

## Maternal low-protein diet during lactation programmes body composition and glucose homeostasis in the adult rat offspring

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Previously we have reported that maternal malnutrition during lactation programmes body weight and thyroid function in the adult offspring. In the present study we evaluated the effect of maternal protein restriction during lactation upon body composition and hormones related to glucose homeostasis in adult rats. During lactation, Wistar lactating rats and their pups were divided into two experimental groups: control (fed a normal diet; 23 % protein) and protein-restricted (PR; fed a diet containing 8 % protein). At weaning, offspring received a normal diet until they were 180 d old. Body weight (BW) and food intake were monitored. Serum, adrenal glands, visceral fat mass (VFM) and carcasses were collected. PR rats showed lower BW (–13 %;  $P < 0.05$ ), VFM (–33 %;  $P < 0.05$ ), total body fat (–33 %;  $P < 0.05$ ), serum glucose (–7 %;  $P < 0.05$ ), serum insulin (–26 %;  $P < 0.05$ ), homeostasis model assessment index (–20 %), but higher total adrenal catecholamine content (+90 %;  $P < 0.05$ ) and serum corticosterone concentration (+51 %;  $P < 0.05$ ). No change was observed in food intake, protein mass or total body water. The lower BW of PR rats is due to a reduction of white fat tissue, probably caused by an increase in lipolysis or impairment of lipogenesis; both effects could be related to higher catecholaminergic status, as well as to hypoinsulinaemia. To conclude, changes in key hormones which control intermediary metabolism are programmed by maternal protein restriction during lactation, resulting in BW alterations in adult rats.

### Lactation: Programming: Insulin: Adrenal hormones

Protein–energy malnutrition is the most prevalent form of nutritional disorder among children in developing countries. It is estimated that more than 3.7 million deaths in 2000 could be attributed to underweight<sup>1</sup>. Protein malnutrition often occurs during gestation, lactation and the first 2 years of life<sup>2</sup>. Despite an overall decrease of stunting in developing countries, child malnutrition still remains a major public health problem<sup>3</sup>.

Barker *et al.*<sup>4</sup> have associated low birth weight with diseases related to the metabolic syndrome (diabetes, obesity and hypertension) in adulthood. This association has been denominated programming, which is defined as the basic biological phenomena that putatively underlie relationships among nutritional experiences of early life and adult diseases<sup>5–7</sup>.

We have shown that adverse situations early in life, such as malnutrition and hormonal changes during lactation, could affect permanently the progeny<sup>8–16</sup>.

Previously we showed that the milk of protein-restricted (PR) mothers had low protein and lipid concentrations<sup>8</sup> and serum albumin is lower in the offspring at the end of lactation, which confirms that these animals during lactation were malnourished.

Our data concerning the adult offspring whose mothers were submitted to protein restriction during lactation support the hypothesis of a hypermetabolic status programming. We demonstrated that protein restriction during lactation programmed the lower body weight, despite no change in food intake in adult offspring<sup>8</sup>, but direct measurements of body composition have not been reported under this condition. Furthermore, those animals showed a hyperthyroid status<sup>9,12,15,17</sup> that could be partly responsible for the hypermetabolic status in the adult age.

The aim of the present study was to characterise the phenotype of these programmed PR animals through the evaluation of their body composition and of some key hormones, such as catecholamines, corticosterone and insulin, that could help us to understand better this hypermetabolic status.

### Experimental methods

#### Animals

Wistar rats were kept in a room with controlled temperature ( $25 \pm 1^\circ\text{C}$ ) and with artificial dark–light cycles (lights on from 07.00 to 19.00 hours). Virgin female rats, aged 3

**Abbreviations:** HOMA, homeostasis model assessment; PR, protein-restricted.

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months, were caged with one male rat at the proportion of 3:1. After mating, each female was placed in an individual cage with free access to water and food until delivery. The use of the animals according to our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based its analysis on the principles described in the Guide for the Care and Use of Laboratory Animals<sup>18</sup>.

At birth, ten lactating rats were randomly assigned to each one of the following groups: control group (*n* 5), with free access to a standard laboratory diet (23% protein); PR group (*n* 5), with free access to an isoenergetic and low-protein diet (8% protein). Generally, pregnant rats produced ten to twelve pups, and so to avoid the influence of the litter size in the programming effect we only used mothers whose litter size was ten offspring. On the first day of birth, litter adjustment was performed and six male pups were kept per control or PR dam, because it has been shown that this procedure maximises lactation performance<sup>19</sup>. Malnutrition was started at birth and ended at weaning (21 d).

