

# Modulation of human cytotrophoblastic leptin secretion by interleukin-1 $\alpha$ and 17 $\beta$ -oestradiol and its effect on HCG secretion

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To investigate the role of leptin during pregnancy, we assessed leptin production by pure cultured human cytotrophoblastic cells (CTB), its regulation by cytokines and 17 $\beta$ -oestradiol and its effects on human chorionic gonadotrophin (HCG) secretion. Purified CTB from first trimester placenta were incubated in duplicates in the presence or absence of cytokines or 17 $\beta$ -oestradiol. Medium was harvested on day 2 and the culture stopped on day 4. Results were corrected for protein content of each individual well and expressed as percent of controls per day (mean  $\pm$  SEM). Basal CTB leptin production was 25.2  $\pm$  2.6 (ng/mg prot). In comparison with controls, leptin production was stimulated to 320  $\pm$  16% ( $P < 0.0001$ ) and 195  $\pm$  3.2% ( $P < 0.0004$ ) by 3 and 10 ng/ml of interleukin-1 $\alpha$  respectively. 17 $\beta$ -oestradiol 10<sup>-6</sup> to 10<sup>-9</sup> mol/l increased basal leptin production 5–9-fold, while 10<sup>-5</sup> mol/l had no such effect. Basal CTB HCG secretion was 5722  $\pm$  1055 (mIU/mg prot). There was a dose-dependent leptin-induced increase in HCG secretion ( $P = 0.0039$ ) reaching a 5-fold increase with a leptin concentration of 1  $\mu$ g/ml ( $P < 0.006$ ). Gonadotrophin-releasing hormone (GnRH) 8.5  $\times$  10<sup>-8</sup> mol/l significantly increased HCG secretion to 140  $\pm$  21% of controls ( $P = 0.031$ ). Cetrorelix (0.1  $\mu$ g/ml) inhibited leptin-induced HCG secretion ( $P = 0.0028$ ).

**Key words:** cytokine/GnRH/HCG/leptin/placenta

## Introduction

The obese gene product, leptin, a 167 amino acid peptide identified in 1994 (Zhang *et al.*, 1994), has provided a potential link between food intake, energy balance and reproductive events (Chehab *et al.*, 1996). A well-recognized site of leptin production is the adipocyte. Thus, the amount of body fat appears as the strongest determinant of plasma leptin concentrations, and factors such as food intake, insulin concentrations, glucocorticoids and catecholamines may influence leptin gene expression (Bray and York, 1997).

Recently, it has been demonstrated that circulating leptin concentrations were elevated during pregnancy, reaching a peak during the second trimester (Hardie *et al.*, 1997; Schubring *et al.*, 1997). However, fat deposition during pregnancy or pregnancy-induced endocrine changes could hardly explain such dramatic changes implying that the fetoplacental unit could be another important source of leptin (Matsuzaki *et al.*, 1997; Senaris *et al.*, 1997). The present work was undertaken to elucidate the potential contribution of first trimester placenta to circulating leptin concentrations during pregnancy and to assess its regulation by factors of trophoblastic and/or endometrial origin. Using pure first trimester human cytotrophoblastic cells (CTB) in culture, CTB leptin production was measured *in vitro* either under basal conditions or stimulated by interleukin-1 $\alpha$  (IL-1 $\alpha$ ), tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) (Li

*et al.*, 1992; Yanushpolski *et al.*, 1993; Mantzoros *et al.*, 1997; Zumbach *et al.*, 1997), macrophage-colony stimulating factor (M-CSF) (Jokhi *et al.*, 1995; Omigbodum *et al.*, 1998) and 17 $\beta$ -oestradiol (Petraglia *et al.*, 1995; Licinio *et al.*, 1998), all well-recognized paracrine/autocrine and endocrine modulators of placental functions.

