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# Study of hypothalamic leptin receptor expression in low-birth-weight piglets and effects of leptin supplementation on neonatal growth and development

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**Attig L, Djiane J, Gertler A, Rampin O, Larcher T, Boukthir S, Anton PM, Madec JY, Gourdou I, Abdennebi-Najar L.** Study of hypothalamic leptin receptor expression in low-birth-weight piglets and effects of leptin supplementation on neonatal growth and development. *Am J Physiol Endocrinol Metab* 295: E1117–E1125, 2008. First published September 9, 2008; doi:10.1152/ajpendo.90542.2008.—Low birth weight resulting from intrauterine growth retardation (IUGR) is a risk factor for further development of metabolic diseases. The pig appears to reproduce nearly all of the phenotypic pathological consequences of human IUGR and is likely to be more relevant than rodents in studies of neonatal development. In the present work, we characterized the model of low-birth-weight piglets with particular attention to the hypothalamic leptin-sensitive system, and we tested whether postnatal leptin supplementation can reverse the precocious signs of adverse metabolic programming. Our results demonstrated that 1) IUGR piglets present altered postnatal growth and increased adiposity; 2) IUGR piglets exhibit abnormal hypothalamic distribution of leptin receptors that may be linked to further disturbance in food-intake behavior; and 3) postnatal leptin administration can partially reverse the IUGR phenotype by correcting growth rate, body composition, and development of several organs involved in metabolic regulation. We conclude that IUGR may be characterized by altered leptin receptor distribution within the hypothalamic structures involved in metabolic regulation and that leptin supplementation can partially reverse the IUGR phenotype. These results open interesting therapeutic perspectives in physiopathology for the correction of defects observed in IUGR.

intrauterine growth retardation; metabolic programming; obesity; catch-up growth; adipose tissue development

LOW BIRTH WEIGHT resulting from intrauterine growth retardation (IUGR) is one of the major causes of perinatal morbidity and increased risk of further adverse health outcomes in adulthood, such as obesity, type 2 diabetes, and cardiovascular diseases (for review, see Ref. 6). In IUGR infants, thinness at birth is associated with a precociously altered metabolic pattern, abnormal tissue development, and accelerated growth during the first few months of life (17, 19). This accelerated growth, currently termed “catch-up growth”, has been shown to predict the future development of metabolic diseases (5, 22, 23). The association between an adverse intrauterine environment and the onset of metabolic disorders during adulthood has led to the concept of “developmental programming.” This

concept supports the idea that environmental influences such as nutritional and hormonal status during critical developmental windows determine the long-term control of energy metabolism, particularly by programming future ingestive behavior and energy-expenditure regulation (8). When fetal nutrient supply is restricted, developmental programming results in endocrine adaptations designed to ensure postnatal survival under poor nutritional conditions by maximizing growth and fuel deposition capacities. However, when fetal and postnatal environmental conditions are mismatched, the risk of developing metabolic diseases increases (14). These epidemiological observations have been reproduced experimentally in several species, such as rodents (43, 48) and sheep (11, 28), by using a range of procedures to compromise fetal growth and obtain experimental IUGR. Such procedures include severe maternal caloric restriction, glucocorticoid exposure, and surgical procedures designed to create placental insufficiency (for review, see Ref. 13). Interestingly, in the pig, IUGR occurs naturally in hyperprolific dams, and recent research has emphasized the advantages of using this animal model, which appears to reproduce nearly all of the phenotypic pathological consequences of human IUGR such as increased adiposity (37), hypertension, cardiovascular risk (34, 36), and glucose intolerance (35).

It has been recently demonstrated in rodents that leptin is involved, during perinatal life, in the establishment of metabolic programming. Leptin is a 16-kDa cytokine produced mainly by the adipose tissue but also by several ectopic sources, such as the placenta (27), mammary glands (44), and stomach (4). It plays a pivotal role, at the hypothalamic level, in food intake regulation and energy expenditure. Vickers et al. (49) have shown that, in female IUGR rat pups, postnatal leptin treatment during the first 2 wk of life corrects the adverse developmental programming and normalizes metabolic pattern, adiposity, and body weight in adulthood. More recently, we have demonstrated that, in normal-birth-weight female rats, blockage of leptin action via the use of a leptin-specific antagonist during neonatal life impacts future sensitivity to this cytokine and predisposes the animals to diet-induced obesity (3). All of these drawbacks could originate from an altered organization of hypothalamic structures involved in metabolic regulation. Several hypothalamic nuclei are implicated in the complex neuronal network that ensures the regulation of food-

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intake behavior and energy expenditure. The arcuate nucleus (ARC) is a primary target for leptin's action of increasing the activity of anorexigenic proopiomelanocortin (POMC)-expressing neurons and decreasing the activity of orexigenic neuropeptide Y (NPY)-expressing neurons. These two neuronal populations send their projections to secondary nuclei, particularly the paraventricular and ventromedial nuclei (PVH and VMH, respectively), for integration and regulation of food-intake behavior (for review, see Ref. 41). It is now clearly established that, at least in rodents, leptin plays a critical developmental role on this hypothalamic organization, by promoting the outgrowth and interconnectivity of neuronal projections among the different nuclei involved in food-intake regulation (7). What remains to be determined is whether these observations on the development of leptin-sensitive circuits can be extended to humans, in which the ontogeny of hypothalamic development is precocious and starts during gestation (21). As recently illustrated (25), the pig appears to closely reproduce the human situation in terms of chronology of neuronal development and brain maturation, suggesting that it may constitute a very useful animal model.

In the present work, we used IUGR piglets to determine whether their unfavorable metabolic programming could be attributed to early defects in hypothalamic organization and to test the effects of leptin supplementation on correcting precocious signs of IUGR phenotype.

