Fetal undernutrition induces overexpression of CRH mRNA and CRH protein in hypothalamus and increases CRH and corticosterone in plasma during postnatal life in the rat

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A B S T R A C T

Prenatal undernutrition induces a variety of cardiovascular alterations in mammals when adults, including hypertension and hypercortisolism, which are thought to be caused by decreased glucocorticoid feedback control of the hypothalamus–pituitary–adrenal (HPA) axis programmed during fetal life. Hypothalamic CRH seems to be involved in blood pressure elevation of spontaneously hypertensive rats and in primary hypertension of humans, but the influence of prenatal undernutrition on CRH expression has deserved little attention. Here, we studied the expression of both CRH mRNA and CRH protein in the hypothalamus of neonatal and juvenile offspring of rats undernourished during fetal life, as well as the plasma levels of CRH and corticosterone. Prenatal undernutrition of pups was induced by submitting pregnant rats to diet restriction (10 g daily of 21% protein standard laboratory diet). Pups born from dams with free access to the standard laboratory diet served as controls. At day 2 of postnatal age, undernourished pups showed lower body and brain weights, but higher plasma CRH and corticosterone than normal pups. At day 40 of age, brain weight was significantly decreased in the undernourished rats, while plasma corticosterone, plasma CRH and systolic pressure were significantly increased in these animals. At days 2 and 40 of postnatal age, increased CRH mRNA expression and CRH concentration were found in the hypothalamus of undernourished rats. Results indicate that, in the rat, prenatal undernutrition led to fetal programming of CRH overexpression, a neuropeptide serving as activating signal to the HPA axis and/or to extrahypothalamic brain regions concerned with cardiovascular regulation.

CRH, a 41-amino acid neuropeptide, is one of the main components of the hypothalamic–pituitary–adrenal (HPA) axis. It is synthesized in the paraventricular nucleus of the hypothalamus (PVN), released into the hypophysial portal blood system and carried out to the anterior pituitary gland. At this site CRH stimulates the synthesis and release of ACTH from pituitary corticotrophs. Within the central nervous system CRH has been detected in many regions outside the PVN, including the locus coeruleus, the inferior oliva nucleus, the central nucleus of the amygdala and the Barrington's nucleus, where it may act as a neurotransmitter or neuromodulator [33]. Thus, in addition to its endocrine effects, CRH is thought to have a wide spectrum of behavioural, autonomic, and immune functions as suggested by studies in animals and humans using CRH, CRH antagonists and other agents [35].

CRH seems to be involved in spontaneous hypertensive states since intracerebroventricular administration of the peptide induced significant elevation of arterial blood pressure in normal but not in spontaneously hypertensive rats (SHR) [4]. Besides, SHR rats exhibited lower CRH concentration in the median eminence, posterior pituitary and cerebral cortex [13] but heightened expression of CRH mRNA in PVN neurons [19] than normotensive rats. Furthermore, the PVN of hypertensive patients who died due to acute cardiac failure showed increased total number of CRH neurons and increased amount of CRH mRNA [12]. Interestingly, basal levels of corticosterone were enhanced in SHR rats [19], thus suggesting that heightened expression of CRH mRNA in PVN neurons in these animals could result in hyperactivity of the HPA axis and thereby in functional peripheral cardiovascular changes.

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Prenatal undernutrition in rats and sheep induces a variety of cardiovascular alterations, including hypertension, which is thought to be caused by decreased glucocorticoid feedback control of the HPA axis programmed during fetal life [3,15,20]. Thus, it could be expected that the HPA axis be hyperactive in prenatally undernourished animals. In this regard, recent data have been shown that undernourished rats exhibited increased spontaneous neuronal activity in the PVN [25] and increased plasma levels of corticosterone [24]. All this is most relevant because in small for gestational age children, increased plasma levels of CRH, ACTH and glucocorticoids have been documented [11], and these children are in high risk to develop hypertension and cardiovascular disease at adulthood [1]. In the present investigation we studied the expression of both CRH mRNA and CRH protein in the hypothalamus of neonatal and juvenile offspring of rats undernourished during fetal life, as well as the plasma levels of CRH and corticosterone in the same animals.

The experimental protocol and animal management were in accordance with the NIH Guide for the Care and Use of Laboratory Animals [23] and were approved by the Committee for the Ethical Use of Experimental Animals, INTA, University of Chile. All animals were reared under controlled laboratory conditions (a 12-h light/dark cycle with lights on at 09:00 h). During the light cycle, light intensity was maintained at 300 lx as measured at the level of the cage floor.

