In vitro effects of chorionic gonadotropin hormone on human adipose development

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Abstract

It is well known that pregnancy is associated with fat weight gain. However, the mechanisms whereby fat mass accumulation is controlled during this period are poorly understood. Therefore, we attempted to determine whether human chorionic gonadotropin (HCG), in vitro, influences human adipose tissue development and/or metabolism. For the first time, HCG/LH receptor was characterized in human adipose cells. We also demonstrated that physiological concentrations of HCG, while unaltering both lipolysis and expression of two markers of lipogenesis (FAS and ADD1) in human mature adipocytes, stimulate human preadipocyte growth via the activation of a protein kinase A-independent mitogen-activated protein kinase/c-fos signaling pathway. HCG also moderately increases the preadipocyte differentiation capacity as reflected by enhanced glycerophosphate dehydrogenase activity and expression of key adipogenic transcriptional factors (C/Enhancer-binding protein α and peroxisome proliferator-activated receptor γ 2). Finally, HCG significantly stimulates the secretion of the proadipogenic factor, leptin, from human adipose tissue. Taken altogether, these data suggest that the proadipogenic effect of HCG in human preadipocytes contributes to explain why increased fat storage occurs during the first trimester of pregnancy.

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Introduction

Human chorionic gonadotropin (HCG) is a placental heterodimeric glycoprotein including an α-subunit, which is common to all the gonadotropins and the hormone-specific β-subunit. This hormone acts through a specific receptor, which is coupled to G proteins, and thus displays the characteristic pattern of the seven-transmembrane-span receptor family (Dufau 1998). Since the early 1990s, many reports have revealed the presence of HCG/humanizing hormone (LH) receptors in non-gonadal tissues. HCG receptors are expressed in human uterus and its related tissues (Menon et al. 2004). The clinical significance of HCG action in the corresponding tissues is not yet defined. In addition to its function in regulating steroidogenesis, HCG was shown to have multiple regulatory actions. In particular, this hormone may have either stimulatory (Czerwiec et al. 1989, Horiuchi et al. 2000) or inhibitory effects (Ku et al. 2002, Rao et al. 2004) on the growth of different cellular types. For example, HCG-induced inhibition of mammary cancer initiation and progression has been reported to be due both to inhibition of cell proliferation and to activation of programmed cell death genes leading to apoptosis (Srivastava et al. 1998, Guo et al. 2004). More recently, HCG has been characterized as a new angiogenic factor in human endothelial cells (Zygmunet et al. 2002).

Blood HCG levels are known to exponentially increase during the first 2 months of pregnancy and then to abruptly decrease to reach basal values at the end of the third month. This period also coincides with important depot-specific changes and more particularly an accumulation of fat mass in the s.c. area in order to cover the energy demands of both the fetus and the mother (Sidebottom et al. 2001). These changes have been demonstrated to be strongly influenced by sex hormones. During pregnancy, progesterone promotes fat deposition by decreasing lipolysis (Sutter–Dub & Corbeta 1997) and by increasing lipogenesis (lipoprotein lipase, fatty acid synthase activities; Shirley et al. 1981, Lacasa et al. 2001). Estrogens also exert multiple effects on adipose tissue metabolism. For example, high concentrations of estrogens increase lipolysis (Palin et al., 2003) and decrease blood lipoprotein levels, whereas low concentrations of estradiol increase lipoprotein expression (Gonzalez et al., 2002). However, little is known concerning the role of HCG on adipose metabolism, especially when this hormone is actively secreted, i.e., during the first trimester of pregnancy.

Fat mass development results from variations in cell size (imbalance between triglyceride storage and mobilization) and/or in cell number (adipogenesis). Adipogenesis corresponds to a sequence of events among which adipose precursor
cells first proliferate until confluence and then differentiate into mature adipocytes. Mitogenesis is regulated by a wide variety of mitogenic signals such as insulin and epidermal growth factor through different signaling pathways (Ailhaud et al. 1994, Butterworth 1994). Among these, the mitogen-activated protein kinase (MAPK) cascade seems to play a crucial role in preadipose growth, in particular in the 3T3-L1 preadipocyte cell line (Spiegelman et al. 1988), and also in rat preadipocytes (Lacasa et al. 1997a,b). Upon activation, MAPK phosphorylates multiple substrates of various subcellular localizations such as the phospholipase A2 in the cytoplasm and several transcription factors in the nucleus like the proto-oncogene c-fos (Janknecht et al. 1995), which plays a pivotal role in preadipocyte growth induction (Lacasa et al. 1997a,b). The adipose differentiation process is characterized by a coordinated expression of adipocyte-specific genes triggered by different interacting transcriptional factors. The most important transcriptional factors involved in this process are the CCAAT–enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor γ2 (PPARγ2; Mac Dougald & Lane 1995), both of which activate a set of adipospecific genes that have functional C/EBP- and PPAR-binding sites in their promoters (Christy et al. 1989).

In order to explain the s.c. fat accumulation observed during pregnancy, the aim of the present study was to establish whether HCG plays a direct and significant role in adipocyte biology and more specifically whether HCG affects in vitro human adipose tissue development (lipolysis, adipogenesis).

