatment with recombinant methionyl leptin resulted in sustained weight reduction and improvement of the metabolic alterations (Farooqi *et al*. 1999). Strobel *et al*. (1998) identified three members of a Turkish family with a homozygous missense mutation in the leptin gene (cytosine → thymine in codon 105, leading to an arginine to tryptophan replacement in the mature protein) resulting in very low plasma leptin concentrations, as the abnormal leptin protein is incapable of being secreted normally (Strobel *et al*. 1998). Human *ob* gene mutations cause severe early-onset obesity, with very low leptin concentrations despite the high fat mass, a marked hyperphagia due to impaired satiety, hyperinsulinaemia and hypothalamic hypogonadism (Montague *et al*. 1997*a*; Strobel *et al*. 1998; Ozata *et al*. 1999). Decreased sympathetic tone and immune system dysfunction are less extensively documented (Ozata *et al*. 1999). Unlike *ob/ob* mice hyperglycaemia, hypercorticism, hypothermia and impairment of linear growth have not been reported in leptin-deficient human subjects (Montague *et al*. 1997*a*; Strobel *et al*. 1998). The reasons for these species differences are unknown, but may suggest substantial differences in the physiological actions of leptin between rodents and man.

Mutations of OB-R are also extremely rare in human subjects. Clement *et al*. (1998) describe a large consanguineous Kabilian family in which three morbidly-obese sisters are homozygous for a splice-site mutation in the OB-R. A substitution in the splice donor site of exon 16 results in a truncated OB-R lacking both the transmembrane and intracellular domains. The mutant OB-R circulates at high concentrations and is capable of binding leptin, but has no signalling function. As with the human *ob* gene mutations, patients who are homozygous for the human *db* mutation suffer from hyperphagia and develop morbid obesity within the first months of life. In addition, pubertal development and functioning of the growth hormone and thyroid axes appear normal in these patients, while the hypothalamic–adrenal axis has not yet been characterised in detail.

The mentioned leptin and OB-R mutations, however, occur very rarely in human subjects, and most obese individuals are neither leptin deficient nor do they lack functional OB-R. Several polymorphisms in the OB-R gene have been identified (Considine *et al*. 1996*a*; Chung *et al*. 1997; Echwald *et al*. 1997; Francke *et al*. 1997; Gotoda

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et al. 1997; Matsuoka *et al*. 1997; Silver *et al*. 1997; Thompson *et al*. 1997; Rolland *et al*. 1998; Chagnon *et al*. 1999), which could possibly cause changes in binding or signalling activity of the receptors. However, until now, none of the studies on these OB-R polymorphisms have shown a major effect on body weight or fat mass (Considine *et al*. 1996*a*; Echwald *et al*. 1997; Francke *et al*. 1997; Gotoda *et al*. 1997; Matsuoka *et al*. 1997; Silver *et al*. 1997; Rolland *et al*. 1998; Chagnon *et al*. 1999).

In most obese patients high leptin concentrations have been found (Hamilton *et al*. 1995; Lönnqvist *et al*. 1995; Maffei *et al*. 1995; Considine *et al*. 1996*b*). Serum leptin concentrations are strongly correlated with estimates of obesity, such as BMI or percentage body fat. In women almost twofold higher leptin concentrations have been found, even when data are adjusted for body fat, revealing a clear gender difference. The hyperleptinaemia observed in obese individuals has been interpreted as a reduced sensitivity to leptin's physiological effects, leading to a compensatory increase in circulating concentrations (Caro *et al*. 1996*b*). Such a resistance can theoretically occur at several levels in the leptin signalling pathway (Fig. 2). Leptin insensitivity may be the result of a production defect, leading to the synthesis of an inactive or less potent form of leptin. Another possibility would be an intravascular defect. Leptin circulates as a monomer in plasma and, other than the single intramolecular disulfide bond, is not post-transcriptionally modified (Cohen *et al*. 1996). In both rodents and human subjects leptin circulates in a free form and also bound to other proteins (Houseknecht *et al*. 1996; Diamond *et al*. 1997; Birkenmeier *et al*. 1998). In human subjects the majority of leptin circulates competitively bound to at least three serum macromolecules with molecular masses of approximately 85, 176, and 240 kDa, which may modulate ligand bioactivity and bioavailability to target tissues (Houseknecht *et al*. 1996). In lean individuals with relatively low adipose tissue depots the majority of leptin is in the bound form, while the proportion of free leptin is increased in serum of obese subjects (Houseknecht *et al*. 1996; Sinha *et al*. 1996*b*). Free leptin may have more rapid turnover because of proteolytic cleavage or increased clearance. This hypothesis is supported by the observation that the half-life of recombinant leptin injected into *ob/ob* mice is much shorter than that in normal mice (Houseknecht *et al*. 1996). During fasting there is a decrease in free leptin concentrations, which is more pronounced in lean subjects as compared with obese individuals, whereas no change is observed in bound leptin in either group (Sinha *et al*. 1996*b*). It is possible that the free:total leptin may not be constant, but rather that a dynamic equilibrium exists between the circulating binding proteins and free leptin, and that this balance may be affected by the metabolic state. Precedent for an important role for binding proteins in the transport or uptake of ligands has been demonstrated for other members of the cytokine family. Additionally, for some cytokines and haematopoietic growth factors, association with binding proteins potentiates ligand activity because of biochemical modifications (Heaney & Golde,