Structural prediction indicates that leptin might fold into a cytokine-like structure (Madej *et al.*, 1995). As the leptin receptor belongs to the class I cytokine receptor superfamily (Tartaglia *et al.*, 1997), it is possible that leptin may act as a paracrine/autocrine regulator of placental function. As cytokines are involved in placental endocrine functions (Masuhiro *et al.*, 1991; Li *et al.*, 1992; Yanushpolski *et al.*, 1993), the present work was also undertaken to elucidate such a role for leptin. Using pure first trimester human CTB in culture, the secretion of human chorionic gonadotrophin (HCG) was assessed *in vitro* by CTB and the influence of recombinant human leptin (rhLeptin) on CTB HCG production was evaluated.

## Materials and methods

CTB were isolated and purified in accordance with a previously described method (Bischof *et al.*, 1995). Briefly, pregnancy trophoblastic tissue was obtained from legal abortions at 6–12 weeks from

the last menstrual period, digested with trypsin, separated from blood cells and syncytia on a discontinuous Percoll gradient and immunopurified by antibody-coated magnetic particles (anti-CD45) (Dyna Beads, Milan, Geneva) in order to eliminate all contaminating leukocytes as previously reported (Bischof *et al.*, 1995). CTB viability was assessed by Trypan Blue (Sigma, Buchs, Switzerland) exclusion and cells diluted to  $10^6$  cells/ml. IL-1 $\beta$ , IL-6, TNF $\alpha$  and M-CSF were purchased from R&D Systems (Bühlmann Laboratories, Basle, Switzerland) and oestradiol from Sigma. RhLeptin was purchased from R&D Systems, gonadotrophin-releasing hormone (GnRH) from Dr J.Rivier (The Salk Institute, La Jolla, CA, USA), and Cetrorelix from Organon, Saffikon, Switzerland.

### Culture conditions

CTB (100  $\mu$ l,  $10^6$  cells/ml, >90% viability by Trypan Blue exclusion) were incubated in duplicates in the presence or absence of IL-1 $\alpha$  (0.01–10 ng/ml), TNF $\alpha$  (1–100 ng/ml), M-CSF (0.1–100 ng/ml) (Simòn *et al.*, 1994) or oestradiol ( $10^{-9}$  to  $10^{-5}$  mol/l) (Petraglia *et al.*, 1995; Licinio *et al.*, 1998), rhLeptin (1–1000 ng/ml), GnRH ( $8.5 \times 10^{-10}$  to  $8.5 \times 10^{-8}$  mol/l) (Bramley *et al.*, 1992), Cetrorelix (0.1–5  $\mu$ g/ml) or rhLeptin (2  $\mu$ g/ml) with or without Cetrorelix (0.1  $\mu$ g/ml). Concentrations of Cetrorelix and rhLeptin were chosen arbitrarily. Incubation was performed in 12-well tissue culture plate (Costar, Cambridge, MA, USA) under a 5% CO<sub>2</sub> and 95% air atmosphere in a humid incubator at 37°C. Culture medium was Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel, Switzerland) containing 2 mmol/l L-glutamine, 4.2 mmol/l magnesium sulphate, 25 mmol/l HEPES, 1% gentamycin, 1% amphotericin B, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin in the absence of serum. Medium was harvested on day 2 and 4 and the culture stopped on day 4. The supernatants were divided into aliquots and stored at  $-20^\circ\text{C}$  until assayed. The cells were lysed with 200  $\mu$ l Triton X-100 (2.5% in water) and stored at  $-20^\circ\text{C}$  for total cell protein measurements. Duplicate wells were run for each treatment condition and the experiments repeated three times with different CTB preparations.

### Hormone and protein assays

Leptin was measured in the supernatants by an enzyme-linked immunosorbent assay (ELISA) (DRG diagnostics, Cis-Medipro, Vernier, Switzerland) with a sensitivity of 0.2 ng/ml and intra- and inter-assay coefficients of variation of 4.3 and 8.6% respectively. The leptin assay neither cross-reacts with mouse, rat leptin nor with human proteins such as proinsulin, C-peptide, glucagon or insulin-like growth factor I. HCG was measured in the supernatants by a microparticle immunoassay (MEIA) (IMX, Abbot USA) with a sensitivity of 0.1 mIU and intra- and inter-assay coefficients of variation of <4%. Total cell proteins were measured in the cell lysate by the Bio-Rad protein assay using bovine serum albumin as the standard (Bio-Rad, München, Germany).

