Cross talk between cAMP and p38 MAPK pathways in the induction of leptin by hCG in human placental syncytiotrophoblasts

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Abstract

Leptin produced by the placental syncytiotrophoblasts participates in a number of processes in pregnancy including implantation, proliferation of the cytotrophoblasts, and nutrient transfer across the placenta. Despite the functional significance of leptin in pregnancy, the regulation of leptin synthesis is poorly understood in human placental syncytiotrophoblasts. In this study, we investigated the role of endogenous human chorionic gonadotropin (hCG) in the regulation of leptin production as well as the underlying mechanism involving the cross talk between cAMP and p38 mitogen-activated protein kinase (MAPK) pathways. We found that neutralization of endogenous hCG with its antibody dose dependently decreased leptin mRNA level and secretion, whereas exogenous hCG increased leptin mRNA level and secretion. Activation of the cAMP pathway with dibutyryl cAMP (db cAMP) or forskolin recapitulated the stimulatory effect of hCG on leptin expression. Inhibition of protein kinase A with H89 not only reduced the basal leptin expression but also attenuated the induced leptin expression by hCG. Treatment of the syncytiotrophoblasts with db cAMP and hCG phosphorylated p38 MAPK. Inhibition of p38 MAPK with SB203580 not only reduced the basal leptin production but also attenuated the leptin-induced production by both hCG and db cAMP. These data suggest that endogenous hCG plays a significant role in maintaining leptin production in human placental syncytiotrophoblasts, and this effect involves a cross talk between cAMP and p38 MAPK pathways.

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Introduction

Leptin is crucial in the regulation of food intake and energy expenditure (Margetic *et al.* 2002). Although adipocytes are regarded as the primary source for leptin in human body, the placental syncytiotrophoblasts provide an additional source during pregnancy (Masuzaki *et al.* 1997). The production of leptin from placental syncytiotrophoblasts increases with gestational age, leading to an increased leptin level in maternal circulation (Masuzaki *et al.* 1997, Highman *et al.* 1998, Lage *et al.* 1999). In pathological conditions of pregnancy such as preeclampsia, gestational diabetes, and intrauterine growth retardation, the level of leptin in maternal circulation is further increased (Hytinanti *et al.* 2000a,b, Jansson *et al.* 2003). Leptin produced by the placental syncytiotrophoblasts has been reported to be implicated in the regulation of implantation of the fertilized ovum, proliferation of the cytotrophoblasts, and nutrient transfer across the placenta to the fetus in a paracrine or intracrine manner via leptin receptor present in the intrauterine tissues (Henson *et al.* 1998, Jansson *et al.* 2003, Henson & Castracane 2006, Forhead

& Fowden 2009). Despite the functional significance of leptin in pregnancy, the regulation of leptin synthesis is poorly understood in human placental syncytiotrophoblasts. It is demonstrated that the transcription of leptin gene in human placenta uses a placenta-selective element in the enhancer (Bi *et al.* 1997, Ebihara *et al.* 1997). Activation of the cAMP pathway leads to an increase of leptin synthesis in human placental trophoblasts (Coya *et al.* 2001, Maymo *et al.* 2010), which is in obvious contrast to the decreased leptin production upon activation of the cAMP pathway in adipocytes (Gettys *et al.* 1996, Szkudelski *et al.* 2005). It is reported that this induction of leptin upon activation of the cAMP pathway involves a cross talk to the extracellular signal-regulated kinase (ERK1/2) in human placental cells (Maymo *et al.* 2010). During pregnancy, placental syncytiotrophoblasts synthesize a large amount of human chorionic gonadotropin (hCG). The role of hCG in the maintenance of pregnancy is well known for stimulating progesterone synthesis, promoting uterine quiescence, regulating implantation, and controlling trophoblastic hormonal secretion and differentiation

