Human Reproduction Update 2000, Vol. 6 No. 3 pp. 290–300

Leptin and reproduction

Ruben Rene González[1], Carlos Simón[2,*], Pedro Caballero-Campo[2], Robert Norman[3], Didier Chardonnens[4], Luigi Devoto[1] and Paul Bischof[4]

[1]Institute of Maternal and Child Research (IDIMI), Hospital 'San Borja Arriaran', Faculty of Medicine, University of Chile, PO Box 226–3, Santiago, Chile, [2]Instituto Valenciano de Infertilidad (IVI), Valencia University, School of Medicine, Spain, [3]Queen Elizabeth Hospital, University of Adelaide, Department of Obstetrics and Gynecology, Woodville, South Australia, and [4]Department of Obstetrics and Gynecology, University of Geneva, Geneva, Switzerland

Leptin, the product of the *ob* gene, is a small peptide molecule synthesized by white adipocytes with an important role in the regulation of body fat and food intake. Leptin and leptin receptor mRNA were first detected in the brain and hypothalamus but now their ubiquitous presence has been demonstrated. Leptin receptor signal transduction involves the activation of signal transducer and activator of transcription (STAT)-3, a member of the transcription family of proteins. Leptin is regulated by hormones and cytokines, interleukin-1, tumour necrosis factor-α and transforming growth factor-β, linking this molecule with the inflammatory response. In addition, emerging evidence has demonstrated that this molecule is related to reproductive function. This small protein is present in the ovary and decidua, in mature oocytes and during embryonic development and trophoblast invasion. Animal models have demonstrated that leptin-deficient *ob/ob* mice are sterile; however, fertility can be restored by exogenous leptin. In addition, embryos implanted in STAT-3-deficient mice degenerate rapidly and are the target disruption of STAT-3-provoked embryonic lethality. Leptin acts as a novel placental hormone participating in the control of fetal growth and development. Leptin could be a modulator for invasive features of cytotrophoblast cells. We postulate that leptin may have an autocrine/paracrine role in human implantation and placentation.

Key words: cytokines/human reproduction/leptin/leptin reception/regulation

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Introduction

Leptin (or OB protein) is a pleiotropic molecule secreted by white adipocytes that plays a role in the regulation of body weight and food intake (Stephens *et al.*, 1995; Messinis and Milingos, 1999). Leptin investigation has increased in many fields: the pathology of obesity, anorexia nervosa, diabetes mellitus, polycystic ovary syndrome (PCOS), acquired immune disease (AIDS), cancer, nephropathy, thyroid disease, Cushing's syndrome and growth hormone deficiency (Fruhbeck *et al.*, 1998). Leptin suppresses the appetite and increases the metabolic rate (Granowitz, 1997). This molecule is known to be correlated with fat mass (Considine *et al.*, 1996) and responds to changes in caloric intake but also recently it has been proposed to be involved in the control of the reproductive function**.** Leptin may be an important signal indicating the adequacy of nutritional status for reproductive function (Conway and Jacobs, 1997). The present review concentrates on the structure, hormonal and paracrine regulation, and the implication of leptin in the reproductive process.

Leptin, leptin receptor and signal transduction

Leptin (from the Greek, leptos, meaning thin) is a small peptide product of the *ob* gene. It is a 16 kDa non-glycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang *et al.* The precursor form of leptin contains 167 amino acids is activated by cleavage of a 21 amino acid residue (Zhang *et al.*, 1994; Ogawa *et al.*, 1995). The study of leptin using a three-dimensional structure database revealed that this protein might adopt a fold

*To whom correspondence should be addressed at: Instituto Valenciano de Infertilidad (IVI), Valencia University, School of Medicine, Spain. Phone: +34 96 362 4399; Fax: +34 96 369 4735; E-mail: centroivi@ivi.es

similar to structures from the family of helical cytokines that includes interleukin (IL)-2 and growth hormone (Madej *et al.*, 1995). Further investigations with nuclear magnetic resonance analysis revealed that it is a four-helix bundle. Helix lengths and disulphide patterns suggest that leptin is a member of the short-helix cytokine family (Kline *et al.*, 1997).

Leptin communicates nutritional status to regulatory centres in the brain and circulates in blood, bound to a family of binding proteins (Campfield *et al.*, 1996). Leptin interacts with the proteinase inhibitor, α_2 macroglobulin. The leptin–α_2 macroglobulin complex is stable and is recognized by the α_2 macroglobulin receptor/low density lipoprotein receptor-related protein (Birkenmeir *et al.*, 1998). In lean subjects, with relatively low adipose tissue, the majority of circulating leptin is protein-bound. In obese individuals, the majority of leptin circulates in free form, presumably the bioactive protein, and thus obese individuals are resistant to free leptin (Hoggard *et al.*, 1998).

Leptin receptor is the product of the *db* gene and belongs to the class I cytokine superfamily of receptors. The full-length receptor has the signalling capabilities of the IL-6 type receptor and its helical structure is similar to this cytokine (Baumann *et al.*, 1996; Tartaglia, 1997). *Leptin receptor* mRNA is expressed in the anterior pituitary, in several areas of the brain and in other tissues (Finn *et al.*, 1998). *Leptin receptor* mRNA has also been detected in granulosa and theca cells (Zachow and Magoffin, 1997). The cloned leptin receptor contains two homologous segments representing potential ligand binding sites. These domains localized to amino acid residues 323–640 and between amino acid residues 428–635, have been identified as a fibronectin type 3 domain (Fong *et al.*, 1998). In humans and rodents, two major forms of leptin receptors (OB-R) are expressed. The short form (OB-RS) is detected in many organs and is considered to lack signalling capability (Wang *et al.*, 1997) as it has a truncated intracellular domain (Campfield *et al.*, 1996). The long form (OB-RL) with the complete intracellular domain, predominates in the hypothalamus, but is expressed in low amounts in peripheral tissues. OB-RL transduces an intracellular signal by activation of the signal transducer and activator of transcription (STAT) proteins, induction of acute-phase plasma proteins, and synergism with IL-1 and tumour necrosis factor (TNF)-α in hepatoma cell lines (Wang *et al.*, 1997). The leptin receptor could also exert a control on its own leptin synthesis in rat adipose tissue (Zhang *et al.*, 1997). Soluble leptin receptor has also been reported in humans (Lewandowski *et al.*, 1999).