The experiments were carried out on male Wistar rats (INTA, Santiago, Chile), born from mothers subjected during pregnancy to one of the two following nutritional conditions: (i) normal pregnant rats, with free access to a 21% protein standard laboratory diet (Champion S.A., Santiago, Chile), (ii) undernourished pregnant rats, with restricted access (10 g daily) to the standard laboratory diet throughout pregnancy; this amount of food is about 40% of that consumed by normal pregnant rats [32], and was given two times daily (5 g at 09:00 h and 5 g at 19:00 h) in order to minimize anxiety for feeding in food restricted pregnant dams. To prevent undernutrition of pups during the postnatal life, prenatally malnourished pups were at birth fostered to well-nourished dams giving birth on that day, according to rearing procedures described elsewhere [32]; pups born from well-nourished mothers were also fostered to well-nourished dams, in order to equalize among groups other factors that may depend on the rearing conditions (i.e. stress due to cross-fostering). During the lactation period all litters were adjusted to eight pups per dam, and all dams continued to receive the standard laboratory diet ad libitum. After weaning, at 22 days of age, all pups were housed eight per cage and fed on the standard laboratory diet. The body weight of pregnant mothers and the body weight of pups were measured daily. At 40 days of age, systolic blood pressure was measured from the rat's tail by means of a non-invasive blood pressure system (XBP 1000 Kent Scientific Apparatus, Torrington, CT, USA).

At 2 and 40 days of postnatal life normal and undernourished animals were killed rapidly by decapitation between 09:00 and 10:00 h in a room separate from that in which the other animals are kept. Trunk blood was collected in heparinized Eppendorf tubes and immediately centrifuged ($1700 \times g$, 10 min, 4 °C) and plasma stored at −80 °C. In these animals the hypothalamic tissue bounded-rostrocaudally by optic chiasm and mamillary bodies, dorsally by the anterior commissure and laterally by the hypothalamic fissures was immediately removed according to Hashimoto et al. [13]. The hypothalamic tissue was homogenized with RNA-solv (Omega Bio-tek, Doraville, GA, USA) and frozen immediately at −80 °C.

Total RNA was isolated from the homogenized hypothalamic tissue by the method of Chomczynski and Sacchi [6], centrifuged at $7500 \times g$ during 5 min at 4 °C, and quantified by spectrophotometry. The 260 nm/280 nm ratio of total RNA was in the 1.8–2.0 range. 1 μg of total RNA was reverse transcribed using 50 U of GeneScript I (Invitrogen Corporation, Carlsbad, CA, USA) and 0.5 μg/μl of oligo-dT primers (Polysciences Inc., Warrington, PA, USA), in accordance with the manufacturer's recommended procedures. Total cDNAs were amplified and quantified for real time PCR using the FastStart DNA Master PLUS SYBR Green I kit and a LightCycler 2.0 (Roche, Basel, Switzerland). The primers for CRH (Genebank V00571) were: forward, 5′-TCC GAG GAG CCT CCC ATC-3′; reverse, 5′-AAT CTC CAT GAG TTT CCT GTT GC-3′. Each PCR reaction was carried out with 2 μl of cDNA in glass capillary. The primers were used at a final concentration of 0.5 μM. The CRH gene was amplified using the following program: 95 °C × 10 min, 45 × 15 s PCR cycles at 95 °C, 60 °C for 40 s, 72 °C for 21 s, according to the manufacturer's protocol (Roche, Basel, Switzerland). At the end of the program, 45 repeats of 12 s each accompanied by a temperature ramp of 0.1 °C/repeat were performed during which melting curve data was collected to verify that only the target sequence was amplified. In each sample, the CRH gene was co-amplified with the standard housekeeping β-globine gene, to control for differences in pipette volume. There were no variations in concentration of β-globine gene between groups. A parallel real-time PCR was conducted using the same CRH primers but substituting the template with PCR-grade water to verify that exogenous DNA was not present. Additionally 1 μg of RNA, isolated by the procedure described above, was replaced with cDNA in the real-time PCR reaction to confirm that there were no genomic DNA contaminants in the RNA samples. For quantification of CRH amplicon concentration, a calibration curve was made by using standards of genomic DNA. The results were expressed as pg of CRH amplicon/ml of PCR mix.

