Hypoadiponectinemia in Lean Lactating Women: Prolactin Inhibits Adiponectin Secretion from Human Adipocytes

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Abstract. Adiponectin is an adipocyte-derived hormone involved in glucose, lipid and energy metabolism. A low plasma adiponectin concentration is associated with insulin resistance, obesity and atherosclerosis. In women, energy homeostasis is remarkably changed during gestation and lactation in order to supply sufficient nutrition for a fetus or newborn. In this study we aimed to elucidate the physiological impact of gestation and lactation on the plasma adiponectin levels and the influence of reproduction-related hormones on adiponectin secretion. We studied the longitudinal changes in plasma adiponectin concentration during pregnancy (1st, 2nd and 3rd trimester) and lactation (3 days and 1 month after the delivery) in lean healthy women (n = 22). The plasma adiponectin level declined slightly as the pregnancy advanced and reached its lowest level during lactation (12.25 ± 0.182 µg/ml at early pregnancy vs. 6.88 ± 0.375 µg/ml at 3 days postpartum, p<0.001). In order to investigate the role of the lactogenic hormone prolactin in the decrease of plasma adiponectin levels during lactation, we further performed in vitro experiments using human primary cultured adipocytes. Western blotting of the adipocyte lysate and ELISA of the culture medium revealed that exogenous prolactin inhibited both production and secretion of adiponectin in a dose-dependent manner. Our results thus suggests that prolactin affects the regulation of maternal metabolism through suppression of adiponectin.

Key words: Adiponectin, Prolactin, Pregnancy, Lactation

Received: February 3, 2006
Accepted: May 15, 2006
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role in the metabolic changes that take place during gestation and lactation.

In order to investigate the association of adiponectin with metabolic changes during pregnancy and lactation, we assessed the longitudinal changes of plasma adiponectin in healthy lean women during menstrual cycles, gestation and lactation.

Materials and Methods

Subjects

Twenty-two healthy non-pregnant and 22 pregnant women participated in the study. The non-pregnant volunteers were interviewed by a physician and confirmed to be normal or underweight (normal weight defined as a body mass index (BMI) 18.5 kg/m$^2$ to 25.0 kg/m$^2$ according to the definition by the Japan Society for the Study of Obesity), have regular menstrual cycles of 27–35 days and no medical complications. Early pregnant women with no history of previous GDM were selected and invited to participate in the longitudinal study. The BMI of the pregnant participants were calculated at the time of their first visit to the prenatal clinic (5–13 gestational weeks). In order to screen GDM in the present pregnancy, 50 g oral glucose tolerance tests were performed between 22–25 gestational weeks and women whose 1 hr blood glucose level was higher than 140 mg/ml were excluded from the study. The study protocol was approved by the institutional review board of Yokohama City University School of Medicine and written informed consent was obtained from all subjects.

All the blood samples were obtained from the subjects in fasting condition. The blood samples were collected from the non-pregnant women at the early follicular phase (3–7 days after the beginning of menstrual blood flow), ovulatory phase (0–1 day after the LH surge, detected by an immuno-chromatographic testing device, Gold Sign LH, Morinaga, Tokyo, Japan) and luteal phase (7 days after the blood collection day at the ovulatory phase). For the pregnant group, the blood samples were collected at 1st, 2nd and 3rd trimester of pregnancy, and 3 days and 1 month after the delivery.

Biochemical analyses

All the human plasma assays (adiponectin, estradiol, progesterone, testosterone, prolactin (PRL) and insulin) were performed by the diagnostic division of Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan. Plasma concentration of adiponectin was measured by an enzyme-linked immunosorbent assay (ELISA) with a minimum detectable dose of 23.4 pg/ml and the coefficients of variation under 10%. The HOMA-R (homeostasis model assessment) index was calculated as follows: fasting plasma insulin (µU/ml) × fasting blood glucose (mmol/l)/22.5 [12].

Pre-adipocyte culture and adipocyte differentiation

The human adipocyte primary culture was performed as described previously [13]. The human adipocyte precursor cells (pre-adipocytes) derived from female subcutaneous adipose tissue were purchased from Cell Applications, Inc., San Diego, CA, USA, and cultured in growth medium (Cell Applications, Inc.) supplemented with 1% of fetal calf serum on plates coated with collagen type I (Toyobo Inc., Tokyo, Japan). When the cells reached semiconfluence, 10 µg/ml insulin, 1 µM dexamethasone, 200 µM indomethacin and 500 µM methylxanthine were added to the medium and the cultures were continued for an additional 10 days until the cells differentiated into an adipocyte phenotype, which was confirmed by the appearance of lipid droplets in the cytosol and expression of adiponectin protein as detected by Western blotting.