## MATERIALS AND METHODS

### *Animal Management*

All procedures were approved by the Animal Ethics Committee (Comité Régional d'Éthique sur l'Expérimentation Animale, Ile-de-France Sud). We used crossbred pigs (1/4 Large White × 1/4 Duroc × 1/4 Pietrain × 1/4 in the Landrace) from our experimental farm (Lasalle Beauvais). Animals were fed different age-appropriate commercial diets to cover their nutritional requirements following the European recommendations for animal welfare in breeding (2001/91/CE, November 9, 2001) and were allowed free access to water. Within 24 h after birth (*day 0*), pigs were cross-fostered to minimize competition between piglets and equalize litter sizes. Piglets were kept with their foster mothers under infrared heating lamps (32°C) until weaning (*day 28*). From weaning to *day 75*, piglets were transferred to a growing room (24–26°C) where they were allotted by groups (a dozen animals in each) and provided free access to a standard diet. After that period, animals were transferred to a larger room at 21°C and fed three times a day until 5.5 mo.

**Phenotypic characterization.** A population of 50 female piglets was monitored for body weight on *day 2* (mean body weight of  $1.47 \pm 0.3$  kg) and split into two groups. We selected low-birth-weight female piglets having body weight between 1.01 and 1.35 kg ( $n = 15$ ) to make up the IUGR group and animals with body weight between 1.62 and 1.92 kg ( $n = 15$ ) to constitute the control group. During the suckling period, body weight was recorded, and blood samples were collected in heparinized tubes on *days 5, 9, 16, and 23* by venopuncture of the subclavian vein. Plasma was stored at  $-20^{\circ}\text{C}$  until analysis to determine metabolic parameters (leptin, glucose, and triglyceride levels) using the methods described further on. After weaning, body weights were recorded on *days 60, 105, 135, and 165*. On *days 135 and 165*, body composition was checked by measuring fat and muscle depths using the Falco-vet ultrasound system (Esaote piomedical SARL, Fontenay-sous-Bois, France). Longitudinal scans were performed at  $\sim 5$  cm to the animal's midline at three different locations (midback, shoulder, and loin). After analysis of the scans, fat and muscle depths were determined from duplicate measurements. Total

fat depth was estimated by adding the data from the three locations and then used to evaluate the general adiposity of the animals.

### *Hypothalamic Leptin Receptor Expression*

**Tissue collection.** Hypothalamic studies were performed in 8-day-old female piglets to determine leptin receptor expression. Animals were killed by intraperitoneal injection of 90 mg/kg sodium thiopental (Nesdonal, Rhône-Mérieux, France). Hypothalami were rapidly dissected from the whole brain, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for further experiments. For *in situ* hybridization studies, piglets were deeply anesthetized with an intraperitoneal injection of 30 mg/kg sodium thiopental. The brain was then perfused, using peristaltic pumps, through the carotid artery, first with 0.9% (wt/vol) NaCl and then with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for fixation. Hypothalamic structures from the optic chiasma to the mamillary bodies were isolated and then post-fixed for 48 h in the same fixative buffer at  $4^{\circ}\text{C}$ . After cryoprotection in a 15% sucrose, 0.1 M phosphate buffer solution at  $4^{\circ}\text{C}$  for 24 h, hypothalami were embedded in Tissue-tek (Euromedex, Souffelweyersheim, France), frozen at  $-50^{\circ}\text{C}$  in isopentane, and stored at  $-80^{\circ}\text{C}$  until sectioning for *in situ* hybridization experiments.

***In situ* hybridization procedure.** Riboprobes for obese receptor B (ObRb) were generated from RNA extracted from hypothalamic homogenates of untreated animals. RNA extraction was performed following the Trizol Reagent protocol (Invitrogen SARL, Cergy Pontoise, France). After a preliminary denaturation for 5 min at  $65^{\circ}\text{C}$  of 4  $\mu\text{g}$  total RNA, reverse transcription reaction was performed at  $42^{\circ}\text{C}$  for 1 h using 200 units of Superscript II reverse transcriptase (Invitrogen SARL). Using the primers 5'-GAAGGAGTAGGGAAACCGAAGA-3' (sense) and 5'-GCCAAAGGTCGCTGATTC-3' (antisense), a 30-cycle PCR amplification of ObRb cDNAs was performed with 3  $\mu\text{l}$  of cDNA and 5 units Taq DNA Polymerase (QBiogene, Strasbourg, France). A 275-bp cDNA was subcloned in PGemT easy vector (Promega, Charbonnières-les-Bains, France), and the sequence was verified (MWG Biotech, Roissy, France). Probes were obtained after plasmid linearization with either *Pst*I or *Eco*RI and labeled after *in vitro* transcription with either T7 or SP6 polymerase using a digoxigenin-labeling kit (DIG RNA Labeling; Roche, Mannheim, Germany). Hypothalamic coronal sections (20  $\mu\text{m}$ ) were prepared in a cryostat from the mamillary bodies to the optic chiasma. Selected serial sections were dehydrated, delipidated, and rehydrated. After prehybridization in a buffer containing  $4\times$  SSC ( $1\times$  SSC: 0.15 M NaCl, 0.015 M sodium citrate) and  $1\times$  Denhardt's solution, adjacent sections were hybridized overnight at  $50^{\circ}\text{C}$  with 1  $\mu\text{g}/\text{ml}$  of either sense or antisense probes diluted in hybridization buffer (50% vol/vol formamide, 10% wt/vol dextran sulfate, 10 mM dithiothreitol). Slices were then washed in  $2\times$  SSC and treated for 1 h at  $37^{\circ}\text{C}$  with 10  $\mu\text{g}/\text{ml}$  RNase A. After additional washing with increasing stringency, slices were incubated for 30 min in a blocking solution (0.1 M phosphate buffer, 1% wt/vol BSA, 0.1% vol/vol Triton X-100) and incubated overnight with anti-DIG Alkaline phosphatase conjugated (Roche). Alkaline phosphatase substrate (NBT/BCIP; Roche) was then applied, and the color reaction was stopped after 24 h.

