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Maternal Undernutrition Induces Neuroendocrine Immune Dysfunction in Male Pups at Weaning

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Key Words
Lipopolysaccharide · ACTH · Glucocorticoid · Carbohydrate · Tumor necrosis factor-alpha · Leptin

Abstract
The present study was designed to assess the effect of maternal undernutrition, during gestation and lactation, on the neuroendocrine [hypothalamo-pituitary-adrenal (HPA)]-immune axis response to endotoxin (LPS) administration. For this purpose, 21-day-old male rats from both well-nourished (WN) and undernourished (UN) mothers were examined 2 h after injection (i.p.) of vehicle alone (VEH) or containing LPS (130 μg/kg BW). Circulating levels of glucose (GLU), ACTH, corticosterone (B), tumor necrosis factor-alpha (TNFα) and leptin were explored. The results indicate that: (a) mother body weight was significantly ($p < 0.05$) reduced, as a consequence of UN, at the second and third weeks of pregnancy; (b) no differences in basal glycemia were found in the two groups of pups, and LPS treatment did not induce hypoglycemia, in either group; (c) basal plasma ACTH, B and TNFα levels were similar in the two groups, and LPS-induced ACTH, B and TNFα secretions, although several-fold higher than respective VEH values ($p < 0.05$) in pups from WN mothers, were fully (ACTH and B) and partially (TNFα) abolished in products from UN mothers; (d) both mean body weights and basal plasma leptin levels were significantly ($p < 0.05$) lower in pups from UN than from WN mothers, and LPS administration did not modify plasma leptin values in products from both groups. In addition, results of dispersed total adrenal cells incubated in vitro indicate that: (a) both basal and ACTH (22 p*M*)-induced B secretion were significantly ($p < 0.05$) lower in cells from UN than WN pups, and (b) leptin (100 n*M*) was able to inhibit partially ACTH-stimulated B output by adrenal gland (AG) cells from WN pups; however, it failed to inhibit ACTH-stimulated glucocorticoid release by AG cells from UN pups. The present results indicate that undernutrition in mothers, during the very critical periods of gestation and lactation, induces in their male pups at weaning: (a) reduced circulating leptin levels and body weight values; (b) metabolic adaptation to normal carbohydrate metabolism; (c) hyporesponsiveness of the HPA and immune (TNFα) axes during endotoxemia, and (d) leptin resistance at the adrenocortical level. This study strongly supports that undernutrition of mothers results in neuroendocrine immune dysfunction of their pups; however, adrenal resistance to the inhibitory effect of leptin on glucocorticoid output is developed, probably as an adaptive mechanism to counteract unfavorable metabolic conditions.

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Introduction

Individual allostasis [1] as a consequence of undernutrition/malnutrition is characterized by, among other effects, immune-neuroendocrine disturbances [2–4]. It has recently been reported that chronic undernutrition, starting after weaning and extending up to 60 days of age, induced an arrest in body weight gain and clearly modified the acute-phase response to endotoxic shock [5], although those alterations can be reversed after refeeding of animals up to normal body weight [5]. It is already known that glucocorticoid is an important factor for the survival of the organism during allostatic states [6]. A bidirectional relationship between the immune and neuroendocrine [hypothalamo-pituitary-adrenal (HPA)] functions [7] is principally maintained by endogenous glucocorticoid [8]. Tumor necrosis factor-alpha (TNFα) is the pathognomonic cytokine of early stages of septicemia [9]. Impaired cytokine production in malnourished children has already been reported [10, 11], and malnutrition has been described as a factor responsible for attenuated febrile [12] and acute-phase protein [13] responses during infection. It is known that there are several factors released during endotoxemia which induce loss of weight and anorexia/cachexia; of these, TNFα [14] and leptin [15] seem to play a very important role. Additionally, leptin is able to inhibit fasting-stimulated HPA axis function [16] and, reciprocally, the lack of endogenous glucocorticoid significantly reduces circulating leptin [17]. Thus, this clear interrelationship between the HPA axis and adipose tissue functions should be of relevance for the acute-phase response to endotoxic shock.

