Regulation of Placental Growth Hormone Secretion in a Human Trophoblast Model—The Effects of Hormones and Adipokines

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ABSTRACT: Placental growth hormone (PGH) is secreted from the human placental syncytiotrophoblast into the maternal circulation. PGH levels in pregnant women correlate with the birth weight of their offspring. We hypothesized that metabolic regulators may alter PGH secretion. BeWo cells as human trophoblast models were treated for 24, 48, and 72 h with insulin, insulin-like growth factor (IGF)-1, cortisol, ghrelin, leptin and visfatin. Cyclic-adenosinmonophosphate treatment served as positive control. PGH concentrations in culture media were measured. Insulin reduced ($p < 0.008$; analysis of variance) PGH secretion from BeWo cells after 72 h. No effect was found when treating cells with IGF-1. Cortisol reduced PGH secretion after 48 h ($p < 0.00118$; analysis of variance) and 72 h ($p < 0.015$). Leptin and ghrelin both suppressed ($p < 0.027$ and $p < 0.017$, paired t test) whereas visfatin increased ($p < 0.014$, paired t test) PGH secretion at 72 h. Cyclic adenosinmonophosphate increased ($p < 0.003$) PGH secretion at 72 h. Our results indicate that *in vitro* PGH secretion by BeWo cells is regulated by hormonal factors and adipokines. We speculate on the existence of a maternal-placental regulatory loop, in which elevated insulin and leptin levels might down-regulate PGH secretion. (*Pediatr Res* 63: 353–357, 2008)

Placental growth hormone (PGH) is synthesized in and secreted from the human placental syncytiotrophoblast (1) into the maternal circulation in a nonpulsatile manner, detectable in the maternal circulation from gestational week 5, and gradually increases in concentration throughout pregnancy. It has a short half-life and is undetectable 3 h after parturition (2). It is only found in the maternal, but not fetal circulation. Its maternal levels rise profoundly between weeks 20 and 30 of gestation, thus progressively replacing pituitary growth hormone in the human maternal circulation from mid-gestation onward (3–5). PGH has somatotrophic effects, acting on liver and adipose tissue to influence gluconeogenesis and lipolysis to help ensure an adequate nutrient supply for the fetoplacental unit (6).

Previously, our group and others have shown a positive correlation between PGH levels in pregnant women and the birth weight of their offspring, stimulating discussions about its possible impact on fetal growth (2,7). Understanding the regulation of fetal growth is important. According to the

“Fetal Origins of Disease” hypothesis, it is well recognized that Type 2 diabetes mellitus (T2DM) and features of the metabolic syndrome are associated with birth weights at the extreme of the growth continuum (either small or large for gestational age) (8). The physiologic role of PGH in pregnancy is not completely understood, but its continuous secretion at high levels during pregnancy sparked speculation that it may be an important placental hormone affecting both mother and, indirectly, fetus (9). *In vivo* experiments have demonstrated that growth hormone, when infused into pregnant ewes late in gestation, can increase fetal growth (10). Transgenic mice overexpressing the human PGH gene become larger than their normal littermates and are hyperinsulinemic and insulin resistant (11). The relationship between fetal growth and PGH raises the possibility that PGH could be involved in the development of insulin resistance in pregnancy and might play an important role in gestational diabetes mellitus (GDM) and pregestational diabetes (12,13).

The regulation of PGH secretion is very different from that of pituitary growth hormone (GH) as growth-hormone-releasing hormone does not modulate PGH *in vivo*. Furthermore, *in vitro* and *in vivo* studies have revealed that alterations in maternal nutrient (glucose) availability may alter PGH secretion (14). However, two decades have passed since the discovery of PGH, and still little is known about the regulation of its secretion.

We hypothesized that PGH secretion is regulated *via* several hormones and adipokines. As speculation, this regulation may lead to changes in PGH secretion in conditions associated with altered levels of hormones and adipokines such as in diabetic pregnancies. Thus, we selected hormones and adipokines, which are known to be closely related to fetal growth and adiposity, and tested their potential to act as PGH secretagogues: insulin as a potent stimulator of fetal growth and lipogenesis (15); IGF-1, which is related to increased birth size in diabetic pregnancies (16), and cortisol, also linked to fetal growth (17). Among the adipokines, we choose leptin, which is closely associated with fetal overgrowth (18) and reflects the mother’s body fat mass (19), ghrelin, which, in low levels, is associated with increased insulin resistance and

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Abbreviations: β -HCG, beta-human chorionic gonadotropin; GDM, gestational diabetes mellitus; PGH, placental growth hormone

inversely related to birth weight (20), and visfatin, which is altered in gestational diabetes (21).