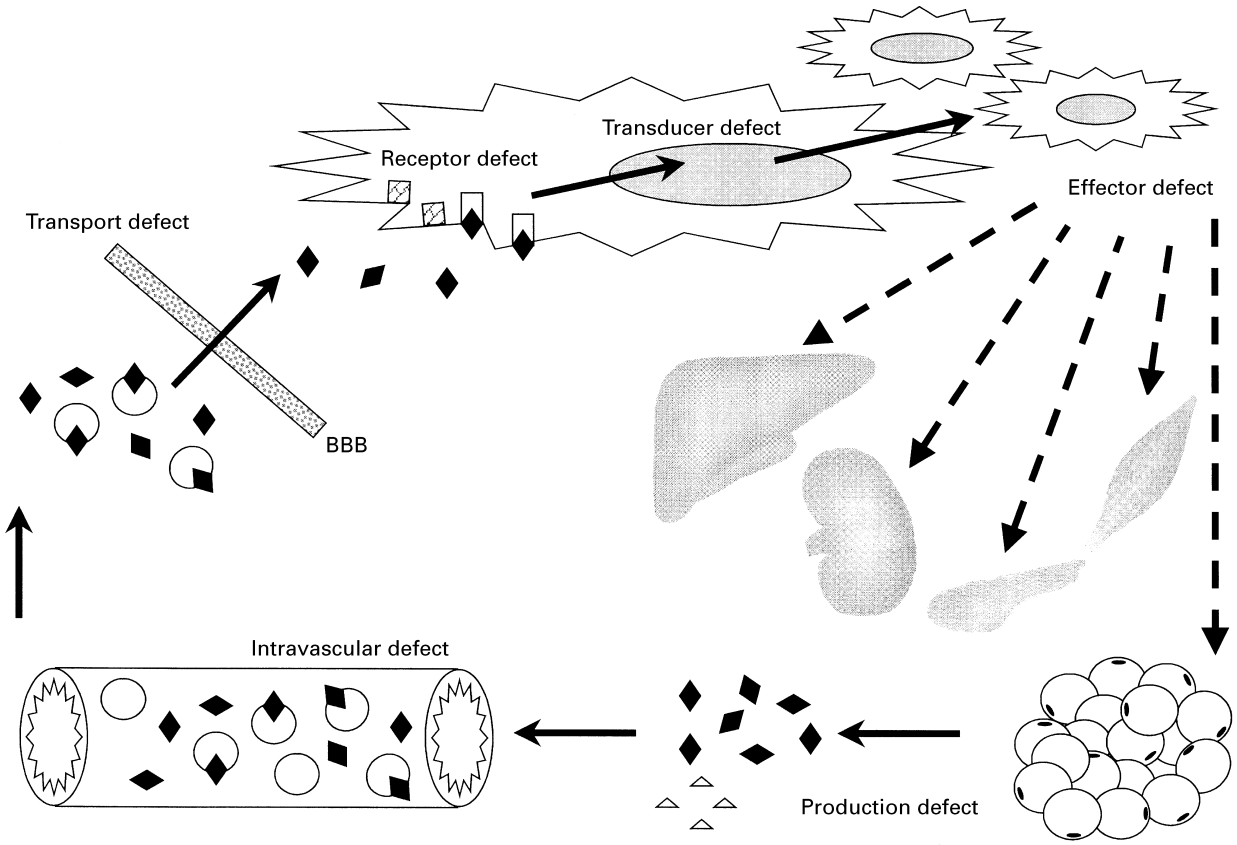

Fig. 2. Potential sites of leptin defects in relation to hyperleptinaemia. BBB, blood–brain barrier.

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1993). These phenomena provide possible explanations for apparent leptin resistance in the context of increased free leptin. Furthermore, the role of binding proteins in regulating the amount of biologically-active leptin may vary by gender and contribute to differences in the physiology of leptin action.

Diurnal and ultradian oscillations are essential physiological characteristics of hormone secretion. Leptin is characterised by nyctohemeral rhythms, with serum leptin levels being highest between midnight and early morning hours and lowest about noon to mid-afternoon (Sinha *et al*. 1996*a*). The nocturnal increase in serum leptin closely resembles the circadian rhythmicity of thyrotropin, prolactin, free fatty acids and melatonin, and precedes those of cortisol and growth hormone (Van Cauter, 1990). Superimposed on the circadian rhythm, total circulating leptin concentrations exhibit a pattern indicative of pulsatile release, with a pulse duration of approximately 30 min, which is inversely related to rapid fluctuations in plasma cortisol and adrenocorticotropic hormone (Sinha *et al*. 1996*c*; Licinio *et al*. 1997). As compared with lean subjects, obese individuals show a sevenfold increase in pulse height, with preservation of both diurnal variation and concentration-independent pulse variables, such as pulse number per 24 h, pulse duration, interpeak interval and pulse height expressed as the percentage of increase over preceding baseline (Licinio *et al*. 1997). Since pulsatility is crucial for the attainment of biological effects in several endocrine systems, it is reasonable to speculate that for the maximal biological effectiveness of leptin, pulsatility may be an important requirement (Frühbeck *et al*. 2000). It is interesting to point out that a high dosage is needed for non-pulsatile administration of leptin to induce weight loss. At present, the physiological significance of pulsatile leptin secretion is unknown, as is the mechanism involved in generating leptin pulses, especially as adipocyte-specific *ob* gene expression and regional differences in adipose tissue have been reported (Masuzaki *et al*. 1996; Montague *et al*. 1997*b*).

A further explanation for the leptin insensitivity observed in the majority of obese individuals is the existence of a transport problem at the blood–brain barrier (Fig. 2). Despite having a fourfold increase in serum leptin concentrations, obese subjects show only a modest increase in cerebrospinal fluid leptin concentrations (Caro *et al*. 1996*a*). A reduced efficiency of brain leptin delivery among obese individuals with hyperleptinaemia may result in the apparent leptin resistance. Leptin insensitivity may also result from a diminished response to leptin at the target cell level due to mutant receptors or deficiencies in the intracellular signalling cascade. Experimental evidence suggests that suppressor-of-cytokine signalling 3 is a leptin-inducible inhibitor of leptin signalling and a potential mediator of leptin resistance (Bjørbæck *et al*. 1998, 1999). Another cause of leptin insensitivity would arise from impairments in the transducer and effector systems. In this sense, it is tempting to speculate that different tissues may exhibit different concentration thresholds for leptin resistance.

Regulation of leptin production

Adipose tissue is the primary site for energy storage and release in response to the changing energy needs of the organism. Since leptin is secreted by fat cells in proportion to body fat stores, it has the potential to play a key regulatory role in fuel homeostasis. The regulation of *ob* gene expression in adipose tissue has been reviewed extensively (Trayhurn *et al*. 1999; Harris, 2000). As for many other physiological processes, leptin production in adipose tissue is under nutritional, hormonal and neural regulation (Table 1). Fasting induces a fall in the level of *ob* mRNA, which is rapidly reversed on refeeding, and circulating leptin concentrations change in a parallel manner to tissue mRNA (MacDougald *et al*. 1995; Saladin *et al*. 1995; Trayhurn

Table 1. Regulation of leptin expression

Localization	Increase	Decrease
White adipose tissue	Positive energy balance	Negative energy balance
	Overfeeding	Fasting
	Insulin	Sympathetic nervous activity
	Glucocorticoids	Thyroid hormones (?)
	Oestrogens	Androgens
	Prolactin	Growth hormone
	Tumour necrosis factor α	Interleukin 6
	Infection, sepsis	β-adrenergic agonists, thiazolidinediones
Placenta	Insulin	Low gestational weight
	Glucocorticoids	Nicotine–smoking
	Hypoxia	
	Pre-eclampsia	
Mammary gland		Inhibition of pulsatile LH secretion
		Prolactin (?)
Gastric fundic mucosa	Refeeding	Fasting
	Distension (vagal afferents)	Cholecystokinin
		Gastrin
Skeletal muscle	Hexosamine pathway	
	Glucose and lipid infusion	

LH, luteinising hormone.