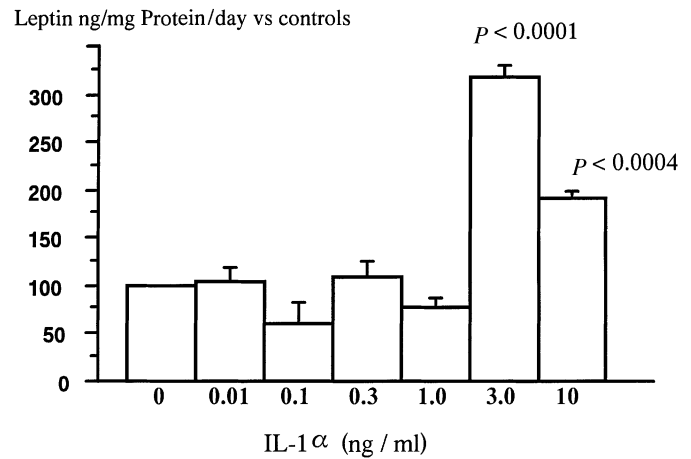
### Statistical analysis

Results were corrected for protein content of each individual well and expressed as percentage of their respective controls per day (CTB in the absence of cytokines or oestradiol or rhLeptin, mean  $\pm$  SEM) or as values per mg/cell protein per day (mean  $\pm$  SEM). Statistical analysis were performed by analysis of variance using Fisher's test on the Statview 4.5 program on a Power Macintosh 7100/66 computer. The different values were compared among them and to the controls.

## Results

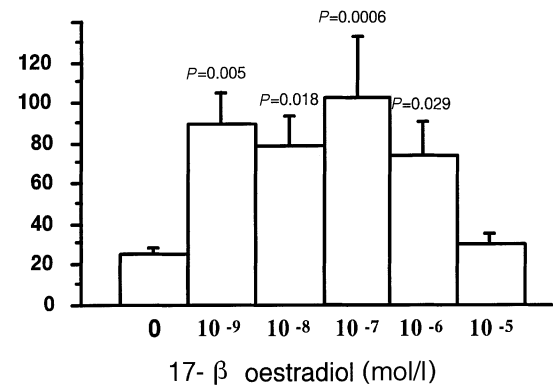
### Effects of cytokines

Mean CTB leptin production under basal conditions was  $25.2 \pm 2.6$  (ng/mg prot). There was no significant change of



**Figure 1.** Effect of interleukin-1 (IL-1 $\beta$ ) on cytotrophoblastic cell (CTB) leptin secretion ( $n = 6$  from three different CTB preparations). Values are expressed as means  $\pm$  SEM.

### Leptin (ng/mg protein)



**Figure 2.** Effect of 17 $\beta$ -oestradiol on cytotrophoblastic cell (CTB) leptin secretion ( $n = 6$  from three different CTB preparations). Values are expressed as means  $\pm$  SEM.

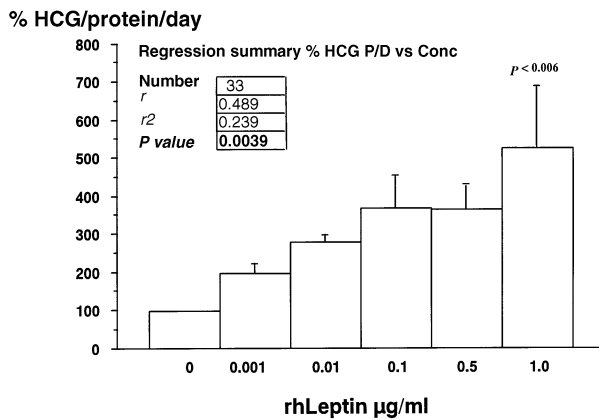
daily basal leptin secretion over the 4-day period. Figure 1 illustrates IL-1 $\beta$ -induced leptin production by CTB. When compared with controls, leptin production was  $320 \pm 16\%$  ( $P < 0.0001$ ) and  $195 \pm 3.2\%$  ( $P = 0.0004$ ) after incubation with 3 and 10 ng/ml of IL-1 $\beta$  respectively. Lower concentrations of this cytokine were without effect. In contrast to IL-1 $\beta$ , 1–100 ng/ml of TNF $\alpha$  or 0.1–100 ng/ml of M-CSF had no significant effect on CTB leptin production (results not shown).