We used a Leica DMR microscope ( $\times 10/\text{HC PL APO 0.4}$  and  $\times 5/\text{NPLAN 0.12}$  objectives) equipped with a DP50 imaging camera (Olympus) for quantification of ObRb mRNA-expressing cells in the ARC and PVH regions. Photographs were taken with a  $\times 10$  objective and analyzed using WCIF ImageJ analysis software (1, 39). For each animal, three slices per region were submitted to quantification. Sense and antisense signals were determined after counting labeled cells in five  $250,000\text{-}\mu\text{m}^2$  areas inside the region of interest and averaged. Specific labeling was calculated after subtraction of the nonspecific signal obtained on the sense slice from the signal obtained on the antisense slice and expressed as cell per square nanometer. These density values, obtained after counting in three different slices, were then averaged to give a single value per animal.

*In Vivo Leptin Treatment*

**Porcine leptin.** Recombinant porcine leptin was prepared as reported previously. The protein was >98% pure by SDS-PAGE and over 95% monomeric. It was fully active in an in vitro bioassay in Baf/3 cells stably transfected with the long form of human leptin receptor (40). Its endotoxin content was <0.02 ng/μg protein.

**Leptin treatment.** IUGR piglets received daily intramuscular injections of either NaCl (IUGR NaCl, *n* = 5) or 0.5 mg/kg leptin (IUGR Lep, *n* = 7) from days 2 to 10. To assess leptin levels, blood samples were collected as already described 1 h after injection on days 2, 5, and 9.

**Analysis of leptin effects on growth performance.** This analysis was performed on IUGR piglets compared with a group of normal-birth-weight animals (control, *n* = 5). Body weight and crown-rump length (CRL) were measured on days 2 and 14. Body composition was estimated on day 14 by measuring total body electrical conductivity (TOBEC) following the method reported by Mostyn et al. (30), and measurements were performed in triplicates. Lean mass was determined using the following formula, taking into account the animal's size (lean mass = √TOBEC × CRL). Piglets were then killed the same day by thiopental administration as already described, and several organs (liver, pancreas, lungs, heart, kidneys, spleen, and brain) were isolated and weighed. Perirenal adipose tissue (PAT) was dissected, weighed, frozen in liquid nitrogen, and stored at -80°C for further analysis.

**Histological analysis of adipose tissue.** Adipose tissue samples were processed in 8-μm-thick sections and stained using a routine hematoxylin-eosin-safranin staining method. Morphometric analysis was performed using a digital camera (Nikon DXM 1200) combined with image-analysis software (Lucia). As many microscopic fields as necessary were randomly selected in adipose tissue samples to observe at least 3,362 adipocytes (64 ± 3 adipocytes/sample). Along with the morphological appearance of brown (BAT) and white adipose tissue (WAT), areas filled by each type of adipocyte were determined by Ferret minimum diameter (variation coefficients of 4.2 and 13.5%, respectively, for WAT and BAT areas after triplicate measurements). White adipocytes were counted (variation coefficient of 4%), and adipocyte density was calculated in cells/μm<sup>2</sup>. Mean adipocyte size was calculated by reporting the area determined for WAT relative to the number of cells found in the same field.

*Measurement of Plasma Hormone and Metabolite Levels*

Leptin levels were measured using a Multispecies Leptin RIA kit (Linco Research, St. Charles, MO). The limit of sensitivity was 1 ng/ml with an intra-assay variation of 2.5%. Glucose and triglyceride concentrations were determined using commercial kits (Roche Diagnostics, Meylan, France).

*Statistical Analysis*

All results were subjected to ANOVA followed by post hoc Student's *t*-test or Newman-Keuls test, as appropriate. Significant differences between two groups were determined by Student's *t*-test. For all analyses, the level of significance was set at *p* < 0.05. All values are expressed as means ± SE.

**RESULTS**

*Postnatal Growth Performance*

On day 2, female IUGR piglets had an average body weight of 1.19 ± 0.11 kg (*n* = 15) compared with 1.76 ± 0.10 kg (*n* = 15) in controls (*P* < 0.0001). The lower body weight was associated with reduced body mass index (6.9 ± 0.1 kg/m<sup>2</sup> in IUGR compared with 8.1 ± 0.1 kg/m<sup>2</sup> in controls, *P* < 0.0001). As shown in Fig. 1, at the end of the suckling period

(day 28), IUGR animals remained around 30% lighter than their control littermates (5.8 ± 0.3 kg in IUGR vs. 8.4 ± 0.4 kg in controls on day 28, *P* < 0.0001). From day 105 until the end of the experiment, there was no longer any significant difference in body weight between control and IUGR animals. As illustrated in Fig. 2A, the recovery of normal weight in the IUGR group was associated with catch-up growth during the first 2 mo after weaning, when animals were given free access to their diets (34.9 and 21.9% more weight gain in IUGR than in control pigs from day 28 to 60 and from day 60 to 105, respectively, *P* < 0.001). Ultrasound measurements of fat depth (Fig. 2B) indicated that as early as day 135, fat deposition tended to be higher in IUGR animals (33.9 ± 1.8 mm for IUGR vs. 29.5 ± 0.9 mm for controls, *P* = 0.052), and they showed a clear increase on day 165 (38.5 ± 1.2 mm in IUGR vs. 32.9 ± 1.7 mm in control, *P* < 0.01). No change in muscle depth values was observed between IUGR and control animals.

*Metabolic Profile During the Suckling Period*

As shown in Table 1, leptin levels measured at different times during the suckling period did not differ significantly between IUGR and control animals. However, there was a clear effect of age on leptin levels (*P* < 0.0001): they were significantly higher in both groups on day 5, decreased on day 9, and increased again on day 16. IUGR piglets also presented transiently higher levels of triglycerides on day 5 (1.33 ± 0.15 g/l vs. 0.80 ± 0.08 g/l in controls, *P* < 0.05), as well as of glucose (1.68 ± 0.16 g/l vs. 1.27 ± 0.03 g/l in controls, *P* < 0.05).