BeWo cells, a human choriocarcinoma cell line, have proven as valuable cell model for the study of the cellular, molecular, and endocrine aspects of human trophoblasts (14). Because they secrete PGH, they were used in this study as *in vitro* system to identify regulators of PGH secretion.

MATERIALS AND METHODS

The study was conducted in accordance with the Guidelines in the Declaration of Helsinki and ethically approved by the Institutional Review Board.

Materials. BeWo cells were generously donated by Prof. R Mortimer's laboratory at the Royal Brisbane and Women's Hospital in Brisbane and were originally purchased from the American Tissue Culture Collection (ATCC, Rockville, MD; ATCC no. CCL98; used at passages 25–30). Reagents for cell culture were supplied by Invitrogen Life Technologies (Sydney, Australia). T 75 cm² flasks and 12-well cell culture plates were obtained from Corning Incorporated (New York, NY). Placental growth hormone enzyme-linked immunosorbent assay (ELISA) kits were purchased from Diagnostic Systems Laboratories (DSL, Webster, TX). OD of the samples was measured in a Multiskan plate reader (Thermo Labsystems, Stockholm, Finland). Beta-human chorionic gonadotropin (β -HCG) concentrations were measured using an immunoassay analyzer (Advia Centaur Immunoassay System, Bayer Diagnostics, Munich, Germany). The amount of cellular protein was determined using a bicinchoninic acid protein assay kit from Pierce Biotechnology (Rockford, IL). Human insulin, cortisol, human recombinant leptin, and cyclic adenosinmonophosphate (cAMP) were purchased from Sigma Chemical Co. Aldrich Inc. (St. Louis, MO), human ghrelin from Bachem AG (Bubendorf, Switzerland) and human IGF-1 from R&D (Minneapolis, MN).

Cell culture. BeWo cells were plated in T 75 cm² flasks (seeding 650,000 cells/mL), cultured in Ham F-12 K nutrient mixture (Kaighn Modification) supplemented with 15% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-glutamine and 2% sodium bicarbonate, at 37°C in a humidified incubator with 5% CO₂ and grown to 70%–80% confluence.

Cell treatment. After seeding, the cells (950,000 cells per well) into 12-well plates, they were treated with insulin, IGF-1, cortisol, leptin, ghrelin, and visfatin. cAMP induces cell differentiation (22), served as a positive control, and was therefore used to monitor cell viability during treatment. Because previous studies have shown that glucose alters PGH secretion (23), cells were grown in Dulbecco's Modified Eagle Medium/No Glucose during treatment supplemented by 10% FCS and 1% L-glutamine. FCS was added to the Dulbecco's Modified Eagle Medium, because pilot experiments had revealed that during time courses in serum-free medium of more than 24 h BeWo cells would lose viability. Both time- (24, 48, and 72 h) and dose-course experiments within, below, and above physiologic concentrations were conducted. During the treatment period cells were kept at 37°C in a humidified incubator with 5% CO₂.

Placental growth hormone quantitation. The cell media was collected at 24, 48, and 72 h after treatment and PGH was quantitated by ELISA. Samples were measured in a plate reader and absorption read at 450 nm with background wavelength correction set at 620 nm. The sensitivity of the assay is 8 pg/mL. The intra-assay and interassay coefficients of variation were 4.7% and 6.2%, respectively. The mean absorbance of two duplicates was calculated for each standard, control and unknown. At the end of each incubation period cell lysates were washed with phosphate-buffered saline (PBS), extracted from the 12-well culture plates, and cell protein content was measured. Data obtained from ELISA was corrected for cell lysate protein content.

Statistical analysis. Within an experiment, treatments were measured in duplicate and experiments were performed 3–5 times. Data are represented as the mean $\pm$ SD (SD). To correct for baseline differences between experiments, the effect of treatment was expressed as a percentage of the control performed for each experiment. Analysis of variance (ANOVA) was used to analyze for treatment effects followed by Tukey's multiple comparison test as *post hoc* test when appropriate. Comparison between treatment and control was analyzed by a Student's paired *t* test. A level of $p < 0.05$ was considered significant.