Malnutrition in children is associated with increased risk of mortality [18, 19]; however, the impact of undernutrition of mothers on the neuroendocrine immune and adipocyte functions in their offspring has not been fully investigated. Thus, we developed a model of undernutrition of mothers (during pregnancy and lactation) to further evaluate, in their male pups at weaning, basal and endotoxin-stimulated neuroendocrine (HPA) and immune (TNFα) axes; the role of adipocyte (leptin) function, as a consequence of negative energetic balance, has also been investigated.

Materials and Methods

Animals, Food Restriction Protocol and Experimental Design

Controlled-pregnant (determined by the presence of sperm in the vaginal smears after mating with an adult male for 24 h) Fisher 344/N rats were individually housed in plastic cages (lights on between 07:00 and 19:00 h) and provided with standard rat pellets either ad libitum (well nourished, WN) or in a restricted fashion (undernourished, UN; table 1). The evolution of body weight was monitored at the end of the first and second weeks of gestation as well as the day before partum. Mothers continued eating either ad libitum (WN) or in a restricted fashion (UN) (table 1) until pups were weaned (day 21). All rats received water ad libitum throughout the entire experiment. On the morning of day 21 of age (between 07:00 and 08:00 h), pups per mother were counted, sexed and body weights of the male pups recorded. Immediately after (between 09:00 and 09:30 h), male pups were injected (i.p.) with either 50 µl of sterile saline solution alone (vehicle) or containing bacterial lipopolysaccharide (LPS; Sigma Chem. Co., L3755, 130 µg/kg body weight) [5]. Pups were killed by decapitation 2 h after treatment and trunk blood was collected in plastic tubes containing 20 mg of EDTA, and kept in an ice bath until centrifuged. Plasma ACTH, glucocorticoid and TNFα concentrations were then measured. Brain tissues were quickly removed immediately after decapitation and the median eminence (ME), the medial basal hypothalamus (MBH) (limits: anterior, border of the optic chiasm; posterior, border of the mammillary bodies; and lateral, hypothalamic border, approximately 2–3 mm deep), the anterior pituitary (AP), the neurointermediate lobe of the pituitary (NIL) and adrenal glands (AG) were dissected as previously described [20]. Tissues were transferred to Eppendorf tubes containing a small volume (100 µl for ME, 500 µl for MBH and 200 µl for AP) of 0.1 *N* HCl, sonicated, boiled for 10 min and centrifuged at 10,000 *g* at 4°C. Acidic extracts were stored at –20°C until determination of CRH and arginine vasopressin (AVP) in different brain areas, AP ACTH and AG corticosterone concentrations.

Table 1. Food intake of undernourished (UN) mothers (n = 19), as a percentage of food intake of well nourished (WN) mothers (n = 11), during pregnancy and lactation

Period		Food intake of mothers, %	
		WN	UN
Pregnancy	First week	100	60–72
	Second week	100	47–63
	Third week	100	47–66
Lactation	Days 1–3	100	83–97
	Days 4–5	100	69–75
	Days 6–7	100	66–70
	Days 8–10	100	55–59
	Days 11–13	100	44–53
	Days 14–15	100	44–49
	Days 16–17	100	48–51
	Days 18–20	100	50–53

Incubation of Adrenal Cells

This method is similar to the one previously described [21]. Briefly, additional male pups (from both groups) were killed by decapitation in basal conditions. AGs dissected free of adipose tissue were enzymatically dispersed and resuspended in 15–20 ml of incubation medium and preincubated at 37°C by shaking for 30 min in a 95%

O_2-5% CO_2 atmosphere. Cells were then centrifuged (10 min at 100 *g*, at room temperature) and resuspended in an appropriate volume of incubation medium in order to obtain a final concentration of 200,000 cells/0.3 ml of medium; this volume was then distributed into 12 × 75-mm polystyrene tubes. Cells were incubated, in similar conditions to those described above, for 1 h in the presence of 0.1 ml of medium alone or containing murine leptin (PrePro Tech, Inc.; 100 n*M* final concentration), then medium alone (0.1 ml) or containing ACTH (Calbiochem-Novabiochem Corp., La Jolla, Calif., USA; 22 p*M* final concentration) was added to the tubes and incubations continued for 2 h. At the end of incubation, tubes were centrifuged at 100 *g* for 10 min at room temperature and supernatants were frozen (–20°C) until the measurement of B concentrations.

