Maternal adaptations to pregnancy in spontaneously hypertensive rats: leptin and ghrelin evaluation

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Abstract

Leptin and/or ghrelin, initially thought to be considered messengers of energy metabolism, are now considered to play a role in normal and complicated pregnancy. In this study, pregnant, spontaneously hypertensive rats (SHR) have been used to evaluate, for the first time, the modification of leptin and ghrelin both at serum and tissue levels. In SHR, we evaluate plasma leptin level and tissue protein expression in both placenta and adipose tissue at the end of gestation (day 20) versus normotensive Wistar–Kyoto (WKY) animals. The expression of functional leptin receptor (Ob-Rb) in peripheral tissues and in the hypothalamus was evaluated. Moreover, we measured plasma ghrelin level and its mRNA expression in the stomach and placenta. SHR strain presented significantly lower plasma leptin levels when compared with those found in pregnant or not WKY controls. Interestingly, in the placenta, leptin gene

expression was higher in SHR than normotensive WKY. Moreover, we demonstrated a resistance to the effects of leptin via 'downregulation' of hypothalamic receptors in pregnant SHR. Conversely, SHR presented significantly higher ghrelin plasma levels when compared with those found in pregnant or not WKY. However, we observed that ghrelin level in the stomach of SHR did not change during pregnancy, and on the opposite, mRNA ghrelin in the placenta of SHR was lower than that of normotensive rats, suggesting a different production of this hormone in the fetal–placental unit. These data gain further insight into metabolic hormone modifications observed in a model of pre-existing hypertension associated with pregnancy.

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Introduction

During pregnancy, several neuroendocrine changes and energy metabolism alterations occur. In this hypermetabolic state, a great increase in maternal body fat and weight and a positive energy balance are well recognized, primarily to prevent the depletion of maternal energy stores (Richard & Trayhurn 1985).

Leptin (Ob), the product of the ob gene, is thought to play a critical role in the regulation of adipose tissue mass, and may directly or indirectly influence reproductive function. It is now recognized that leptin is produced in several organs and tissues besides white adipose tissue, such as heart, mammary epithelial cells, and placenta. This hormone inhibits food intake, regulates energy expenditure, is a permissive signal to the reproductive system, and is a metabolic hormone affecting insulin secretion, lipolysis, and sugar transport (Trayhurn *et al.* 1999). Leptin has been proposed as a lipostatic factor that regulates the amount of body fat stores by means of a closed feedback loop involving the hypothalamus (Erickson *et al.* 1996, Rohner Jean Reaneud *et al.* 1996).

Leptin affects body weight and reproduction mainly by interacting with specific receptors (Ob-R), which use the JAK/STAT pathway of signal transduction. Different isoforms of the Ob-R exist, including a long isoform (Ob-Rb) with signaling capacity and short isoforms with several and not completely known functions. In particular, Ob-Rb is expressed in a wide range of tissues including hypothalamus (Tartaglia 1997).

Recent reports have demonstrated that leptin levels are elevated in serum during human and rodent gestation (Henson & Castracane 2006). In particular, during pregnancy in rats, the high levels of leptin indicate the existence of a physiological state of central leptin resistance that might explain the increased food intake observed during gestation (Garcia *et al.* 2000).

In humans, a potential role of maternal leptin concentration as a marker of pre-eclampsia, a form of pregnancy-induced hypertension, has been suggested. In fact, maternal leptinemia is significantly higher in pregnancies complicated by pre-eclampsia than in gestational age-matched controls (Teppa *et al.* 2000). Moreover, the increase of hormone level

predates the development of pre-eclampsia (Anim-Nyame *et al.* 2000), and it has been shown that leptin gene expression in the placenta is augmented in severe pre-eclampsia (Laivouri *et al.* 2006) and that a dysregulation of autocrine and paracrine function of leptin in fetal-maternal interface can be implicated not only in pregnancy-induced hypertension, but also in gestational diabetes and in the intrauterine growth retardation, including disturbance of fetal bone (Bajoria *et al.* 2002).