*Hypothalamic Leptin Receptor Expression*

Figure 3 illustrates leptin receptor expression and localization within the hypothalamus by in situ hybridization. Leptin receptor was expressed mainly in the ARC, PVH, and VMH

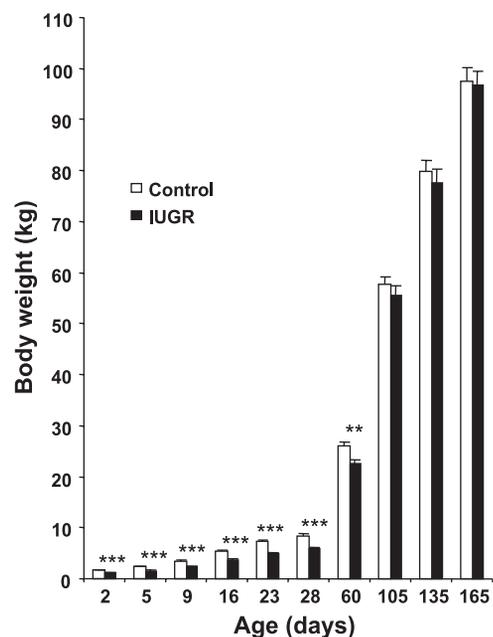


Fig. 1. Body weight evolution in intrauterine growth retardation (IUGR) and control animals from day (d) 2 to d165. Animals were weighed on d2, d5, d9, d16, and d23 and then weighed monthly from weaning (d28) until 5.5 mo of age (d165). Values are means ± SE, *n* = 15/group. \*\*\**P* < 0.001 and \*\**P* < 0.01 for comparison between age-matched control and IUGR animals.

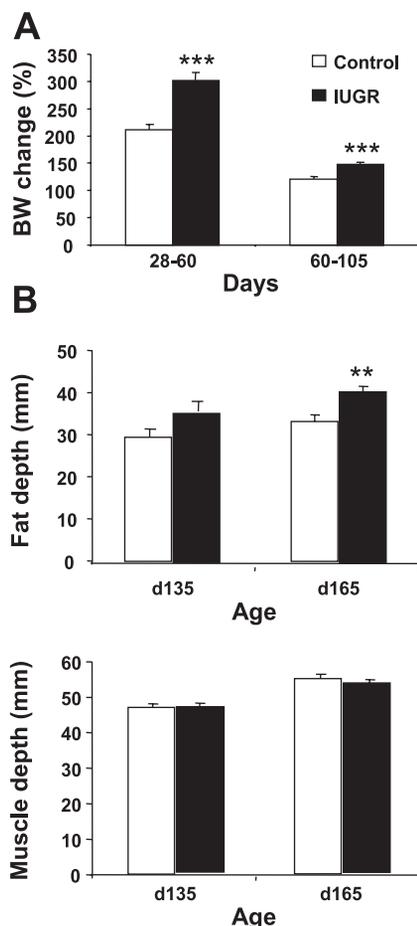


Fig. 2. Postnatal growth performance in IUGR and control animals. *A*: body weight (BW) change during the first 2 mo after weaning from d28 to d60 and from d60 to d105. BW change was calculated as a percentage of the difference in BW recorded between the end and the beginning of each period considered relative to initial BW. *B*: body composition on d135 and d165 in control and IUGR pigs. Ultrasound measurements of fat and muscle depths were performed in duplicate and averaged to give a single value. Fat depth represents the sum of the shoulder, midback, and loin fat depth values. Values are means  $\pm$  SE,  $n = 15/\text{group}$ . \*\*\* $P < 0.001$  and \*\* $P < 0.01$  for comparison between control and IUGR animals.

(data not shown). Major differences were observed in the distribution of leptin receptor expression in ARC and PVH between control and IUGR animals. In ARC, the signal from ObRb mRNA-expressing cells was lower in IUGR animals than in controls. In contrast, in PVH, there were more cells labeled for ObRb mRNA in IUGR than in controls. A quantitative method was applied to more precisely evaluate this phenomenon (Fig. 4, *A* and *B*). For an equivalent number of total labeled cells counted in PVH and ARC, the number of ObRb-expressing cells in ARC was found to be lower in IUGR piglets ( $39 \pm 2$  cells/ $\text{nm}^2$  in IUGR vs.  $66 \pm 6$  cells/ $\text{nm}^2$  in controls,  $P < 0.001$ ), whereas it was higher in the PVH of these piglets ( $34 \pm 2$  cells/ $\text{nm}^2$  in IUGR vs.  $19 \pm 1$  cells/ $\text{nm}^2$  in controls,  $P < 0.05$ ). Interestingly, the distribution among the two nuclei was completely different in the two types of animals. In control animals, ObRb-expressing cells were predominantly present in ARC ( $77.1 \pm 5.1\%$  labeled cells compared with  $23.2 \pm 2.5\%$  in PVH,  $P < 0.001$ ). In IUGR piglets, on the other hand, ObRb-expressing cells were partitioned

Table 1. Metabolic parameters during the suckling period

	Leptin, ng/ml	Triglycerides, g/l	Glucose, g/l
<i>Day 5</i>			
Control	$8.9 \pm 0.9$	$0.80 \pm 0.08$	$1.27 \pm 0.03$
IUGR	$8.6 \pm 0.5$	$1.33 \pm 0.15^*$	$1.68 \pm 0.16^*$
<i>Day 9</i>			
Control	$3.9 \pm 0.7$	$0.78 \pm 0.07$	$1.19 \pm 0.09$
IUGR	$4.0 \pm 0.7$	$0.80 \pm 0.11$	$1.26 \pm 0.07$
<i>Day 16</i>			
Control	$6.7 \pm 1.12$	$0.67 \pm 0.07$	$1.38 \pm 0.06$
IUGR	$6.2 \pm 0.9$	$0.66 \pm 0.07$	$1.20 \pm 0.10$
<i>Day 23</i>			
Control	$2.3 \pm 0.3$	$0.90 \pm 0.09$	$1.33 \pm 0.05$
IUGR	$2.1 \pm 0.1$	$0.81 \pm 0.14$	$1.18 \pm 0.07$