Metabolite Determinations

Plasma glucose (GLU) concentrations were determined by enzymatic assays from Wiener Laboratories. Circulating levels and AP content of ACTH were measured by a specific immunoradiometric assay [21]. Plasma, medium and adrenal gland corticosterone (B) concentrations were determined by a specific radioimmunoassay [20]. The intra-assay coefficients of variation were 2–3 and 4–7%, for ACTH and B, respectively; the inter-assay coefficients of variation were 5–8 and 8–10%, for ACTH and B, respectively. CRH and AVP tissue concentrations were determined by specific radioimmunoassays previously reported [21]; the intra- and inter-assay coefficients of variation of the assays ranged between 5–8 and 10–12%, respectively. Plasma leptin concentrations were determined by a RIA developed in our laboratory and validated, for rat and mouse leptin, against a commercial kit (Linco Research, Inc., St. Charles, Mo.; Cat. # RL-83K). Briefly, synthetic murine leptin (PrePro Tech, Inc.) was used for both labelled peptide and standards as well as for the development of anti-leptin serum. Leptin was labelled ($Na^{125}I$) by the chloramine-T method and purified by elution, after loading the radioiodination mixture into a Sephacryl S-300 (Sigma Chem. Co., St. Louis, Mo., USA) (1.5 × 60 cm) column with sodium phosphate (0.05 *M*)-BSA (2 g/l)-sodium azide (10 mg/l) solution (pH 7.4). The anti-leptin serum was developed by rabbit immunization with leptin coupled to BSA. The detection range of the standard curve was 0.4–50 ng/ml. Unknowns or standards (100 µl) were incubated overnight at room temperature (RT) in the presence of 50 µl of anti-leptin serum (final dilution 1:15,000) and 50 µl (30,000 cpm) of tracer. Separation of bound and free hormone fractions was achieved by addition, first, of 200 µl of normal saline solution containing anti-rabbit γ-globulin, and then 500 µl of a solution of polyethylene glycol 6,000 (10% w/v in normal saline), incubated for 30 min at RT before centrifuging at 5,000 rpm for 30 min at 4°C. Supernatants were aspirated and bound radioactivity was counted. The assay displayed 2% and zero cross-reactivity with human leptin and mouse/rat anterior pituitary (LH, FSH, GH, PRL) hormones, respectively. The intra- and inter-assay coefficients of variation were 5–8% and 10–13%, respectively.

Finally, plasma TNFα concentrations were determined by evaluation of the cytolytic effect of TNFα on L929 cells as previously reported [21]. The intra- and inter-assay coefficients of variance ranged between 7–9 and 9–11%, respectively.

Statistical Analysis

Results are expressed as mean ± SEM. Data were analyzed by analysis of variance, followed by Fisher's test for comparison of different mean values [22].