It has recently been reported that ghrelin, a gastric-derived peptide, plays an important role in the reproductive function both in animals (Gualillo *et al.* 2002, Caminos *et al.* 2003) and in humans (Gaytan *et al.* 2003). In the CNS, this hormone induces growth hormone (GH) release and interacts with hypothalamic nuclei, stimulating feeding and determining body weight gain (Wren *et al.* 2000). Ghrelin augments neuropeptide Y gene expression and blocks leptin-induced feeding reduction, implying that there is a competitive interaction between ghrelin and leptin in feeding regulation (Nakazato *et al.* 2001).

Ghrelin is also synthesized by placenta and its secretion might influence the fetal growth and maturation. During pregnancy in rats, the maternal concentration of plasma ghrelin is significantly lower than that of nonpregnant animals, even if ghrelin peptide concentrations in the stomach did not change significantly during pregnancy (Shibata *et al.* 2004).

Since pre-existing hypertension is included among hypertensive disorders of pregnancy, we used spontaneously hypertensive rats (SHR) in order to evaluate prospective metabolic and hormonal differences or modifications. In particular, here we compared plasma leptin and its protein expression in placenta and adipose tissue at the end of gestation in normal (Wistar-Kyoto, WKY) and SHR animals. Differential regulation of the Ob-R expression in peripheral tissues and in the hypothalamus of SHR and WKY rats was also evaluated. Moreover, we also measured plasma ghrelin level at the end of pregnancy and mRNA in the stomach and placenta.

Material and Methods

Animals

Twenty-week-old female normotensive WKY (average body weight of the cohort, 208 ± 3 g) and SHR (average body weight of the cohort, 212.0 ± 2 g; Harlan Italy, San Pietro al Natisone, Udine, Italy) were housed one per cage in stainless steel cages in a room kept at 22 ± 1 °C with a 12 h light:12 h darkness cycle. The animals had access to tap water and rodent standard diet and were allowed to feed *ad libitum*.

Estrus cycle was assessed to determine the reproductive status. SHR and WKY virgin female rats ($n = 5$ per group) were mated at estrous, and the day of mating determined by the presence of spermatozoa after a vaginal smear, being considered day 0 of pregnancy. Same number of virgin WKY and SHR of the same age served as controls.

All animal experiments complied with the Italian D.L. no. 116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC).

Body weight, food intake, and body gain in fat

Throughout pregnancy, body weight and food intake were monitored once a week (6, 14, and 20 days). At the end of the experimental period, food intakes were cumulated. Bio-electrical impedance analysis (BIA) was applied to body composition assessment at day 20 by a BIA 101 analyzer, modified for rat (Akern, Florence, Italy). Free fat mass was calculated by the BIA (50 kHz) prediction equation of Ilagan *et al.* (1993), and fat mass content was obtained as the difference between body weight and free fat mass.

Measurement of arterial blood pressure indirectly in conscious rat

Mean arterial blood pressure in conscious rats was measured by a Blood Pressure Recorder (Ugo Basile, Biological Research Apparatus, 21025 Comerio, Italy) using a technique described by Gerold & Tschirky (1968). After 2 weeks training period, blood pressure was measured before mating and at 6, 14, and 20 days of pregnancy. To measure arterial blood pressure, rats were housed for 30 min in a warmed room (28–30 °C), then a tail cuff, placed about 2 cm from the base of the tail, measured arterial blood pressure.

Tissue collection and blood parameters

At day 20 of pregnancy, animals were anesthetized by enflurane and killed by cervical dislocation. Control animals were killed at the second day of diestrus. Blood collected by cardiac puncture was centrifuged at 1500 g, at 4 °C for 15 min, and sera were stored at -70 °C for later biochemical and hormonal measurements.

Glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and cholesterol were quantified using nonfasting blood sample. The serum leptin and ghrelin (the bioactive form of the hormone, *n*-octanoyl modified in Ser³) concentrations were measured by RIA kits according to the manufacturer's instruction (Linco Research Inc., St Charles, MO, USA).