Values are means  $\pm$  SE,  $n = 12$  piglets/group. IUGR, intrauterine growth retardation. Plasma levels of leptin, glucose, and triglyceride were measured on days 5, 9, 16, and 23 in repeated blood samples collected in 24 piglets. \* $P < 0.05$  between control and IUGR animals.

almost equally between the two nuclei ( $42.8 \pm 4.2\%$  in PVH vs.  $57.1 \pm 2.3\%$  in ARC,  $P < 0.05$ ).

#### Postnatal Leptin Administration

In a preliminary experiment, we determined the optimal dose of porcine leptin administration to obtain the longest duration of high leptin levels. We found that administration of 0.5 mg/kg leptin leads to circulating leptin concentrations of up to 40 ng/ml 1 h after injection, which persist for at least 6 h (Fig. 5A). Figure 5B shows a dramatic increase in leptin levels in IUGR animals 1 h after leptin injection (0.5 mg/kg) on days 2, 5, and 9. A slight progressive decrease in induced levels was observed as the treatment progressed, suggesting more rapid clearance of the injected hormone with time, but after 7 days of treatment (day 9), leptin levels were still >10-fold higher than

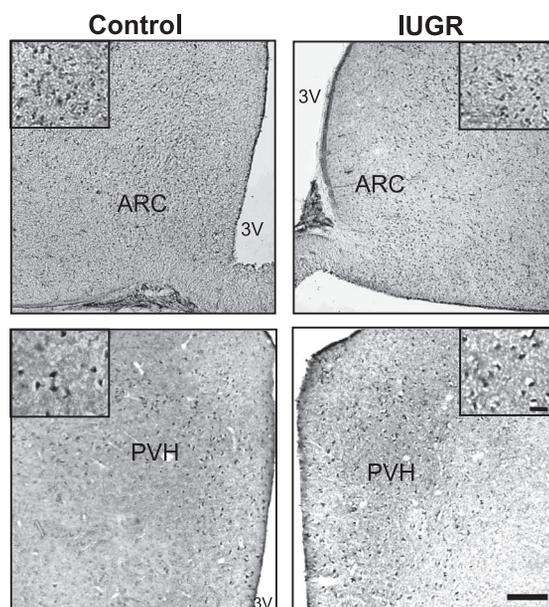


Fig. 3. Hypothalamic obese receptor B (ObRb) expression in control and IUGR female piglets. Representative in situ hybridization signal of hypothalamic ObRb-expressing cells in paraventricular hypothalamic nucleus (PVH) and arcuate nucleus (ARC) of 8-day-old control and IUGR piglets. Magnification =  $\times 5$ . Bar = 500  $\mu\text{m}$ . In each corner on top, a  $\times 10$  magnification taken inside the region of interest is shown; bar = 100  $\mu\text{m}$ .

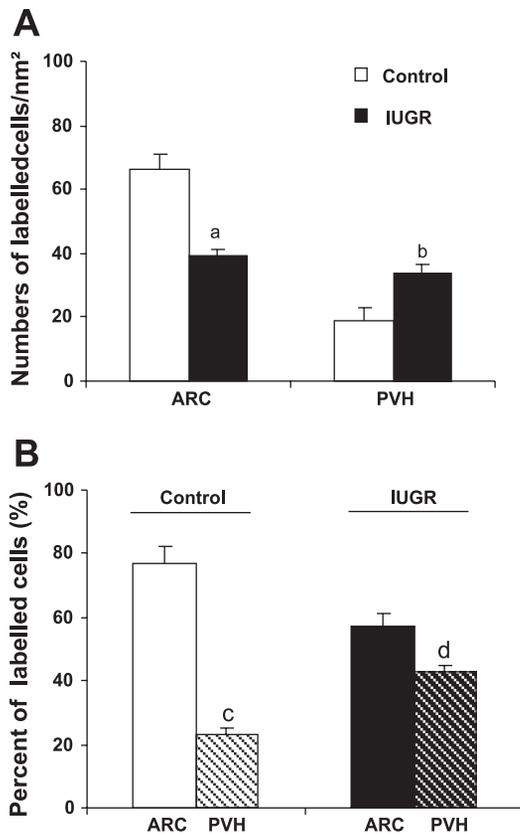


Fig. 4. Hypothalamic ObRb distribution within ARC and PVH in control and IUGR piglets. Quantification of labeled cells, using the  $\times 10$ -magnified photographs taken in the ARC and PVH regions indicates altered partitioning of ObRb-expressing cells in IUGR animals compared with control piglets. *A*: representation of nos. of ObRb-expressing cells/nm<sup>2</sup> in ARC and PVH of IUGR and control animals. *B*: distribution of labeled cells between ARC and PVH in control and IUGR animals. Values are means  $\pm$  SE,  $n = 5$ /group. <sup>a</sup> $P < 0.001$  and <sup>b</sup> $P < 0.05$  for difference between control and IUGR. <sup>c</sup> $P < 0.001$  and <sup>d</sup> $P < 0.05$  for difference between ARC and PVH.