(Shi *et al.* 1993, Cronier *et al.* 1994, Kraiem *et al.* 1994, Kurtzman *et al.* 2001). It is well established that hCG shares the same receptor with pituitary LH and the hCG/LH receptor is coupled to G α s protein using the cAMP pathway in its signal transduction (Strauss *et al.* 1992). Exogenous hCG is reported to stimulate leptin production in human placental explants and immortalized cell lines (Maymo *et al.* 2009). However, it is not known whether the endogenous hCG plays any role in the maintenance of leptin production in human placental syncytiotrophoblasts. In addition to ERK, there are other members in the mitogen-activated protein kinase (MAPK) family, including p38 MAPK and SAPK/JNK (Roux & Blenis 2004). As activation of both p38 MAPK and ERK1/2 has been reported to be associated with placental trophoblast differentiation (Daoud *et al.* 2005), and activation of p38 MAPK is known to increase leptin synthesis in adipocytes (Trujillo *et al.* 2006), we postulated that activation of p38 MAPK may also be involved in hCG/cAMP-induced leptin production in human placental syncytiotrophoblasts. In this study, we addressed the above issues in cultured primary human placental syncytiotrophoblasts.

Results

Effect of exogenous and endogenous hCG on leptin production in human placental syncytiotrophoblasts

Our previous study has shown that the placental trophoblasts achieved extensive syncytialization 3 days after plating in medium containing 10% newborn calf serum (NCS; Ni *et al.* 2009). The viability and syncytialization of the cells were not affected by the treatments in serum-free medium thereafter. Measurement with quantitative real-time PCR (qRT-PCR) revealed that treatment of the syncytiotrophoblasts with exogenous hCG increased leptin mRNA levels in a concentration-dependent manner with the maximal effect observed around 10 IU/ml (Fig. 1A). hCG at 10 IU/ml significantly increased both leptin mRNA level and secretion (Fig. 1B and C). Neutralization of endogenous hCG with its antibody decreased leptin mRNA level in a dose-dependent manner (0, 1:250, 1:125, 1:62.5; Fig. 2A). The leptin level in the culture medium was also significantly decreased by hCG antibody (1:62.5; Fig. 2B). These results suggest that both exogenous and endogenous hCG play a significant role in upregulating leptin expression and secretion in human placental syncytiotrophoblasts.

Role of the cAMP pathway in the upregulation of leptin production by hCG in human placental syncytiotrophoblasts

HCG/LH receptor is coupled to the cAMP signal transduction. Our previous study also demonstrated

Figure 1 Effect of exogenous hCG on the production of leptin in human placental syncytiotrophoblasts. (A) Exogenous hCG increased leptin mRNA level in a concentration-dependent manner. (B) Exogenous hCG (10 IU/ml) significantly increased leptin mRNA level. * $P < 0.05$ versus control, $n = 8$. (C) Exogenous hCG (10 IU/ml) significantly increased leptin level in the culture medium. ** $P < 0.01$ versus control, $n = 4$.

that neutralization of endogenous hCG decreased the cAMP level in the syncytiotrophoblasts (Ni *et al.* 2009). Thus the effect of activation or inhibition of the cAMP pathway on the induction of leptin production by hCG was investigated in the syncytiotrophoblasts. Activation of the adenylyl cyclase with forskolin (100 μ M) or mimicking the effect of cAMP with dibutyryl cAMP (db cAMP; 100 μ M) significantly increased leptin mRNA level (Fig. 3A and B). Inhibition of protein kinase A (PKA) with H89 (10 μ M) not only decreased the basal leptin mRNA level but also attenuated the stimulation of leptin mRNA expression by hCG (10 IU/ml; Fig. 3C). The dramatic reduction of leptin expression by H89 alone may derive from the inhibition of the basal leptin expression maintained by endogenous hCG. These data suggest that the cAMP pathway plays a significant role in the upregulation of leptin expression by hCG in human placental syncytiotrophoblasts.

Role of p38 MAPK in the induction of leptin production by hCG/cAMP in human placental syncytiotrophoblasts

Treatment of the syncytiotrophoblasts with db cAMP (100 μ M) or hCG (10 IU/ml; 0, 15, 30, and 60 min) induced the phosphorylation of p38 MAPK in a time-dependent manner with the maximal effect observed at 30 min (Fig. 4). SB203580 (10 μ M), an inhibitor of p38 MAPK, not only decreased the basal leptin mRNA level

Figure 2 Effect of hCG antibody on the production of leptin in human placental syncytiotrophoblasts. (A) hCG antibody decreased leptin mRNA level in a dose-dependent manner. * $P < 0.05$, ** $P < 0.01$ versus normal serum control (0), $n = 3$. (B) hCG antibody decreased leptin level in the culture medium. * $P < 0.05$ versus normal serum control (0), $n = 3$.

and secretion but also attenuated the induction of leptin mRNA expression and secretion by either hCG (10 IU/ml; Fig. 5A and B) or db cAMP (100 μ M) significantly (Fig. 6A and B), suggesting that the induction of leptin mRNA expression and secretion by hCG/cAMP is, at least in part, mediated via p38 MAPK in human placental syncytiotrophoblasts.