Subcutaneous white adipose tissue, stomach, placenta, and hypothalamus (dissected according to the map of Glowinski & Iversen (1966)) were excised and immediately frozen in liquid nitrogen.

Western blot analysis

The s.c. adipose tissue, hypothalamus, and placenta obtained from each animal were disrupted by homogenization on ice in lysis buffer (20 mM Tris-HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 10 µg/ml leupeptin

and trypsin inhibitor). After 1 h, tissue lysates were obtained by centrifugation at 100 000 *g* for 15 min at 4 °C. Protein concentrations were estimated by the Bio-Rad protein assay (Bio-Rad Laboratories) using BSA as standard.

For western blot analysis, 100 µg protein of all tissue lysates were dissolved in Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE (8 or 12% polyacrylamide). The blot was performed by transferring proteins from a slab gel to nitrocellulose membrane at 240 mA for 40 min at room temperature. The filter was then blocked with 1 × PBS, 5% nonfat dried milk for 40 min at room temperature, and probed with a goat polyclonal anti-C-term Ob-Rb antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or with rabbit polyclonal anti-Ob antibody (1:1000; Santa Cruz Biotechnology Inc.) in 1 × PBS, 5% nonfat dried milk, and 0.1% Tween 20 at 4 °C overnight. The secondary antibody (anti-rabbit or anti-goat IgG-horseradish peroxidase conjugate 1:2000 dilution) was incubated for 1 h at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions, and exposed to Kodak X-OMAT film (Eastman Kodak Co). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the β-actin protein (Sigma). The protein bands of Ob and Ob-Rb (~16 and 130 kDa respectively) on X-ray film were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories).

RT-PCR analysis

Total RNA was extracted by a modified method of Chomczynski & Sacchi (1987), using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed by a standard procedure (Brenner *et al.* 1989) using 2 µg total RNA. After reverse transcription, 2 µl RT products were diluted in 48 µl PCR mix, to give a final concentration of 50 U/ml Taq DNA polymerase (Life Technologies), 4 µM 5' and 3' primers, 50 µM each dNTP, 1.5 mM MgCl₂, and 1 × PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl). The following oligonucleotides were used: ghrelin (forward primer 5'-TTGAGCCCAGAGCAC-CAGAAA-3' and reverse primer 5'-AGTTGCAGAGGAGG-CAGAAGCT-3') and β-actin (forward primer 5'-TAC-AACTCCTTGCAGCTCC-3' and reverse primer 5'-ATCTTCATGAGGTAGTCAGTC-3'). PCR primers were synthesized by 'Servizio di Biologia Molecolare, Stazione Zoologica A. Dohrn' (Naples, Italy). The amplification profile for rat ghrelin and β-actin was: denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A 35-cycle amplification was completed with an additional step at 72 °C for 10 min. The amplification was performed in an automatic thermal cycler (Biometra, Göttingen Germany). To assure that PCR was performed in the linear amplification range, samples were taken after 15, 20, 25, 30, 35, and 40 cycles,

showing that the reaction was linear over this range (data not shown). Then 10 µl RT-PCR products were separated by 1.5% agarose gel electrophoresis in TBE 1 × (10.089 M Tris base, 0.089 M boric acid) containing 0.2 µg/ml ethidium bromide. Fragments of DNA were seen under u.v. light. β-Actin was used as an internal reference. PCR generates a single 347 bp product for rat ghrelin and a single 603 bp product for the β-actin gene. The bands of ghrelin were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories).

Statistical analysis

All data were presented as mean ± S.E.M. Statistical analysis was performed by ANOVA test for multiple comparisons, followed by Bonferroni's test. Statistical significance was set at $P < 0.05$.

To better understand statistical significance, we conventionally used the following symbols: to compare the examined group versus WKY-NP; °, to compare SHR-P versus SHR-NP; and #, to compare SHR-P versus WKY-P.