### Effects of oestradiol

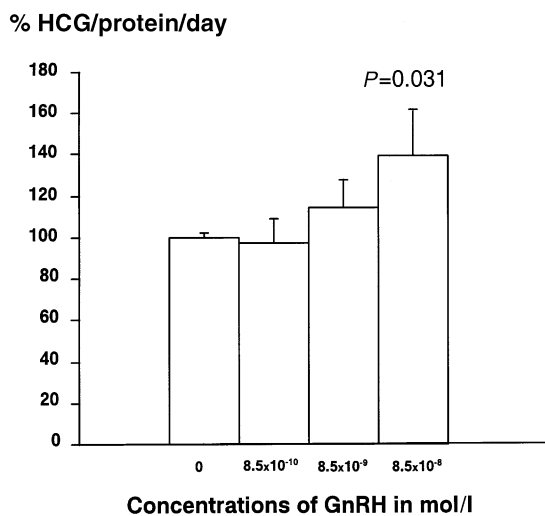
Figure 2 demonstrates the effects of oestradiol on CTB leptin production. Oestradiol  $10^{-9}$  to  $10^{-6}$  mol/l did stimulate CTB leptin production while  $10^{-5}$  mol/l did not.

### Effects of recombinant human leptin

Mean CTB HCG production under basal conditions was  $5722 \pm 1055$  mIU/mg prot. There was no significant change of daily basal HCG secretion over a 4- and even an 8-day period of CTB culture (results not shown). RhLeptin stimulated HCG production by CTB in a concentration-dependent manner ( $P = 0.0039$ ), as illustrated in Figure 3. When compared with controls, HCG production was  $527 \pm 159\%$  ( $P < 0.006$ ) after



**Figure 3.** Effect of human recombinant leptin on human chorionic gonadotrophin (HCG) secretion by cytotrophoblastic cells (CTB) ( $n = 6$  from three different CTB preparations). Values are expressed as means  $\pm$  SEM.



**Figure 4.** Effect of gonadotrophin-releasing hormone (GnRH) on human chorionic gonadotrophin (HCG) secretion by cytotrophoblastic cells (CTB) ( $n = 6$  from three different CTB preparations). Values are expressed as means  $\pm$  SEM.

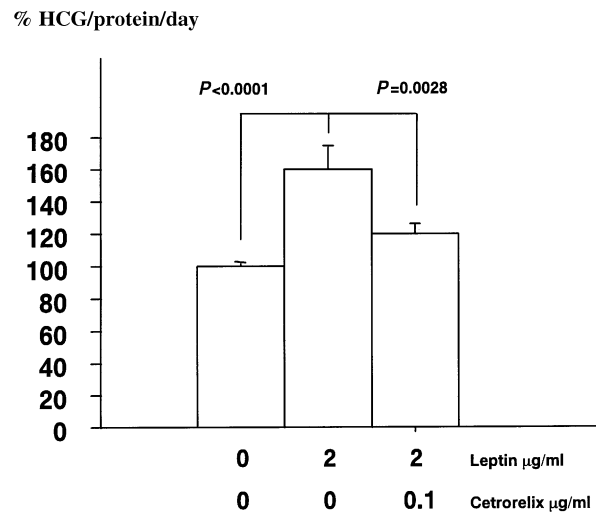
incubation with 1000 ng/ml of rhLeptin. There was no observed syncytium formation when stained with desmoplakin (results not shown).

#### Effects of GnRH and GnRH antagonists

Figure 4 displays the effects of GnRH on HCG secretion by CTB. GnRH  $8.5 \times 10^{-8}$  mol/l increased HCG secretion which was  $140 \pm 21\%$  of controls ( $P = 0.031$ ). Cetrorelix did not modify basal HCG secretion.