physiological levels ( $30.7 \pm 1.85$  ng/ml in the IUGR Lep group vs.  $2.6 \pm 0.2$  ng/ml in the IUGR NaCl group). The 8-day leptin treatment had pronounced effects on both postnatal growth (Fig. 6A) and body composition (Fig. 6B), as evidenced on *day 14*, when body weight and size as well as lean mass were almost completely normalized in the treated piglets. In agreement with these changes, the growth of several organs was increased by leptin supplementation (Fig. 7). Pancreas, liver, and lung weights were particularly increased by the leptin treatment (by 53.2, 31.7, and 27.6%, respectively,  $P < 0.05$ ). A slight, albeit nonsignificant, increase in kidney weight (18.9%) was also observed in the leptin-treated animals ( $P = 0.09$ ). In contrast, there were no effects on brain, heart, or spleen weights, which remained significantly lower in IUGR-treated animals than controls. There were no differences in the total amount of PAT collected from the three groups ( $6.0 \pm 1.1$ ,  $4.8 \pm 1.2$ , and  $5.4 \pm 0.6$  g, respectively, in control, IUGR NaCl, and IUGR Lep), but, interestingly, the histological structure of the adipose tissue was highly modified after the leptin treatment (Fig. 8A). Morphometric analysis of adipose tissue on *day 14* (Fig. 8B) showed that IUGR NaCl animals present a higher number of small-sized adipocytes compared with normal-weight animals (controls). Additionally, IUGR

NaCl animals tended to have less BAT than the controls ( $P = 0.07$ ). Leptin treatment (IUGR Lep) strongly modified the histological organization of the adipose tissue by inducing a significant increase in adipocyte size as well as a reduction in white adipocyte numbers per unit surface (Fig. 8). Among the seven treated animals, five showed a marked increase in the surface occupied by BAT.

DISCUSSION

The results of the present study show, for the first time, that, in pigs, IUGR is associated just a few days after birth with dramatic changes in the partitioning of leptin receptor between the different hypothalamic nuclei involved in metabolic regulation. It is tempting to speculate that these changes are at the origin of most of the adverse effects observed in these animals in terms of body weight gain, metabolic programming, and susceptibility to obesity. This study was performed only in females, and it remains to be seen whether the same phenomena also occur in males. Furthermore, the potential reversibility of these metabolic defects remains an essential question in the development of new strategies to counteract the adverse effects of IUGR.

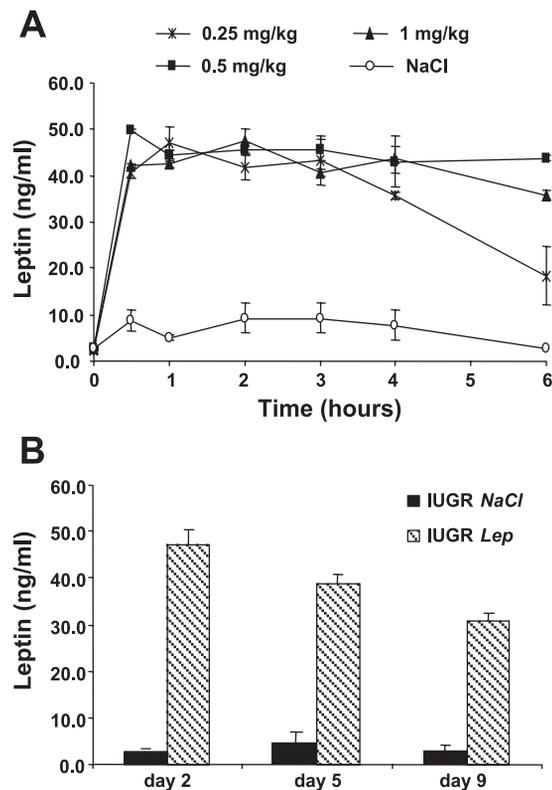


Fig. 5. Leptin injections in newborn piglets. *A*: determination of optimal dose of leptin. Eight normal-weight piglets were injected intramuscularly on d2 with either recombinant porcine leptin (0.25, 0.5, or 1 mg/kg) or NaCl ( $n = 2$  in each group). Repeated blood samples were taken before injection [*time 0* ( $t_0$ )] and at 30 min and 1, 2, 3, 4, and 6 h after injection. The experiment was repeated the day after injection, and leptin profiles were similar. *B*: leptin levels in leptin or NaCl-treated IUGR animals. Animals were injected 1 time/day with either saline (IUGR NaCl) or leptin (0.5 mg/kg; IUGR Lep) from d2 to d10. Leptin levels were assessed in blood samples collected 1 h after injection on d2, d5, and d9.

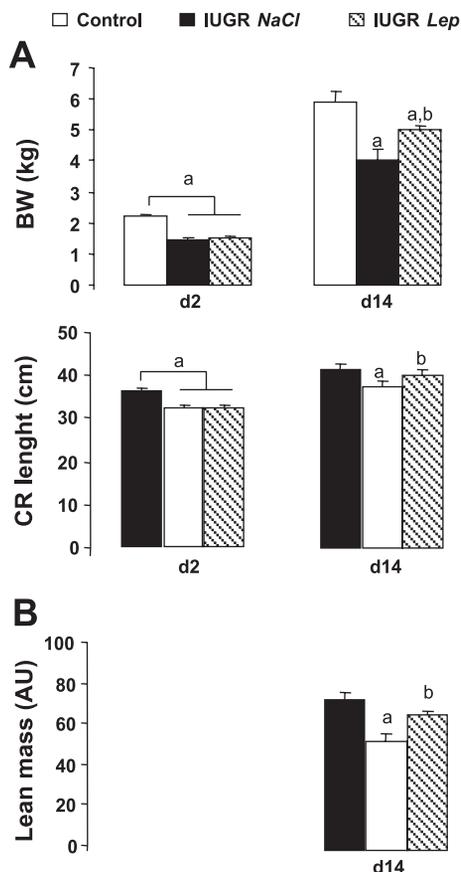


Fig. 6. Morphometric characteristics in normal-birth-weight and leptin- or NaCl-treated IUGR piglets. *A*: BW and size [crown-rump (CR) length] measured on d2 and d14 in normal-birth-weight (control,  $n = 5$ ) or IUGR piglets treated with either saline (IUGR NaCl,  $n = 5$ ) or leptin (IUGR Lep,  $n = 7$ ) from d2 to d10. *B*: determination of lean mass by total body electrical conductivity (TOBEC) analysis on d14 in control, IUGR NaCl, and IUGR Lep animals. Values are means  $\pm$  SE. <sup>a</sup> $P < 0.05$  between control and IUGR animals. <sup>b</sup> $P < 0.05$  for effect of leptin treatment in IUGR piglets.