Materials and Methods

Materials

DMEM–Ham’s F12 (50:50 mix), penicillin, streptomycin, leupeptin, aprotinin, AEBSE, vitamin E, BSA, and human recombinant chorionic gonadotropin were purchased from Sigma Chemical Co., urine-extracted HCG from Organon (Eragny sur Epte, France), and collagene from Roche Molecular Biochemicals. Superscript II RNase H-RT was provided by Gibco BRL, Taq polymerase and RNA guard by Pharmacia Biotechnology, and the antisem specific for active MAPK and the MEK inhibitor, U0126, by Promega Corp. The antisem specific for non-phosphorylated forms of MAPK was obtained from Transduction Laboratories (Lexington, WI, USA). Monoclonal antibody against the human HCG/LH receptor was kindly provided by Dr Hugues Loosfelt (Paris, France). Fetal calf serum (FCS) was obtained from Gibco-BRL. Human insulin was from Nova Nordisk (Boulogne-Billancourt, France). A RIA kit specific for human leptin was purchased from Linco (St Charles, MO, USA), [3H]thymidine from Amersham, and reagents for glycerol determinations from Boehringer Mannheim. Human ovary tissue lysate was purchased from Imgenex (San Diego, CA, USA).

Subjects

The adipose tissue donor group included seven non-pregnant pre-menopausal women (age 41.8 ± 5.5 years; BMI 27.1 ± 1.9 kg/m²) and 14 men (age 64.5 ± 16 years; BMI 25.7 ± 3.2 kg/m²) undergoing surgical intervention. None of these patients suffered from endocrine malignant or chronic inflammatory diseases. This study was approved by the patient’s written consent and by the local ethical committee.

Cell culture and treatment

The adipose tissue samples (50–100 g) obtained from s.c. fat depots were collected in saline (NaCl 150 mM) and immediately transferred to the laboratory. After removing blood vessels and connective tissue, adipose tissue was rinsed in saline containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Preadipocytes and adipocytes were obtained by collagenase digestion as previously described (Deslex et al. 1987). The floating adipocytes were washed three times with Dalbeco’s modified Eagle’s medium (DMEM)/F12 containing 1% BSA. Preadipocytes were plated in DMEM/F12 supplemented with streptomycin (0·1 mg/ml), penicillin (100 U/ml), and 10% FCS and maintained at 37 °C under 5% CO₂ 95% air atmosphere. After plating at a density of 2–3 × 10⁵ cells/cm², cells that exhibited a fibroblast-like morphology were extensively washed and maintained (i) for cell growth experiments, in DMEM/F12 supplemented with antibiotics and 10% FCS until confluence (3–4 days after plating), or (ii) for differentiation studies, in DMEM/F12 with antibiotics, insulin (80 nM), transferrin (10 μg/ml), triiodothyronin (0·2 nM), 3-isobutyl-1-methylxanthine (IBMX; 0·2 mM), and troglitazone (1 μg/ml) for 2–3 days and then in DMEM/F12 with antibiotics, insulin (80 nM), transferrin (10 μg/ml), triiodothyronin (0·2 nM), and cortisol (100 nM; insulin, transferrin, triiodothyronin (ITT) medium) in the absence or presence of HCG, as described in Wabitsch et al. (1995) until the full differentiation state was achieved, which corresponded to 80–90% of cells accumulating intracellular lipid droplets (12–14 days after plating). Medium was changed every other day for control and HCG-treated cells.

Cyclic AMP (cAMP) production in human confluent preadipose cells in culture

Confluent preadipocytes were rinsed three times with Hank’s solution and then incubated in Krebs–Ringer solution containing 2% albumin and 0·1 mM IBMX. After 5-min preincubation at 37 °C, the cells were treated with HCG (5000 mU/ml) for 5, 15, 30, and 60 min. In addition, a dose–response experiment was also performed by treating cells with either 50, 500, or 5000 mU/ml HCG for 15 min. At the end of the incubation, cells were lysed in Tris 50 mM, 4 mM EDTA and kept for 30 s in a boiling water bath. cAMP concentrations were measured following a radioimmunoassay assay as described in Gaben–Cogneville et al. (1984). Forskolin

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(10 µM), the adenylyl cyclase activator, was used as a positive control at the 15 min time point.

**Lipolysis and cAMP production in human mature adipocytes**

Glycerol release and cAMP production were measured as previously described in Lacasa et al. (1986). Briefly, human adipocytes were incubated for 1 h (lipolysis) or 30 min (cAMP) at 37 °C in Krebs-Ringer-25 mM Tris buffer (pH 7.4) containing 5 mM glucose, 3% albumin and when indicated 0.1 mM IBMX and 50 µM/ml adenosine deaminase. Forskolin (10 µM) and isoproterenol (1 µM) were used as positive controls.

**Leptin secretion**

To study the effects of HCG on leptin secretion, adipose tissue fragments were prepared after careful dissection of the fat depots discarding the connective tissue. The resulting fragments were placed in 12-well dishes (300 mg adipose tissue/well) containing 3 ml DMEM/F12 supplemented with antibiotics, BSA (5%), 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF; 20 µg/ml), leupeptin (4 µM), vitamin E (4 mg/ml), and the hormones tested at 37 °C under 5% CO₂ and 95% air atmosphere. Twenty-four hours later, aliquots were taken from the culture media and kept at –20 °C until used for leptin RIA as described elsewhere (Machinal et al. 1999).

[^3H]thymidine incorporation

Human preadipose cells were suspended in 12-well plates containing DMEM–Ham’s supplemented with 10% FCS. During the exponential phase of growth, the culture medium was replaced by DMEM–Ham’s containing 2% charcoal-stripped FCS for 24 h. For the next 24 h, [^3H]thymidine (1 mCi/ml) was added to the culture medium in the presence or absence of HCG from Organon Laboratories at different concentrations (5–5000 mU/ml) or human recombinant HCG from Sigma at 5000 mU/ml. After washing thrice with saline, cells were lysed for 5 min with 1% SDS and 0.1 mM EDTA, 0.5 mM AEBSF, 30 mM β-glycerophosphate, 5 µg/ml aprotinin, and 12.5 µg/ml leupeptin. After centrifugation at 100 000 g for 10 min at 4 °C, supernatants were diluted in Laemmli’s buffer (vol/vol). Equal amounts (10 µg) of cellular extracts were subjected to SDS-PAGE (12.5%). Proteins were transferred to polyvinylidenedifluoride (PVDF) membrane and blocked in buffer A (20 mM Tris HCl, 137 mM NaCl, and 1% Tween 20, pH 7-6) with 2.5% gelatin for 2 h. Then membranes were incubated overnight at room temperature with the primary antibody diluted in buffer A with 2.5% gelatin (1:7000 dilution).