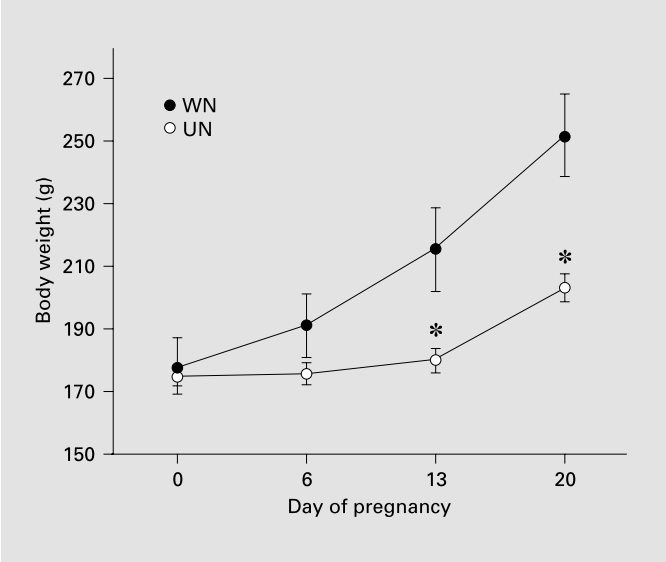
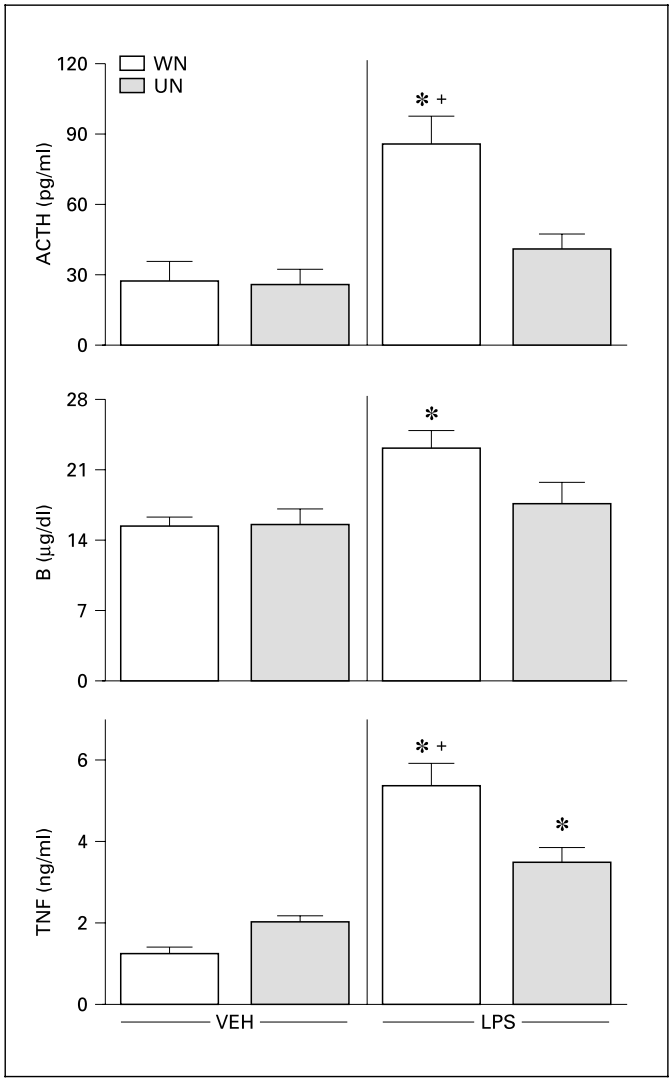


Fig. 1. Body weight values of well nourished (WN) and undernourished (UN) mothers at different days of pregnancy. Values are the mean ± SEM (n = 11 and 19 for WN and UN rats, respectively). * $p < 0.05$ vs. the respective values for WN mothers.

Results

Effects of Food Restriction on the Evolution of Body Weight in Pregnant Mothers and on the Number and Body Weights of Weaned Pups

Body weights of mothers during gestation are shown in figure 1. The evolution of body weights in WN mothers was characterized by a time-related increase, when monitored week after week from the day before pregnancy (day zero) up to the day before delivery (day 20). Conversely, body weight values in UN mothers did not significantly increase, compared to the respective departing values, in the first and second weeks of pregnancy; however, on the day before delivery, a significant ($p < 0.05$ vs. day 0 values) increase occurred. It should be noticed that in the second week of pregnancy (day 13) and the day before delivery (day 20) body weight values in UN mothers were significantly ($p < 0.05$) lower than those of WN mothers. One hundred and fourteen pups (of both sexes) were born from 11 WN mothers and 146 were born from 19 UN mothers, 10.36 ± 0.72 pups per WN mother and 7.68 ± 0.51 pups per UN mother ($p < 0.05$). Interestingly, the number of male pups per mother was similar in the two groups [4.91 ± 0.49 (n = 54) and 3.95 ± 0.33 (n = 75) from WN and UN, respectively]; however, a significantly ($p < 0.05$) lower number of female pups per mother was



found in the UN group [3.67 ± 0.41 (n = 71)] than in the WN group [5.55 ± 0.57 (n = 60)]. When the body weights of weaned pups were analyzed, we found a significantly ($p < 0.05$) lower body weight in male and female pups from UN (16.51 ± 1.56 and 16.73 ± 1.91 g, respectively) than from WN (26.43 ± 0.96 and 26.76 ± 1.14 g, respectively) mothers; no significant sex-related differences were observed in either group.