Results

Body weight gain, food intake, and body fat modifications in hypertensive and normotensive rats during pregnancy

Body weight gain, food intake, and fat mass of the normotensive and hypertensive rats are presented in Fig. 1. Pregnancy in normotensive as well as hypertensive rats induced a significant increase ($P < 0.001$) in body weight gain (Fig. 1A), food intake (Fig. 1B), and fat mass (Fig. 1C).

Interestingly, SHR-NP showed a significant increase of food intake when compared with WKY-NP ($P < 0.001$). This parameter was evaluated as area under curve of the amount of food consumed in 20 days. However, in SHR-P, there was a significant increase in body weight gain when compared with that of WKY-P ($P < 0.01$), that was paralleled by a significant increase in fat mass content ($P < 0.001$).

Systolic blood pressure in pregnant or not WKY and SHR animals

The mean values of systolic blood pressure detected during the experimental period for both SHR and WKY rats are shown in Fig. 2. No differences in blood pressure were observed in WKY-P when compared with WKY-NP rats (panel A). In SHR-P, this parameter was significantly reduced at day 14 of pregnancy when compared with SHR-NP ($P < 0.05$), and the fall was more marked at day 20 ($P < 0.001$), where blood pressure reading became similar to normotensive pregnant rats.

Hormone levels and blood parameters

Determination of plasma leptin and ghrelin levels of all groups was shown in Fig. 3A and B respectively. In our experimental

Figure 1 Changes in (A) body weight gain, (B) food intake, and (C) fat mass of nonpregnant (-NP) or pregnant (-P) hypertensive (SHR) and normotensive (WKY) rats. Values are mean $\pm$ S.E.M. of five animals. AUC, area under the curve. *** $P < 0.001$ versus WKY-NP; ooo $P < 0.001$ versus SHR-NP; ## $P < 0.01$ and ### $P < 0.001$ versus WKY-P.

conditions, SHR strain animals presented a lower level of plasma leptin versus that of the respective WKY controls ($P < 0.001$). Leptin level (ng/ml), measured at gestational day 20, did not significantly change in comparison with nonpregnant respective controls (3.30 ± 0.10 vs 3.00 ± 0.21 in WKY rats, and 1.16 ± 0.14 vs 0.56 ± 0.57 in SHR). Conversely, plasma ghrelin is higher in SHR when compared with WKY animals ($P < 0.001$). Neither pregnant group had different plasma ghrelin levels than their respective nonpregnant controls.

Figure 2 Systolic blood pressure levels of nonpregnant (-NP) or pregnant (-P) hypertensive (SHR) and normotensive (WKY) rats measured at 0, 6, 14, and 20 days. The results are expressed as mean $\pm$ S.E.M. of five animals. ° $P < 0.05$, and ooo $P < 0.001$ versus SHR-NP; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ versus WKY-P.

In Table 1, modifications of serum parameters are reported. Cholesterol, triglycerides, and glucose were similar in nonpregnant SHR and WKY rats. Conversely, SHR-NP presented a significantly reduced plasma LDL cholesterol and a higher HDL cholesterol when compared with that of WKY-NP ($P < 0.05$ and $P < 0.01$ respectively). In normotensive animals, pregnancy induced a significant decrease of total cholesterol ($P < 0.01$), LDL cholesterol ($P < 0.001$), and glucose ($P < 0.05$), and an increase of plasma triglycerides ($P < 0.05$). In SHR-P, all these parameters were similarly modified; in particular, a very strong increase of triglycerides was observed when compared with WKY-P ($P < 0.001$).

Ob expression in adipose tissue and placenta from WKY and SHR animals

Figure 4A shows that s.c. adipose tissue from SHR presented a slight reduction of leptin expression when compared with that of WKY rats; moreover, pregnancy in normotensive as well as hypertensive rats induced a significant increase ($P < 0.01$ and $P < 0.05$ respectively) in Ob expression versus their respective nonpregnant controls. Ob protein in WKY-P tissue lysates was more expressed than in SHR-P. Conversely, as appears in Fig. 4B, Ob expression in placentas of SHR-P was increased ($P < 0.01$) in comparison with WKY-P animals.