Leptin receptor activation involves the activation of STAT3, a member of the STAT family of proteins. STAT3 can also be activated by a variety of cytokines, e.g. IL-6, granulocyte-colony stimulating factor (G-CSF) and epidermal growth factor (EGF) (Takeda *et al.*, 1998). The OB-R might exert a signalling effect similar to G-CSF receptor (G-CSFR), leukaemia inhibitory factor receptor (LIFR) or the glycoprotein, gp130, through the activation of receptor-associated kinases of the Janus kinase family. Activation of these kinases phosphorylate and activate in turn DNA binding activity of signal transducers and activators of transcription proteins (STAT1, STAT3 and STAT5) (Baumann *et al.*, 1996).

Neuroendocrine, hormonal and paracrine regulation of leptin

Many leptin effects on food intake and energy expenditure are thought to be mediated centrally via neurotransmitters, e.g. neuropeptide Y (Houseknecht *et al.*, 1998). Catecholamine metabolism seems also to have a role in the regulation of leptin. Plasma epinephrine has been negatively correlated with leptin concentrations (Mills *et al.*, 1998). However, using an inhibitor of catecholamine synthesis (α-methyl-para-tyrosine) it was not possible to demonstrate that noradrenaline represents the afferent signal from the central nervous system which modulates leptin release from adipocytes in human (Zimmermann *et al.*, 1998).

Glucocorticoids and insulin participate in the regulation of leptin metabolism. Glucocorticoids and insulin act as long-term regulators of leptin in omental (Om) and sub-abdominal (Sc) adipose tissue (Rusell *et al.*, 1998). Obese children treated with a single dose of dexamethasone showed a significant increase in leptin concentration, indicating that glucocorticoids up-regulate human leptin (Kiess *et al.*, 1996). An inverse correlation between cortisol and leptin has been reported in lean or obese subjects, either after food intake or fasting (Korbonits *et al.*, 1997). This study revealed that leptin is a powerful indicator of insulin values and body mass index (BMI); however, leptin concentrations did not change acutely after food administration. Interestingly, insulin did not regulate acute leptin concentrations in lean or obese subjects, nor was leptin secretion different in diabetic obese and lean patients. Basal concentrations of insulin and leptin were positively correlated only in insulin-sensitive individuals (Sinha and Caro, 1998). High concentrations of soluble leptin receptor might be involved in the leptin resistance found in insulin-dependent diabetic women (Lewandowski *et al.*, 1999). Some experimental data suggest that leptin could regulate the expression of insulin. The molecular mechanisms underlying the effect of leptin on insulin secretion are unknown. It has been suggested that physiological concentrations of leptin in normal rodents modulate a potentiation of glucose-induced insulin secretion involving cyclic AMP or phospholipase C/protein kinase activation (Poitout *et al.*, 1998). In obese mice, leptin inhibits transcription of the proinsulin gene by altering the transcription factor binding (STAT5) element, conferring glucose responsivity and secretion of insulin by activation of ATP-sensitive potassium channels. Leptin may inhibit insulin gene transcription and secretion in pancreatic β-cells by different mechanisms (Seufert *et al.*, 1999). However, in ovariectomized ewes, leptin treatment raised plasma lactate and fatty acid concentrations but had no effect on glucose or insulin concentrations (Henry *et al.*, 1999).

Leptin secretion in humans shows gender differences, independent of adiposity. Leptin concentrations are three times higher in women than in men (Sinha and Caro, 1998). Dexamethasone and oestradiol stimulate leptin release only in females. *In vitro*, Om adipose tissue of women secretes more leptin than that of men. However, regardless of gender, progesterone or oestrone did not modify leptin secretion (Casabiell *et al.*, 1998). The sexual dimorphism of leptin concentrations could be due to the effect of androgens. Obesity in women (opposite to that of men), is associated with increased ovarian androgen production, and this difference might be due to the action of leptin (Conway and Jacobs, 1997). In addition, rapid

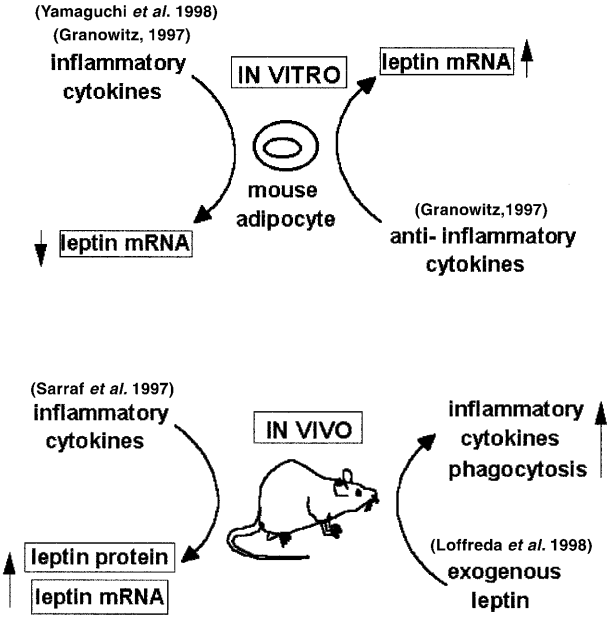

Figure 1. Leptin and inflammatory response. In-vitro mouse adipocytes differentially regulate *leptin* mRNA expression by actions of inflammatory or anti-inflammatory cytokines. In-vivo response of genetically deficient leptin-related mouse by cytokines actions is contrary to in-vitro response. Leptin is up-regulated by inflammatory cytokines, i.e. interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) and also it could up-regulate macrophage synthesis of these cytokines. Leptin is linked to transforming growth factor-β (TGF-β) action (Granowitz, 1997; Sarraf *et al.*, 1997; Loffreda *et al.*, 1998; Yamaguchi *et al.*, 1998).

fluctuations in plasma concentrations of leptin have been found to be negatively correlated with those of adrenocorticotropic hormone (ACTH) (Licinio *et al.*, 1997).