#### Effects of GnRH antagonists on leptin-induced HCG secretion

Figure 5 illustrates the effects of Cetrorelix on CTB HCG secretion induced by rhLeptin (2  $\mu$ g/ml). In that set of experiments, rhLeptin raised HCG secretion to  $160.2 \pm 13.3\%$  of controls. Leptin-induced HCG secretion was not as high with 2  $\mu$ g/ml rhLeptin when compared with a 1  $\mu$ g/ml concentration. However, we know that variability from one cell preparation to the other and different gestational ages between



**Figure 5.** Effects of Cetrorelix on leptin-induced human chorionic gonadotrophin (HCG) secretion by cytotrophoblastic cells (CTB) ( $n = 6$  from three different CTB preparations). Values are expressed as means  $\pm$  SEM.

preparations may explain such a result. The addition of Cetrorelix at a concentration of 0.1  $\mu$ g/ml together with rhLeptin inhibited leptin-induced HCG secretion which was then  $119.5 \pm 6.4\%$  ( $P = 0.0028$ ).

#### Discussion

Circulating peripheral leptin concentrations are elevated during pregnancy (Hardie *et al.*, 1997; Schubring *et al.*, 1997) and the placenta has been suggested to contribute to this pregnancy-related increase. Several recent reports have demonstrated leptin gene expression by human placental tissue or the placenta-derived cell line BeWo (Matsuzaki *et al.*, 1997; Senaris *et al.*, 1997). However, those only provide indirect evidence of placenta leptin production. This study represents the first direct evidence of leptin secretion by primary cultures of human cytotrophoblast.

Little is known about the regulation of leptin secretion in the placenta. Placental age may be important as placental leptin gene expression is higher in the first trimester villi when compared with third trimester villi (Matsuzaki *et al.*, 1997). This fits rather well with longitudinal measurements of peripheral concentrations of leptin during pregnancy which are highest in the second trimester (Hardie *et al.*, 1997). Recently, insulin has been suggested as another important regulator of placental leptin secretion (Lepercq *et al.*, 1998). Another important modulator of leptin secretion in the placenta is cell differentiation. Immunohistochemical studies have demonstrated a more intense staining for leptin in syncytiotrophoblast in comparison with cytotrophoblast (Matsuzaki *et al.*, 1997; Senaris *et al.*, 1997). Moreover, forskolin-induced syncytium formation in BeWo cells was shown to increase leptin secretion (Matsuzaki *et al.*, 1997). In this study, basal leptin secretion remains constant over a 4-day period. This was observed in the absence of serum, a known promoter of syncytium formation. Staining of CTB with desmoplakin after incubation with leptin did not demonstrate syncytium formation (results not

shown). Taken together, this makes syncytium formation an unlikely hypothesis to explain the results of this study. However, M-CSF has been shown to induce differentiation of cytotrophoblast (Saito *et al.*, 1993). No such results appear to be available for IL-1. This would warrant further investigation.

The results of this study indicate that IL-1 $\beta$  is a regulator of leptin secretion in first trimester CTB. Its effect seems to be specific, as neither TNF $\alpha$  nor M-CSF changed leptin secretion. IL-1 $\beta$  is known to stimulate in-vivo leptin secretion in humans (Janik *et al.*, 1997) but it remains unclear whether this is a direct action of IL-1 $\beta$  on the adipocyte or via an endocrine cascade involving corticosteroids. In this study, we provide evidence of a direct effect of IL-1 $\beta$  on CTB leptin secretion. The cellular mechanism of such an effect remains unclear. However, IL-1 receptors are present on the trophoblast (Simòn *et al.*, 1994). A placenta-specific enhancer element (placental leptin enhancer, or PLE) has recently been identified in the promoter region of the leptin gene (Bi *et al.*, 1997), with at least three protein binding sites. Protein binding to the PLE3 motif up-regulates the transcription of the leptin gene in the human placenta. Since IL-1 exerts similar effects and since, in other cell systems, IL-1 effects are mediated through the NF- $\kappa$ B transcription factor (Friedman *et al.*, 1996; Bird *et al.*, 1997; Reddy *et al.*, 1997), it is tempting to speculate that IL-1 $\beta$  may induce leptin expression through an NF- $\kappa$ B-dependent activation of PLE3. The specificity of IL-1 $\beta$  is interesting because the complete IL-1 system is present at the feto-maternal interface and is clearly involved in implantation and placentation (Simòn *et al.*, 1994). This raises the important question as to whether leptin is also involved in these crucial events.