#### *IUGR Piglets are Characterized by Abnormal Leptin Receptor Distribution Within the Hypothalamus*

One of the most interesting results stemming from this study concerns hypothalamic organization, illustrated by the differences in the partitioning of leptin receptor expression among the different hypothalamic nuclei involved in metabolic regulation in IUGR vs. normal piglets. In animals with normal body weight, ObRb expression follows the adult-type pattern and is preferentially localized in the ARC. In contrast, in IUGR, we found ObRb expression to be almost equally distributed between ARC and paraventricular nucleus (PVN). The preferential localization of ObRb in secondary nuclei such as PVN or VMH has been attributed, in sheep, to a lower stage of hypothalamic structural development (33). The same idea was illustrated by Grove et al. (15), who demonstrated that, in contrast to adults, during an early stage of development, NPY is not preferentially expressed in ARC but more widely distributed among the different hypothalamic structures. In addition to these changes in ObRb partitioning, we observed a lower level of ObRb expression in the ARC of IUGR vs. control animals. The ARC is well known to be the primary site for leptin's effects on food-intake behavior and energy expen-

diture, and the lower expression of leptin receptor in this nucleus suggests the animal's lower sensitivity to leptin's action. Along the same lines, POMC, one of the major leptin-dependent neuropeptides, has been reported to exhibit lower expression in the ARC of IUGR male rat pups (12). Based on these observations, the important issue becomes whether this apparent stage of immaturity found in IUGR newborns is permanently established, thus resulting in further disturbances of food intake behavior and leading to long-term unfavorable metabolic programming.

#### *Neonatal Leptin Treatment in IUGR Piglets Exerts a General Developmental Burst*

IUGR piglets showed a general developmental delay, as evidenced by their reduced body weight and size and the diminished growth of almost all organs. These developmental defects were associated with early deficiency in metabolic regulation and altered body composition and adipose tissue accumulation in later life. In our experiments, leptin administration induced, after just a few days, a rapid increase in the weight and size of IUGR animals. Analysis of the individual organ weights showed an apparent improvement in the growth of organs involved in metabolic regulation, such as the pancreas, liver, and, to a minor extent, kidneys. In addition, leptin was able to increase lung weight, which may reflect better maturation of this organ. These results are in direct contrast to

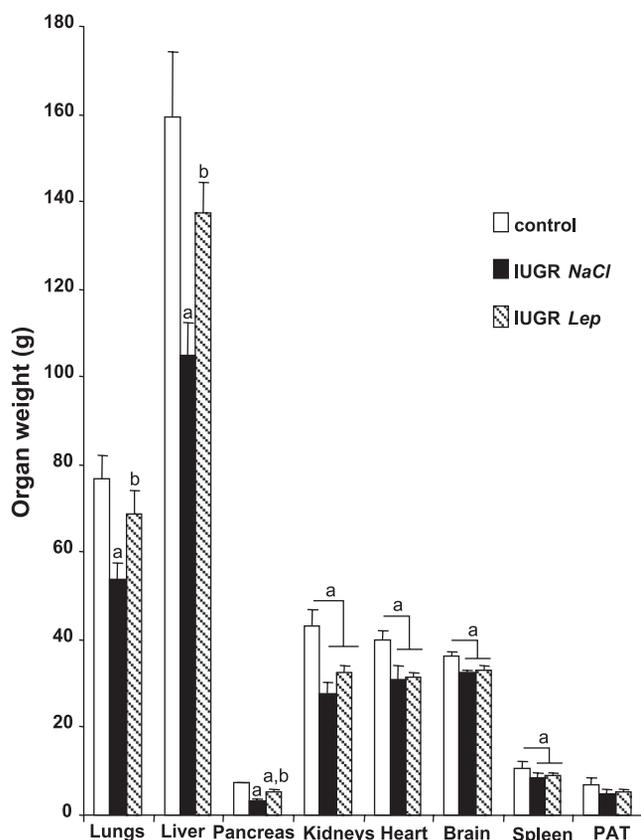


Fig. 7. Organ weight in control and leptin- or NaCl-treated IUGR animals on d14. The different organs [lungs, liver, pancreas, kidneys, heart, brain, spleen, and perirenal adipose tissue (PAT)] were rapidly collected after death and weighed. Values are means  $\pm$  SE. <sup>a</sup> $P < 0.05$  between control and IUGR animals. <sup>b</sup> $P < 0.05$  for effect of leptin treatment in IUGR piglets.