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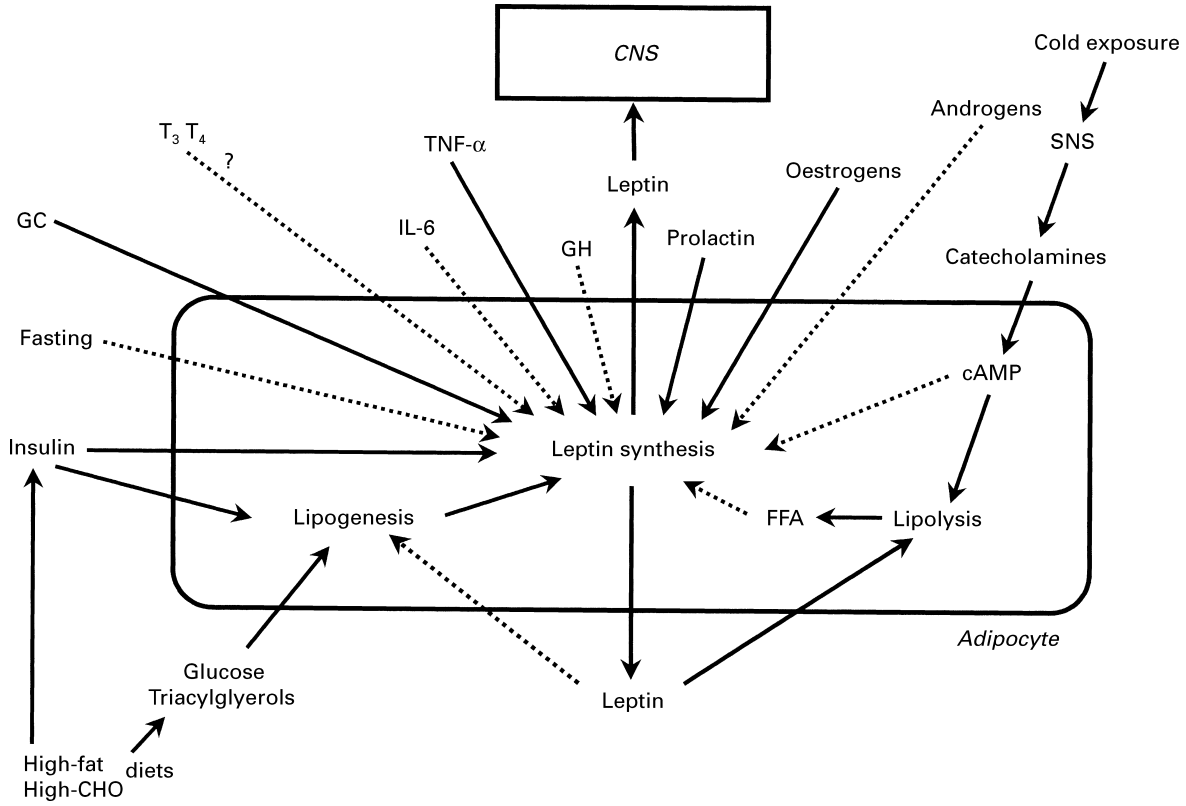

Fig. 3. Regulation of leptin production in white adipocytes. (—), Stimulation of leptin synthesis; (…), hormone release inhibition. GC, glucocorticoids; CHO, carbohydrate; T_3, T_4, thyroid hormones; IL-6, interleukin 6; TNF-α, tumour necrosis factor α; GH, growth hormone; CNS, central nervous system; FFA, free fatty acids; SNS, sympathetic nervous system. (Modified from Frühbeck *et al*. 1998*c*.)

et al. 1995*b*; Weigle *et al*. 1997). High-fat diets as well as high-carbohydrate diets are known to increase lipogenesis and, consequently, stimulate leptin synthesis (Ahren *et al*. 1997; Jenkins *et al*. 1997). As illustrated in Fig. 3, insulin stimulates *ob* gene expression, as do glucocorticoids, with the effects of the latter being maintained during chronic treatment (MacDougald *et al*. 1995; Saladin *et al*. 1995; Frühbeck & Salvador, 2000*a*). Although plasma leptin, thyroid-stimulating hormone, and adiposity correlate in euthyroid patients, there are conflicting reports on the effect of hypo- and hyperthyroidism on *ob* gene expression (Ahima & Flier, 2000). Some studies have described an increase in plasma leptin in hypothyroid patients and a decrease in hyperthyroidism, but other studies have shown no significant alteration in leptin concentrations in these conditions or in response to thyroxine-replacement treatment (Mantzoros *et al*. 1997*c*; Diekman *et al*. 1998; Ozata *et al*. 1998; Pinkney *et al*. 1998). The striking sexual dimorphism is evident in both *ob* mRNA expression and the correlation between leptin concentrations and fat mass. Some researchers attribute the observed gender differences to the stimulating role of oestrogens and/or the suppressive effect of circulating androgens (Rosenbaum *et al*. 1996; Kennedy *et al*. 1997), but other investigators have not been able to ascribe the sexual dimorphism to sex hormones (Saad *et al*. 1997). Recently, prolactin has also been demonstrated to induce *ob* mRNA in white adipose tissue as well as to stimulate leptin synthesis and secretion (Gualillo *et al*. 1999). An inverse relationship between leptin and growth hormone concentrations has been reported. Circulating leptin has been observed to fall promptly in response to growth hormone-replacement therapy, even in the absence of changes in BMI (Fisker *et al*. 1997; Florkowski *et al*. 1996).

Cold exposure induces a sympathetically-mediated suppression of the *ob* gene, leading to a rapid decrease in both *ob* mRNA and serum leptin concentrations (Trayhurn *et al*. 1995*a*; Trayhurn, 1996). Furthermore, a positive and independent association between tumour necrosis factor α levels and circulating leptin concentrations has been reported (Mantzoros *et al*. 1997*b*). Tumour necrosis factor α induces the release of both interleukin 6 and leptin from adipose tissue (Grunfeld *et al*. 1996). Knockout mice for the tumour necrosis factor α gene show a hypoleptinaemia compared with wild-type mice (Kirchgessner *et al*. 1997). While tumour necrosis factor α has a stimulatory effect, interleukin 6 exerts an inhibitory action on leptin production. Like many other adipocyte genes, the *ob* gene promoter is positively regulated through a functional binding site for CCAAT/enhancer-binding protein α (He *et al*. 1995; Miller *et al*. 1996). In contrast, thiazolidinedione, a ligand for peroxisome proliferator-activated receptor γ transcription factors, suppresses leptin expression (De Vos *et al*. 1996). This process may partly involve a functional antagonism between CCAAT/enhancer-binding protein α and peroxisome proliferator-activated receptor γ on the leptin promoter.

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'Geocentric *v.* heliocentric' view of leptin

Before Copernicus and Galileo the geocentric model placed the earth at the centre of the universe and all celestial bodies, including the sun, were thought to revolve around it. The heliocentric model proposed by these two astronomers, on the contrary, identified the sun as the centre of the universe, asserting that the earth and all other planets travel around the sun. This proposal changed forever the understanding of the cosmos and, in a way, a close parallelism can be drawn to the present knowledge of leptin. At the beginning, in what we can call the 'geocentric view' of leptin, the brain was considered the centre of all leptin effects. However, this first concept has evolved to a 'heliocentric view' in which leptin is being placed at the centre and the different organs target for this hormone. Obviously, in this model the peripheral effects of leptin are considered equally relevant to the actions exerted at the hypothalamic level.

Pleiotropic effects of leptin

The 'heliocentric view' of leptin is supported by the almost universal distribution of OB-R, which reflects the multiplicity of biological effects in extraneural tissues. The initial, rather simplistic, notion that leptin participates only in food intake and body weight has evolved considerably. Leptin was discovered on the basis of a very specific biological action consisting in its involvement in body-weight and appetite regulation. Many cytokines, originally isolated on the basis of a particular biological action, have subsequently been shown to be capable of stimulating a variety of biological responses in a wide spectrum of cell types. Thus, leptin shares with other cytokines an extreme functional pleiotropy and has been shown to be involved in quite diverse physiological functions, such as reproduction (Holness *et al*. 1999), haematopoiesis (Cioffi *et al*. 1996), angiogenesis (Sierra-Honigmann *et al*. 1998), immune responsiveness (Lord *et al*. 1998), blood pressure control (Frühbeck, 1999) and bone formation (Ducy *et al*. 2000).