The resulting blots were extensively washed with buffer A and incubated with the secondary antiserum coupled to peroxidase (1:10 000 dilution in buffer A) for 1 h at room temperature and washed in buffer A. Finally, an enhanced chemiluminescence kit was used for signal detection. Reprobing of the membranes gave identical results. Specificity of the immunoreactive proteins was verified by the loss of sample immunoreactivity when incubated with the antiserum neutralized with the corresponding specific peptide. Control experiments with various amounts of protein (5–50 µg) were performed to ensure that the densitometric signal intensity was proportional to the amount of protein loaded. To normalize the results, membranes were stripped and reprobed with antiserum specific for non-phosphorylated forms of MAPK.

Protein concentrations were measured according to Bradford (1976) using BSA as standard.

**HCG/LH receptor protein expression**

Mature adipocytes and preadipocytes were sonicated in cold buffer containing 10 mM Tris (pH7-4), 0.25 M sucrose, 5 mM EDTA, 0.5 mM AEBSF, 25 µg/ml aprotinin, and 105 µM leupeptin. After centrifugation at 21 000 g for 20 min at 4 °C, the pellet was resuspended and denatured with Laemmli buffer (vol/vol) and stored at –20 °C. Membrane extracts (50 µg) and human ovary tissue lysate (25 µg) were resolved by SDS-PAGE. Proteins were transferred to PVDF membrane and blocked in buffer A with 2.5% gelatin for 2 h. Then, membranes were incubated overnight at room temperature with a mouse monoclonal antibody (1:100 dilution in buffer A) for 12 h. Membrane blots were stripped and reprobed with antiserum specific for non-phosphorylated forms of MAPK. Protein concentrations were measured according to Bradford (1976) using BSA as standard.

**Glycerol-3-phosphate dehydrogenase (GPDH) assay**

Cell differentiation was followed by measuring the GPDH activity according to Wise & Green (1979) in cell homogenates as previously described (Lacasa et al. 1997a,b).
**Isolation of RNA**

Total RNA was isolated from human preadipocytes and adipocytes according to the method of Chomczynski & Sacchi (1987). RNA recovery and quality were checked by measuring the 260/280 nm optical density ratio and by electrophoresis under denaturing conditions on 2% agarose gel.

**RT-PCR**

Total RNA (2.5 μg) were denatured for 10 min at 72 °C and reverse transcribed to cDNA in 20 μl total volume of reverse transcription mixture containing 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl₂, 20 mM dithiothreitol, 0.5 mM of each dNTP, 16 μl RNA guard, 50 ng random hexamers, and 200 U Superscript II reverse transcriptase. Incubation was performed at 42 °C for 60 min, heated to 95 °C for 5 min, and then quickly chilled on ice.

In this semi-quantitative RT-PCR method, two different primer sets were used. One couple of primer sets designed for the amplification of the human HCG/LH-R was used. To ensure that amplification of this gene was within the exponential range, different numbers of PCR cycles (25–40 cycles) were run. Finally, 30 cycles of PCR amplification were chosen to study HCG/LH-R mRNA expression. The second primer set was specific for the 18S cDNA that was used as internal standard. In the same way, different numbers of PCR cycles (25–40 cycles) were run. Finally, 40 cycles of PCR were found to be optimal for detection of the 18S mRNA. PCRs were performed with a thermocycler Gene Amp PCR 2400 (Perkin–Elmer, MA, USA).

PCR products were analyzed on a 2% agarose gel in 90 mM Tris–borate, 2 mM EDTA buffer (TBE; pH8), and visualized by staining with ethidium bromide and u.v. transillumination. Quantification was realized with the Bio-1D software (Vilber Lourmat, Marne la Vallée, France). Controls without reverse transcriptase were systematically performed in order to detect eventual genomic DNA contaminations.

**Real-time RT-PCR**

Total RNA (0.5 μg) was reverse transcribed as previously described (Machinal-Quelin et al. 2002b). Quantitative PCR was performed using a LightCycler instrument (Roche Diagnostics) with QuantiTect SYBR Green PCR Master Mix (Qiagen). Primer sets used are indicated in Table 1. cDNA calibrators were prepared by PCR amplification run to saturation (35 cycles) with the appropriate primers. The resulting cDNAs were purified by QIAquick PCR purification Kit (Qiagen). The samples showed a unique band in agarose electrophoresis. The numbers of cDNA copies were calculated from the absorbance at 260 nm. Calibrators were defined to contain arbitrary units of human c-fos, Ob, adiponectin, PPARγ2, C/EBPα, fatty acid synthase (FAS), ADD1, p53, bcl2, bax, and GAPDH mRNAs, and all calculated concentrations are relative to GAPDH concentrations. In accordance with Gorzelnia et al. (2001), the choice of GAPDH as housekeeping gene was based on the observation that GAPDH mRNA expression is insensitive to various hormones in human adipose cells. Separate calibration curves for human Ob, c-fos, adiponectin, PPARγ2, C/EBPα, FAS, ADD1, p53, bcl2, bax, and GAPDH were constructed from serial dilutions from 10⁸ to 100 copies of cDNA calibrators. Calibration curves were log linear over the quantification range with correlation coefficients (r²) ≥ 0.99 and slopes ranging from −3.5 to −3.8. The intra-assay variability of duplicate crossing point (Cp) values never exceeded 0-2 cycles and the inter-assay variability (coefficient of variation (CV) value) ranged from 1 to 5% CV values for the three or four runs of each transcript.