Leptin is related to inflammatory response

Leptin is a key hormone coupling the immune system and energy balance (Finck *et al.*, 1998). Inflammatory cytokines influence leptin concentrations, but leptin may also induce the synthesis of inflammatory cytokines *in vivo* and *in vitro* (Figure 1). Mouse 3T3-L cells that differentiated into adipocytes, expressed higher levels of *leptin* mRNA. In these cells, IL-1β, IL-6, IL-11, TNF-α (inflammatory cytokines) decreased but transforming growth factor (TGF)-β (anti-inflammatory cytokine) increased *leptin* mRNA (Loffreda *et al.*, 1998). Mouse parametrial adipocytes can produce TNF-α, which could in turn inhibit *leptin* mRNA expression through TNF receptor (TNF-R1). Similar results have been found with cultures of human adipocytes. The addition of human recombinant TNF-α to adipocytes from the subcutaneous fat of pregnant women inhibited leptin secretion (Yamaguchi *et al.*, 1998). In contrast, other investigators have found a stimulatory effect of TNF-α on leptin secretion from mouse 3T3-L1 adipocytes. A rapid stimulation of leptin accumulation in cultures of mouse adipocytes was observed after 6 h of TNF-α treatment and this release of leptin was completely inhibited by brefeldin A, an inhibitor of TNF-α. These findings indicate that TNF-α can act directly on adipocytes, regulating the release of a preformed pool of leptin (Kirchgessner *et al.*, 1997).

Inflammatory cytokines also induce the synthesis of leptin *in vivo*. In female mice, administration of TNF, IL-1 and LIF increased serum leptin concentrations and mRNA expression in fat (Sarraf *et al.*, 1997). Lethal effects of TNF in leptin-defective mice were reduced by exogenous leptin. Thus, leptin could participate in protective mechanisms against the autoaggressive effects of the immune system (Takahashi *et al.*, 1999).

TNF-α and IL-1 are mediators of response to lipopolysaccharide (LPS), inducing anorexia and increasing *leptin* mRNA expression in hamster adipose tissue. Induction of leptin during the host response to infection may contribute to the anorexia associated with infection (Grunfeld *et al.*, 1997). Injection of LPS or turpentine into IL-1β-deficient (–/–) mice did not elevate plasma leptin concentrations, unlike IL-1β (+/+) mice. This finding demonstrated that IL-1β is essential for leptin induction by both LPS and turpentine in mice (Fanggioni *et al.*, 1998). In humans, administration of recombinant human IL-1α to cancer patients increased serum leptin concentrations (Janik *et al.*, 1997). TNF-α infusion to patients with solid tumours also increased serum leptin concentrations (Zumbach *et al.*, 1997).

Studies in rodents with genetic abnormalities in leptin (*ob/ob*) or leptin receptor (*db/db*) demonstrated that exogenous leptin up-regulated both macrophage phagocytosis and the production of inflammatory cytokines (Loffreda *et al.*, 1998). It has been also reported that leptin enhanced cytokine production and phagocytosis by murine peritoneal macrophages, although human leptin has exhibited no capacity to stimulate cell survival or proliferation in cultures of murine or human marrow cells. These data demonstrate that leptin may also be able to regulate aspects of haemopoiesis and macrophage function (Gainsford *et al.*, 1996).

Leptin may be implicated in the regulation of apoptosis in the adipose tissue. *Leptin* mRNA was higher in Sc than in Om adipocytes (Montague *et al.*, 1997, 1998). This relationship was inverse for cellular inhibitor of apoptosis, the protein-2 (*cIAP2*) mRNA. Depot-specific differences (in leptin and cIAP2) could play a role in the regulation of apoptosis in adipose tissue (Montague *et al.*, 1998). In addition, reduction of adipose tissue through apoptosis has been observed after intra-cerebroventricular administration of leptin in rats. The adipose tissue of leptin-treated rats showed the characteristic features of apoptosis, e.g. internucleosomal fragmentation of genomic DNA, increased amounts of DNA strand breaks and a reduction in total DNA content and cell volume (Qian *et al.*, 1998). However, the effect of leptin on apoptosis differs in other cell types. In myeloid leukaemia cell lines, leptin had proliferative and anti-apoptotic properties. Leptin reduced apoptosis induced by cytokines in these cell lines (Konopleva *et al.*, 1999).

Leptin as a signal of nutritional status linked to the reproductive process

The amount of body fat stored is known to influence fertility, indicating a link between adipose tissue and the reproductive system (Frisch, 1990). An interesting hypothesis is that leptin is a peripheral signal indicating the adequacy of nutritional status for reproductive function (Tataranni *et al.*, 1997). Therefore, it seems possible that low leptin concentrations indicate a status of inadequate nutritional stores and could prevent an unwanted