Hypothalamic CRH: Following the organic phase described in hypothalamic tissue collection, total protein was purified, precipitated with isopropanol, centrifuged at $1700 \times g$ during 10 min at 4 °C, and washed with 0.3 M of guanidine monohydrochloride. An aliquot of 100 μl of total purified protein was used for quantification of hypothalamic CRH, in duplicate, by using a commercial RIA kit (Phoenix Pharmaceuticals Inc., Saint Joseph, MO, USA). The antiserum is specific for human and rat CRHs. Total count, non-specific binding, total binding, negative controls, as well as the standard curve for CRH (1–128 pg/tube range) were made. Counting the tube (100 μl sample) in a gamma counter (Riastar Packard, CT, USA) then yielding a number that converts by way of a calibration curve to a measure of the CRH present in the sample. The coefficient of variation was approximately 4% at normal physiological levels. The CRH concentration in the hypothalamic tissue was expressed as pg/ml of homogenized tissue.

Plasma CRH: Tubes with plasma stored at −80 °C were acidified with an equal amount of buffer A (1% trifluoracetic acid in water), mixed and centrifuged at $6000 \times g$ for 20 min at 4 °C, and the supernatant kept. A SEP-COLUMN containing 200 mg of C_{18} was equilibrated by washing with buffer B (60% acetonitrile in 1% trifluoracetic acid) followed by buffer A. The acidified plasma solution was load onto the pre-treated C_{18} SEP-COLUMN. The peptide was slowly eluted with buffer B and the eluent was collected in a polypropylene tube. The eluent was evaporated in a lyophilizer and the residue dissolved in buffer of the CRH RIA kit and quantified following the manufacturer's instructions (Phoenix Pharmaceuticals Inc., Saint Joseph, MO, USA). The coefficient of variation was approximately 5% at normal plasma levels. The CRH concentration in plasma was expressed as pg/ml plasma.

Plasma corticosterone: Plasma corticosterone was measured using a RIA based on ^{125}I-labelled rat corticosterone that was performed according to the manufacturer's instructions (Diagnostic Products Corporation, Los Angeles, CA, USA). This Coat-a-Count rat corticosterone kit is a solid-phase RIA, in which rat ^{125}I-

Table 1
Effect of the maternal dietary treatment on body weight and brain/body weights ratio, plasma corticosterone, plasma CRH, and systolic pressure of 2- and 40-day old normal and undernourished pups.

	Normal	Undernourished
Day 2		
Body weight (g)	6.6 ± 0.08	5.7 ± 0.09***
Brain weight/body weight (mg/g)	34.2 ± 0.7	36.7 ± 0.9*
Plasma corticosterone (ng/ml)	<5.7#	58.3 ± 4.5***
Plasma CRH (pg/ml)	<10#	38.1 ± 4.4*
Day 40		
Body weight (g)	155.3 ± 2.4	150.9 ± 3.2
Brain weight/Body weight (mg/g)	8.8 ± 0.16	8.3 ± 0.17*
Plasma corticosterone (ng/ml)	231.4 ± 28.6	340.2 ± 29.5**
Plasma CRH (pg/ml)	29.1 ± 4.2	66.7 ± 5.5***
Systolic pressure (mmHg)	127.6 ± 3.7	145.0 ± 7.3*

Data are the mean ± SEM. $N = 10–12$ pups per group. Statistical analysis was performed using unpaired two-tailed Student's t-test. The symbol (#) denotes the detection limit of the RIA kits; for statistical comparison between plasma corticosterone or CRH levels from normal and malnourished pups of 2 days of age, the SEM of each detection limit was considered as higher as the corresponding detection limit itself.

* $P < 0.05$.

** $P < 0.025$.

*** $P < 0.001$.

corticosterone competes for antibody sites with corticosterone in the sample during a fixed time (120 min). The antibody is coated on the wall of a polypropylene tube. Decanting the supernatant is sufficient to terminate the competition and to isolate the antibody-bound fraction of the radiolabelled corticosterone. Counting the tube in a gamma counter (Riastar Packard, CT, USA) then yields a number that converts by way of a calibration curve to a measure of the corticosterone present in the sample. Intra- and interassay coefficients of variation were approximately 5%. Corticosterone concentration was expressed as ng/ml plasma.

To determine the changes induced by the malnutrition regimen during pregnancy on the various parameters measured, values obtained from undernourished animals were expressed as means ± SEM and compared to the corresponding values found in normal animals using unpaired two-tailed Student's t-test.