Real-time PCR was performed in a total reaction volume of 20 μl per capillary for the LightCycler format. Each provided cDNA preparation (50 ng/μl) was diluted 1:10 in

**Table 1 Primer pairs used for RT-PCR**

<table>
<thead>
<tr>
<th>Primer sets</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Ob gene</td>
<td>5’ CCA AGA TGG ACC AGA CAC TG 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ GGC ACC ACC TCT GTG GAG TA 3’</td>
</tr>
<tr>
<td>Antisense C/EBPα</td>
<td>5’ GAG TGG CGG CAG CGG C 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CAG TTC GCC GCT CAG CTG TT 3’</td>
</tr>
<tr>
<td>Antisense Adiponectin</td>
<td>5’ GCT ATG CTC TTC ACC TAT 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ GTC ATT GT CAC ATG ACG C 3’</td>
</tr>
<tr>
<td>Antisense 18S</td>
<td>5’ AAC TCC AAG GAC ACA GTC ACC AT 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CAG CTC CAC GAA CTC AA 3’</td>
</tr>
<tr>
<td>Antisense GAPDH</td>
<td>5’ GAT ACA CTG TCT GCA AAC ATCA CAA 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CCA CGG AGC TGA TCC CAA 3’</td>
</tr>
<tr>
<td>Antisense p53</td>
<td>5’ ATT CCA GCT CCA ATA GCG 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CAC TCA GCT AAG AGC ATG G 3’</td>
</tr>
<tr>
<td>Antisense Bcl2</td>
<td>5’ ACC CAC TCC TCC ACC TTT G 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CTC TGT GTC TCT TGC TGG G 3’</td>
</tr>
<tr>
<td>Antisense Bax</td>
<td>5’ ACT AAG CGA CGA CTG CCC AA 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ ATG GCG GGA GGT AGA AC 3’</td>
</tr>
<tr>
<td>Antisense FAS</td>
<td>5’ ATG TGT GTG GAC AGC GTG AAC C 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ TGA GCA GAG TCT TCA GAG ACG GCC 3’</td>
</tr>
<tr>
<td>Antisense GAPDH</td>
<td>5’ CAA ACT GGT GCT CAA GGC C 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ GCA CTC CCG CCA CAA AGA T 3’</td>
</tr>
<tr>
<td>Antisense Human HCG/LH-R</td>
<td>5’ GAC CGC TTC CGA GAT TCT AT 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ CAG GCT CAC AAA CGA ATG GA 3’</td>
</tr>
<tr>
<td>Antisense ADD1</td>
<td>5’ ACC TCC AAC TGC AAA AAT GG 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ TTA CGT GTG GCA GTG ATC AT 3’</td>
</tr>
<tr>
<td>Antisense GAPDH</td>
<td>5’ GGA GCC ATG GAT TGC ACA TT 3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’ AGG AAG GCT TCC AGA GAG GA 3’</td>
</tr>
</tbody>
</table>

water. The reaction buffer contained 10 μl 2X QuantiTect SYBR Green PCR Master Mix (including HotStar Taq DNA polymerase, reaction buffer, deoxyNTP mixture, and SYBR Green 1), 0.5 μM of each primer, and 4 μl diluted cDNA or calibrator. To verify that fluorescence generated by SYBR green incorporation into double-stranded DNA was not overestimated by contaminations resulting from residual genomic DNA amplification and/or from primer–dimer formation, controls without reverse transcriptase, and without DNA template or reverse transcriptase were included in each experiment.

After PCR, a melting curve was constructed by increasing the temperature from 65 to 95 °C with a transition rate of 0.1 °C/s to verify the specificity of the desired PCR products and the absence of primer–dimers. To validate the melting curve results, representative samples of PCR products were separated by 2% agarose gel electrophoresis.

The second derivative maximum method was used to automatically determine the CP for the individual samples. For each sample, ΔCP values were determined (CP of the target gene minus CP of the GAPDH gene). Fold changes in expression were calculated according to the transformation: fold increase = 2^(-ΔCP).

Statistical analysis

All values were expressed as means ± S.E.M. of four to seven different experiments. Statistical analyses were performed using the non-parametric paired Wilcoxon test or unpaired Mann–Whitney U test because only two populations (hormonal incubation versus control incubation) were compared. For real-time PCR data, statistical analysis was performed on ΔCP values, rather than on fold changes.

Results

In the following investigations, the effects of HCG were essentially the same whatever the sex of the donors was. Therefore, most of the data shown herein are means of the results of pooled experiments performed in both men and women cells. Moreover, as the effects of HCG were the same (cell proliferation, MAPK activation, etc.), whatever the origin of this hormone was (recombinant or extracted), all the following experiments were performed with extracted human HCG for economic reasons.

HCG/LH receptor expression in human adipose cells

Using RT-PCR analysis, we found that HCG/LH-receptor (HCG/LH-R) mRNA is expressed in both human confluent and differentiated preadipocytes as well as in human adipocytes whatever the sex was (Fig. 1a). Moreover, HCG/LH-R expression is comparable with that observed in mammary epithelial cancer cells (MCF-7) used as a positive control (Lojun et al. 1997, Meduri et al. 1997). Interestingly, HCG/LH-R mRNA abundance in human adipose cells is dependent on the stage of adipocyte differentiation, being three times higher in fully differentiated adipocytes than in confluent preadipocytes or early differentiating preadipocytes (Fig. 1a).