Table 1 shows the composition of the diets, which follow recommended standards. The PR diet was made in our laboratory using the control diet and replacing part of its protein with maize starch. The amount of starch was calculated so as to make up for the decrease in energy content due to protein reduction<sup>8,12,15</sup>.

After weaning (21 d lactation), control and PR offspring received a normal diet until they were age 180 d. Both groups of rats were killed by decapitation, to collect blood, adrenal glands, visceral fat mass and carcasses. We chose decapitation, since this a quick method and also to avoid catecholamine and glucocorticoid changes induced by anaesthetics commonly used for rats.

#### Nutritional evaluation

During lactation, each pup's body weight was monitored daily. From weaning until day 180, body weight and relative food intake (g/100 g body weight) were monitored every 4 d.

**Table 1.** Composition of the diets

|                               | Control* | Low-protein† |
|-------------------------------|----------|--------------|
| Ingredients (g/kg)            |          |              |
| Soyabeans + wheat             | 230.0    | 80.0         |
| Maize starch                  | 676.0    | 826.0        |
| Soyabean oil                  | 50.0     | 50.0         |
| Vitamin mix‡                  | 4.0      | 4.0          |
| Mineral mix‡                  | 40.0     | 40.0         |
| Macronutrient composition (%) |          |              |
| Protein                       | 23.0     | 8.0          |
| Carbohydrate                  | 66.0     | 81.0         |
| Fat                           | 11.0     | 11.0         |
| Total energy (kJ/kg)          | 17 038.7 | 17 038.7     |
| Mean energy intake (kJ/d)     | 512.2    | 311.8        |

\* Standard diet for rats (Nuvilab-NUVITAL Nutrientes LTDA, Paraná, Brazil).

† The low-protein diet was prepared in our laboratory using the control diet and replacing part of its protein with maize starch. The amount of the latter was calculated so as to make up for the decrease in energy content due to protein reduction.

‡ Vitamin and mineral mixtures were formulated according the AIN-93G recommendation for rodent diets<sup>46</sup>.

#### Body composition

Visceral fat mass was excised and weighed for the evaluation of central adiposity – mesenteric, epididymal and retroperitoneal<sup>20</sup>.

Body composition (fat and protein mass, total body water) was determined by carcass analysis<sup>21,22</sup>. After the rats were killed, control and PR animals were eviscerated, the carcasses were weighed, autoclaved for 1 h and homogenised in distilled water (1:1). The homogenate was stored at 4°C for analysis.

Homogenate (3 g) was used to determine fat content gravimetrically<sup>23</sup>. Samples were hydrolysed in a shaking water-bath at 70°C for 2 h with 30% KOH and ethanol. The total fatty acids and non-esterified cholesterol were removed with three successive washings with petroleum ether. After drying overnight in vacuum, all tubes were weighed and data were expressed as g fat/100 g carcass.

Protein content was determined in 1 g homogenate. Tubes were centrifuged at 2000 g for 10 min. The total protein concentrations were determined by the Lowry method<sup>24</sup>. Data were expressed as g protein/100 g carcass.

Total body water was determined by drying 1 g homogenate (duplicate), overnight, at 90°C to a stable weight<sup>25</sup>. Data were expressed as g water/100 g carcass.

#### Serum hormone levels

Blood samples were centrifuged (3000 rpm for 20 min, at 4°C) to obtain serum, which was individually kept at –20°C until assay. All measurements were performed in one assay.

Insulin was determined by RIA, using a commercial kit (ImmuChem<sup>TM</sup> <sup>125</sup>I, coated tube; ICN Biomedicals Inc., Aurora, OH, USA) with an assay sensitivity of 0.1 ng/ml and a range of detection of 0.1–10 ng/ml. The intra-assay variation was 8.9%.

Total corticosterone was measured by a specific murine RIA kit (ImmuChem<sup>TM</sup> <sup>125</sup>I, double antibody; ICN Biomedicals, Inc.). The intra-assay variation was 7.1%.

#### Glucose measurement

Glycaemia was determined in blood samples from the tail vein of fasting rats using a glucometer (ACCU-CHEK<sup>®</sup> Advantage; Roche Diagnostics, Mannheim, Germany).

#### Insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: insulin (μIU/ml) × serum glucose (mmol/l)/22.5<sup>26</sup>.

#### Catecholamine assays

The total catecholamine (adrenaline and noradrenaline) content in adrenal medulla was measured by the trihydroxyndole fluorescence method<sup>27,28</sup>.

Left adrenal glands were homogenised in 500 μl 10% acetic acid using an ultrasonic processor and centrifuged (10 000 g for 1 min). To assay, 50 μl supernatant fraction was mixed with 250 μl 0.5 M-buffer phosphate (pH 7.0) and 25 μl potassium ferricyanate (0.5%), followed by incubation

(20 min). The reaction was stopped with 500  $\mu$ l ascorbic acid–10 M-NaOH (1:19 proportion). The fluorometer parameters were: 420 nm to excitation and 510 nm to emission.

Results were obtained by plotting the values into a linear regression of the standard adrenaline curve. Data were expressed as  $\mu$ M catecholamines/mg gland. Protein concentration was determined by the Bradford method<sup>29</sup>.

### Statistical analysis

Results are reported as mean values with their standard errors. Statistical significance was determined by mixed-model ANOVA to analyse body weight and food intake. The other experimental data were analysed by Student's unpaired *t* test and differences were considered significant at  $P < 0.05$ .

## Results

### Nutritional evaluation

Body weight and food intake of pups whose mothers were submitted to protein restriction during lactation are shown in Fig. 1. PR offspring showed lower body weight than control animals ( $F(9, 162) = 10.95$ ;  $P < 0.0001$ ) from lactation until adulthood (Fig. 1 (A) and (B)), but no change in food intake (Fig. 1 (C)), as previously reported by our group<sup>8,11,12,15</sup>.

As shown in Fig. 2 (A), the PR offspring aged 180 d showed lower content of visceral fat mass ( $-33\%$ ;  $P < 0.01$ ). Adult PR animals also presented lower ( $-33\%$ ;  $P < 0.01$ ) body fat mass (Fig. 2 (B)), but no change was observed in body protein mass (Fig. 2 (C)) and body water content (Fig. 2 (D)).

### Glucose homeostasis

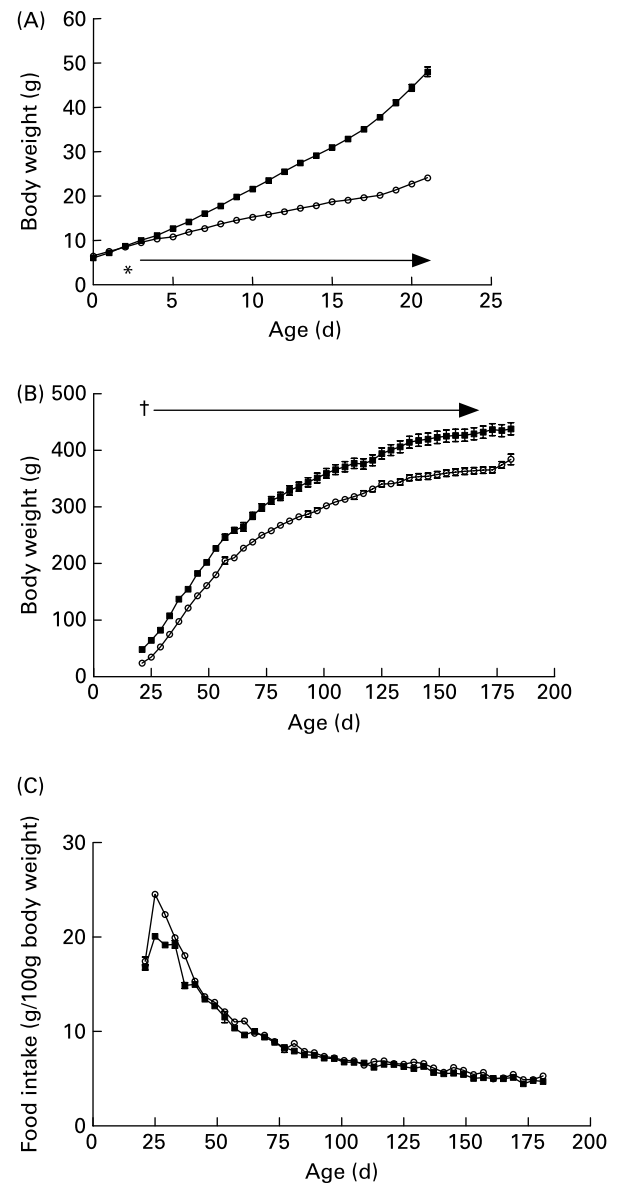
Fig. 3 shows serum glucose (Fig. 3 (A)) and insulin (Fig. 3 (B)) in 180 d old offspring whose mothers were submitted to protein restriction during lactation. The PR group presented lower glycaemia ( $-7\%$ ;  $P < 0.05$ ) and insulinaemia ( $-26\%$ ;  $P < 0.05$ ) than control animals. The evaluation of insulin resistance is shown in Fig. 4. There was a trend for the PR group to have a lower HOMA index ( $-20\%$ ), suggesting higher insulin sensitivity.