Undernourished pups exhibited, from day 2 of postnatal life until weaning (on day 22), a significant body weight deficit compared with normal pups ($P < 0.001$). Table 1 shows that, at day 2 of postnatal age, undernourished pups showed statistically lower body weight ($P < 0.001$) but higher brain/body weights ratio ($P < 0.05$) than normal pups. At this same age, corticosterone and CRH protein were not detected by RIA in plasma from normal pups, while in plasma from the undernourished group corticosterone amounted near to 60 ng/ml ($P < 0.001$, in respect with normal pups) and CRH near to 40 pg/ml ($P < 0.05$, in respect with normal pups). Table 1 also shows that, at day 40 of age, the body weight of undernourished rats did not significantly differ from that of normal ones, while the brain/body weights ratio was lower ($P < 0.05$) in the undernourished animals. Plasma corticosterone ($P < 0.025$), plasma CRH ($P < 0.001$) and systolic pressure ($P < 0.05$) were significantly increased in the undernourished group as compared to normals.

Fig. 1A shows that at days 2 and 40 of postnatal age, CRH mRNA expression was significantly higher ($P < 0.01$) in the hypothalamus of undernourished rats compared to expression levels found in normal rats. Increased hypothalamic concentration for CRH protein (Fig. 1B) was also found at days 2 and 40 of postnatal age, the CRH levels in the undernourished group being in this case about twice than those found in the normal group ($P < 0.001$ and $P < 0.01$, at days 2 and 40, respectively).

Reduction of food intake during pregnancy resulted in a significant body weight deficits of pups during the lactation period, which is indicative of fetal growth retardation. At day 2 of age, the brain/body weights ratio was found to be slightly but significantly enhanced in the malnourished animals indicating fetal brain sparing during prenatal malnutrition, which is in accordance with published data [22]. At day 40 of age, no significant body weight difference between undernourished and eutrophic controls was observed, but now the brain/body weights ratio was found to be lower in the undernourished animals. This is probably due to catch-up growth of body weight, but not of brain weight, of malnourished pups during postnatal life. As reported previously, prenatal malnutrition could result in long-lasting brain weight deficits through a mechanism involving losses of neurons, glia and myelin, and impaired dendritic differentiation, among other factors [22].

At days 2 and 40 of age, enhanced expression of CRH mRNA and CRH protein in the hypothalamus, as well as increased plasma level of CRH and corticosterone were observed in the undernourished rats. Overexpression of CRH mRNA is probably the result of decreased feedback control of the HPA axis by glucocorticoids programmed during prenatal life. As discussed elsewhere [29], fetal growth restriction leads to a reduction of the glucocorticoid negative feedback by means of a series of sequential events, including: (i) decreased activity of placental 11β-hydroxysteroid dehydrogenase type 2 which catalyses the rapid metabolism of cortisol and

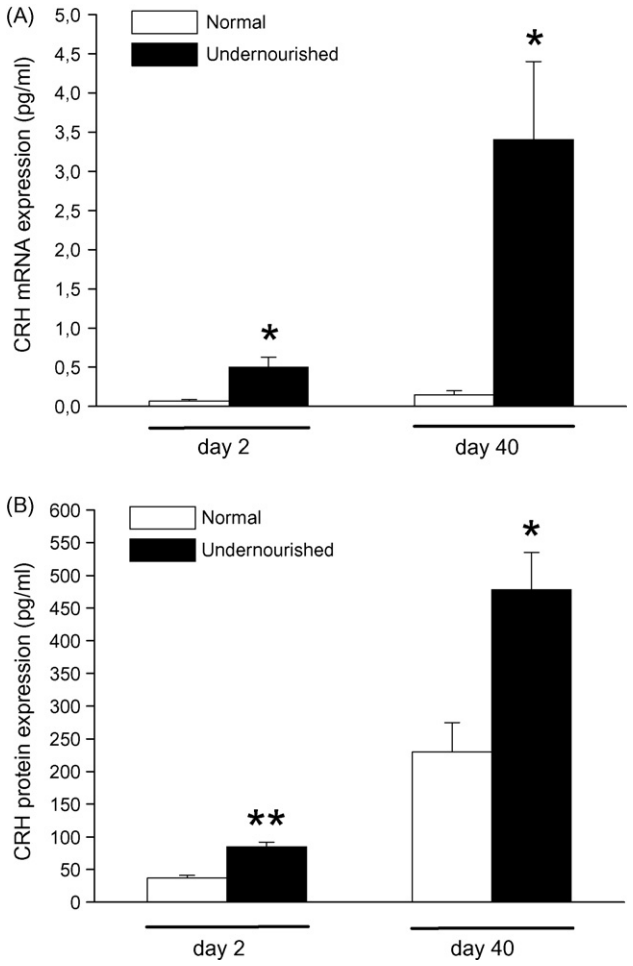

Fig. 1. Expression of CRH mRNA (A) and CRH protein (B) in hypothalami of normal and undernourished rats of 2 and 40 days of age. Values are means ± SEM. $N = 16$ rats per group. Asterisks indicate significant differences between normal and undernourished rats (*$P < 0.01$, **$P < 0.001$, unpaired two-tailed Student's t-test).