Expression analysis of PRL receptor by RT-PCR

The expression of the PRL receptor (PRLR) in primary cultured adipocytes and adipose tissues was studied by RT-PCR analysis. The subcutaneous and visceral adipose tissues from non-pregnant premenopausal women were collected during surgery for benign gynecological diseases such as uterine fibroma or ovarian cyst. Total RNA from adipose tissue, pre-adipocytes, adipocytes and OVSAAYO (a PRLR-negative ovarian carcinoma cell line used as a negative control [14]) were extracted using RNaseasy Mini Kit with DNase treatment (QIAGEN, Hilden, Germany) according to the manufacturer’s directions. Complementary DNA (cDNA) was synthesized from one microgram of total RNA using Omniscript RT kit
1/25th of the cDNA reaction was used as template for PCR amplification with Taq PCR Master Mix Kit (QIAGEN). Synthetic oligonucleotides used for PCR primers were as follows: β-actin, 5'-atcggcac cacaccttctacaatgcteg3' (forward) and 5'-cgtcatactc ctcctgtgtctaccctgc-3' (reverse); and PRL receptor (PRLR), 5'-gatctctgctgtcctttctg-3' (forward) and 5'-cagtgtcaggatccggtatgtg-3' (reverse). These sequences span nucleotides 294–1131 of human β-actin cDNA (accession no. X00351) and nucleotides 938–1266 (accession no. XM_003883) of human PRLR cDNA, and yield PCR products of 838 and 329 base pairs, respectively. The PCR conditions were 94°C for 2 min, followed by cycles of 94°C for 60 sec, 59°C for 60 sec and 74°C for 60 sec with a final extension of 74°C for 4 min. The numbers of the PCR cycles were 20 cycles for β-actin and 40 cycles for PRLR. One fourth of each reaction mixture was separated on a 2% agarose gel and visualized by ethidium bromide staining.

PRL treatment and measurement of adiponectin

The medium was changed to serum-free medium one day prior to the experiment, and then the differentiated adipocytes were treated with various doses of human recombinant PRL (Genzyme-Technne Corp., Minneapolis, MN, USA) and either 60 ng/ml of the mouse monoclonal PRLR-blocking antibody B6.2 [15] (a gift from Dr. Barbara K. Vonderhaar, Breast Cancer Faculty, Center for Cancer Research, National Cancer Institute, NIH, MD, USA) or non-immune mouse IgG1 (Ancell Corp., Bayport, MN, USA). Twenty-four hours after the PRL treatment, the medium of the human adipocytes treated with PRL was collected and the concentration of adiponectin in the medium was measured using Human Adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) following the manufacturer’s instructions. The sensitivity of the assay was 60.0 pg/ml and the interassay coefficient of variation was under 10%. The cells were lysed in a lysis buffer (50 mM pH 8.0-Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 100 µg/ml phenylmethylsulfonyl fluoride and 1 µg/ml aprotinin), followed by 10,000 × g centrifugation at 4°C for 10 min. The protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK). Ten µg of protein was separated using 15% SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked in 3% wt/vol bovine serum albumin and then probed by rabbit anti-adiponectin polyclonal antibody (1:1000 dilution, Chemicon International, Inc., Temecula, CA, USA) or mouse anti-β-actin (1 µg/ml dilution, Sigma, St Louis, MO, USA). Peroxidase-conjugated donkey anti-rabbit antibodies or rabbit anti-mouse antibodies were used as secondary antibodies (Amersham Biosciences), respectively. Immunoreactive bands were visualized by ECL-detection (Amersham Biosciences) and analyzed using KODAK 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA). The experiment was performed in triplicate and repeated 3 times.

Results

Longitudinal changes of plasma adiponectin in non-pregnant and pregnant women

The clinical and endocrine characteristics of the women studied are summarized in Table 1. There were no significant differences in BMI and age between non-pregnant and pregnant participants. In addition, the BMI of the pregnant women at early pregnancy and one month postpartum were not statistically different. All of the women at postpartum were interviewed by midwives and confirmed to breastfeed the infants with or without occasional use of artificial milk at the time of blood collection.

The plasma adiponectin levels during the menstrual cycles, gestation and lactation are shown in Fig. 1. In spite of the significant increases in estradiol and progesterone at the ovulatory and luteal phases, plasma adiponectin levels remained steady during the menstrual cycle. In contrast, the plasma adiponectin levels decreased slightly, but gradually, during the gestation. Interestingly, the suppression of adiponectin reached its maximum after delivery (12.25 ± 0.182 µg/ml at early pregnancy vs. 6.88 ± 0.375 µg/ml at 3 days postpartum, p<0.001 by Wilcoxon signed-ranks test) and remained at a low level even one month postpartum. No significant correlation was observed between decline in plasma adiponectin levels and body weight gain in late gestation and lactation (p>0.1 by Pearson’s test, Fig. 2). The remarkable decrease in adiponectin levels in lean lactating women led us to speculate that the lactogenic hormone PRL would affect the secretion...
of adiponectin by the adipocytes. The subsequent in vitro experiments were performed in order to assess the effects of PRL on adiponectin production by human adipocytes.