Reproductive physiology

Leptin quickly proved to play an important role in reproductive physiology (Hoggard *et al*. 1998; Holness *et al*. 1999; Chehab, 2000). Sterility was a well-recognized feature in *ob/ob* mice. Exogenous administration of leptin to these mice increased the weight of ovaries and uterus, which is consistent with a trophic action of leptin on gonadal function. Long-term injections of leptin have been shown to correct the sterility of both female (Chehab *et al*. 1996) and male (Mounzih *et al*. 1997) adult *ob/ob* mice, which does not appear to be a consequence of weight change *per se*, since weight loss in control *ob/ob* animals due to food restriction did not ameliorate their infertility (Chehab *et al*. 1996; Mounzih *et al*. 1997). In addition, leptin has been shown to accelerate the onset of puberty in normal mice. Normal prepubertal female mice injected with leptin experienced an earlier maturation of the reproductive tract accompanied by a precocious onset of classic pubertal signs like vaginal opening, oestrus and cycling (Chehab *et al*. 1997). In accordance with these findings, leptin is increased

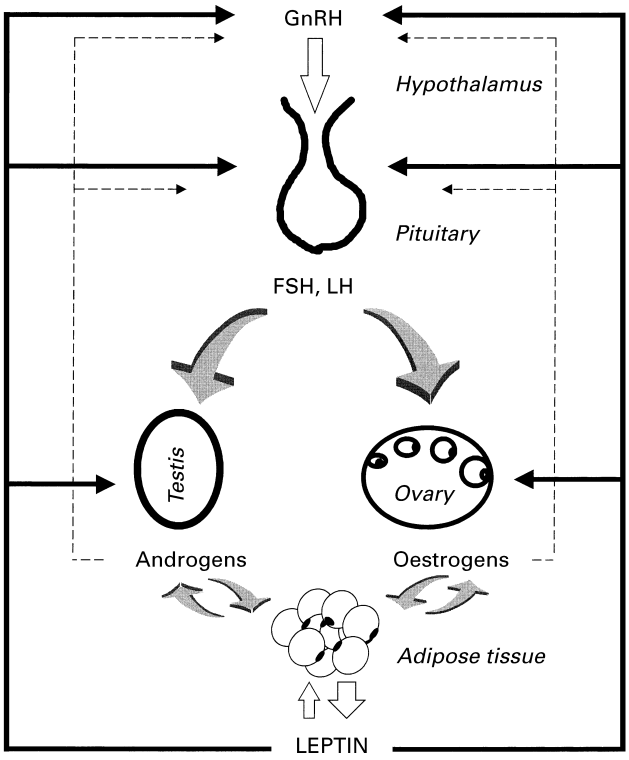

Fig. 4. Diagram of the probable involvement of leptin in the hypothalamic–pituitary–gonadal axis. (—) Enhancement of endocrine secretion; (– – –), hormone-release suppression. GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinising hormone.

in both boys and girls before the appearance of other reproductive hormones related to puberty (Mantzoros *et al*. 1997*a*; Garcia-Mayor *et al*. 1997). Thus, leptin signals the adequacy of energy stores and seems to be needed for the initiation of puberty and establishment of secondary sexual characteristics by interacting with different target organs in the hypothalamic–pituitary–gonadal axis (Fig. 4; Frühbeck, 1997; Frühbeck *et al*. 1998*c*).

In human subjects serum leptin concentrations have been shown to be higher in the luteal phase than in the follicular phase (Hardie *et al*. 1997; Shimizu *et al*. 1997). The relationship between BMI and circulating leptin has been observed to vary during the course of spontaneous cycles, the best correlation occurring during the luteal phase when progesterone and leptin concentrations are highest (Hardie *et al*. 1997). The leptin peak follows the surge of oestradiol and luteinising hormone. Moreover, leptin concentrations were found to be higher in premenopausal women than in post-menopausal women (Rosenbaum *et al*. 1996; Shimizu *et al*. 1997), indicating that oestrogens are implicated in the regulation of leptin production. However, other researchers were unable to detect differences in leptin concentrations in relation to menopause (Saad *et al*. 1997).

Since pregnancy entails many physiological changes, in part as a consequence of endocrine adaptation, leptin's role in pregnancy has been addressed. In pregnant women plasma leptin concentrations have been shown to be augmented, especially during the second and third trimesters (Hardie *et al*. 1997), but they do not correlate with maternal

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weight or BMI at the beginning of pregnancy and at term (Butte *et al*. 1997; Masuzaki *et al*. 1997; Schubring *et al*. 1997). Within 24 h of delivery plasma leptin concentrations return to normal. Potential explanations for the elevated leptin concentrations in pregnancy include an increased production by maternal fat depots, as it is known that during pregnancy there is increased secretion of a number of hormones which have a stimulatory effect on leptin expression in adipocytes, such as oestradiol, insulin and cortisol. Another possibility could be increased circulating concentrations of binding proteins. The soluble form of the OB-R is increased in maternal serum, binding circulating leptin (Gavrilova *et al*. 1997). This factor may protect leptin from degradation or excretion. Moreover, placental leptin may contribute to the increased maternal concentrations (Masuzaki *et al*. 1997).

Body weight and body composition change dramatically in fetuses and newborn infants. In view of the rise in fetal leptin observed in fetal cord blood (Matsuda *et al*. 1997; Schubring *et al*. 1997), a possible involvement for leptin in fetal development has been suggested. Hoggard *et al*. (1997, 1998) have observed high levels of gene expression for leptin and OB-Rb in the placenta as well as in fetal tissues, pointing to the fact that leptin may play a role in the growth and development of the fetus. High levels of leptin gene expression have been observed in cartilage and bone, in particular in the vertebrae, ribs and hindlimb digits. This finding may imply a role for leptin in fetal bone development, which may be linked also with haematopoiesis, while the presence of leptin in hair follicles may be related to leptin's role in thermoregulation (Hoggard *et al*. 1997, 1998). In brain the OB protein was found in leptomeninges and the choroid plexus. However, the absence of leptin does not prevent fetal growth, as two children with leptin deficiency are known to have grown at a normal rate (Montague *et al*. 1997*a*). Leptin may be involved in the control of maternal nutrient availability as well as in fetal energy homeostasis, as serum leptin has been shown to correlate with fetal body-weight gain (Harigaya *et al*. 1997). It is conceivable, therefore, that leptin predetermines a body-weight set point imprint, which is carried forward postnatally and into adulthood.

Immunity

Interestingly, OB-R have been detected in tissues related to immunity, such as spleen, thymus, lymph nodes, haematopoietic cells and T-cells (Fig. 1). The functional OB-R has been shown to be capable of signalling for cell survival, proliferation and differentiation into macrophages (Cioffi *et al*. 1996; Gainsford *et al*. 1996). In addition, leptin appears to be able to enhance the production of cytokines in macrophages and to increase the attachment and subsequent receptor-mediated process of phagocytosis (Gainsford *et al*. 1996). This activity may be mediated by an up regulation of macrophage receptors or by an increased phagocytic activity. More than 20 years ago researchers showed that *ob/ob* and *db/db* mice have an impaired cell-mediated immunity. Lord *et al*. (1998) explored the potential immunomodulatory effects of leptin and showed a marked dose-dependent alloproliferative increase in T-cells. Furthermore, leptin was reported to oppose the inhibitory effects of starvation on T-cell priming, revealing an adaptive response of this hormone to enhance the immune competence of the organism against the immunosuppression associated with starvation. Thymic atrophy is a prominent feature of malnutrition. Starvation of normal mice for 48 h has been reported to reduce the total thymocyte count to 13% of that observed in freely-fed controls, predominantly due to a decrease in the cortical thymocyte subpopulation (Howard *et al*. 1999). Prevention of the fasting-induced fall in leptin concentrations by exogenous administration of the recombinant hormone has been shown to protect mice from these starvation-induced thymic changes. In *ob/ob* mice a marked reduction in the size and cellularity of the thymus has been observed together with a high level of thymocyte apoptosis, resulting in a cortical:precursor thymocytes that was fourfold lower than that observed in wild-type mice. Peripheral administration of recombinant leptin to *ob/ob* mice has been shown to reduce thymocyte apoptosis and substantially increase both thymic cellularity and cortical:precursor thymocytes. These findings indicate that reduced circulating leptin concentrations are pivotal in the pathogenesis of starvation-induced lymphoid atrophy (Howard *et al*. 1999).