RESULTS

cAMP treatment resulted in increased secretion of β -HCG into the media indicating differentiation of the BeWo cells as

Figure 1. Dose-dependent suppression of PGH secretion in BeWo cells after insulin treatment (72 h), ANOVA $p = 0.008$; * $p < 0.05$, ** $p < 0.01$, (Tukey's multiple comparison test). cAMP treatment resulted in up-regulated PGH secretion, § $p = 0.003$ (paired *t* test), $n = 4$ individual experiments each run in duplicates.

previously reported by others (24) and therefore served as a positive control for viability. PGH secretion was also increased by cAMP ($p = 0.003$) (Fig. 1).

Insulin suppressed PGH secretion from BeWo cells at 72 h in a dose-dependent fashion (ANOVA $p = 0.008$) with a $33 \pm 8\%$ suppression at 100 $\mu\text{g/mL}$ ($p < 0.01$) (Fig. 1). PGH secretion was not altered by IGF-1 at concentrations of 100, 200, 400 and 800 ng/mL (data not shown). Cortisol dose-dependently reduced PGH secretion in BeWo cells at 72 h (ANOVA $p = 0.015$) with $38 \pm 19\%$ suppression at a concentration of 100 $\mu\text{g/dL}$ ($p < 0.05$) (Fig. 2). A similar effect (ANOVA $p = 0.0118$) was found already at 48 h at concentrations of 5, 25, and 50 $\mu\text{g/dL}$ (data not shown).

Both leptin and ghrelin showed a tendency toward a dose-dependent suppression of PGH secretion at 72 h without reaching significance for treatment (ANOVA $p = 0.183$ and $= 0.193$, respectively; data not shown). However, at 72 h both leptin (50 ng/mL) and ghrelin (0.1 ng/mL) suppressed PGH secretion significantly by $10 \pm 6\%$ ($p = 0.027$) and $18 \pm 7\%$ ($p = 0.017$), respectively (Fig. 3). Conversely, visfatin tended to dose-dependently increase PGH secretion at 72 h without reaching significance (ANOVA $p = 0.148$) (Fig. 4).

Figure 2. Dose-dependent suppression of PGH secretion in BeWo cells after cortisol treatment (72 h), ANOVA $p = 0.015$, Tukey's multiple comparison test: * $p < 0.05$, ** $p < 0.05$, $n = 3$ individual experiments each run in duplicates.

Figure 3. PGH secretion in BeWo cells after leptin (* $p < 0.05$) and ghrelin (** $p < 0.05$) treatment (72 h), (paired t test), $n = 4$ individual experiments each run in duplicates.

Figure 4. Dose-dependent increase of PGH secretion in BeWo cells after visfatin treatment (72 h), ANOVA $p = 0.148$; Tukey's multiple comparison test: * $p < 0.05$), (paired t test), $n = 4$ individual experiments each run in duplicates.

However, after 72 h visfatin increased PGH secretion significantly by $18 \pm 8\%$ ($p = 0.014$) at a concentration of 5 ng/mL.

None of the hormones and adipokines altered β -HCG secretion into the media (data not shown). Over the time period of the experiments, *i.e.*, up to 72 h, no changes of PGH levels in the culture medium of the controls were observed.

DISCUSSION

The present study demonstrates for the first time that PGH secretion is regulated *in vitro* by a number of hormones and adipokines. In the past, serum level of hormones, adipokines, and PGH in pregnancy and their relationship to fetal growth have been investigated by correlation analyses (25), but to the best of our knowledge, a direct effect of hormones or adipokines on PGH secretion *in vitro* has never been studied.

Most treatments have resulted in suppression of PGH secretion. Because this could be accounted for by reduced functional activity of the cells, cell viability was carefully monitored. This was also necessary, because the cells were cultured in the absence of glucose to avoid any cross-regulation (22). Differentiation of the cells by cAMP-treatment (23) (Fig. 1) was accompanied by the well-known elevation of β -HCG levels in the culture medium. PGH levels also increased to a similar extent as β -HCG, demonstrating that PGH secretion increases with differentiation of BeWo

cells. Like in the controls, none of the treatments altered β -hCG secretion over the time course of the experiments. Therefore, we conclude that the cells maintained their functional viability and that neither PGH nor any of the treatments alters the differentiation status of the cells. Functional integrity of the cells is also indirectly reflected by the stimulatory effect of visfatin on PGH secretion.