Discussion

By using cultured primary human placental syncytiotrophoblasts, we demonstrated that endogenous hCG played a significant role in maintaining leptin expression and secretion, and a cross talk between the cAMP and the p38 MAPK pathways was involved in this process.

Although there is little doubt about the functional significance of leptin in pregnancy, it is less clear how leptin expression and secretion are regulated in the placenta. Studies have shown that proinflammatory cytokines, estrogen, glucocorticoids, and activation of the cAMP pathway are all inducers of leptin synthesis in placental syncytiotrophoblasts (Chardonnnens *et al.* 1999, Coya *et al.* 2001, Nuamah *et al.* 2004, Marinoni *et al.* 2008, 2010, Gambino *et al.* 2010). Recently it was demonstrated that exogenous hCG stimulated leptin expression in human placental cell line as well as in human placental explants (Maymo *et al.* 2009), which is confirmed by the findings in this study that exogenous hCG or activation of cAMP pathway with forskolin and db cAMP induced leptin production in primary placental syncytiotrophoblasts. Studies have shown that activation of cAMP pathway inhibited exogenous hCG-induced leptin production despite that both hCG and activation of

cAMP pathway were found to induce leptin production (Maymo *et al.* 2009, 2010). This apparent contradiction may not reflect the physiological situation as the placental syncytiotrophoblasts produce abundant endogenous hCG, and the combination of exogenous hCG and other inducers of cAMP pathway activation might result in non-physiological outcome. HCG is one of the most abundant hormones secreted by the placental syncytiotrophoblasts in pregnancy, which may influence syncytiotrophoblasts in a paracrine or an autocrine manner (Kurtzman *et al.* 2001). Thus the cAMP pathway may have already been activated by endogenous hCG via the $G_{\alpha s}$ -protein coupled-hCG/LH receptor at the basal state (Bahl 1977). This notion is supported by the findings that both hCG antibody and PKA inhibitor H89 could significantly decrease leptin expression and secretion. HCG has important implications in the maintenance of pregnancy (Shi *et al.* 1993, Kurtzman *et al.* 2001). Leptin is also reported to be involved in implantation, proliferation of the cytotrophoblasts, and nutrient transfer across the placenta (Jansson *et al.* 2003, Henson & Castracane 2006, Magarinos *et al.* 2007, Forhead & Fowden 2009). The induction of leptin production by hCG in the syncytiotrophoblasts may thus contribute to the maintaining effect of hCG on pregnancy.

As placental leptin is mainly synthesized by the syncytiotrophoblasts, the process of syncytium formation

Figure 3 Effect of cAMP pathway on leptin mRNA expression in human placental syncytiotrophoblasts. (A) Activation of adenylyl cyclase with forskolin (100 μ M) increased leptin mRNA level. ** $P < 0.01$, $n = 5$. (B) Dibutyryl cAMP (db cAMP, 100 μ M) increased leptin mRNA level, $n = 3$. ** $P < 0.01$. (C) H89 (PKA inhibitor, 10 μ M) attenuated both basal and hCG (10 IU/ml)-induced leptin mRNA expression. ^a $P < 0.05$ versus control, ^b $P < 0.01$ versus hCG, $n = 3$.

Figure 4 Phosphorylation of p38 MAPK (P-p38) induced by dibutyryl cAMP (100 μ M) (A) and hCG (10 IU/ml) (B) in human placental syncytiotrophoblasts. Upper panels of A and B are the representative blots. Bottom panels are the average data of three experiments. * $P < 0.05$, ** $P < 0.01$ vs 0 min.

may promote the production of leptin (Masuzaki *et al.* 1997, Ashworth *et al.* 2000). In addition, both hCG and cAMP pathway have been demonstrated to promote trophoblast syncytialization (Strauss *et al.* 1992, Shi *et al.* 1993, Yang *et al.* 2003), which may, at least in part, account for the effect of hCG/cAMP on leptin production during syncytialization. However, in this study, the effect of hCG/cAMP on leptin production was studied in the placental trophoblasts 3 days after plating in medium containing 10% NCS. Under this incubation condition, maximal syncytialization is known to have taken place (Kliman *et al.* 1986, Ni *et al.* 2009). Therefore we believe that hCG/cAMP may also increase leptin production via induction of its mRNA expression in syncytiotrophoblasts, which is supported by the findings that hCG/cAMP is capable of stimulating leptin promoter activity (Maymo *et al.* 2009, 2010).