Neuroendocrine Immune Axis Response to Endotoxin Administration

Basal glucose levels in weaned male pups were similar in both groups: 1.11 ± 0.02 and 1.09 ± 0.04 g/l, in WN and UN pups, respectively. Two hours post-LPS, plasma glucose levels (1.01 ± 0.04 and 1.12 ± 0.07 g/l, in WN and UN groups, respectively) were similar in the two groups and not significantly different vs. respective basal values. Table 2 shows the results of basal neuropeptides content in different brain areas in the two groups examined. A significantly ($p < 0.05$) higher MBH CRH and AVP and ME AVP were found in UN vs. WN rats, accompanied by a significantly ($p < 0.05$) lower ME CRH and NIL AVP in UN vs. WN pups. Figure 2 (upper panel)

Fig. 2. Plasma ACTH (upper), corticosterone (middle) and TNFα (lower) levels, 2 h after intraperitoneal injection of vehicle (VEH) alone or containing LPS (130 µg/kg body weight), in different groups of male pups at weaning. Values are the mean ± SEM (n = 6–8 rats per group condition). * $p < 0.05$ vs. the respective VEH values; $^{+}$ $p < 0.05$ vs. UN group values in similar conditions.

Table 2. MBH and ME neuropeptide (CRH and AVP; ng per tissue), AP ACTH (µg per tissue), NIL AVP (µg per tissue) and AG B (µg per gland) contents in 21-day-old WN and UN rats killed in basal conditions

	MBH	ME	AP	NIL	AG
WN					
CRH	11.65 ± 1.17	15.23 ± 3.23	–	–	–
AVP	6.05 ± 0.98	4.97 ± 1.23	–	0.14 ± 0.02	–
ACTH	–	–	0.34 ± 0.02	–	–
B	–	–	–	–	0.15 ± 0.01
UN					
CRH	16.53 ± 1.19*	10.74 ± 1.01*	–	–	–
AVP	13.62 ± 2.49*	17.09 ± 4.51*	–	0.09 ± 0.01*	–
ACTH	–	–	0.26 ± 0.01*	–	–
B	–	–	–	–	0.08 ± 0.01*

Values are the mean ± SEM (n = 6–8 rats per group).
* $p < 0.05$ vs. values for WN rats.

shows plasma ACTH values in basal and post-LPS conditions. Despite no group-related differences in basal values, LPS-stimulated ACTH secretion was severalfold higher ($p < 0.05$) than basal levels in WN pups, while UN pups displayed no ACTH response. It is important to note that basal AP ACTH content was significantly ($p < 0.05$) lower in UN than in WN pups (table 2). Figure 2 (middle panel) shows the characteristically high (statistically similar in the two groups) basal corticosterone levels in both groups of 21-day-old pups and, similarly to findings for the ACTH responses, LPS-elicited adrenal response in WN but not in UN male pups. As for basal AG glucocorticoid, a significantly ($p < 0.05$) lower B content in UN than in WN counterparts (table 2) was found. Basal plasma leptin levels concorded with body weights and LPS treatment failed to modify plasma leptin levels in both groups (table 3). Finally, figure 2 (lower panel) shows plasma TNFα concentrations in basal and post-LPS conditions; basal cytokine levels were similar in both groups and LPS-induced cytokine output was significantly ($p < 0.05$) higher than the respective basal values, also for both groups; however, TNFα response to endotoxin administration was significantly ($p < 0.05$) lower in UN than in WN pups.

In vitro Effects of Leptin on Adrenal Function

Figure 3 shows the results of basal and ACTH-stimulated corticosterone secretion by dispersed AG cells, from WN and UN pups, incubated in vitro. Spontaneous (basal) glucocorticoid output was significantly ($p < 0.05$) higher from WN than from UN cells. These AG cell functions were related to the amount of glucocorticoid contained in the AGs of each group. When cells were stimulated with ACTH, a significant ($p < 0.05$) increase in B secretion vs. the respective baseline was found in both cell groups; however, while a 35% (approximately) increase in B output over the baseline was found after ACTH incubation with cells from WN pups, only an 11% (approximately) increase in B secretion over the baseline was found in cells from the UN group stimulated with a similar concentration of ACTH. When the effect of exogenous leptin was investigated, the results indicated that the adipocyte product failed to modify spontaneous glucocorticoid output, in either cell group. Interestingly, while leptin significantly ($p < 0.05$ vs. ACTH alone) inhibited ACTH-stimulated B secretion by adrenal cells from WN pups, AG cells from UN donors displayed total leptin resistance when stimulated with ACTH.