corticosterone to inert steroids, resulting in increased exposure of the fetal brain to glucocorticoids of maternal origin; (ii) this leads to decreased glucocorticoid receptor expression during fetal life in regions concerned with the regulation of the HPA axis, such as the hypothalamus [3], the pituitary [15] and the hippocampus [20], this later structure showing the highest density of corticosteroid binding sites in the brain and being an important site of feedback control upon the HPA axis [21]. Reduced number of glucocorticoid receptors in these brain areas would constitute, at first sight, a sufficient condition to explain the increased hypothalamic expression of CRH mRNA and of CRH protein. However, caution must be exercised regarding this interpretation because the negative glucocorticoid regulation of CRH expression seems to be specific for the PVN, while CRH mRNA expression appears as unaffected or upregulated in, for example, the supraoptic nucleus or other extrahypothalamic nuclei after glucocorticoid challenge [34]. Another plausible explanation for the increased CRH mRNA expression in undernourished rats is provided by data showing that these animals have enhanced synthesis and release of noradrenaline in the brain [31,32], a neurotransmitter that stimulates CRH gene transcription very rapidly in the PVN [8] via the α1 adrenoceptor subtype [16].

Increased plasma levels of corticosterone in prenatally undernourished rats could be consequence of the enhanced hypothalamic expression of CRH, which may lead to a hyperactive HPA axis via stimulation of adrenocorticotropin hormone (ACTH) secretion. Previous studies have reported greater plasma levels of ACTH and corticosterone/cortisol in mice, rats and lambs that underwent different forms of prenatal undernutrition [14,17,27]. Increased plasma ACTH and corticosterone in undernourished rats could also be the result of enhanced arginine vasopressin secretion, which is colocalized and cosecreted with CRH in parvocellular PVN neurons [16]. CRH and AVP act synergistically on ACTH secretion in the anterior pituitary and synthesis of these two peptides in the parvocellular division of the PVN is suppressed by glucocorticoids in a parallel manner [16]. However, prenatal undernutrition significantly decreases the number of vasopressin-expressing hypothalamic cells in the rat, at least in the suprachiasmatic nucleus [28]. On the other hand, the increased levels of circulating CRH in undernourished animals could reflect enhanced CRH release into the portal circulation and/or increased CRH production from diverse peripheral sources (i.e. immunocompetent and reproductive peripheral tissues [18]). Moreover, the enhanced plasmatic levels of CRH in undernourished animals could not necessarily reflect increased CRH synthesis, but also decreased plasma CRH-binding protein which clears the peptide from the blood [2]. The effect of prenatal malnutrition on the circulating level of CRH-binding protein is still unknown.

Together with the increased expression of CRH during the postnatal life of prenatally undernourished rats, a significant increase of the systolic pressure was observed in these animals. Irrespective of the primary cause for CRH overexpression in prenatally undernourished rats, i.e. reduced feedback control by glucocorticoids or enhanced CRH synthesis by central noradrenaline, it is known that CRH is a peptide serving as a positive signal to the HPA axis (thereby causing increased plasma levels of corticosterone) but also to extra-hypothalamic brain regions such as the locus coeruleus [10,25]. Both factors, enhanced plasma corticosterone and increased activity of coerulear neurons, may account for the hypertensive state showing prenatally undernourished rats, the first by acting directly on vascular glucocorticoid receptors [37] and the later by activating the sympathoadrenomedullary system [9] and/or by depressing the baroreceptor reflex [5]. As shown in the present experiments, plasma corticosterone was increased on days 2 and 40 of postnatal life in the undernourished rats. This is in line with previous reports showing that maternal food restriction during gestation resulted in high plasma levels of corticosterone in adult offspring [30], suggesting a maternal malnutrition programming for permanent alterations in glucocorticoid secretion in the progeny. In agreement with animal studies, low human birth weight, as an approximation for undernutrition in utero, is associated with increased urinary glucocorticoid secretion in children [7] and increased plasma levels of glucorcorticoids in adult men [36]. Further studies involving pharmacological manipulations of glucocorticoid and noradrenergic receptors in the PVN are required for a better understanding of the central mechanisms by which maternal malnutrition programmes increased hypothalamic CRH expression and the functional consequences in later life. As an example, microinjection of the α1 adrenoceptor prazosin into the PVN abolished the hypertensive state in prenatally undernourished rats but did not induce lowering of blood pressure in normal ones [26].

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