Cross talk between cAMP and MAPK pathways has been documented at multiple levels in a number of cell types (Bhat *et al.* 2007, Rey *et al.* 2007, Sengupta *et al.* 2007). It was demonstrated that the induction of leptin expression by the cAMP pathway was mediated via the activation of ERK/MAPK in a placental cell line (Maymo *et al.* 2010). Furthermore it was found that activation

of p38 MAPK by forskolin could be inhibited by H89 in osteoblastic cells (Rey *et al.* 2007). Moreover induction of CREB phosphorylation by forskolin was found to be mediated through activation of p38 MAPK in oligodendrocytes (Bhat *et al.* 2007). Of interest, it was reported that hCG induced neuronal differentiation through activation of both ERK and p38 MAPK via hCG receptor activation of cAMP pathway (Meng *et al.* 2007). As activation of p38 MAPK is known to increase leptin synthesis in adipocytes (Trujillo *et al.* 2006), we postulated that cross talk between cAMP and p38 MAPK pathway is possibly involved in the induction of leptin production by hCG in placental syncytiotrophoblasts. This notion is supported by our findings that both basal and leptin-induced production by either hCG or db cAMP could be attenuated by p38 MAPK inhibitor as well as by the observation that the phosphorylation of p38 MAPK was significantly increased by db cAMP and hCG in the syncytiotrophoblasts.

In summary, we presented evidence in this study that endogenous hCG maintains leptin production via activation of the cAMP pathway. Subsequent phosphorylation of p38 MAPK following activation of cAMP pathway may account, at least in part, for the increased leptin production by hCG in cultured human placental syncytiotrophoblasts.

Materials and Methods

Human placental trophoblast cell culture

Human placentae were obtained from uncomplicated normal-term (38–40 weeks) pregnancies after elective cesarean section

Figure 5 (A) Effect of SB203580 (p38 MAPK inhibitor, 10 μ M) on the basal and hCG (10 IU/ml)-induced leptin mRNA expression in human placental syncytiotrophoblasts. ^a $P < 0.01$ versus control, ^b $P < 0.01$ versus hCG, $n = 3$. (B) Effect of SB203580 (p38 MAPK inhibitor, 10 μ M) on the basal and hCG (10 IU/ml)-induced leptin secretion in human placental syncytiotrophoblasts. ^a $P < 0.05$, ^b $P < 0.01$ versus control, ^c $P < 0.01$ versus hCG, $n = 4$.

Figure 6 (A) Effect of SB203580 (p38 MAPK inhibitor, 10 μ M) on the basal and dibutyl cAMP (db cAMP, 100 μ M)-induced leptin mRNA expression in human placental syncytiotrophoblasts. ^a $P < 0.01$, ^b $P < 0.05$ versus control, ^c $P < 0.01$ versus db cAMP, $n = 3$. (B) Effect of SB203580 (p38 MAPK inhibitor, 10 μ M) on the basal and dibutyl cAMP (db cAMP, 100 μ M)-induced leptin secretion in human placental syncytiotrophoblasts. ^a $P < 0.01$, ^b $P < 0.05$ versus control, ^c $P < 0.01$ versus db cAMP, $n = 5$.