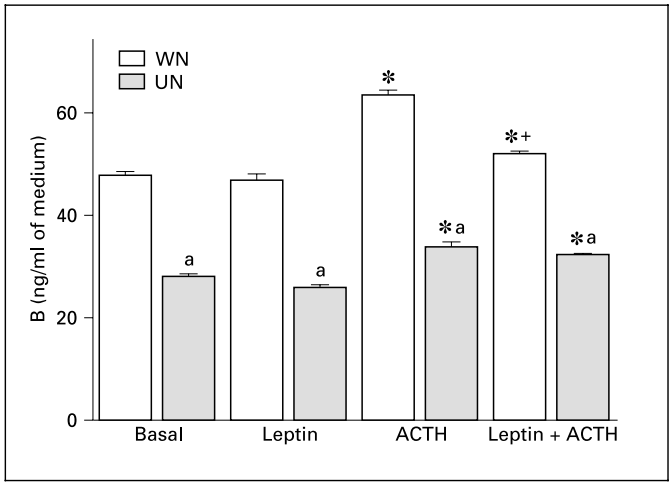


Fig. 3. Effects of leptin (100 n*M*) on spontaneous (Basal) and ACTH (22 p*M*)-induced corticosterone (B) release by dispersed total adrenal gland cells, from different (WN and UN pups) donors, incubated in vitro. Each value represents the mean of 3 different experiments (n = 6–7 tubes per condition in each experiment). [a] $p < 0.05$ vs. the respective values in cells from WN donors. * $p < 0.05$ vs. the respective basal values; [+] $p < 0.05$ vs. ACTH values in the absence of leptin, in cells from WN donors.

Table 3. Plasma leptin levels, 2 h after intraperitoneal administration of vehicle (VEH) alone or containing LPS (130 μg/kg body weight), in 21-day-old male pups from WN and UN mothers

Group		Leptin, ng/ml
WN	VEH	1.12 ± 0.14
	LPS	1.03 ± 0.13
UN	VEH	0.58 ± 0.06*
	LPS	0.54 ± 0.09*

Values are the mean ± SEM (n = 6–8 rats per group condition).

* $p < 0.05$ vs. WN values in similar condition.

Discussion

The results of the present study indicate that severe undernutrition of mothers, during gestation and lactation, induces in their male offspring at weaning: (1) adaptive mechanisms to maintain normal basal carbohydrate me-

tabolism, corticotrope-adrenocortical activity and TNFα secretion; (2) reduced body weight and basal circulating leptin level; (3) a full and partial abolishment of HPA axis and cytokine responses, respectively, to endotoxin administration, and (4) in vitro adrenocortical dysfunction and leptin resistance.

Although significant arrest in body weight gain occurred in undernourished mothers, the normal rat delivery time was not modified by the reduction in food intake. It should be noted that a significantly lower offspring number was obtained per UN than per WN dam due to differences in gender distribution, with a significantly lower number of female than male pups per UN dam. However, the development of adaptive mechanisms in male pups from UN mothers were limited. First, body weight and plasma leptin concentration were significantly reduced in 21-day-old pups from UN dams; nevertheless, UN pups showed normal basal glucose levels; this fact tallies with reports of 4 weeks' undernutrition during early life which did not induce changes in basal glucose concentrations [23]. Conversely, chronically undernourished rats, beginning at weaning and evaluated at 60 days of age, did develop basal hypoglycemia [5].