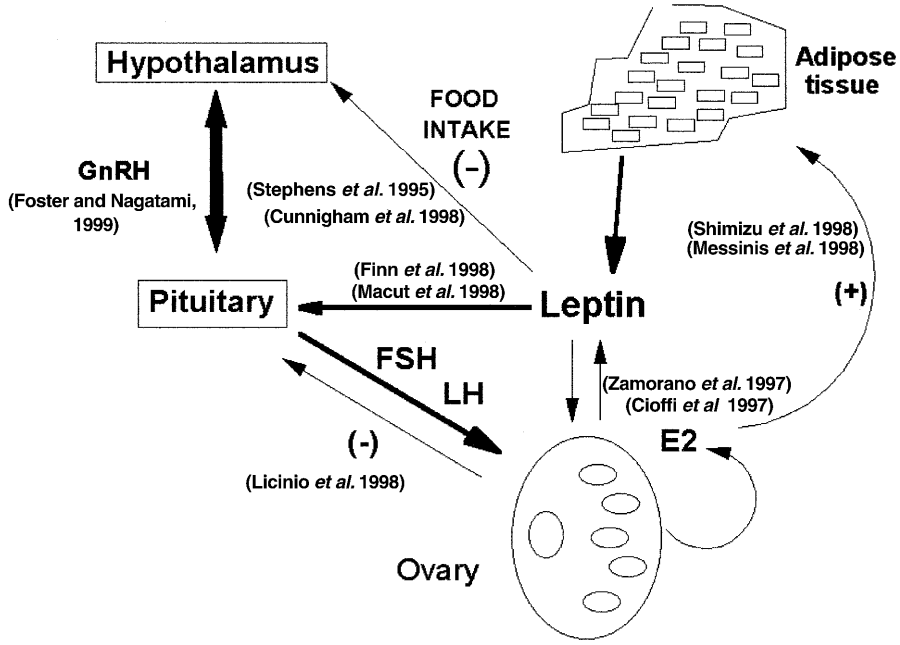

Figure 2. Hypothetical model of relationships between leptin and the hypothalamus–pituitary–ovary axis. Leptin is a molecular signal from adipose tissue that regulates the food intake, presumably through neuropeptide Y actions. Recent data support the idea that leptin binds to the short form of its receptor to transport itself into the arcuate hypothalamic region. Neuropeptide Y synthesis is decreased, diminishing the appetite and increasing whole-body energy expediture and weight loss. Therefore, leptin communicates the size of the adipose reserve to the hypothalamus. Leptin is also synthesized in the reproductive tissues and it is related to the hypothalamus–pituitary–ovary axis function. Gonadotrophin-releasing hormone (GnRH) and LH pulses can be related to leptin actions. Leptin may directly regulate the function of the reproductive organs and, via paracrine effects, may regulate oestradiol synthesis. In addition, oestradiol concentrations could also influence leptin synthesis. Leptin may be a signal of metabolic status to the reproductive system (Stephens *et al.*, 1995; Cioffi *et al.*, 1997; Shimizu *et al.*, 1997; Zamorano *et al.*, 1997; Finn *et al.*, 1998; Licinio *et al.*, 1998; Macut *et al.*, 1998; Messinis *et al.*, 1998; Cunningham *et al.*, 1999; Foster and Nagatami, 1999).

pregnancy which demands additional energy to support a growing fetus. However, leptin is not a sensitive marker of nutritional status (Korbonits *et al.*, 1997).

In starved mice, the lack of reproductive function coincides with a fall of plasma leptin concentrations and several neuroendocrine changes. Exogenous leptin injections to these mice restore fertility (Ahima *et al.*, 1997). In addition, leptin infusions are able to restore ovulatory function in an animal model of starvation. However, fasting-induced anoestrus was not reversed by leptin when glucose oxidation was blocked with 2-deoxy-D-glucose and fatty acid oxidation with methyl palmoxirate (Schneider *et al.*, 1998). Nevertheless, differences in leptin plasma concentrations in long-term conditions need not indicate different fertility rates. Clark and Henry (1999) have recently discussed whether chronic undernutrition or differences in the long-term maintenance of body fat could affect the reproductive function. At the present time, the mechanism and reproductive consequences of low leptin status remain unknown (Miller *et al.*, 1998).

On the other hand, human obesity is not characterized by leptin deficiency. An attractive idea is that obesity could be a state of leptin resistance, but evidence for this assumption is limited (Conway and Jacobs, 1997). A few cases of congenital leptin deficiency have been reported in humans. These were due either to low leptin synthesis (Strobel *et al.*, 1998), or to splice-site mutation in the leptin receptor, which leads to a truncated form of the receptor with no signalling function (Clément *et al.*, 1998).

The *ob/ob* mutant female mouse does not produce an active form of leptin, and is acyclic and sterile. This sterility is reversed by treatment with recombinant leptin, but not by food restriction, suggesting that leptin is required for normal reproductive function. Moreover, impaired reproductive function of *ob/ob* male mice was corrected only with leptin treatment (Mounzih *et al.*, 1997).

Leptin could be a regulator of hypothalamic–pituitary–ovarian function (Figure 2). It has been suggested that leptin is a metabolic signal to the reproductive axis in primates, where leptin increases the plasma concentrations of LH and follicle stimulating hormone (FSH) (Finck *et al.*, 1998; Cunningham *et al.*, 1999), and LH pulse frequency and amplitude (Licinio *et al.*, 1998). Different degrees of subnormal gonadotrophin secretion with lower LH compared with FSH secretion have been found in patients suffering from diverse degrees of severity of weight loss (Conway and Jacobs, 1997). Severely food-restricted animals have reduced circulating concentrations of leptin, which are associated with markedly reduced secretion of LH and FSH (Cunningham *et al.*, 1999). Reducing the amount of nutrition during adulthood can lead to infertility, primarily through reduction of gonadotrophin-releasing hormone (GnRH) secretion (Foster and Nagatami, 1999). Furthermore, patients with hypothalamic amenorrhoea are characterized by lower leptin concentrations than in eumenorrhoeic controls (Licinio *et al.*, 1998). FSH administration induces a parallel increase in serum oestrogen and leptin concentrations (Mannucci *et al.*, 1998; Messinis *et al.*, 1998).

To date, the mechanism linking leptin, LH and oestradiol concentrations has not yet been elucidated. It has been suggested that LH and oestradiol oscillations are under the regulation of

leptin (Licinio *et al.*, 1998), and these authors have proposed that the nocturnal rise of leptin determines the change in nocturnal LH in the mid- to late follicular phase. Thus, these data could explain the altered hypothalamic–pituitary–ovarian function in anorexia nervosa and cachexia, when leptin is decreased (Licinio *et al.*, 1998). However, an important role for oestradiol as regulator of leptin production by the adipocyte has also been suggested (Shimizu *et al.*, 1997; Messinis *et al.*, 1998). Most recent data demonstrate a significant reduction in leptin concentrations in normal women following bilateral ovariectomy (Messinis *et al.*, 1999). A significant reduction in leptin values was observed in both phases of the cycle during the week following the operation. This reduction was preceded by a rapid increase during the first 24 h after the operation. However, the precise mechanism leading to the temporal increase in leptin values is unknown. These authors reported that in these patients BMI seems to be the predominant factor related to leptin concentrations and also suggested that oestradiol and progesterone may participate in the control of leptin production during the human menstrual cycle. Overall, leptin seems to be a signal to the neuroendocrine reproductive system and, when low concentrations of leptin are secreted due to inadequate energy reserves, this protein acts as a metabolic gate to inhibit the neuroendocrine reproductive axis in both sexes (Cunninhgan *et al.*, 1999).