Oestradiol plays a role in the regulation of fetal growth (Abdul-Karim *et al.*, 1991), onset of parturition (Chibbar *et al.*, 1995), placental steroidogenesis (Petraglia *et al.*, 1995, Grimes *et al.*, 1996; Babishkin *et al.*, 1997), release of neuropeptides (Petraglia *et al.*, 1990), release of glycoproteins (Wilson *et al.*, 1984; Petraglia *et al.*, 1995) and leptin secretion (Sivan *et al.*, 1998). Administration of oestradiol antagonist reverses oestradiol placental actions, pointing towards a receptor-mediated effect. However, the exact mechanisms of oestradiol action within the placenta are still a matter of debate. Classical cytosol oestradiol receptors seem to be absent or in an extremely low number, thus escaping classical immunological and molecular detection (Rossmann *et al.*, 1997). This raises the possibility that oestradiol actions within the placenta are mediated by a non-classical membrane-bound receptor. This study demonstrated a concentration-dependant bimodal pattern of oestradiol on the regulation of leptin secretion. There was an increase in leptin secretion of up to 100 nmol/l of oestradiol, which is close to its physiological concentration during late pregnancy (30–50 nmol/l) (Tulchinski *et al.*, 1972). This corroborates in-vivo studies where leptin concentrations were correlated with oestradiol concentrations during pregnancy, especially during the first trimester (Hardie *et al.*, 1997). However, higher doses of oestradiol did not affect basal leptin values. Neither the underlying molecular mechanisms, nor the biological or pharmacological significance of this biphasic pattern of oestradiol response are known. One possible

mechanism for the decrease in leptin secretion at 10  $\mu$ mol/l of oestradiol may be down-regulation of the oestradiol receptor as it occurs in other reproductive tissues (Medlock *et al.*, 1991; Simerly *et al.*, 1991; Simòn *et al.*, 1994) and with glucocorticoid and thyroid hormone receptors (Raaka *et al.*, 1981; Rosewicz *et al.*, 1988).

No clear role for placental leptin secretion has been demonstrated. So far, cord blood concentrations have been found to be correlated with birth weight and placenta weight (Harigaya *et al.*, 1997; Hassink *et al.*, 1997; Mantzoros *et al.*, 1997; Matsuda *et al.*, 1997; Schubring *et al.*, 1997; Helland *et al.*, 1998; Machini *et al.*, 1998; Tamura *et al.*, 1998), implying that the feto-placental leptin production may be involved in placental and fetal growth regulation. One putative role for leptin could be related to follicular growth, egg and embryo polarity (Antczak and van Blerkom 1997; Cioffi *et al.*, 1997; Edwards and Beard, 1997; Antczak *et al.*, 1998). This study provides the first direct evidence for a definite role of leptin in a fundamental placental function, i.e. HCG production.

During pregnancy, trophoblast differentiates in a multistep process that converts cytotrophoblast to syncytiotrophoblast (Ringle *et al.*, 1990). As the placenta grows and matures, there is increased formation of syncytiotrophoblast with an associated secretion of HCG. In models of trophoblast differentiation *in vitro*, the onset of HCG production appears to parallel the formation of syncytiotrophoblast (Kliman *et al.*, 1986). In this study, basal HCG secretion remained constant over time. This was observed in the absence of serum, a known promoter of syncytium formation and, indeed, cultured CTB showed no syncytium formation.