Angiogenesis and wound healing

Leptin has to be included in the list of angiogenic factors, as it has been shown to cause cultured endothelial cells to aggregate, form tubes and display a reticular array reminiscent of tissue vasculature (Bouloumie *et al*. 1998; Sierra-Honigmann *et al*. 1998). These effects, tested in both *in vitro* and *in vivo* models of angiogenesis, indicate that leptin, via activation of the endothelial OB-R, generates a growth signal involving a tyrosine kinase-dependent intracellular pathway that contributes to the promotion of angiogenic processes.

Topically-administered as well as systematically-administered leptin has been reported to improve re-epithelialization of wounds in *ob/ob* mice (Frank *et al*. 2000). Leptin completely reversed the atrophied morphology of the migrating epithelial tongue observed at the wound margins of leptin-deficient animals into a well-organised hyperproliferative epithelium. Moreover, topically-supplemented leptin accelerated normal wound-healing conditions in wild-type mice. Proliferating keratinocytes located at the wound margins specifically expressed the functional OB-R subtype during skin repair. Additionally, leptin has been shown to mediate *in vitro* a mitogenic stimulus to human keratinocytes (Frank *et al*. 2000).

Inflammation and vascularization play an important role in tissue healing after injury. In this sense, the activation of the immune system by leptin together with the angiogenic and wound-healing effects of the hormone may prove to be of extraordinary physiological relevance. Leptin may participate in the development of an inflammatory reaction in infarcted tissue and accelerate tissue repair. The involvement of leptin in the signalling cascade following myocardial infarction is feasible both from a molecular and functional point of view (Frühbeck & Salvador, 2000*b*).

Interestingly, a worse clinical outcome after acute myocardial infarction is observed in obesity, where a state of leptin resistance has been proposed. The study of the potential participation of leptin may provide valuable information concerning cooperativity among different signalling systems and further the understanding of how the induction of cytokines operates in a cascade fashion.

Bone development

The expression of high levels of leptin and OB-R in fetal bone and cartilage implies a role for leptin in skeletal development. Recently, leptin has been identified as a potent inhibitor of bone formation, acting through the central nervous system (Ducy *et al*. 2000). Despite suffering from hypogonadism and hypercortisolism, known inducers of increased osteoclast number and bone resorption activity, leptin-deficient and OB-R-deficient mice exhibit a high bone mass phenotype. Interestingly, this phenotype is not secondary to obesity, but is directly related to the lack of leptin signalling. Intracerebroventricular infusion of leptin to *ob/ob* and wild-type mice has been shown to be followed by a significant bone mass reduction ($P < 0{\cdot}05$).

Lipolysis

Since functional OB-R are present in white adipose tissue, the potential role of leptin in regulating lipolysis has also been studied. Adenoviral transfer of the leptin gene into rats has been shown to dramatically reduce tissue triacylglycerol stores compared with pair-fed controls; evidence of a role for leptin in lipid metabolism beyond its appetite-reducing properties (Chen *et al*. 1996; Shimabukuro *et al*. 1997). The lipopenic action of hyperleptinaemia on adipocytes has been reported not to be mediated by neurotransmitted signals from the central nervous system (Wang *et al*. 1999). Moreover, the same group has demonstrated a novel form of lipolysis by which the leptin-induced glycerol release is not accompanied by a rise in plasma free fatty acids (Wang M-Y *et al*. 1998). Previous studies had shown an autocrine–paracrine lipolytic effect of leptin on white adipose tissue both *in vitro* and *in vivo* (Frühbeck *et al*. 1997, 1998*a*). In addition, leptin has been shown to repress acetyl-CoA carboxylase gene expression, fatty acid synthesis and lipid synthesis; biochemical reactions that contribute to lipid accumulation without the participation of centrally-mediated pathways (Bai *et al*. 1996; Wang M-Y *et al*. 1998). Thus, leptin is involved in the direct regulation of adipose tissue metabolism by both inhibiting lipogenesis and stimulating lipolysis. The mechanisms of leptin-induced lipolysis, however, still remain to be completely elucidated.

Until recently, the adipocyte has been considered to be only a passive tissue for the storage of excess energy in the form of fat (Flier, 1995). However, there is now compelling evidence that adipocytes act as endocrine, secretory cells (Flier, 1995; Lau *et al*. 1996; Serrero & Lepak, 1996). It has been shown that several hormones, growth factors, cytokines and their respective soluble receptors are actually expressed in white adipose tissue, with a wide range of signals emanating from adipocytes (Fig. 5). NO synthase has been also reported to be expressed in rat white adipose tissue, indicating that adipocytes are a potential source of

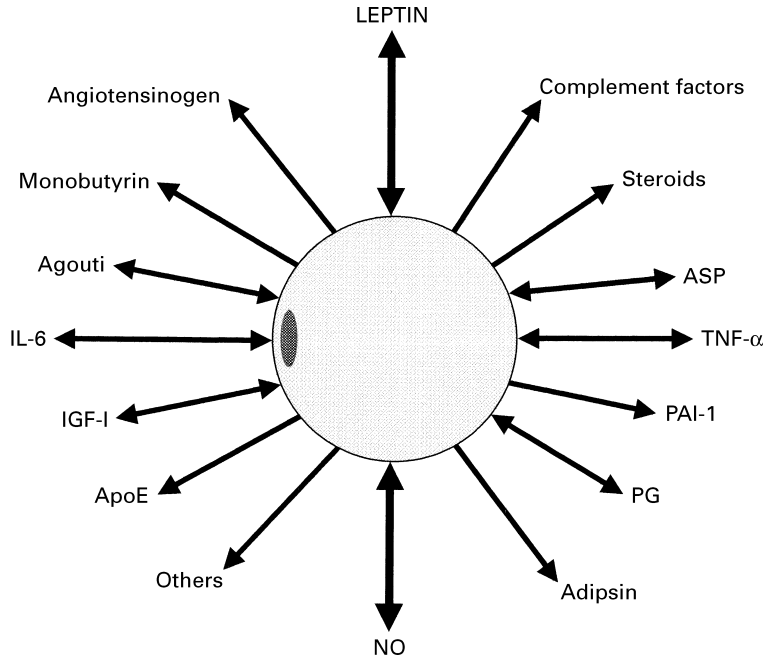

Fig. 5. Dynamic view of white adipose tissue in which a wide range of signals emanating from and impinging on the adipocyte are represented. IL-6, interleukin 6; IGF-I, insulin-like growth factor-I; ApoE, apolipoprotein E; ASP, acylation-stimulating protein; TNF-α, tumour necrosis factor α; PAI-1, plasminogen-activator inhibitor-1; PG, prostaglandins.