**PRLR expression in human adipocytes**

PRLR mRNA was detected in both pre-adipocytes and adipocytes as well as in the visceral and subcutaneous adipose tissue obtained from non-pregnant premenopausal women (Fig. 3). PRLR protein was not detectable in adipocyte cell lysate by immunoprecipitation-Western blotting using anti-PRLR extra cellular domain antibody, while the breast cancer cell line T47D, over-expressing PRLR, was positive for the 97 KDa PRLR protein (data not shown).

**Table 1.** The clinical and endocrine characteristics of the non-pregnant and pregnant participants

<table>
<thead>
<tr>
<th></th>
<th>Non pregnant women (N = 22)</th>
<th>Pregnant women (N = 22)</th>
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<tbody>
<tr>
<td></td>
<td>follicular phase</td>
<td>ovulatory phase</td>
</tr>
<tr>
<td>age (yr)</td>
<td>29.18 ± 4.68</td>
<td>20.08 ± 2.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.86 ± 1.71</td>
<td>5.09 ± 2.09</td>
</tr>
<tr>
<td>E₂ (pg/ml)</td>
<td>8.18 ± 19.93</td>
<td>100.23 ± 79.88*</td>
</tr>
<tr>
<td>P (ng/ml)</td>
<td>0.54 ± 6.21</td>
<td>3.30 ± 5.05*</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>0.20 ± 0.13</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>7.05 ± 6.17</td>
<td>10.38 ± 8.23</td>
</tr>
<tr>
<td>glucose (mmol/1 × 10³)</td>
<td>4.78 ± 0.81</td>
<td>4.77 ± 0.39</td>
</tr>
<tr>
<td>insulin (µU/ml × 10³)</td>
<td>8.72 ± 9.02</td>
<td>9.27 ± 11.63</td>
</tr>
<tr>
<td>HOMA-R</td>
<td>1.83 ± 2.00</td>
<td>1.95 ± 0.24</td>
</tr>
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Data are presented as means ± SD. B.W. gain: body weight gain vs. early pregnancy. E₂: estradiol, P: progesterone, T: testosterone. *: p<0.05 vs. follicular phase, **: p<0.05 vs. early pregnant phase by Student’s t-test. N.E.: not evaluated.

**Fig. 1.** Plasma adiponectin concentration during menstrual cycle and pregnancy. Error bar: SD. *: p<0.001 by Wilcoxon signed-ranks test. Plasma adiponectin levels decreased slightly at 3rd trimester pregnancy and reached the lowest level after delivery.

**Fig. 2.** Correlations between changes in plasma adiponectin levels and body weight gain at 3rd trimester pregnancy (A) and 1 month postpartum (B). X-axis: body weight gain (kg) from 1st trimester pregnancy. Y-axis: changes in plasma adiponectin levels as presented by the ratio of plasma adiponectin levels at 3rd trimester pregnancy (A) or 1 month postpartum (B) to the level at 1st trimester pregnancy. No significant correlation was observed between changes in plasma adiponectin levels and body weight gain in either 3rd trimester pregnancy or 1 month postpartum. Statistical analysis was performed by Pearson’s correlation test.
Suppression of adiponectin production in human adipocytes by PRL

The ELISA analysis of the medium was performed in order to examine the effect of PRL on extracellular secretion of adiponectin protein. As shown in Fig. 4-A, adiponectin secretion into the medium decreased up to approximately 40% by PRL treatment in a dose-dependent manner. The inhibition of adiponectin secretion by PRL treatment was significantly abolished by the presence of the PRLR-blocking antibody B6.2 in the medium in addition to PRL. Western blotting using adipocyte whole cell lysate was performed in order to examine whether PRL inhibited the production of adiponectin. As shown in Fig. 4-B, the intracellular protein level of adiponectin was markedly decreased by PRL treatment in a dose-dependent manner, concurrent with the decrease of extracellular adiponectin levels, as analyzed by ELISA. Blockage of PRLR by B6.2 inhibited, at least partially, the decrease of adiponectin production by PRL as demonstrated in the histogram of Fig. 4-B.

Discussion

Although a number of studies have been done regarding the correlation between the decrease of adiponectin secretion and female reproductive disorders, such as gestational diabetes mellitus and polycystic ovarian syndromes [16–19], the physiological changes in plasma adiponectin levels in healthy lean women during reproductive activities have not been fully clarified.