Trophoblast secretion of HCG is regulated by a multitude of endocrine and paracrine/autocrine factors. GnRH (Siler-Khodr *et al.*, 1986), epidermal growth factor (EGF) (Morrish *et al.*, 1987), IL-1 and IL-6 (Masuhiro *et al.*, 1991), leukaemia inhibitory factor (Sawai *et al.*, 1995), TNF (Li *et al.*, 1992), activin (Petraglia *et al.*, 1989) and M-CSF (Saito *et al.*, 1991) are recognized as promoters of HCG secretion while progesterone (Szilágyi *et al.*, 1993), inhibin (Petraglia *et al.*, 1989), transforming growth factor (Matsuzaki *et al.*, 1992), GnRH antagonist (Siler-Khodr *et al.*, 1983) and RU 486 (Das *et al.*, 1987; Szilágyi *et al.*, 1993) are inhibitors of HCG secretion. However, fine tuning of HCG secretion during pregnancy is still incompletely understood and the dynamic pattern of HCG may involve an up- and down-regulation of the GnRH receptor (Lin *et al.*, 1995).

The results of this study confirm the role of GnRH in HCG secretion with a maximal stimulatory effect at  $8.5 \times 10^{-8}$  mol/l. The need for such a high concentration of GnRH probably reflects the presence of low affinity GnRH receptors within the placenta (Bramley *et al.*, 1992). Changes in intracellular phosphoinositol turnover have been proposed (Conn *et al.*, 1986) as a possible mechanism of GnRH effect. Likewise, GnRH action within the placenta may involve Ca<sup>2+</sup> ions, similar to the situation described in the pituitary (Mathialagan *et al.*, 1989). A high dose of GnRH may quickly desensitize the local receptors at the post-receptor level. Alternatively, high concentrations of GnRH have been demonstrated to down-regulate GnRH receptor without altering its receptor

mRNA content (Tstsumi *et al.*, 1990) but with diminished polysome formation (Tstsumi *et al.*, 1993), raising the possibility that translational modulation may contribute to homologous down-regulation. This may explain why pulsatile GnRH is more efficient in eliciting HCG secretion in the placenta (Barnea *et al.*, 1991).

The leptin receptor mRNA has been demonstrated within the murine placenta (Hoggard *et al.*, 1997) and leptin receptor has been demonstrated by immunohistochemistry in the human trophoblast (Castellucci, personal communication). However, it is not yet known which isoform of the leptin receptor predominates in CTB. This receptor seems functional as, in this study, leptin increases HCG secretion in CTB in a concentration-dependent way. Thus, leptin can now be added to the growing list of HCG secretion enhancers. GnRH antagonist Cetrorelix (0.1 µg/ml) inhibited leptin-induced HCG secretion. Taken together, the results suggest that the GnRH pathway may be involved in leptin-induced HCG secretion which could be put into perspective with the release of leptin-induced luteinizing hormone-releasing hormone (LHRH) from the median eminence from male rats and luteinizing hormone (LH) release from their anterior pituitaries which is thought to be mediated by nitric oxide (Yu *et al.*, 1997). However, the full-length leptin receptor has the signalling capabilities of the IL-6-type cytokine receptors (Baumann *et al.*, 1996), and structural predictions indicate that leptin would be expected to fold into an IL-6-like structure (Madej *et al.*, 1995) and that IL-6 would stimulate HCG secretion. These considerations can only partly explain the effects of leptin on HCG secretion since IL-6-induced HCG secretion seems different from GnRH-induced HCG secretion (Matsuzaki *et al.*, 1992). Moreover, IL-1, a known activator of HCG secretion, acts via IL-6 and the IL-6 receptor (Masuhiro *et al.*, 1991), and we have also observed that it stimulates trophoblast leptin secretion. Thus, the precise mechanism underlying the action of leptin on HCG secretion needs further investigation.

In conclusion, these data demonstrate that human cytotrophoblastic cells secrete leptin and that IL-1 $\alpha$  and oestradiol are important factors involved in the regulation of leptin secretion. Moreover, these results demonstrate a clear and important role of leptin in HCG secretion by CTB. GnRH appears to be involved in the pathway leading to leptin-induced HCG secretion but further investigations are required.

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