The insulin-induced suppression of PGH secretion is an important observation. The underlying mechanism is unclear, but preliminary data using the PI3-kinase inhibitor LY294002 showed an even bigger inhibitory effect of insulin (not shown). If confirmed this would strongly argue for a PI3-kinase-independent insulin effect. *In vivo* trophoblast insulin receptors are located exclusively at the microvillous membrane of the syncytiotrophoblast (26,27). This location strongly argues for the maternal circulation as the sole insulin source regulating PGH secretion. PGH can cause insulin resistance by changing the subunit composition of phosphatidylinositol 3-kinase17 (11). The contribution of PGH to maternal insulin resistance makes it tempting to hypothesize a maternal-placental feedforward-feedback loop as proposed recently (28). In this model, elevated insulin levels associated with insulin resistance would lead to a reduced PGH secretion, a contributing factor to this condition, which in turn would alleviate insulin resistance. It remains to be determined whether insulin resistance often associated with maternal obesity or pregnancies complicated by gestational or Type-2 diabetes reflects dysregulation of this insulin-PGH feedback loop.

In contrast to insulin, IGF-1 had no significant effect in our experiments. Insulin and IGF-1 can stimulate each other's receptors at different potencies (29). Absence of IGF-1 effect in BeWo cells that express IGF-I receptors and respond to IGF-1 (30), suggests specific signaling of the insulin effect through insulin receptors only. IGF-1 and growth hormone are often involved in feedback loops with opposing actions—often described as “direct” and “indirect” growth hormone effects (31). Our data might suggest there is no feedback system of IGF-1 on PGH secretion *in vivo*. However, this has to be confirmed in primary human trophoblast cells.

We have shown for the first time that cortisol treatment of BeWo cells decreases PGH secretion into the cell media (Fig. 2). The concentration range chosen included the range of cortisol levels present in cord blood in *in vivo* studies (13–23 μ g/dL) (32). Previous studies in lambs have suggested that application of repetitive maternal glucocorticoid exposure leads to symmetric growth retardation in lambs delivered prematurely and a decreased fetal size persisted to term (33). Furthermore, increased cortisol levels have been noted in SGA fetuses (34). Further experiments are necessary to help understand the significance of the findings for pregnancy and fetal growth.

The expression of leptin receptor transcripts in the syncytiotrophoblast makes the placenta one of the target tissues for leptin. The suppression of PGH *in vitro* after treatment with leptin conforms to *in vivo* studies identifying maternal leptin as a negative predictor of PGH (35). Increased leptin levels are found in obese pregnant women, who have a higher risk of adverse pregnancy outcomes including gestational diabetes (36). The treatment effect was not significant at 72 h, but was

significant at the highest concentration tested (50 ng/mL), a level which occurs in very obese pregnant women (37). This suggests that a concentration range up to much higher concentrations might have resulted also in significance of the treatment effect. As both insulin and leptin have similar effects of PGH suppression, it is possible that both regulate PGH secretion in a diabetic and/or obese metabolic state.

Ghrelin has been discovered as a hormone related to pituitary growth hormone secretion, but is also recognized as an anorexogenic hormone with elevated levels increasing appetite. However, ghrelin's role in pregnancy is far from understood (19). Recently, ghrelin was found in cord blood samples with increased levels in smaller fetuses (18,38). However, the consequences of the direct suppressive effect of ghrelin on PGH as found are unclear at present.

Visfatin treatment resulted in a nonsignificant tendency toward a dose-dependent increase of PGH secretion at 72 h (Fig. 4). Concentrations here resembled those of maternal venous blood in GDM pregnancies between the 24th and 28th week of gestation, which are lower in GDM women than in a healthy control group (38), although elevated visfatin levels in GDM women were also found (20). The significance, if any, for normal or GDM pregnancies of the present findings remains to be determined in future studies.

In conclusion, our data indicate that PGH secretion in BeWo cells that serve as a model for human trophoblast can be regulated *in vitro* by a number of adipokines and hormones. These results are in line with previous *in vivo* studies and lead us to conclude that PGH may be directly regulated by certain factors rather than just associated with these factors as previously demonstrated *in vivo*. Although the present study was not designed to identify the fetal or maternal origin of the regulation in some instances the placental location of the hormone or adipokine receptors demonstrated in other studies has allowed some conclusions. Further *in vitro* and *in vivo* experiments are warranted. It remains to be determined whether the effects of adipokines and hormones on PGH secretion are altered in pregnancies associated with diabetes and altered fetal growth.

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