As for HPA axis function, we found similar basal plasma ACTH and glucocorticoid concentrations in both groups. Although significant ACTH and glucocorticoid responses to endotoxic shock were developed by pups from WN mothers, LPS-induced corticotrope and adrenal responses were absent in weaned male offspring from UN dams. It should be noted that in vivo basal HPA axis function was similar in UN and WN pups, even though AP ACTH and AG B contents were lower in UN than in WN rats; thus, an increased output of both CRH, from the ME, and AVP, of magnocellular origin, could have developed as compensatory mechanisms to assure normal basal HPA axis function in UN rats. These results agree fully with recent data showing that undernutrition of mothers induces HPA axis dysfunction during development in sheep [24]. It has also been reported that athymic mice could develop adrenal insufficiency, among other abnormalities, which could be compensated by increased vasopressinergic activity during adulthood, thus resulting in normal basal HPA axis function [25]; however, although we found increased hypothalamic and diminished NIL AVP contents, respectively, data of circulating vasopressin levels in basal condition and the characteristics of water and mineral metabolism remain to be determined in UN male pups. Our study also provides evidence of significant changes in the ontogeny of the hypothalamic CRH system [26] as a result of maternal UN. It has been reported that, in normal male rat offspring, hypothalamic CRH mRNA increases persistently after birth up to 21 days of age [27]; our findings indicate that UN in mothers significantly enhanced, over WN values, MBH CRH in 21-day-old male pups. This observation found support in the data reported by Heiman et al. [28], and in fact long-acting low leptin plasma levels should be able to enhance hypothalamic CRH-ergic activity. We also found that hypothalamic AVP content was severalfold higher in UN than in WN rats. Both facts could probably be integrated since, as mentioned above, they could compensate mechanisms regulating the normal basal circulating glucocorticoid level [25, 29]. It is important to add that UN female littermate (at weaning) treated with insulin, a pure neuroendocrine stressor, developed HPA axis hyporesponse 15 and 45 min postinsulin, despite similar insulin-induced hypoglycemia [30]; this observation, coupled with the present results, clearly indicate an impaired HPA axis response as a consequence of maternal undernutrition, regardless of the stressor applied. Whether the abnormality induced by maternal undernutrition could be affecting the time lasting the classical hyporesponsiveness period of HPA axis response is still open to research [31].

As expected, the diminished body weight of pups from UN dams was accompanied by hypoleptinemia [32], that the stimulatory effect of low concentrations of ACTH on the adrenal gland resulted from this reduced inhibitory leptin effect [33]. This fact is also strongly supported by the observation of in vitro adrenal leptin resistance in the UN rat. Our in vitro results indicate a potentially impaired adrenal function since both spontaneous and ACTH-stimulated glucocorticoid release is lower in cells from UN than from WN pups. This would indicate an endogenous disadvantage for UN pups to muster appropriate glucocorticoid secretion in inflammatory and other stress conditions. Since ex vivo basal corticosterone secretion was diminished, perhaps in a direct relationship with decreased adrenal gland corticosterone content, and because basal plasma circulating levels of glucocorticoid are similar to those of WN animals, the hypothesis for the development of several compensatory mechanisms for regulating normal basal HPA axis function in UN pups could be strongly sustained [28, 29]. It is important to mention that the development of adrenal leptin resistance occurred even with hypoleptinemia. Low circulating leptin levels would be expected to induce an upregulation of leptin receptors; however, our results indicate that from the functional point of view adrenal leptin receptors are nonresponsive in UN pups. Therefore, the mechanisms whereby this phenomenon takes place remain open for

investigation. However, it should be accepted that this pathophysiological change at the adrenal level is relevant for the survival of the UN pup in unfavorable conditions.

The neuroendocrine dysfunction of 21-day-old rats from UN mothers occurred simultaneously with a TNFα hyporesponsiveness post-LPS, despite unchanged basal plasma cytokine and glucocorticoid levels. Decreased immune function as a result of severe protein calorie malnutrition and undernutrition is an accepted fact [13]. It is known that macrophage-derived TNFα, among other cytokines, is a key factor to trigger HPA axis activity during the acute phase to endotoxic shock [34]; therefore, it is plausible to speculate that decreased peripheral mononuclear cell function after endotoxin administration could be additionally contributing to lower HPA axis stimulation during endotoxemia [34, 35]. However, changes in the sensitivity of HPA axis response to cytokine stimulation, as a consequence of UN, should not be discarded.

This study further suggests a clear disadvantage of offspring derived from undernourished mothers for survival of infection/injury or any other stress situation [6]. It remains to be determined whether the recovery of normal body weight, as a consequence of the reinsertion of the individual in a program of normal food intake, or the cross-foster of UN pups to WN mothers during lactation, could result in a correction of the immune neuroendocrine dysfunction [5].

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