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Gene expression of placental hormones regulating energy balance in small for gestational age neonates☆

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ABSTRACT

Objective: Fetal growth restriction is associated with an increased risk for metabolic and cardiovascular disease in later life. To further elucidate mechanisms that might be involved in the process of prenatal programming, we measured the adipokines leptin, resistin, and adiponectin and the GH-releasing hormone ghrelin in the placenta of small for gestational age (SGA) neonates.
Study design: The control group included 24 placentas of appropriate for gestational age (AGA) newborns, in the study group were 16 placentas of SGA neonates. Gene expression of leptin, resistin, adiponectin, and ghrelin was examined. For hormones showing alterations in gene regulation placental protein expression was measured by Western blot.
Results: Placental mRNA expression of leptin was significantly increased in SGA placentas ($p = 0.0035$, related to β-actin). Protein concentration was increased, as well. There were no differences in placental resistin, adiponectin, or ghrelin gene expressions between SGA neonates and controls. Leptin was the only hormone to demonstrate a significant inverse correlation with birth weight ($r = -0.44$, $p = 0.01$). Adiponectin correlated significantly with leptin ($r = 0.53$, $p = 0.0023$) and ghrelin ($r = 0.50$, $p = 0.0045$).
Conclusions: Placental leptin gene expression and protein concentration showed the expected increase in the SGA group. Leptin was inversely correlated with birth weight. Positive correlation of adiponectin with leptin and ghrelin expression suggests an interaction between these hormones in the placenta. However, the unchanged expression of resistin, adiponectin, and ghrelin in SGA placentas and the absence of correlation with birth weight cast doubt whether these hormones produced in the placenta play a key role in fetal programming.

1. Introduction

Intrauterine growth-restricted (IUGR) infants have an increased risk of developing disease of the Metabolic Syndrome complex in adulthood including obesity, insulin resistance, hypercholesterolemia, and hypertension. Altered intrauterine hormonal and metabolic conditions are thought to play an important role in fetal programming by increasing susceptibility to adult disease in humans and in animals [1]. Due to their partially common, partially antagonistic regulatory effects on metabolic functions and on growth, adipokines and ghrelin are probably key regulators of this process. The adipokines leptin, resistin, and adiponectin are secretory hormones mainly originating from adipose tissue, but also from the placenta [2].

The main function of leptin is to decrease food intake, to increase energy expenditure, and to increase insulin sensitivity. During gestation, leptin plays an important role in regulation of fetal and placental growth [3]. In the placenta, leptin is predominantly expressed in the syncytiotrophoblast and in villous vascular endothelial cells [4]. Gene expression and maternal plasma levels of leptin have been shown to be increased in IUGR [5].

In contrast, resistin is thought to be an anti-adipogenic factor and an inducer of insulin resistance. It is expressed in human placenta mainly in trophoblast cells, more prominently at term than in the first trimester [6]. In parallel to its placental gene expression maternal serum levels rise in the third trimester of pregnancy [7]. Resistin has been postulated to play a role in regulating energy metabolism in pregnancy [7].

Adiponectin is a novel hormone exerting anti-diabetic, anti-inflammatory, and anti-atherogenic actions. In cord blood, adiponectin levels are higher than in blood samples of adults. Whether

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there is a correlation between adiponectin and birth weight remains controversial [8–10]. Adiponectin has been shown to be produced by amniotic epithelial cells and the syncytiotrophoblast [11].

Ghrelin is a peptide of predominantly gastric origin with potent GH-releasing activity functioning as an opponent to leptin. It serves as an indicator for a lack of energy by stimulating food intake and fat deposition. Besides its expression in stomach and placenta, ghrelin shows a widespread tissue distribution [12]. In cord blood, ghrelin levels are higher than in venous blood samples from adults, and an inverse correlation to birth weight has been shown by some authors [13] but not by others [14,15].

To the best of our knowledge, no data on placental gene expression of resistin, adiponectin, and ghrelin in small for gestational age (SGA) neonates have been published yet. Therefore, the aim our study was to measure mRNA expression of these hormones in placentas of SGA newborns and, finally, to examine their interrelationship in SGA pregnancies.

2. Subjects and methods

2.1. Subjects

Tissue for the study was collected in collaboration with the Department of Obstetrics and Gynecology at the University of Erlangen-Nuremberg. After removal of amniotic membranes and maternal decidua full-thickness samples were obtained from two different locations in the middle of the placenta immediately post-partum after primary cesarean section. Both samples were analyzed in parallel to control for placental inhomogeneity. Data were only considered significant if measurements from both placental localizations showed significant results. For comparison of SGA and control neonates, gestational age-matched pairs were formed. Matching was achieved within 1 week gestation. Gestational age dating was performed considering last menstrual period and ultrasound. Twelve matched pairs were analyzed as not for every patient an appropriate pair could be found.