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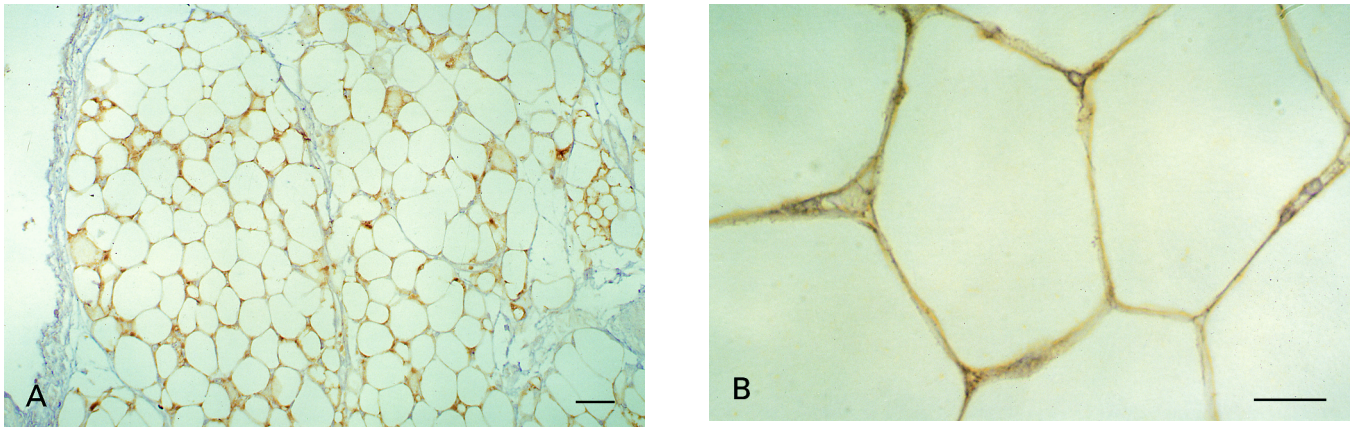

Fig. 6. Paraffin sections of rat visceral white adipose tissue immunostained for inducible NO synthase. The brownish stain appears in the thin cytoplasmic rim of the adipocytes while connective tissue, which does not contain inducible NO synthase is blue. Note that the stain is more intense in some adipocytes at a multilocular stage of differentiation. A, × 440 (scale bar 40 μm); B, ×1100 (scale bar 10 μm).

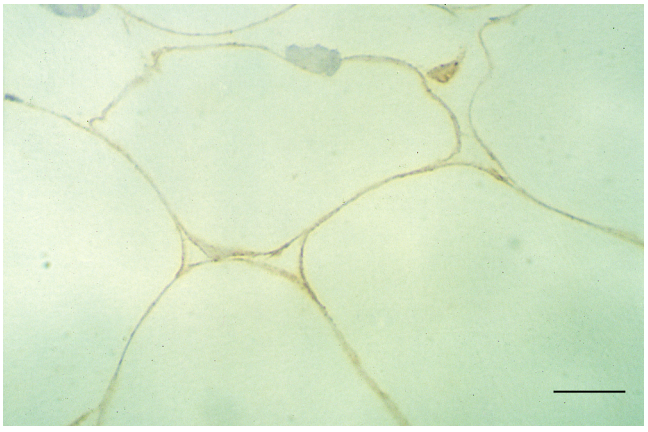

Fig. 7. Semithin section of leptin immunolabelling of rodent adipocytes. Note that the staining pattern is very similar to that of inducible NO synthase (×1100; scale bar 10 μm).

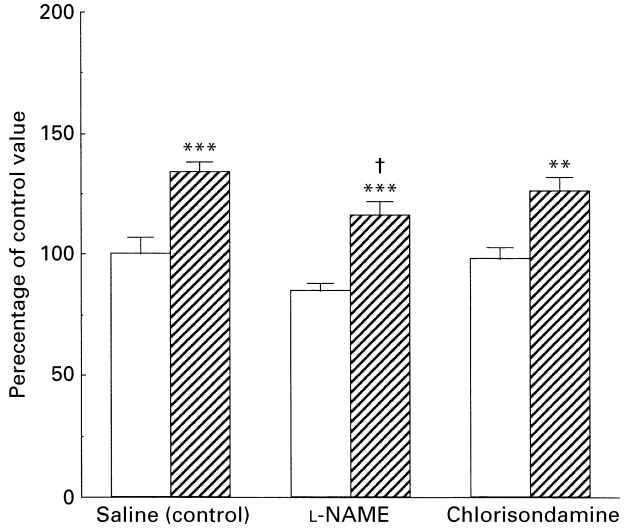

Fig. 8. Effect on basal lipolysis of fat cells obtained from Wistar rats under pharmacological pretreatment consisting of intravenous administration of vehicle (saline; 9 g NaCl/l), nitric oxide synthase inhibition (N$^{\omega}$-nitro-L-arginine methyl ester; 30 mg/kg body weight) or acute ganglionic blockade (chlorisondamine; 30 mg/kg body weight) followed by injection of either saline (□) or leptin (▨; 100 μg/kg body weight). The lipolytic activity was measured as the amount of glycerol released after 90 min by isolated adipocytes. Results are expressed as the percentage of basal lipolysis of fat cells from saline-treated control animals and are means with their standard errors represented by vertical bars for eight rats per group (lipolytic experiments were performed in duplicate). Statistical comparisons were made by ANOVA and Scheffe's *post hoc* pair-wise comparisons. Mean values were significantly different from those for saline-treated controls within the same pharmacological pretreatment group: $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$. Mean values were significantly different from those for leptin-treated control animals: $\dagger P<0{\cdot}05$.

NO production (Ribière *et al*. 1996). Recently, evidence for an involvement of NO in both rat and human lipolysis has been published (Gaudiot *et al*. 1998; Andersson *et al*. 1999). Interestingly, leptin immunolabelling of white adipocytes exhibits an absolutely superimposable staining pattern to that of inducible NO synthase, as can be observed in histological sections (Figs. 6 and 7).

Taking into consideration the morphological and physiological resemblance between NO and leptin, the potential role of NO in the leptin-induced effects on lipolysis was investigated (Frühbeck & Gómez-Ambrosi, 2000*a,b*). Leptin administration significantly increased ($P<0{\cdot}001$) serum NO concentrations in a dose-dependent manner. Simultaneously, a statistically significant ($P=0{\cdot}0001$) dose-dependent increase in the basal lipolytic rate was observed 1 h after exogenous leptin administration. Simple linear regression analysis showed that the lipolytic rate measured in white adipose tissue was significantly correlated with serum NO concentrations (r 0·52; $P=0{\cdot}0025$), with 27% of the variability taking place in lipolysis being attributable to the changes in NO concentrations. Under NO synthesis inhibition by N$^{\omega}$-nitro-L-arginine methyl ester pretreatment, the leptin-induced stimulation of lipolysis was significantly reduced compared with the leptin-treated control animals ($P<0{\cdot}05$). Conversely, the effect of leptin on adipocytes obtained from rats under acute ganglionic blockade, achieved by chlorisondamine injection, did not show differences with the lipolytic activity observed in control rats treated with leptin (Fig. 8).