### Adrenal hormones

Serum corticosterone and intra-glandular catecholamine content of offspring aged 180 d whose mothers were submitted to protein restriction during lactation are shown in Fig. 5 (A) and Fig. 5 (B), respectively. PR rats showed higher corticosteronaemia ( $+51\%$ ;  $P < 0.05$ ) and adrenal medulla catecholamine content ( $+90\%$ ;  $P < 0.05$ ).

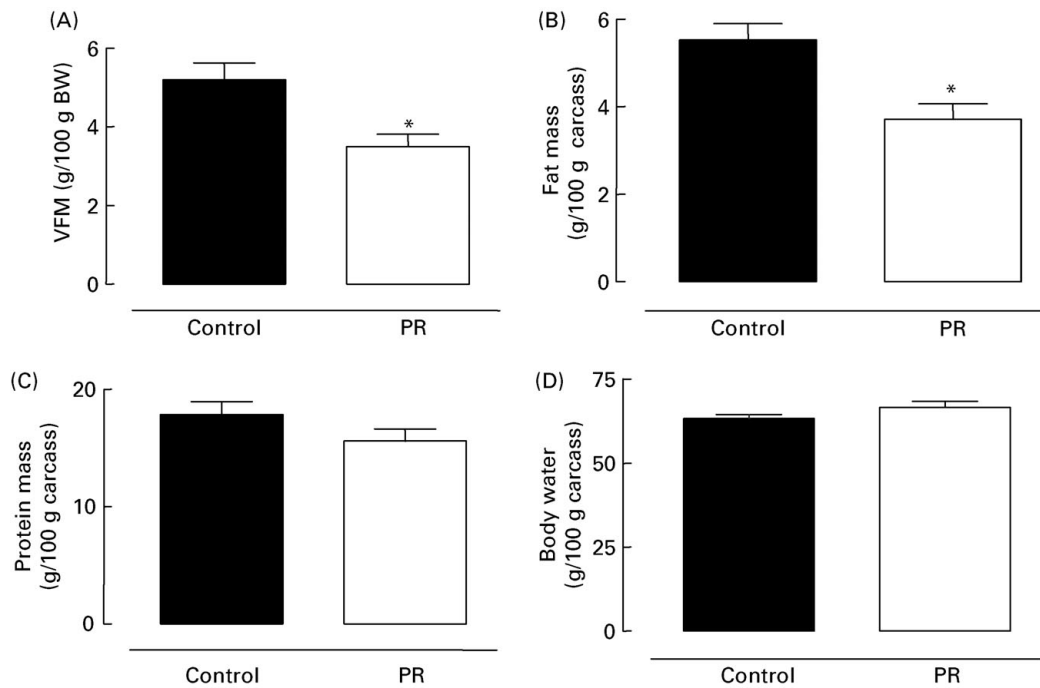
## Discussion

The present study showed that protein restriction during lactation programmed lower visceral fat mass and total body fat, which are responsible for the lower body weight in the adult offspring, without altering food intake, reinforcing previous studies from our group<sup>8,11,12,15</sup>. In addition, those PR animals present, at age 180 d, hypoinsulinaemia, hypercorticosteronaemia and higher total catecholamine content, which may inform