Tissue samples were taken from 24 healthy women (one smoker) with normal weight babies delivered prematurely or at term (10–90 weight centile, 15 males, 9 females, 7 preterm) and from 16 mothers (4 smokers) with SGA babies (<10 weight centile, 7 males, 9 females, 9 preterm). Two of the mothers with SGA newborns had preeclampsia, the others were healthy. The weight centiles of Voigt et al. for German newborns were used in the study [16]. All mothers were of Caucasian origin. Patients with documented fetal anomalies or chromosomal abnormalities were excluded from the study. Birth weight SDS was calculated using the following formula: birth weight SDS = (newborns' birth weight − mean birth weight)/S.D.

The study was approved by the ethics committee of the University of Erlangen. Informed consent was provided by all mothers. Tables 1a and 1b shows the patient characteristics.

Table 1a
Characteristics of patients from whom placental tissue was obtained.

	SGA group $n = 16$	Control group $n = 24$
Maternal age (years)	30.2 ± 5.4	30.7 ± 6.1^{NS}
Pre-pregnancy body mass index (kg/m^2)	22.0 ± 3.6	22.7 ± 2.7^{NS}
Gestational age (weeks)	36.3 ± 3.4	37.9 ± 1.9^{NS}
Placental weight (g)	313 ± 96	$562 \pm 99^{*}$
Newborns' weight (g)	1796 ± 688	$3052 \pm 454^{*}$

Values are shown as mean ± S.D.; SGA: small for gestational age; significant differences between the SGA group and the control group are marked by asterisks; NS: not significant.
* $p < 0.0001$.

Table 1b
Patient characteristics of 12 gestational age matched pairs.

	SGA group	Control group
Maternal age (years)	30.2 ± 5.3	30.5 ± 6.5^{NS}
Pre-pregnancy body mass index (kg/m^2)	21.8 ± 3.5	23.1 ± 2.2^{NS}
Gestational age (weeks)	37.4 ± 3.0	37.7 ± 2.6^{NS}
Placental weight (g)	313 ± 96	$560 \pm 102^{*}$
Newborns' weight (g)	1796 ± 688	$2962 \pm 412^{**}$

Values are shown as mean ± S.D.; SGA: small for gestational age; significant differences between the SGA group and the control group are marked by asterisks; NS: not significant.
* $p < 0.005$.
** $p < 0.0001$.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from the tissues using guanidine–thiocyanate acid phenol (TRIzol®, WAK Chemie, Medical GmbH, Bad Homburg, Germany). RNA concentrations were determined spectrophotometrically. One microgram of RNA was reversely transcribed in a volume of 20 μl at 39 °C for 60 min (chemicals from Boehringer, Mannheim, Germany).

2.3. TaqMan real-time PCR

During the extension phase of the PCR, the Taq polymerase cleaves a probe releasing the reporter dye (i.e. 6-carboxy-fluorescein, FAM). An automated photometric detector combined with a special software (ABI Prism 7700 Sequence Detection System, PerkinElmer, Foster City, CA) monitors the increasing reporter dye emission. The algorithm normalizes the signal to an internal reference (ΔRn) and calculates the threshold cycle number (C_T), when the ΔRn reaches 10 times the standard deviation of the baseline. The C_T values of the probes are interpolated to an external reference curve constructed by plotting the relative amounts of a serial dilution of a known template versus the corresponding C_T values.

Commercial reagents (TaqMan PCR Reagent Kit, PerkinElmer) and conditions according to the manufacturer protocol were employed. 2.5 μl of cDNA (reverse transcription mixture) and oligonucleotides with a final concentration of 300 nM (β-actin, leptin, resistin), 600 nM (β2-MG), or 900 nM (adiponectin, ghrelin) of primers and 200 nM of TaqMan hybridization probe were added to 25 μl reaction mix. All of the primers and probes were purchased from Eurogentec (Belgium) and Sigma (Germany). The thermocycler parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles (45 for adiponectin) of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions of one of the samples served as reference providing relative quantification of the unknown samples. Leptin, ghrelin, resistin, and adiponectin gene expression was related to the housekeeping genes β-actin and β2-MG. The primers and TaqMan probes used are listed in Table 2.

2.4. Western blot detection of leptin protein

Protein was extracted from cells using RIPA buffer. Protein concentration was determined using a protein assay kit (Pierce). Protein samples containing 75 μg total protein were denatured by boiling for 5 min in Laemmli sample buffer and separated on a 10% denaturing SDS-PAGE gel. The molecular weight marker used was peqGold Protein Marker V (PeqLab). After electrophoresis, the gels were electroblotted onto nitrocellulose membranes (Whatman), blocked with 5% dry milk/2% BSA in TBS/0.05% Tween 20 overnight and incubated for 1 h with the polyclonal leptin antibody (Sigma). Leptin was visualized with a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (Promega), using the ECL