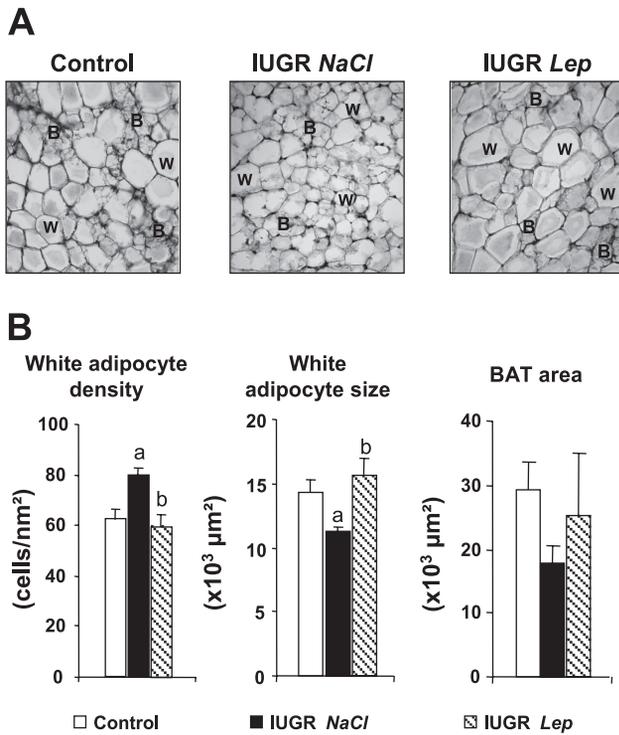


Fig. 8. Histological organization of adipose tissue in 14-day-old piglets. A: histological analysis of PAT in control ( $n = 5$ ) and IUGR piglets treated with either saline (IUGR NaCl,  $n = 5$ ) or leptin (IUGR Lep,  $n = 7$ ) at  $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from d2 to d10. W and B indicate white and brown adipocytes, respectively. B: determination of white adipocyte density and cell size and brown adipose tissue (BAT) area. Values are means  $\pm$  SE. <sup>a</sup> $P < 0.05$  between control and IUGR animals. <sup>b</sup> $P < 0.05$  for effect of leptin treatment in IUGR piglets.

those found previously in normal body weight piglets, where leptin injections appeared ineffective in increasing organ weights although it improves global growth (26). One interesting interpretation of this discrepancy might be that leptin acts as a growth-promoting factor only when organ maturation is incomplete, as in IUGR. The notion that organ maturation can be influenced postnatally by the hormonal milieu reveals interesting new perspectives for therapeutic approaches. The mechanisms by which leptin acts remain a matter of speculation. However, at least two hypotheses can be proposed, one involving direct leptin action on peripheral target organs to improve cell differentiation and organ maturation and the second involving centrally mediated action via stimulation of the hypothalamopituitary axis to promote general growth. A direct leptin effect is supported by the fact that the long form of the leptin receptor has been identified in all investigated peripheral organs in several different species (9, 24). Leptin has been shown to stimulate cell proliferation, particularly in the pancreas (45) and kidneys (50), and to increase surfactant synthesis and enhance lung maturity (20). At the central level, one of the consequences of leptin stimulation may be an increase in growth hormone secretion. Studies performed in vivo in rat and pig have clearly demonstrated this stimulatory effect (38, 46). Interestingly, the somatotrophic axis is known to be deficient in the case of IUGR (16, 31, 51), and it can be speculated that leptin's action on general growth and organ weight is mediated by stimulation of this axis. Taken

together, the growth-promoting effects of leptin appear to be complex, involving at least central and peripheral actions, and they may only be effective in the case of IUGR.

*Neonatal Leptin Supply to IUGR Animals Restores Normal Adipose Tissue Development*

We were particularly interested in studying the adipose tissue, for which we found no change in total weight but suspected structural histological modifications since its development has been shown to be sensitive to leptin in fetal and newborn sheep (32, 52). One of the consequences of IUGR in humans, as verified in this study in pigs, is the induction via unknown mechanisms of catch-up growth, leading to further excess fat deposition. We observed, as early as 2 wk after birth, an alteration in adipose tissue structure in IUGR relative to controls, i.e., a high density of small white adipocytes, probably resulting from a high level of cell proliferation. In contrast, in normal-weight animals, the number of white adipocytes was lower, and they were morphologically different. One interesting suggestion is that, in IUGR, active cell proliferation may continue after birth, whereas in normal situations this phenomenon may be much more limited. Indeed, it is well documented that, in pigs, like in humans, most fat cells are generated before birth (for review, see Ref. 2). The excessive proliferation of fat cells in IUGR may be designed to improve long-term capacity for lipid storage, thereby constituting an adaptive advantage in a restricted-food environment. However, if nutrient supply becomes abundant, this "adaptation" will promote excess adiposity. The observed catch-up growth that occurred after weaning, when animals had free access to food, and their subsequently higher adiposity were most probably consequences of this adaptive phenomenon. However, at birth, fat cells in IUGR are probably less effective at ensuring lipid storage, as reflected by their smaller size and the presence of higher levels of circulating triglycerides. This inability of IUGR animals to adequately store and use energetic substrates may compromise neonatal survival (47). Our results demonstrate that leptin treatment can normalize the structural organization of the adipose tissue by directing it toward a more differentiated stage. This results in an apparently equal number of white adipocytes in treated IUGR and control animals, which may correct the former's susceptibility to the development of obesity related to an excess number of primary adipocytes. In addition, leptin increased BAT content, probably leading to improved thermogenic capacity as shown in fetal and newborn sheep (29, 52), which is important for neonatal survival. Again, the question of direct or indirect effects of leptin on adipose tissue can be raised. Direct effects have been evidenced by the identification of leptin receptors and their biological effects in adipocytes (42). However, the involvement of neuronal connections between the hypothalamus and adipose tissue cannot be excluded. It has been demonstrated that ObRb-expressing neurons in hypothalamic areas known to control energy homeostasis, such as the ARC, the VMH, and the PVN, are *trans*-synaptically connected to adipose tissue, thus demonstrating a regulatory feedback pathway between food-intake behavior and fat-tissue metabolism (10). One interesting hypothesis is that the altered leptin receptor distribution in the hypothalamus in IUGR may render this feedback regulation less effective.

In conclusion, the data presented in this paper provide strong support for leptin's crucial role during the last steps of fetal development and organ maturation. Both central and peripheral effects are probably exerted in a specific perinatal window to adequately match the newborn's physiology to its future environment. A better understanding of the mechanism of leptin action in the developmental patterns of the different target organs, particularly the hypothalamus, is expected to provide some interesting new perspectives on counteracting the adverse effects of IUGR via metabolic programming.

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