Western blot analysis confirmed the presence of the HCG/LH-R in human preadipocytes (Fig. 1b). As shown in this Figure, several bands were detected in preadipose membrane extracts: a major protein of 68 kDa and minor proteins of 45–48 kDa and 85 kDa. According to Misrahi et al. (1996), the profile of these bands corresponds to different variant mRNAs generated by alternative splicing. The mass of the detected component co-migrated with the ovary tissue extracts that served as positive control.

HCG influence on lipolysis, lipogenesis, and cAMP production of human mature adipocytes

The influence of various concentrations of HCG (5–50 000 mU/ml) on human adipose lipolysis was next investigated. Lipolysis was studied for 1 h in the presence of
adenosine deaminase in order to suppress the antilipolytic effect induced by the release of endogenous adenosine (Lacasa et al. 1991). Under these conditions and at all doses tested, HCG failed to affect lipolysis of human adipocytes (data not shown). Under the same experimental conditions, however, forskolin (10 μM) and isoproterenol (1 μM), two activators of the lipolytic cascade, induced a modest but significant increase (1.4- and 1.3-fold respectively), in the amount of glycerol released from human adipocytes.

As the fat cell size depends on a balance between lipolysis and lipogenesis, we decided to study the direct effect of HCG on the expression of FAS and ADD1, two markers of lipogenesis (Butterwith 1994). No effect, however, was seen after exposure of mature adipocytes to HCG (5000 mU/ml) for 24 h (data not shown).

Since the classical HCG signal transduction pathway is the activation of the adenylyl cyclase–cAMP–protein kinase A (AC–cAMP–PKA) pathway (Segaloff & Ascoli 1993), we tested the influence of HCG on human adipocyte cAMP production. These experiments were performed after adipocyte exposure for 30 min to different doses (5–5000 mU/ml) of HCG followed by cell incubation in the presence of adenosine deaminase and IBMX, a potent inhibitor of cAMP phosphodiesterase. Under these conditions, HCG failed to elicit any effect on human adipocyte cAMP production (data not shown). This lack of effect of HCG could not be attributed to the non-functionality of the AC enzyme, since under the same experimental design, forskolin (10 μM) and isoproterenol (1 μM), two activators of AC, induced a four- and tenfold increase in cAMP production respectively.

**HCG modulation of human preadipocyte proliferation**

Cell proliferation was studied by measuring changes in the rate of DNA synthesis ([3H]thymidine incorporation). As shown in Fig. 2, preadipocyte exposure to HCG (5000 mU/ml) for 24 h resulted in an increased [3H]thymidine incorporation (+59 ± 11%, n = 5) for HCG from Organon Laboratories and (+68 ± 12%, n = 5) for human recombinant HCG from Sigma. The latter effect was confirmed by direct cell counting (+47 ± 3%, n = 3). For comparison, the magnitude of the increase in [3H]thymidine incorporation caused by 10% FCS, used as a positive control, was equivalent to +90 ± 22% (n = 5). For the following studies, we have chosen this concentration of HCG (5000 mU/ml), which is in the range of those tested in vitro by other investigators (Nalbant et al. 1998, Zygmunt et al. 2002).

**HCG action on different cytoplasmic signaling pathways in human confluent preadipocytes**

In human confluent preadipocytes, we first tested the influence of HCG on cAMP production following HCG stimulation for 10, 20, 40, and 60 min. As in human endometrial stromal cells (Kasahara et al. 2001), stimulation of preadipocytes with HCG (5000 mU/ml) revealed no significant changes in cAMP at any of the tested times. Here, again, this negative result could not be attributed to the non-functionality of AC, since forskolin (10 μM) induced a 3.5 ± 0.5-fold increase in cAMP production under the same experimental conditions (data not shown).

MAP kinases (MAPKs), especially p42 and p44 MAPK, play an important role in cell growth in general, and in rat preadipose cell proliferation in particular (Lacasa et al. 1997a,b, 1998). In a recent review discussing HCG/LH actions, it was reported that MAPK may be an important signaling pathway in non-gonadal sites expressing HCG/LH-R (Rao 2001). We therefore investigated the ability of HCG to activate the p42/p44 MAPK pathway by performing western blot analysis of human preadipocyte cellular extracts using antibodies recognizing specifically the activated phosphorylated forms of p42/p44 MAPK among total p42/p44 MAPK (activated plus inactivated).

As shown in Fig. 3, exposure of human preadipocytes to HCG (5000 mU/ml) induced a clear activation of the p42/p44 MAPK pathway in non-gonadal sites expressing HCG/LH-R (Dos Santos et al. 2002). The expression of the p42 isoform in human preadipocytes is higher than that of the p44 isoform. Reproducing the membranes using an antibody that recognizes the non-phosphorylated forms of p42/p44 MAPK confirms that HCG specifically alters MAPK activity but not their expression levels.

In order to determine, among the upstream elements of MAPK pathway, the target(s) of HCG, we next tested two inhibitors, U0126, a selective MAPK kinase (MEK) inhibitor

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**Figure 2** Effect of HCG on DNA synthesis in human confluent preadipocytes. Growing human preadipocytes were exposed for 24 h to different concentrations of HCG from Organon Laboratories (5–5000 mU/ml) or to human recombinant HCG from Sigma at 5000 mU/ml or to 10% FCS used as a positive control in the presence of [3H]thymidine as described under ‘Materials and Methods’. Assays were performed in triplicates for each sample. Results are means ± S.E.M. of five experiments and are normalized as percentages of the control value (without HCG): 1705 ± 320 c.p.m./24 h. *P ≤ 0.03, ns = non-significant compared with control values, Wilcoxon test.
(Favata et al. 1998), and H89, a PKA inhibitor. As shown in Fig. 4, pretreatment of human preadipocytes with U0126 significantly reduced the magnitude of MAP kinase phosphorylation in response to HCG. Conversely, pretreatment with H89 failed to influence HCG stimulation of MAPK. These results imply the involvement of a PKA-independent signaling pathway in the positive MAPK response to HCG in human preadipocytes.