Initiation of puberty in girls may occur when sufficient leptin concentrations are reached (Palmert *et al.*, 1998). Genetic studies have shown that leptin is needed for the initiation of puberty (Clément *et al.*, 1998; Strobel *et al.*, 1998). Earlier menarche in obese girls as compared with normal girls could depend on leptin action. Leptin can serve as a metabolic cue in the neuronal activation of GnRH at the end of the prepubertal period (Macut *et al.*, 1998). Initiation of sexual maturation is associated with body growth of chronological age. Longevity and growth is reduced in many species by energy restriction. These organisms may not even attain puberty before they die (Foster and Nagatami, 1999). However, whether leptin is a primary stimulus of the reproductive axis or acts as a permissive factor, remains an open debate (Clarke and Henry, 1999; Cunningham *et al.*, 1999).

Many studies have demonstrated that leptin has physiological fluctuations during the menstrual cycle, and its concentrations were significantly lower in the early follicular phase (Hardie *et al.*, 1997; Shimizu *et al.*, 1997; Lukaszuk *et al.*, 1998; Mannucci *et al.*, 1998; Messinis *et al.*, 1998, 1999). However, other investigators have reported a different pattern for serum leptin concentrations during the menstrual cycle. Leptin concentrations were found to be similar during the early and late follicular and late secretory phases (Teirmaa *et al.*, 1998). Moreover, leptin concentrations were not influenced by oral contraceptives, indicating that oestrogen and/or progesterone did not influence peripheral serum leptin concentrations. These women however, showed an association between leptin and LH (Teirmaa *et al.*, 1998).

Menstrual abnormalities in young, healthy women are notably related to adiposity and leptin. Adiposity <15% and leptin <3 ng/ml have been associated with impaired reproductive function. Even the degree of alteration is correlated with leptin concentrations: amenorrhoeic < anovulatory < eumenorrheic women (Tataranni *et al.*, 1997). In addition, effects of leptin in obese women have been associated with increased ovarian androgen production (Conway and Jacobs, 1997). Although leptin metabolism has been related to PCOS, its potential contribution to the pathogenesis of PCOS is unknown at the present time. Serum leptin concentrations are elevated in anovulatory women with PCOS (Conways and Jacobs, 1997). It has been suggested that high leptin values may contribute to infertility in PCOS by counteracting the sensitizing effects of insulin-like growth factor-I (IGF-I) in dominant follicles (Zachow and Magoffin, 1997). However, other investigators did not confirm the hypothesis that leptin is closely related to PCOS. Circulating leptin concentrations patients with PCOS did not differ from those in age and weight-matched controls (Mantzoros *et al.*, 1997; Gennarelli *et al.*, 1998).

Furthermore, oestrogens did not affect leptin concentrations in young oral contraceptive users or post-menopausal women receiving hormone replacement therapy. Young women have higher leptin concentrations than men, but similar concentrations to post-menopausal women and, in all groups, leptin values are related to BMI (Castracane *et al.*, 1998). However, other investigators have found lower concentrations of leptin in post-menopausal women compared with premenopausal women, but still higher than those in men (Rosenbaum *et al.*, 1996; Shimizu *et al.*, 1997). The mechanism(s) whereby leptin modulates reproductive function are unknown, however it is possible that, in addition to its action on GnRH, leptin may directly regulate the function of reproductive organs (Conway and Jacobs, 1997; Zamorano *et al.*, 1997).

Using the reverse transcription–polymerase chain reaction (RT–PCR) in humans, *leptin* mRNA has been found in granulosa and cumulus cells, and protein has been detected in mature oocytes (Cioffi *et al.*, 1997). However, other investigators have not been able to detect *leptin* mRNA in ovary using the same technique (Karlsson *et al.*, 1997). Granulosa and theca cells express *leptin receptor* mRNA (Zachow and Magoffin, 1997; Agarwal *et al.*, 1999).

Follicular fluid contains similar concentrations of leptin as serum (Karlsson *et al.*, 1997; Agarwal *et al.*, 1999). Highly specialized sub-populations of granulosa and cumulus oophorus cells can accumulate and sequester leptin, TGF-β and vascular endothelial growth factor (VEGF). Interestingly, the release of leptin and growth factors from these cells may be pulsatile. These findings suggested a novel apocrine-like mechanism within the human ovarian follicle (Antczak *et al.*, 1997).

In-vitro studies have revealed some effects of leptin on the regulation of steroidogenesis in ovary. Leptin effects seem to be dependent on LH. In human granulosa cell cultures, LH production was induced by oestradiol and this effect was inhibited by leptin. However, no effect on oestradiol production was found when LH was not present (Karlsson *et al.*, 1997). Similarly, Agarwal *et al.* (1999) have found that oestradiol production was not influenced by leptin in granulosa cells in either the presence or absence of FSH. Furthermore, leptin did not effect androstenedione synthesis in granulosa or theca cells. These investigators found that leptin could also directly inhibit IGF-I action in theca cells at concentrations commonly present in obese women (Agarwal *et al.*, 1999). In agreement with these findings, Zachow and Magoffin (1997) had previously reported that leptin could directly inhibit IGF-I action in rat ovarian granulosa cells. Moreover, leptin can directly impair the IGF-I-mediated increase

of FSH stimulation of oestradiol synthesis in rat granulosa cells, but progesterone synthesis was unchanged (Zachow and Magoffin, 1997). Furthermore, leptin can directly influence insulin-induced steroidogenesis of bovine ovarian theca cells and stimulates proliferation. In these cells, the inhibitory effect of leptin on insulin action appears to be mediated through leptin binding to its own receptor (Spicer and Francisco, 1998). Recent studies have demonstrated that leptin time- and dose-dependently inhibited human chorionic gonadotrophin (HCG)-stimulated progesterone production by human luteinized granulosa cells, but did not alter basal steroidogenesis. This inhibitory effect was only observed when granulosa cells were cultured in the presence of insulin, apparently by antagonizing insulin action. Leptin suppression of insulin-supported steroidogenesis was also time- and dose-dependent (Brannian *et al.*, 1999).