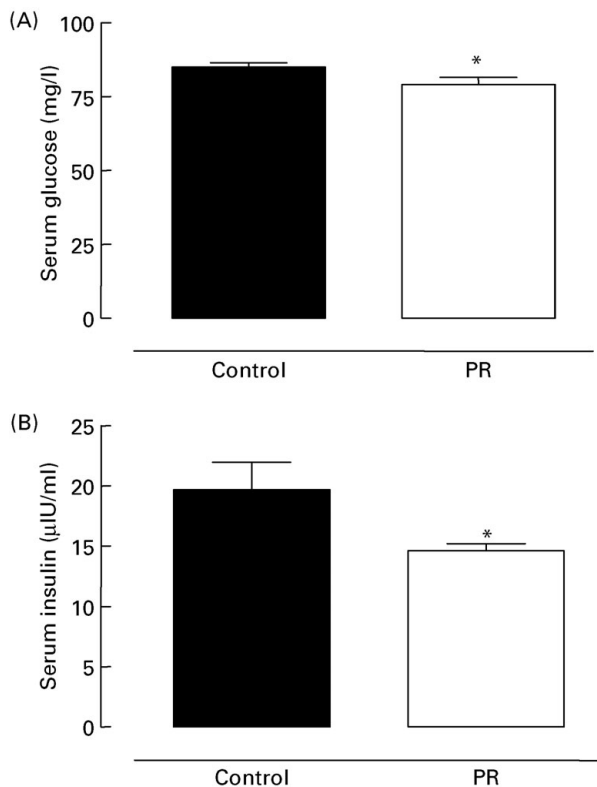


**Fig. 1.** Body weight and food intake evolution. (A) Body weight during lactation of pups whose mothers were fed a control (■) or protein-restricted (PR; ○) diet. (B) Body weight after weaning until adult age of rats whose mothers were fed a control (■) or PR (○) diet during lactation. (C) Food intake after weaning until adult age of rats whose mothers were fed a control (■) or PR (○) diet during lactation. Values are means for thirty animals per group, with standard errors represented by vertical bars. Mean values for the PR animals were significantly different to those of the control animals: \*  $P < 0.05$ , †  $P < 0.0001$  (ANOVA).

us about their energetic metabolism profile and suggested a metabolic dysfunction. Also, our previous findings showed that PR animals presented lower pituitary growth hormone mRNA expression ( $-29\%$ ) and lower body length ( $-20\%$ ) at weaning, as well as at age 90 d ( $-19$  and  $-5\%$ , respectively), but no changes in body length when they were age 180 d, even continuing with lower ( $-17\%$ ) growth hormone mRNA expression<sup>17</sup>. Strangely, those animals had lower fat mass, even though growth hormone deficiency is associated with higher adiposity<sup>30,31</sup>. Further, we also showed that neonatal protein restriction programmes for hyperthyroidism



**Fig. 2.** Body composition of adult offspring. (A) Visceral fat mass (VFM) (g/100 g body weight (BW)), (B) body fat mass, (C) body protein mass and (D) body water of adult animals whose mothers were fed a normal (control; ■) or protein-restricted (PR; □) diet during lactation. Values are means for ten animals per group, with standard errors represented by vertical bars. \*Mean value was significantly different from that of the control group ( $P < 0.01$ ).



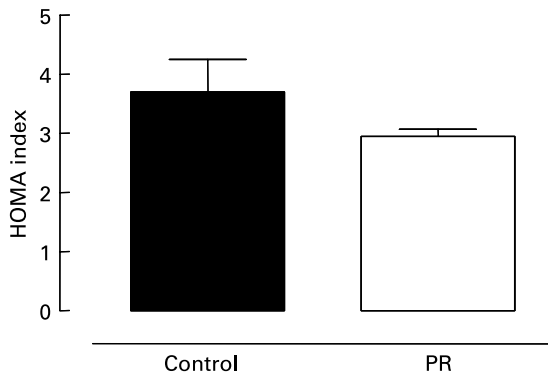
**Fig. 3.** Glycaemia and insulinaemia of adult offspring. (A) Serum glucose and (B) insulin of adult animals whose mothers were fed a normal (control; ■) or protein-restricted (PR; □) diet during lactation. Values are means for ten animals per group, with standard errors represented by vertical bars. \*Mean value was significantly different from that of the control group ( $P < 0.05$ ).

in adult offspring<sup>9,12</sup>, evidencing that our programmed animals become hypermetabolic, which could increase cardiovascular risk.