MAPK pathway implication in HCG-induced mitogenesis of human confluent preadipocytes

To evaluate the role of MAPK in the proliferative effect of HCG on human preadipocytes, further experiments were performed using the selective MEK inhibitor, U0126. As shown in Fig. 5, HCG stimulation of [3H]thymidine incorporation was abrogated when the MAPK pathway was inhibited. As can also be seen in Fig. 5, proliferation of preadipose cells was altered by U0126 as well. A similar effect of U0126 per se on DNA synthesis was also described in human vascular smooth muscle cells (Somjen et al. 2005).

Altogether, these results strongly suggest that the mitogenic effect of HCG on human preadipocytes is mediated via MAPK pathway activation.

A apoptotic and antiapoptotic gene expressions in response to HCG in growing undifferentiated human preadipocytes

In order to further examine the mechanism whereby HCG increases the number of human preadipocytes, the expressions of the antiapoptotic \textit{bcl2} gene and the proapoptotic \textit{Bax} and \textit{Bax} genes were analyzed (Figures 6 and 7). The results showed that HCG treatment upregulated the expression of \textit{bcl2}, while downregulating the expression of \textit{Bax}. This suggests that HCG may play a role in the survival of preadipocytes via the \textit{bcl2} gene.
p53 genes were compared in preadipocytes exposed or not to 5000 mU/ml HCG. Under the same experimental conditions used in the thymidine incorporation studies (see Fig. 2), we failed to see any significant change in Bax, p53, and bc12 mRNA after the exposure of growing preadipocytes to HCG for either 1 or 24 h (data not shown).

c-fos mRNA expression in response to HCG in human confluent preadipocytes

Once activated, MAPK rapidly translocates into the nucleus in order to affect target genes and, in particular, the expression of immediate early genes (Janknecht et al. 1995). As c-fos proto-oncogene induction plays an important role in adipogenesis (Spiegelman et al. 1988) and as induction of c-fos gene expression by HCG has already been observed in vitro in various non-adipose cells (Schultz et al. 1995, Suzuki et al. 1998), we studied the influence of HCG on c-fos mRNA expression in human preadipocytes. As shown in Fig. 6, HCG (5000 mU/ml) induced a robust and transient induction of c-fos mRNA expression. c-fos mRNA, which was almost undetectable before HCG stimulation, reached a peak level after 30 min of HCG exposure (14.8 ± 3.8, n = 3), and then rapidly decreased. For comparison, 10% FCS as a control enhanced c-fos mRNA expression to 33.5 ± 3.1 (n = 3). These findings indicate that the c-fos proto-oncogene is a nuclear target of HCG-R signaling in human preadipocytes.

**HCG modulation of human adipocyte differentiation**

The influence of HCG on the capacities of human preadipocytes to differentiate was next investigated by measuring GPDH activity, a late marker of adipose conversion (Wise & Green 1979). As shown in Fig. 7, adipogenic capacities of human preadipocytes were moderately but significantly increased after 12–14 days of exposure to HCG, whatever the concentrations tested were (5–5000 mU/ml). The maximal effect was observed with 50 mU/ml HCG concentration (+38 ± 6%, n = 3). As negative control, we used 10 ng/ml tumor necrosis factor α (TNFα; Prins et al. 1997) and, as can be seen in Fig. 7, this factor strongly inhibited the differentiation process (−72 ± 4%, n = 5).

To gain more information about the molecular basis underlying this HCG-stimulated adipogenesis, expression of two early transcriptional factors (PPARγ2 and C/EBPz) playing a key role in the adipose conversion process (MacDougald & Lane 1995, Fajas et al. 1998) was studied. As depicted in Fig. 8, PPARγ2 and C/EBPz mRNA expressions were almost doubled in human differentiated preadipocytes after their exposure to HCG (5000 mU/ml) for 6–8 days. In comparison, TNFα treatment resulted in an almost complete inhibition of the expression of these two transcriptional factors.

**HCG action on leptin secretion in human adipocytes**

For the following in vitro studies, secretion of the proadipogenic factor, leptin, the Ob gene product, was investigated in a culture media of human adipose tissue fragments and not of fat cells. This choice was based on the observation that leptin amounts secreted by isolated adipocytes and preadipocytes are far below the detection range of the leptin RIA, which is not the case when whole adipose tissue fragments are studied (Machinal et al. 1999). Data in Fig. 9 show a significant enhancement of the amount of leptin secreted into the culture media after a 24-h incubation in the presence of 500 or 5000 mU/ml HCG. In addition, the leptin increase observed with 5000 mU/ml HCG (+120 ± 50%, n = 6) was similar to that induced by 10 nM 17β-estradiol (E2; +160 ± 50%, n = 6), used as a positive control in these experiments (Machinal-Quelin et al. 2002a).

Surprisingly, under the same experimental conditions, we failed to observe any significant effect of HCG on the Ob gene expression in human mature adipocytes whatever the
concentration of HCG tested was (Fig. 10). In contrast, 10 nM E2, used as positive control (Machinal-Quenlin et al. 2002a), induced a significant increase in the Ob gene expression (Fig. 10).

In parallel, we also studied the influence of HCG on the expression of adiponectin, another factor secreted by adipocytes playing an important role in glucose homeostasis and insulin sensitivity (Lin et al. 2004). As for the Ob gene, we failed to observe any significant change in adiponectin mRNA after a 24-h exposure to HCG (data not shown).