without labor following a protocol approved by the Ethics Committee of School of Life Sciences of Fudan University. Subjects gave informed, written consent. Placental cytotrophoblast cells were prepared using a modified method of Kliman (Kliman *et al.* 1986) as described previously (Sun *et al.* 1997). In brief, tissue aliquots were randomly removed from the maternal side of the placenta and digested with 0.125% trypsin (Sigma Chemical Co.) in DMEM (Gibco). The placental cytotrophoblasts were purified using a 5–75% Percoll (Sigma) gradient at step increments of 5%. The cytotrophoblasts were plated at a density of 1.5×10^6 cells/well in 6-well plates for culture at 37 °C in 5% CO₂, 95% air in DMEM containing 10% NCS (Gibco) to allow syncytialization *in vitro* for 3 days. The culture medium was replaced with serum-free medium 3 days after plating and the cells were treated with forskolin (an adenylyl cyclase stimulator, Sigma), db cAMP, an analog of cAMP (Sigma), hCG antibody (C8543, Sigma) or normal serum, hCG (Sigma) in the presence and absence of PKA inhibitor H89 (Sigma) or p38 MAPK inhibitor SB203580 (Sigma), and db cAMP in the presence and absence of SB203580. The concentrations of these reagents are shown in the Results section and in the corresponding figure legends and were chosen according to the literature report as well as our pilot study. The culture medium was collected for leptin measurement with enzyme immunoassay (EIA) 24 h after treatment and cells were lysed for total RNA extraction and leptin mRNA measurement with qRT-PCR. To measure the phosphorylation of p38 MAPK on activation of cAMP pathway, the syncytiotrophoblasts, after washing, were incubated in serum-free medium with db cAMP or hCG for 0, 15, 30, and 60 min, total cellular protein was then extracted for the analysis of phosphorylated, and total p38 MAPK levels with western blotting.

Extraction of RNA and measurement of leptin mRNA level with qRT-PCR

Total RNA was extracted from the placental syncytiotrophoblasts 24 h after treatment using UNIQ-10 RNA extraction kit (Sangon Biotech, Shanghai, China). After determination of RNA concentration, mRNA was reverse transcribed to cDNA with oligo (dT) 12–18 primer using Moloney murine leukemia virus reverse transcriptase (Promega), and cDNA was used for subsequent measurement of leptin level with qRT-PCR using power SYBR green PCR master mix (Toyobo, Osaka, Japan). Primers for amplifying leptin were 5'- GATGACACCA-AAACCCTCATC- 3' (sense) and 5'- GGCCACCACCTCTGTG-GAGTA- 3' (antisense). The annealing temperature was set at 61 °C. The absolute mRNA levels in each sample were calculated according to a standard curve set up using serial dilutions of known amounts of specific PCR product templates against corresponding cycle threshold values. To control for sampling errors, qRT-PCR for the housekeeping gene β -actin was routinely performed on each sample. Primers for amplifying β -actin were 5'-GGGAAATCGTGCGTGACAT-TAAG-3' (sense) and 5'-TGTGTTGGCGTACAGGTCTTTG-3' (antisense). The ratio of the copy number of target gene over the copy number of β -actin in each sample was obtained to normalize the expression of the target gene.

Extraction of protein and measurement of p38 MAPK level with western blotting

Total cellular protein was extracted from human placental syncytiotrophoblasts 0, 15, 30, and 60 min after stimulation with db cAMP or hCG using an extraction kit from Active Motif (Carlsbad, CA, USA). Phosphatase inhibitor was included in all the buffers used in protein extraction. The levels of phosphorylated p38 MAPK and total p38 MAPK were measured following a standard western blotting protocol. Briefly, after determination of protein concentration with Bradford assay, 50 μ g sample protein was electrophoresed in 10% SDS-polyacrylamide gel and transferred to the nitrocellulose blot. After blocking with 5% nonfat milk buffer, the blot was incubated with 1:500 dilution of antibodies recognizing phosphorylated p38 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and total p38 MAPK (Santa Cruz) overnight. After washing, the blot was incubated with appropriate secondary antibody conjugated with HRP (Santa Cruz) for 1 h. The enhanced chemiluminescent detection system (Amersham) was used to detect the bands with peroxidase activity. The level of phosphorylated p38 MAPK is expressed as the ratio of band densities of phosphorylated p38 MAPK over total p38 MAPK.

EIA for leptin

Serum-free medium removed from the placental syncytiotrophoblasts 24 h after treatment was assayed for leptin level using an EIA kit (Millipore Corporation, Billerica, MA, USA) following a protocol provided by the manufacturer.

Statistical analysis

All data are reported as mean + s.e.m. of repeated experiments on the placental syncytiotrophoblasts prepared from different placentae. Paired Student's *t*-test or one-way ANOVA test followed by the Student–Newman–Keuls test was used where appropriate to assess significant differences. Significance was set at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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