In-vivo studies showed that leptin treatment of *ob/ob* mice increased cholesterol side chain cleavage and *17α hydroxylase* mRNA. Expression of these genes in reproductive tissues can be regulated by leptin through a direct or indirect mechanism. (Zamorano *et al.*, 1997). Taken together, these data suggest an apparent autocrine mechanism involving leptin in the human ovary that may influence pre- and post-ovulatory follicular development.

Embryonic development and implantation

An elegant study (Antczak and Van Blerkom, 1997) demonstrated that leptin and STAT3 proteins are immunolocalized in mouse and human oocytes, and preimplantation embryos. Both leptin and STAT3 were found in a polarized manner in the oocyte, and differences in allocation of these proteins between blastomeres occurred after the first cell division (2–4-cell stage). These authors observed a unique pattern of cellular domains consisting of leptin/STAT3-rich and leptin/STAT3-poor populations of cells. A cell-borne concentration gradient of these proteins extended along the surface of the embryo at morula stage. A potential role of these proteins in early development has been suggested for the morula stage where inner blastomeres contain little leptin/STAT3, while outer cells contain both leptin/STAT3-rich and -poor cells. This pattern was observed through to the hatched blastocyst stage (Antczak and Van Blerkom, 1997). In addition, mouse oocytes in the metaphase II stage (MII) expressed *leptin receptor* mRNA and protein (Matsuoka *et al.*, 1999). Moreover, leptin at physiological concentrations caused tyrosine phosphorylation of STAT3 in mouse MII stage oocytes. Therefore, leptin could have a role in several aspects of oocyte maturation through the activation of STAT3 signal transduction (Matsuoka *et al.*, 1999).

Mutant *ob/ob* mice, characterized by obesity and sterility, synthesize a truncated version of leptin (Zhang *et al.*, 1994), and had their fertility restored by exogenous leptin (Chehab *et al.*, 1996). Embryos from STAT3-deficient mice can implant, but the embryos degenerate after implantation (Takeda *et al.*, 1997) and disruption of STAT3 in mice produced embryonic death prior to gastrulation (Akira, 1998). Fertilized oocytes from these STAT3-deficient mice could still contain the protein of maternal origin, as they were generated from STAT3+/– parents. Thus, the presence and polarized distribution of regulatory proteins (leptin/STAT3) in mammalian oocytes and embryos may be derived from the oocyte itself or from a maternal source (Antczak and Van Blerkom, 1997). Although leptin is present in the mature oocyte, an association between follicular fluid leptin concentration and embryo development has not been observed. It has been postulated that a post-ovulatory increase in serum leptin concentration could be associated with implantation potential (Cioffi *et al.*, 1997). However, Mounzih *et al.* (1998) reported from studies with *ob/ob* mice pre-treated with exogenous leptin to restore fertility that leptin does not seems to be an important factor for implantation in mice. The relevance of leptin for implantation can not be deduced from this investigation, since leptin is present in the oocyte and preimplantation embryo (Antczak and Van Blerkom, 1997).

More recent data suggest a role for leptin during the preimplantation phase in humans. By using a human in-vitro model to study interactions between the human embryo and endometrial epithelial cells (De los Santos *et al.*, 1996; Simón *et al.*, 1999), it was observed that leptin was present in conditioned media from human blastocyst whether or not co-cultured with endometrial epithelial cells (González *et al.*, 1999a). Endometrial epithelial cell cultures and arrested blastocysts cultured alone secreted similar amounts of leptin. Moreover, significant differences were found between arrested and competent embryos cultured *in vitro*. Hatched blastocysts cultured alone secreted significantly higher concentrations of leptin than arrested blastocyst alone or endometrial epithelial cells alone. Nevertheless, when competent blastocysts were co-cultured with endometrial epithelial cells, leptin concentrations were lower than when arrested blastocysts were co-cultured with endometrial epithelial cells. This finding suggests two possibilities: (i) leptin produced by a competent blastocyst may bind to endometrial epithelial cells, or (ii) these cells can inhibit the secretion of leptin by the human blastocyst. The higher leptin secretion found in competent blastocyst cultures, compared with arrested blastocysts, suggests that this molecule may be marker of cell viability. Differences between arrested and competent blastocysts suggest autocrine/paracrine regulation of leptin secretion during the cross-talk between endometrial epithelial cells and preimplantation embryos (González *et al.*, 1999a).

Leptin and IL-1 system actions might be related during the early phases of human embryo implantation. It has been suggested that the IL-1 system could play an important role in the cross-talk established between the preimplantation embryo and the receptive endometrium during the early phase of human implantation process (Simón *et al.*, 1994). Endometrial epithelial cells express maximal IL-1 receptor (at protein and mRNA levels) during the implantation window, in the mid-secretory phase of the menstrual cycle, where the highest endometrial receptivity to embryo implantation is expected (Simón *et al.*, 1993). The addition of IL-1 receptor antagonist decreased the implantation rate of mouse embryos (Simón *et al.*, 1994). IL-1 synthesis is higher in competent human embryos co-cultured with endometrial epithelial cells, compared with arrested embryos. Moreover, IL-1 secreted by preimplantatory embryos co-cultured with endometrial epithelial cells induces the expression of β_3 integrin in endometrial epithelial cells (Simón *et al.*, 1997). The expression of β_3 epithelial integrin has been proposed as a reliable marker of endometrial receptivity (Lessey *et al.*, 1995; González *et al.*,

1999b). These data underline the putative role of this cytokine system as paracrine/autocrine mediator in local intercellular interaction during embryo implantation (Simón and Polan, 1994; De los Santos *et al.*, 1996). An inverse association between concentrations of leptin and IL-1 in conditioned media from arrested and competent human blastocyst could indicate a sophisticated molecular dialogue among the maternal endometrium and human embryo preceding and during the implantation process. In addition, IL-1 system and leptin have evident relationships during the trophoblastic invasion. This subject will be further discussed.