Discussion

Pregnancy is a hypermetabolic state associated with a physiological increase in maternal body fat and weight (Herrera et al. 1991). In order to gain a better understanding of the factors regulating body fat mass during gestation and more particularly during the first trimester, we have examined

Figure 7 Influence of HCG on GPDH activity in human differentiated preadipocytes. (a) Morphological aspect of primary cultures of human preadipocytes. Growing preadipocyte cells (3–4 days after plating) were maintained in ITT differentiation medium for 12–14 days in the absence (CONT) or presence of 50 mU/ml HCG or 10 ng/ml TNFα. The medium was changed every other day for control and treated cells as described under ‘Materials and Methods’. (b) GPDH activity was measured in cytosolic fractions from human preadipocytes after chronic treatment with various concentrations of HCG or TNFα (10 ng/ml) for 12–14 days of exposure to ITT differentiation medium. Data are means ± s.e.m. of five separate experiments performed in triplicate and are normalized as percentages of the control value (without HCG or TNFα). *P ≤ 0.03 compared with control values, Wilcoxon test.

Figure 8 Influence of HCG on C/EBPα and PPARγ2 mRNA expressions in human differentiated preadipocytes. Human preadipocytes were allowed to differentiate as described under ‘Materials and Methods’. After 6–8 days of exposure to HCG (5000 mU/ml), total RNAs were extracted and subjected to real-time RT-PCR to determine C/EBPα and PPARγ2 mRNA levels. Values are the mean ± s.e.m. obtained from three separate experiments. *P ≤ 0.05, **P ≤ 0.01 compared with control values, Mann–Whitney test.
Let's start by examining the contents of the document. We have a series of statements and findings related to the effects of HCG on adipose tissue and the mechanisms involved in its action. The text is divided into several sections, each discussing different aspects of HCG's impact on adipose cells.

### Section 1: Effect of HCG on Adipose Tissue Secretion

- **Figure 9**: In vitro effects of HCG on leptin secretion from human adipose tissue. Fragments from human adipose tissue were incubated for 24 h in the absence (control) or presence of HCG (500 or 5000 mU/ml) or 17β-estradiol (E2; 10 nM). At the end of the incubation, samples of the culture medium were collected and assayed for leptin by RIA. Results are expressed as arbitrary units. Each bar represents the mean ± S.E.M. of duplicate assays from three to four separate experiments and is normalized as the percentage of the control value: 8.8 ± 1.3 ng/g lipid/24 h. *P≤0.03, **P≤0.01 compared with control values, Wilcoxon test.

### Section 2: The Effects of HCG on Adipose Development and Metabolism

For this purpose, we first attempted to determine whether human adipose cells express the HCG/LH-R gene. For the first time, our results reveal that both human adipocytes and their precursors express HCG/LH-R, a finding that strongly suggests that fat tissue is a target for this placental hormone.

Under our experimental conditions, HCG did not affect lipolysis of human adipose tissue. This result is consistent with another in vitro study that did not reveal an effect of HCG on the release of glycerol from rodent adipose tissue (Farmer et al. 1972). The hypothesis of an eventual control of adipocyte metabolism by HCG was also investigated herein, but our results failed to show any increase in the expression of the two markers of lipogenesis, FAS and ADD1 (data not shown).

It is well established that the adipocconversion process in a human is regulated by various growth factors, cytokines, sex steroids, etc. (Bjorntorp 1996). More particularly, estrogens have been reported to increase in vitro human preadipocyte replication (Roncari & Van 1978, Anderson et al. 2001) without altering the differentiation process (Hauner et al. 1987). However, no direct effects of HCG on human adipogenesis have been so far explored. Our experiments show that, at physiological concentrations, HCG in vitro has a direct and positive influence on both the proliferation and the differentiation capacities of human preadipocytes.

Concerning the mitogenic action of HCG, different hypotheses can be put forward: i) HCG could act as an antiapoptotic factor by reducing the expression of apoptotic genes and/or by inducing antiapoptotic genes in human preadipocytes as was reported in human breast epithelial cancer cells (Srivastava et al. 1998, Guo et al. 2004). However, this hypothesis is not supported by our present experiments revealing unaltered expressions of the apoptotic p53 and Bax genes or of the antiapoptotic bcl2 gene in HCG-exposed growing preadipocytes; ii) HCG could also act indirectly via a crosstalk between HCG and estrogens as suggested by the following observations. First, in human endometrial cells in vitro, HCG modulation of cyclooxygenase 2 mRNA expression requires a pretreatment of these cells with estradiol, probably because this estrogen increases HCG/LH-R number and/or induces, at the nucleus level, the recruitment of cofactors, which are necessary for HCG action (Munir et al. 1999). Secondly, the repressive effect of HCG on the estrogen receptor α (ERα) expression has been considered as the mechanism responsible for the defective growth of HCG-treated estrogen-dependent mammary cancer cells (Lojun et al. 1997). However, our preliminary experiments failed to show any effect of HCG on ERα mRNA expression in human preadipocytes (unpublished observation), which thus excludes this mechanism to explain how HCG exerts its mitogenic effect on human preadipocytes; iii) HCG could act directly on the preadipocyte cell cycle by modulating via HCG/LH-R the expression and/or the activity of cyclin-dependent kinases (CDK) and cyclins. As a matter of fact, in two recent studies, HCG was reported to directly promote the growth of human uterine myometrial cells (Horiuchi et al. 2000) and rodent granulosa cells (Cannon et al. 2005) through increased expression of cyclin E and cdk2, two important cell cycle-related proteins. Further experiments will be needed to establish whether HCG interferes with the human preadipocyte cell cycle.