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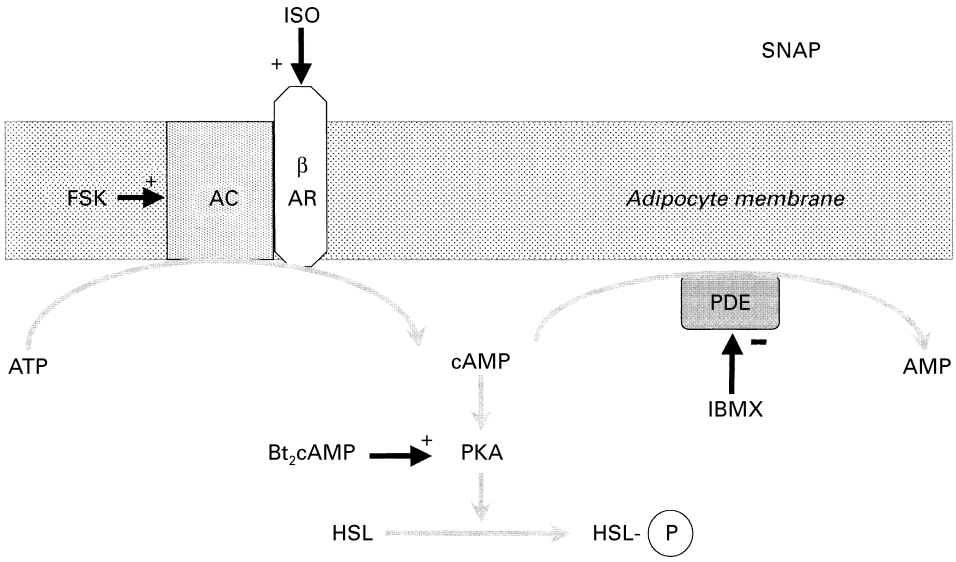

Fig. 9. Schematic representation of the site of action of diverse pharmacological agents at different levels of the lipolytic pathway. FSK, forskolin; AC, adenylate cyclase; β AR, β-adrenoceptor; ISO, isoproterenol; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; PDE, phosphodiesterase E; Bt_2-cAMP, dibutyryl-cAMP; PKA, protein kinase A; IBMX, isobutylmethylxanthine; HSL, hormone-sensitive lipase; P, phosphate.

Lipolysis can be stimulated by a rise in cAMP resulting from either adenylate cyclase activation or phosphodiesterase inhibition. In order to gain insight into the likely mechanisms implicated, lipolysis was stimulated *in vitro* in fat cells isolated from age- and weight-matched non-treated rats using a number of agents acting at different levels of the lipolytic pathway (Fig. 9): (1) at the β-adrenoceptor (isoproterenol); (2) at adenylate cyclase (forskolin); (3) at phosphodiesterase E (isobutylmethylxanthine); (4) at protein kinase A (dibutyryl-cAMP). To further validate the underlying assumption that NO is involved in the modulation of the leptin-induced lipolysis the effect of *S*-nitroso-*N*-acetyl-penicillamine (SNAP), a known NO donor, was assayed *in vitro* with leptin, isoproterenol and combinations of the different lipolytic agents in fat cells isolated from age- and weight-matched non-treated control rats. The stimulatory effect of leptin, SNAP and catecholamines was further studied in adipocytes of obese Zucker diabetic fatty (*fa/fa*) rats to examine the effect of defective OB-R on the stimulation of lipolysis.

Administration of leptin did not alter the lipolytic rate of white adipocytes obtained from *fa/fa* rats. However, addition of SNAP or isoproterenol to the incubation medium of fat cells from obese Zucker animals produced a marked lipolytic response, thus showing that the adipocyte preparations from these rats are not defective to other known lipolytic agents. The simultaneous presence of leptin and SNAP in the incubation medium of adipocytes isolated from Wistar rats exerted an additive effect on *in vitro* lipolysis compared with the effect elicited by the products acting individually. Only SNAP exerted a statistically significant inhibitory effect on isoproterenol-stimulated lipolysis ($P < 0{\cdot}001$). Neither SNAP nor leptin modified forskolin-, dibutyryl-cAMP- and isobutylmethylxanthine-stimulated lipolysis in lean rats. The lack of effect of leptin on isoproterenol-induced lipolysis in the *in vitro* assays adds further weight to the *ex vivo* experiments performed with the ganglion-blocking agent. Altogether these findings suggest that leptin does not interfere with catecholamine-mediated lipolysis. A direct effect of leptin on adenylate cyclase appears unlikely since the OB protein failed to reduce forskolin-induced lipolysis. Furthermore, the lack of effect on dibutyryl-cAMP-mediated lipolysis suggests that leptin does not interfere at the protein kinase A level either. Although a marked decrease in the release of glycerol was observed in isobutylmethylxanthine-treated adipocytes after exposure to leptin it did not reach statistical significance. However, the possibility that leptin may interfere at the phosphodiesterase level should not be completely ruled out. It can be concluded from our studies that NO may function as an important autocrine physiological regulator signal controlling lipolysis by facilitating leptin-induced lipolysis and simultaneously being capable of inhibiting catecholamine-induced lipolysis.

Blood pressure regulation

The presence of functional OB-R in brain regions as well as in peripheral organs that are important in cardiovascular control, such as heart, kidneys and adrenals (Fig. 1), led to the suggestion that leptin might affect blood pressure regulation (Tartaglia *et al*. 1995; Tartaglia, 1997). Intracerebroventricular (Dunbar *et al*. 1997; Casto *et al*. 1998) as well as intravenous (Shek *et al*. 1998) administration of leptin have been shown to increase both mean arterial pressure and heart rate. Furthermore, leptin administration has been reported to increase sympathetic nerve activity to kidneys, adrenals and brown adipose tissue (Dunbar *et al*. 1997; Haynes *et al*. 1997). However, this generalized sympathoexcitation was not always followed by an increase

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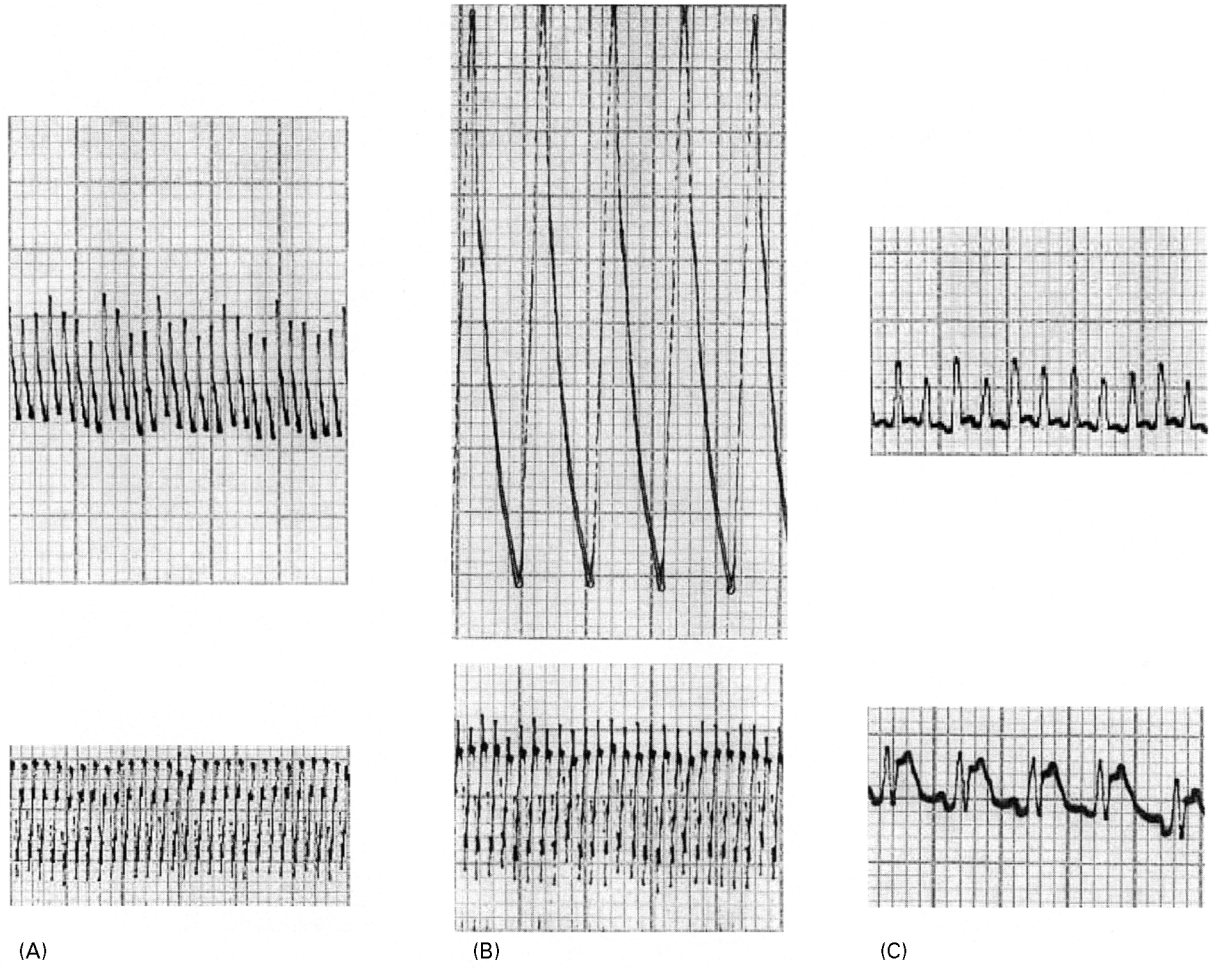