Fetal development

Leptin is synthesized and secreted by placental trophoblast (Mazusaki *et al.*, 1997). From in-vitro studies, it has been proposed that leptin plays an important role in the regulation of HCG production by cytotrophoblastic cells (CTB) (Chardonnens *et al.*, 1999). In BeWo cells (a choriocarcinoma cell line), the trophoblast-specific transcription of the human leptin gene involved the promoter activity in the 208 bp region (Ebihara *et al.*, 1997). In these cells, leptin secretion is increased by forskolin (an inductor of CTB differentiation into syncytium) (Mazusaki *et al.*, 1997).

In rodents, leptin does not seem to be a critical molecule for implantation, gestation and parturition. The pregnant rat placenta is not a major source of leptin. The total amount of *leptin* mRNA is only significantly increased in rat maternal adipose tissue during pregnancy (Kawai *et al.*, 1997; Tomimatsu *et al.*, 1997). *Leptin receptor* mRNA increased in the uterus of pregnant rats concomitantly with serum leptin (Chien *et al.*, 1997). Female *ob/ob* mice, previously treated with exogenous leptin and mated to similarly treated *ob/ob* males, developed a normal pregnancy without further administration of leptin. Interestingly, in most of these pregnant mice, a prolonged parturition was observed (Mounzih *et al.*, 1998). In contrast, other investigators have reported high levels of gene expression (mRNA) for leptin, the leptin receptor, and the long splice variant (OB-RL) in the murine placenta and fetus (Hoggard *et al.*, 1997). Amico *et al.* (1998) found a significant increase of serum leptin concentrations from days 14 to 21 of gestation. This rise in leptin concentration was concomitant with an increase (4–5-fold) of placental *leptin* mRNA.

The human situation is probably different. Leptin can be considered as a novel placental hormone (Masuzaki *et al.*, 1997); its concentration increases throughout gestation, especially in the second trimester, and is correlated with oestradiol and HCG (Hardie *et al.*, 1997) concentrations. RT–PCR and immunohistochemical analysis showed the presence of leptin in the cytoplasm of syncytiotrophoblast but not in the villous core, indicating that the human placenta is a source of leptin (Senaris *et al.*, 1997).

A role in intrauterine fetal development has been proposed for this placental leptin (Hassink *et al.*, 1997). During the last weeks of gestation, higher leptin concentrations have been observed in the female fetus, compared with males (Tamura *et al.*, 1998), suggesting that sexual differentiation may be involved in leptin regulation (Jaquet *et al.*, 1998). At time of delivery, cord plasma leptin concentrations were not influenced by gender difference. During the early postnatal period, leptin concentrations decreased in both sexes and higher concentrations were found in female newborns (Hytinantti *et al.*, 1999). In addition, preterm neonates had significantly lower serum leptin concentration than full-term neonates. Thus, leptin derived either from placenta or fetal adipose tissue may regulate fetal growth and development. It has been suggested that testosterone could be a suppresser of leptin synthesis in preterm male infants (Ertl *et al.*, 1999).

Leptin has been also related to pre-eclampsia, a hypertensive disorder of late pregnancy. Placental production of leptin is increased in severe pre-eclampsia, suggesting that leptin is a possible marker of placental hypoxia (Mise *et al.*, 1998). Increased *leptin* mRNA and protein was found in placentas from insulin-treated diabetic women. Insulin is likely to play a critical role in leptin regulation (Lepercq *et al.*, 1998).

Trophoblast invasion

Secretion of proteases by maternal and fetal tissues and changes of the integrin repertoire in both maternal and fetal tissues characterize the invasion phase of embryo implantation. Gelatinase B (92 kDa) or matrix metalloproteinase 9 (MMP-9) is a major protease secreted by CTB cultured *in vitro* (Bischof *et al.*, 1995a). Invasive CTB express the integrin $\alpha_6\beta_4$ (a laminin receptor) in a non-polarized manner. A switch of integrin expression in CTB is associated with the invasion of trophoblast into decidual tissue. MMP-9 secretion is higher in $\alpha_6\beta_4$-positive CTB (Bischof *et al.*, 1995b). Trophectodermal cells, once they reach the endometrial basement membrane, express $\alpha_6\beta_4$ integrin and induce secretion of gelatinases. These proteases digest the basement membrane, allowing the embryo to make contact with the stromal extracellular matrix (ECM). Integrin $\alpha_5\beta_1$ (a fibronectin receptor) anchors the embryo into the ECM, and induces secretion of collagenases that digest the ECM allowing the embryo to burrow into the endometrium. Invasive trophoblast cells are thus characterized by protease secretion and $\alpha_6\beta_4$ integrin expression This process is under the paracrine control of endometrial cytokines and ECM glycoproteins (Bischof and Campana, 1997).

CTB cultured in media conditioned by in-vitro decidualized stromal cells show a reduced gelatinolytic activity but an increased secretion of tissue inhibitor of metalloproteinase (TIMP-1) and fetal fibronectin. In contrast, IGF binding protein-1 (IGFBP-1; the main secretory product of decidualized stromal cells) increased the gelatinolytic activity of CTB. It has been suggested that the effects of IGFBP-1 are mediated through binding of this protein to the $\alpha_5\beta_1$ integrin through the Arg-Gly-Asp (RGD) integrin recognition sequence (Bischof *et al.*, 1998).