Our data showed that the plasma adiponectin levels decreased only after the 3rd trimester of pregnancy reaching the lowest level after the delivery and remained low for one month. Recently, Fuglsang et al. studied longitudinal changes in plasma adiponectin levels through normal gestation and postpartum [20]. They reported plasma adiponectin changes during pregnancy that were similar to ours, and proposed that the decline in plasma adiponectin levels might be due to increase in maternal fat stores during pregnancy and lactation [20]. However, our cases showed a minimal average body weight gain during pregnancy and lactation, with the result that the BMI of the participants during lactating was not significantly greater than that at early pregnancy (Table 1). Furthermore, plasma levels of adiponectin at 3rd trimester pregnancy and lactation decreased with no correlation to the degree of gestational weight gain (Fig. 2). Therefore, the gestational adipose store at a physiological level appears to have a minimal effect on the regulation of adiponectin secretion.

It is well recognized that gestational insulin resistance rapidly improves after delivery [3, 21]. Thus it is inconsistent with our primary hypothesis that adiponectin plays a key role in the development of insulin resistance during gestation. Recently, adiponectin has been reported to have a variety of functions. Using a mouse model, Qi et al. reported that adiponectin increased the basal energy expenditure and that a decrease in plasma adiponectin levels suppressed the basal metabolism, thus accelerating the energy storage in mice [11]. It is also known that brown adipose tissue thermogenesis is suppressed in rodents during lactation under the influence of PRL [22], resulting in a substantial reduction in the energy requirement of the mother, which is an energy-sparing advantage for milk production. Therefore, it is possible that the decrease in plasma adiponectin influences the energy metabolism of a lactating mother, although more studies are re-
required to verify this hypothesis.

In vitro experiments in this study showed that PRL reduced adiponectin levels concurrently in culture medium and whole cell lysate, suggesting that PRL may regulate the production of adiponectin by adipocytes rather than affect the release of the protein from the cells. Furthermore, the suppression of adiponectin production by PRL was remarkably impaired by the PRLR-blocking antibody, which suggests that PRL directly affected the adiponectin secretion through PRLR. PRLR is expressed in adipose tissue as shown by our current study and those of others [23, 24]. Ling et al. reported that human subcutaneous abdominal adipose tissue expressed PRLR protein more abundantly than the ovary, suggesting that adipose tissue may also be an important target organ of PRL [25].

The mechanisms underlying the suppressive effects of PRL on adiponectin secretion remain to be clarified. Only a few studies have been reported regarding the effect of PRL on adipocytes [24, 26, 27]. Using mouse adipose tissue, Ling and Billing demonstrated that PRL induced suppressors of cytokine signaling (SOCS), a family of genes involved in suppressing cytokine signal transduction, and inhibited insulin-induced secretion of leptin, suggesting an anti-insulin effect of PRL on adipocytes [26]. Binding of PRL to PRLR stimulates a number of signaling pathways; the janus kinase2-signal transducers and activators of transcription 5 (JAK2-STAT5) pathway, Ras-mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol-3OH kinase-AKT-protein kinase B (PI3K/ AKT/PKB) pathway, and SOCS, which are involved in
proliferation and differentiation of different kinds of cells [28]. An involvement in so many different pathways suggests that PRL may perform a variety of functions in different kinds of cells. Further studies are required in order to clarify which signaling pathway of PRL is the most important for adiponectin secretion, and whether PRL inhibits adiponectin production by directly suppressing the transcription or by affecting the expression or function via some other substance.

Recent two studies have demonstrated that exogenous PRL inhibited adiponectin secretion in mice in vivo [29], and that PRL also suppressed adiponectin secretion by cultured human adipose tissue, and decreased the serum adiponectin level in PRL transgenic mice [30]. Our results are in agreement with these findings and showed further evidence that hyperprolactinemia induces hypo-adiponectinemia, suggesting that adiponectin affects metabolism of lactating women.

In our data, the plasma adiponectin level remained low at 1 month postpartum in spite of relatively low levels of blood PRL (47.16 ng/ml ± 38.88 SD). The blood samples from most of the participants at 1 month postpartum were collected just before their appointments with midwives for breastfeeding consultation, which take place immediately before the mother breastfeeds the infant. It has been reported that the blood PRL concentration returned to prepartment levels within a week, but that PRL secretion increased in response to suckling for up to three months after delivery [31]. Therefore, spiky increases of PRL alone may be sufficient to suppress adiponectin secretion.

In summary, our study demonstrated that hypo-adiponectinemia in lactating women was possibly induced by hyper-prolactinemia. Although the mechanism of PRL suppression of adiponectin secretion and the physiological role of hypo-adiponectinemia in postgestational women remain to be investigated further, our results suggest the involvement of adiponectin in the alterations of the maternal metabolism during lactation under the influence of PRL.

**Acknowledgements**

We thank Dr. Barbara K. Vonderhaar of providing the anti-PRLR antibody B6.2, Mrs. M. Inada, N. Kawada, C. Kusaka for technical assistance and Dr. Ken Sato of critical comments.

**References**