In gonadal tissues, HCG via its receptors stimulates the AC-cAMP-PKA pathway (Segaloff & Ascoli 1993). However, as already observed in human endometrial epithelial cells and osteoblasts (Munir et al. 1999, Srisuparp et al. 2002, Yarram et al. 2003), the present study fails to reveal any HCG activation of this signaling pathway in human preadipose cells. Exposure of human preadipocytes to HCG leads rather to a rapid PKA-independent activation of the MEK/MAPK/c-fos signaling pathway. The latter finding indicates that HCG activates a different set of signal transduction in these cells and thus provides a solid argument...
in favor of the functionality of the HCG/LH-R. The lack of stimulation of the AC–cAMP–PKA pathway in human preadipocytes could be explained by the presence of some but not all known AC isoforms (Serazin Leroy et al. 2001), an increased phosphodiesterase activity or a deficient HCG/LH-R coupling to Gs. Among these hypotheses, the latter seems to be the most plausible explanation since: i) like in peripheral non-gonadal tissues, where various HCG/LH-R transcript isoforms differing from the Gs-coupled 85 kDa full-length transmembrane species and generating truncated receptors have been described (You et al. 2000), our western blots detected various immunoreactive proteins of lower molecular weight with the major fraction of 68 kDa and a protein doublet between 45 and 48 kDa, ii) a new receptor variant for another gonadotropin (FSH) with a single transmembrane domain and acting as a growth factor receptor has recently been identified in ovarian granulosa cells (Babu et al. 2000), and iii) it was demonstrated that gonadotropin–releasing hormone receptor initiates multiple signaling pathway and more particularly MAP kinase activation by an exclusive coupling to the pertussis toxin-insensitive Gq/G11 (Grosse et al. 2000). Hence, we could postulate a link between HCG/LH-R and Gq/G11 rather than Gs.

Little is known concerning the regulation of transcriptional factors by HCG. In some studies, C/EBP transcriptional factors were reported to be regulated by HCG during follicular development. In rodent granulosa cells, for instance, C/EBPβ and C/EBPα mRNA expressions were reciprocally induced and down-regulated by HCG (Sirois & Richards 1993, Sterneck et al. 1997). Other investigators also described an HCG regulation of C/EBPα expression in rat primary cultured Leydig cells (Nalbant et al. 1998). Our study thus provides another example of cells where expression of the C/EBPα factor is under the positive control of HCG. Moreover, the HCG-induced increased capacity of human preadipocytes to differentiate, which is particularly obvious in cell morphological studies (Fig. 7), is associated, at the molecular level, with the induction of both the C/EBPα and PPARγ2 genes, two key factors regulating the adipogenic process.

In accordance with the observations made in human cytrophoblasts (Chardonnens et al. 1999, Islami et al. 2003), this study demonstrates that an in vitro exposure to HCG for 24 h is able to induce leptin secretion from human adipose fragments. Moreover, in our laboratory, it has recently been demonstrated that leptin increased both the proliferation and the differentiation of rat s.c. preadipocytes in vitro (Machinal et al. 2002b). These observations suggested that HCG could directly or indirectly via leptin production modulate adipogenesis in human. However, under the present experimental conditions, HCG did not affect Ob gene mRNA level. This negative finding suggests that, in human adipose tissue, HCG enhances the secretion and/or the stability of the leptin protein as was already observed in fully differentiated 3T3-L1 preadipocytes (Sivan et al. 1998) and with sex steroid hormones in rat and human adipocytes (Machinal et al. 1999, Machinal-Quelin et al. 2002a). These findings also support the hypothesis that the increased circulating leptin concentration observed during the first trimester of pregnancy corresponds to increased leptin production not only by the placenta itself (Chardonnens et al. 1999) but also by adipose tissue through direct estrogen (Machinal-Quelin et al. 2002a) and HCG effects on this tissue.

Otherwise, although controversial, some physicians have pointed out the importance of HCG in the approach to fat reduction. In particular, when combined with a severe diet (500 calories per day; severe calorie-restricted diet (SRCD)), daily HCG injections seem to facilitate a substantial weight reduction (Simeons 1954, Asher & Harper 1973, Ballin & White 1974). These different observations suggest an in vivo preventive action of HCG toward adipose mass development. However, the present experiments (lipolysis, cAMP production, FAS, and ADD1 mRNA expression) lead to discard the hypothesis of an eventual and direct HCG control of lipogenesis and lipolysis in human adipose tissue in vitro. These negative findings, which are consistent with another in vitro study (Farmer et al. 1972), allow postulating another site of action for HCG to explain the beneficial effects of the HCG–SRCD association reported by some investigators (Vogt & Belluscio 1987). HCG administration in humans stimulates the secretion of growth hormone, which is a potent lipolytic factor in human adipose tissue (Felig et al. 1971). We can thus speculate that the HCG-induced secretion of growth hormone in vivo is responsible for the effects observed in some but not all clinical investigations dealing with HCG treatment of patients undergoing SRCD.

In summary, we have demonstrated that HCG has direct regulatory effects on proliferation, differentiation, and secretion of the proadipogenic factor, leptin, in human adipose tissue in vitro. We thus suggest that these HCG effects could contribute to explain, at least in part, the increased s.c. fat deposition occurring during the first trimester of pregnancy.

Acknowledgements

The authors express their sincere gratitude to Dr Hugues Loosfelt from the Faculté de Médecine Paris Sud (France) for the monoclonal antibody used to detect the HCG/LH receptor by western blotting. We are particularly indebted to the General Surgery Department of the Centre Hospitalier de Poissy for their courtesy in making human adipose tissue available. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 22 May 2007
Accepted 24 May 2007
Made available online as an Accepted Preprint 29 May 2007