Ob-Rb expression in s.c. adipose tissue and hypothalamus from WKY and SHR animals

The expression of the functional isoform of Ob-R, Ob-Rb, in the adipose tissue and hypothalamus is reported in Fig. 5A and B. In adipose tissue, there was no significant modulation of Ob-Rb expression in any group, but a trend of Ob-Rb decreased expression was revealed in both pregnant groups

Figure 3 Changes in circulating plasma (A) leptin and (B) ghrelin in nonpregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean $\pm$ S.E.M. of five animals. *** P <0.001 versus WKY-NP; and ### P <0.001 versus WKY-P.

versus their respective controls (Fig. 5A). Hypothalamus from SHR strain animals had a reduced expression of Ob-Rb (P <0.05) when compared with that of normotensive WKY rats. The same pattern was found during pregnancy, where Ob-Rb continued to be underexpressed when compared with WKY-P (P <0.05).

Ghrelin mRNA expression in stomach and placenta from pregnant WKY and SHR animals

In stomach, no difference in ghrelin mRNA expression was observed between normotensive and hypertensive rats (Fig. 6A). Conversely, a significant reduction (P <0.05) of ghrelin expression in placenta from SHR-P was evidenced (Fig. 6B).

Discussion

During pregnancy, physiological adaptation in the nutritional and hormonal setting is necessary for fetal growth and maternal well-being. When pathological conditions, such as obesity or hypertension, occur, further modifications are needed to avoid maternal complications or fetal programming. Moderate hypertension tends to happen at the end of the pregnancy and does not represent a serious disturbance. Hypertensive disorders of pregnancy include pre-existing hypertension and pregnancy-induced hypertension. In this study, the SHRs were used for an experimental model of pregnancy associated with pre-existing hypertension in order to evaluate some metabolic and hormonal modifications. SHRs are a genetic animal model of hypertension widely used in medical research because of the features they share with idiopathic hypertension in humans (Okamoto & Aoki 1963). Even if, differently from pre-eclampsia, no edema and only a small amount of proteinuria were observed in SHR (Yamada *et al.* 1981), the behavior of blood pressure during pregnancy has been a matter of controversy in the literature.

Unlike another study (Tanigawa *et al.* 1999), the normotensive pregnant rats that we examined showed no change in blood pressure during the gestational period. Conversely, we found that in SHR, pregnancy remarkably reduced pressure at delivery, confirming previous data (Zamorano *et al.* 1980, Lindheimer *et al.* 1983, Ahokas & Sibai 1990). The pressure decrease near term was not associated with an alteration of insulin resistance (Tanigawa *et al.* 1999) and could be related to an increase of nitric oxide in pregnancy (Conrad *et al.* 1993), or the release of hypotensive substances by the placenta (Nakanishi *et al.* 1980), or the modification of the renin-angiotensin system (Ahokas *et al.* 1989).

Leptin and/or ghrelin, initially thought to be considered messengers of energy metabolism, are now deemed to play a role in normal and complicated pregnancy (Makino *et al.* 2002, Henson & Castracane 2006). During pregnancy, the increase of leptin level in human correlates with a higher expression of the leptin gene in placenta (Masuzaki *et al.* 1997). Contrasting data are reported about the main source of leptin in rat; some authors indicate adipose tissue as the main source of leptin also during pregnancy because lack of leptin expression was reported in placenta (Kawai *et al.* 1997). Other

Table 1 Modifications of serum parameters of nonpregnant (-NP) or pregnant (-P) spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Values are the mean $\pm$ S.E.M.

	Cholesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	Triglycerides (mg/dl)	Glucose (mg/dl)
Groups					
WKY-NP	124.0 $\pm$ 4.7	77.8 $\pm$ 4.4	29.8 $\pm$ 0.9	82.2 $\pm$ 8.1	152.20 $\pm$ 12.00
WKY-P	102.8 $\pm$ 1.4 [†]	37.6 $\pm$ 0.8 [‡]	33.0 $\pm$ 1.0	165.4 $\pm$ 2.2*	99.60 $\pm$ 2.25*
SHR-NP	120.0 $\pm$ 4.1	63.0 $\pm$ 2.5*	37.8 $\pm$ 1.5 [†]	86.2 $\pm$ 6.7	165.40 $\pm$ 12.45
SHR-P	97.6 $\pm$ 3.5 [†]	NC	31.6 $\pm$ 1.5	440.8 $\pm$ 40.0 ^{,¶}	124.60 $\pm$ 8.29

* P <0.05, [†] P <0.01, and [‡] P <0.001 versus WKY-NP; ^{||} P <0.001 versus SHR-NP; [¶] P <0.001 versus WKY-P; NC, not calculable.

Figure 4 Western blot analysis of Ob expression and densitometric analysis of protein bands in (A) s.c. adipose tissue and (B) placenta. (A) Shows the modulation of Ob expression in adipose tissues prepared from nonpregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. In B, the modulation of Ob expression was shown in placentas from pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean $\pm$ S.E.M. of five animals. Equal loading was confirmed by β -actin staining. ** P <0.01 versus WKY-NP; ° P <0.05 versus SHR-NP; ## P <0.01 versus WKY-P.

authors provide evidence, using RT-PCR, that placenta may be a further source of leptin in rodents and may explain the decrease of leptin levels after delivery (Hoggard *et al.* 1997, Garcia *et al.* 2000).

As reported by Kawai *et al.* (1997), during pregnancy in rats, maternal plasma leptin levels, measured every 3 days, gradually increased on days 9–19, when the peak leptin levels occur. Thereafter, maternal leptin levels sharply declined to less than those of nonpregnant animals. In our experimental conditions, at day 20 of pregnancy, we did not find differences between pregnant or not WKY leptinemia (3.30 ± 0.10 vs 3.00 ± 0.21 respectively), even if a significant increase of leptin protein expression (P <0.01) was evidenced in s.c. adipose tissue. Moreover, we observed, for the first time using hypertensive animals, at day 20, a twofold increase of serum leptin level (1.16 ± 0.14 SHR-P vs 0.56 ± 0.57 in SHR-NP, $t=2.965$) that paralleled a significant increase of leptin gene expression in s.c. adipose tissue (P <0.05). This accounts for the highest

Figure 5 Western blot analysis of Ob-Rb expression and densitometric analysis of protein bands in (A) s.c. adipose tissue and (B) hypothalamus prepared from nonpregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean $\pm$ S.E.M. of five animals. Equal loading was confirmed by β -actin staining. * P <0.05 versus WKY-NP; # P <0.05 versus WKY-P.

amount of adipose tissue in the body and contributes to circulating leptin levels. However, SHR strain animals presented significantly lower plasma leptin when compared with those determined in WKY-NP and WKY-P controls. Interestingly, in the placenta compared with the plasma or adipose tissue, the opposite seemed to happen: leptin gene expression was higher in SHR-P than in normotensive animals. The increased plasma leptin concentration during pregnancy in SHR may be due to increased placental production. In pregnant SHR, increased serum leptin concentrations and expression of placental leptin in late gestation may seem paradoxical. If the organism were to respond to this satiety signal (e.g. increased leptin), food consumption would decline, and nutritional support for the mother and fetus would be compromised. Since food intake is not diminished in late pregnancy, an adaptive process appears to have evolved to maintain maternal and fetal well-being. In SHR-P, the increase of leptin in serum and tissues (adipose and placenta) is not accompanied by a food intake modification. One possible explanation of this discrepancy may be the resistance to the effects of leptin via 'downregulation' of

Figure 6 RT-PCR analysis of ghrelin mRNA and its densitometric analysis in (A) stomach and (B) placenta from pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean $\pm$ S.E.M. of five animals. β -Actin was used as internal reference. * $P < 0.05$ versus WKY-P.

hypothalamic leptin receptors in pregnant SHR, as evidenced by the marked reduction of the hypothalamic expression of Ob-Rb protein bands (Fig. 5). However, the possible involvement or alteration of other peptides or peripheral hormones, which regulate food intake, cannot be excluded. No significant modification of hypothalamic Ob-Rb expression was seen at day 20 of pregnancy in WKY, even if previous data are suggestive for leptin resistance in the hypothalamus and pituitary (Szczepankiewicz *et al.* 2006). Moreover, in our conditions, no significant modification of

Ob-Rb expression was seen in adipose tissues either in normotensive or in hypertensive animals.