Fig. 10. Representative records of arterial blood pressure (upper panel) and heart rate (lower panel) obtained in anaesthetised Wistar rats under baseline conditions (A). Effect of intravenous leptin administration (100 μg/kg body weight) in the setting of nitric oxide synthase inhibition (N$^{\omega}$-nitro-L-arginine methyl ester pretreatment; 30 mg/kg body weight; B) or acute ganglionic blockade (chlorisondamine, 30 mg/kg body weight; C).

in arterial pressure (Haynes *et al*. 1997; Jackson & Li, 1997; Casto *et al*. 1998).

The finding of functionally-competent OB-R in endothelial cells provided evidence that the endothelium is also a target for leptin action (Sierra-Honigmann *et al*. 1998). The vascular endothelium is known to play a critical role in blood pressure homeostasis, in part by its ability to produce potent vasoactive factors, principal among these factors being the vasodilator NO. Some of my own research therefore, has taken the approach of studying the potential role of NO in the leptin-induced effects on blood pressure regulation (Frühbeck, 1999). Intravenous administration of leptin to Wistar rats was followed by a statistically significant dose-dependent increase in serum NO concentrations ($P < 0{\cdot}001$). Under NO synthesis inhibition, performed by N$^{\omega}$-nitro-L-arginine methyl ester administration, leptin produced an increase in both systolic and diastolic blood pressure, resulting in a sharp rise in mean arterial pressure (Fig. 10). However, in the absence of sympathoactivation, achieved by pretreatment with the ganglion-blocking agent chlorisondamine, leptin administration significantly reduced both blood pressure and heart rate ($P < 0{\cdot}01$).

The effect of N$^{\omega}$-nitro-L-arginine methyl ester injection in the setting of acute ganglionic blockade and leptin treatment was also studied to validate the underlying assumption that the hypotensive effect of leptin administration observed during ganglionic blockade is caused by the release of NO. Under these circumstances the inhibition of NO synthase by N$^{\omega}$-nitro-L-arginine methyl ester blocked the leptin-mediated decrease in blood pressure during pharmacologically-induced acute ganglionic blockade by chlorisondamine. Thus, leptin appears to have a balanced effect on blood pressure, with a pressor response attributable to sympathetic activation and a depressor response attributable to NO release. This study was the first to show that leptin is involved in the control of vascular tone by simultaneously producing a neurogenic pressor action and an opposing NO-mediated depressor effect (Frühbeck, 1999).

Obesity is associated with increased incidence of hypertension and cardiovascular mortality (Ascherio *et al*. 1992; Hall, 1994; Hsueh & Buchanan, 1994). However, the mechanisms that link obesity with high blood pressure have not been fully elucidated. The adipocyte-derived hormone,

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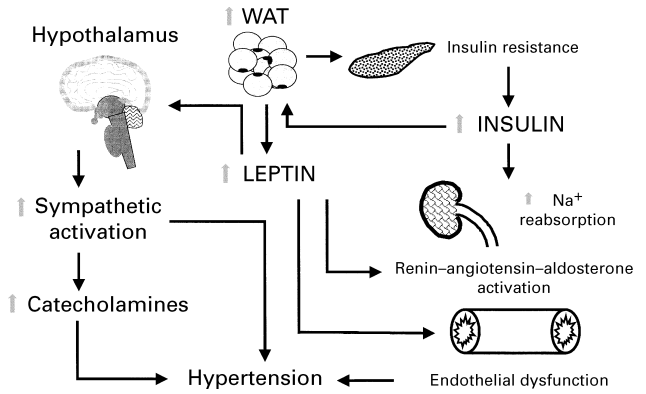

Fig. 11. Potential link between well-established risk factors and the adipocyte-derived hormone, leptin. WAT, white adipose tissue.

leptin, has been suggested to be implicated in obesity-related hypertension, as it provides a link with well-established risk factors such as sympathetic activation, insulin resistance, increased Na^+ reabsorption, stimulation of the renin–angiotensin–aldosterone system and endothelial dysfunction (Fig. 11; Bornstein & Torpy, 1998; Schorr *et al*. 1998; Suter *et al*. 1998; Villarreal *et al*. 1998; Hall *et al*. 1999; Mark *et al*. 1999; Ozata *et al*. 1999; Ruige *et al*. 1999; Zimmet *et al*. 1999). Since leptin's effects on NO synthesis appear to be protective against the development of high blood pressure, it may be argued that if the vasculature is resistant to the actions of leptin, it may be involved in the development and maintenance of arterial hypertension. Thus, a defect in the leptin system may contribute to hypertension as well as obesity. The increased incidence of hypertension observed in obesity may be explained by a hampered NO modulation of a compensatory hypertensive response. This possibility is supported by findings made in both animal models and human subjects. It has been reported that obesity-related hypertension is associated with attenuated arterial dilation (Wu *et al*. 1996). Furthermore, NO synthase activity has been shown to be decreased in obese Zucker rats compared with littermate controls (Morley & Mattammal, 1996) and the JCR:LA corpulent rat shows a defective NO-mediated vascular relaxation (Russell *et al*. 1997). In human subjects an impaired endothelium-derived NO synthesis in obesity has been shown (Cardillo *et al*. 1998). In addition, an impaired NO-mediated vasodilation has been reported in healthy elderly subjects; this being high blood pressure more commonly associated with old age (Lyons *et al*. 1997). In this context, further studies on the relationship between adipose tissue, blood pressure homeostasis and leptin appear warranted.

Concluding remarks

The leptin system is like a dynamic puzzle; as more pieces of the puzzle are found, more questions arise and more pieces are needed. At this early juncture in the course of leptin research, much has been discovered. The tip of the iceberg has become visible now. However, there is still much more below the sea surface, and much remains to be learned about leptin's physiology and clinical relevance. Given leptin's versatile and ever-expanding list of activities, additional and unexpected consequences of leptin are sure to emerge. The intense efforts underway on many different frontiers of leptin research will undoubtedly add more information to the already large body of knowledge.

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