The mechanism involved in the switching from non-invasive villous CTB to invasive extravillous CTB are still speculative (Bischof and Campana, 1996). Many cytokines and leptin have different effects on the expression of metalloproteinases in CTB cultures (Librach *et al.*, 1994; Shimonovitz *et al.*, 1996; Simón *et al.*, 1996). For example, TGF-β inhibits HCG production by CTB in a dose-dependent manner (Song *et al.*, 1996), inhibits CTB invasion and stimulates trophoblastic TIMP synthesis (Graham and Lala, 1991), whereas IGF-1 is an important regulator

of proliferation and differentiation of trophoblast (Murata *et al.*, 1994).

Human CTB cultured *in vitro* synthesize leptin and this production is modulated by IL-1β and 17β-oestradiol, providing evidence for an autocrine/paracrine regulation of leptin production in the human placenta (Chardonnens *et al.*, 1999). The results of this study indicate that IL-1β is a regulator of leptin secretion in first trimester CTB. The cellular mechanism of such an effect remains unclear. These authors postulated that IL-1β may induce leptin expression through an activation of PLE3, a placenta-specific enhancer element that was identified in the promoter region of the leptin gene (Bi *et al.*, 1997). In addition, IL-1 receptors are present on the trophoblast (Simón *et al.*, 1994) and IL-1β is known to stimulate in-vivo leptin secretion in humans (Janik *et al.*, 1997). Furthermore, the study made by Chardonnens *et al.* (1999) demonstrated a concentration-dependent bimodal pattern of oestradiol on the regulation of leptin secretion. An increase in leptin secretion was observed with oestradiol concentrations similar to those found during late pregnancy. The molecular mechanism of this regulation is unknown. However, up-regulation of leptin secretion by oestradiol in CTB cultured *in vitro* was in agreement with in-vivo observations that leptin and oestradiol concentrations are correlated during pregnancy (Hardie *et al.*, 1997).

Leptin is a regulator of protease synthesis and integrin expression in CTB (Figure 3). *In vitro*, leptin, TGF-β and IL-6 did not affect $\alpha_2\beta_1$ integrin (laminin/collagen receptor) expression in cultured-CTB from first trimester placenta. However, IL-1α, IL-6, TGF-β and leptin up-regulate the expression of $\alpha_5\beta_1$ integrin. TGF-β and leptin up-regulated $\alpha_6\beta_4$ integrin expression in 80% of CTB cultured *in vitro*. Moreover, IL-1α and leptin also increased MMP-9 activity in these cells without affecting MMP-2 activity (González *et al.*, 1999c).

Thus, a novel role for leptin as an autocrine/paracrine regulator of the invasion phase of human implantation may be proposed. Trophoblastic leptin could be an inductor of the secretion of invasive metalloproteinases and a modulator of the expression of integrins conferring an invasive phenotype to CTB (Figure 3). Further investigations should be performed to establish the autocrine/paracrine effects of leptin and cytokines on the invasive behaviour of CTB during implantation and placentation.

Conclusions

Leptin, a novel molecule, initially implicated in food intake and obesity, participates in many important physiopathological events in reproductive function, fetal development and immune responses. Recent data support the idea that leptin might also have an important role in human implantation, as an autocrine/paracrine regulator in the invasion phase. Leptin is present in the pre-implantation embryo and has been identified in conditioned media from blastocyst and endometrial epithelial cell cultures. Moreover, leptin is actively produced by the invasive trophoblast, and modulates the invasive behaviour of cytotrophoblast cells. Further investigations are clearly needed to establish the specific role of leptin in the early phases of human embryo implantation.

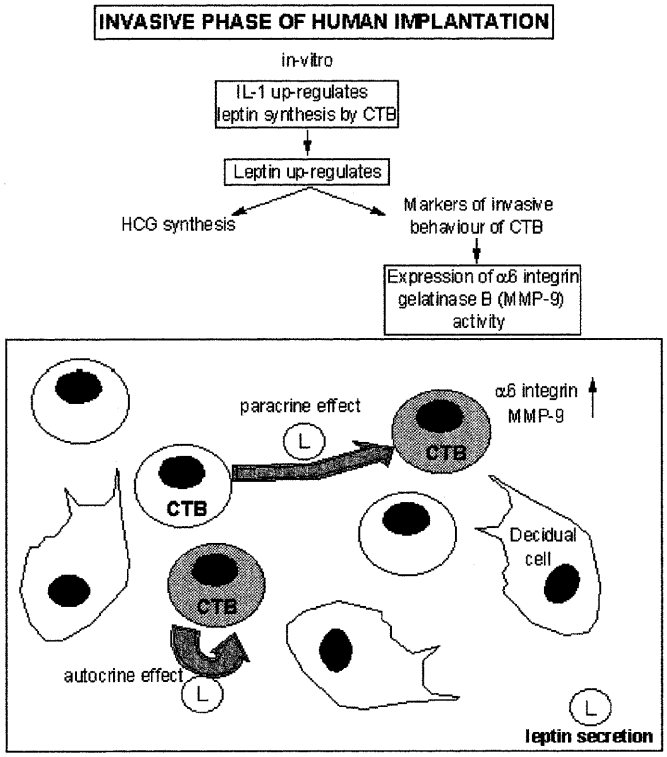

Figure 3. Leptin could be an autocrine/paracrine regulator of the invasive phenotype of cytotrophoblast cells (CTB) during the invasive pathway. Interleukin-1 (IL-1) induces the in-vitro secretion of leptin by CTB (Chardonnens *et al.*, 1999) and leptin up-regulates α_6 integrin expression and matrix metalloprotein-9 (MMP-9) activity in CTB cultures (González *et al.*, 1999c).

Acknowledgements

This work was supported in part by the following grants and organizations. WHO/HRP fellowship (R.R.G), a grant from the Rockefeller Foundation RF 94025 (L.D), Programa de Cooperación Científica con Iberoamérica (C.S & L.D) and FISS 98/0855 from the Spanish Government (C.S.), a grant from the Ernst Schering Research Foundation (DI).

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Received on March 1, 1999; accepted on March 6, 2000