Previous data demonstrated that SHR is a strain that easily manifests hyperlipemia and hyperlipoproteinemia during pregnancy when compared with the Wistar rat (Yoshioka *et al.* 1986). In fact, many data suggest that in late pregnancy, liver lipid metabolism may be directed toward VLDL secretion at the expense of biliary secretion. This would supply lipids, especially triglycerides, to the placenta for fetal energy requirements and to the mammary gland for milk lipids in anticipation of suckling (Smith *et al.* 1998). This study compared the changes in the level of serum lipid concentration in pregnant and nonpregnant SHR and WKY rats. The serum triglyceride concentration is significantly higher in the last period of pregnancy, i.e. twofold higher in WKY and fivefold higher in SHR. It has been noted that elevated plasma lipid and lipoproteins may induce endothelial dysfunction secondary to oxidative stress and that dyslipidemia may impair trophoblast invasion, thus contributing to a cascade of pathophysiological events that lead to the development of pre-eclampsia (Lorentzen & Henriksen 1998). Consistent with our results on the lower leptin serum levels in SHR strain, Anderson *et al.* (2005) found a significant decrease of leptinemia in reduced uterine perfusion pressure (RUPP) animals. RUPP is an animal model of pre-eclampsia characterized by persistent elevation of arterial pressure, reduced litter size, fetal, and placental weights.

In opposite to leptinemia, our data show an increase of ghrelin plasma level in SHR strain when compared with that of WKY. In particular, in SHR at the end of day 20 pregnancy, we determined the bioactive (*n*-octanoyl modified) form of ghrelin and showed a slight increase of plasma hormone. Ghrelin has been shown to participate in cardiovascular and sympathetic regulation (Matsumura *et al.* 2003). Intravenous injection of human ghrelin elicits a decrease in blood pressure (Nagaya *et al.* 2001) with direct vasodilatory effects possibly through GH or nitric oxide-independent mechanisms (Okumura *et al.* 2002). However, in our experimental condition, this weak hormone increase could not account the deep fall of blood pressure evidenced in SHR-P before delivery. The full explanation for the fall in blood pressure occurring in pregnant SHR is unclear: probably other factors and mechanisms may be involved in pressure homeostasis, such as the placenta-derived hypotensor substances, modification of angiotensin-renin system or of endothelium-derived relaxing factors.

Confirming previous data in normotensive animals (Shibata *et al.* 2004), we observed that in SHR, as well as in WKY, the level of ghrelin peptide in the stomach did not change at the end of pregnancy in both strains. On the contrary, mRNA ghrelin in the placenta of SHR was lower than that of normotensive rats, suggesting not only that in the fetal-placental unit there is a different production of this hormone, but also that circulating levels are influenced by the release of ghrelin by other regions (i.e. intestinal tract, pancreas, or hypothalamus or pituitary). Therefore, in SHR,

the production of placental ghrelin was not related to the plasma ghrelin concentration during pregnancy.

In conclusion, we observed an opposite profile of serum concentration of the examined hormones between WKY and SHR strains, showing a marked decrease of leptin and an increase of ghrelin level in either pregnant or not SHR. The inverse occurs in placenta expression of leptin: the higher production of leptin in pregnant SHR than in WKY might compensate the downregulated leptin system in SHRs. During pregnancy, SHRs presented an increase of body weight gain, paralleled to an increase of fat mass when compared with pregnant WKY animals, without a modification of food intake. Moreover, we found significantly higher plasma ghrelin levels in SHR that is not related to gastric or placental contribution. In contrast to the defined role of leptin and ghrelin in metabolic adaptations and dietary intake during pregnancy, their involvement in energy balance in hypertensive animals remains to be clarified.

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