Concerning catecholamines, the present findings corroborate another study<sup>32</sup>, which showed an increase in serum catecholamines in rats at age 90 d whose mothers were fed a PR diet during gestation and lactation. However, the increase in medullary catecholamines could be due to a decrease in their secretion. Thus, a more direct evaluation turns out to be necessary, such as experiments of *in vitro* secretion or measurement of serum catecholamines or their metabolites.

Evidence that reinforces our main hypothesis that catecholamines could be increased is the fact that body fat mass of PR animals is lower. In addition, glucocorticoids stimulate catecholamine synthesis, mainly through the effect upon the enzyme phenylethanol amine-*N*-methyl transferase, responsible for noradrenaline to adrenaline conversion in the cytoplasm of chromaffin cells<sup>33</sup>. Thus, the hypercorticosteronaemia detected in these animals could contribute to the increase of adrenal medullary catecholamine content in PR animals.

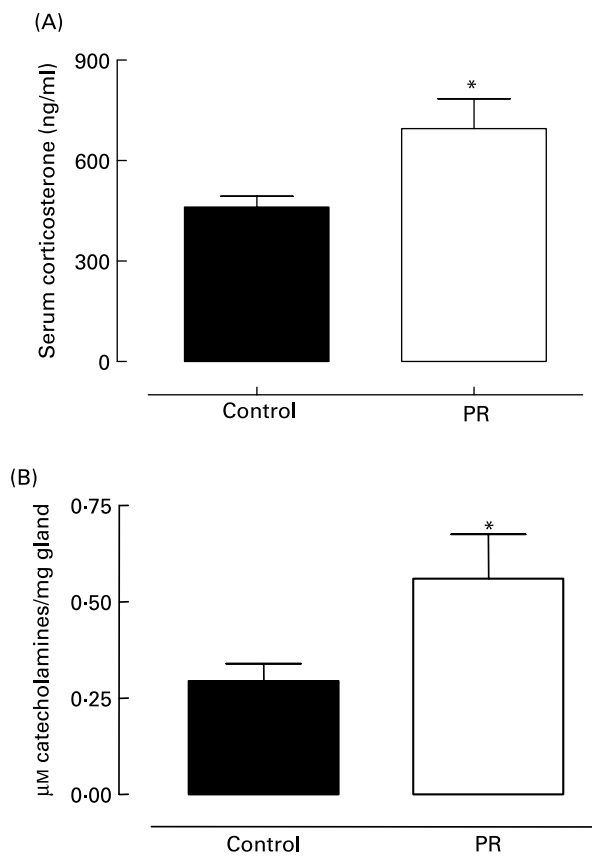
The adult programmed PR animals showed lower serum insulin levels and a slight lower glycaemia than control animals. Furthermore, there was a trend for HOMA index to be lower, which may indicate that despite hypoinsulinaemia, there is normal or even higher insulin sensitivity, since a higher HOMA index indicates insulin resistance<sup>26,34</sup>. These results corroborate previous studies<sup>35–37</sup> about lower insulin secretion and its higher sensitivity in adult rats submitted to severe postnatal protein restriction (0 or 4% protein content). Recently, Zambrano *et al.*<sup>38</sup> also described a lower glycaemia and higher sensitivity in adult offspring whose mothers were



**Fig. 4.** Homeostatic model assessment (HOMA) index of adult offspring. HOMA index of adult animals whose mothers were fed a normal (control; ■) or protein-restricted (PR; □) diet during lactation. Values are means for ten animals per group, with standard errors represented by vertical bars.

submitted to protein restriction (10% protein content) during lactation.

A rat model of maternal protein restriction (80 g/kg v. 200 g/kg control) during gestation and lactation showed that offspring were born smaller and were programmed for hyperinsulinaemia and tissue insulin resistance, developing diabetes in adult life<sup>39</sup>.



**Fig. 5.** Corticosteronaemia and catecholamines of adult offspring. (A) Serum corticosterone levels and (B) adrenal catecholamine content of adult animals whose mothers were fed a normal (control; ■) or protein-restricted (PR; □) diet during lactation. Values are means for ten animals per group, with standard errors represented by vertical bars. \*Mean value was significantly different from that of the control group ( $P < 0.05$ ).

The main difference between this model and the present data, concerning insulin levels and its sensitivity, seems to be the different period of undernutrition, which suggests that during gestation organogenesis can also be affected.

The slight decrease in glycaemia associated with higher insulin sensitivity in programmed PR rats could be causing a contra-regulatory response to insulin, suggested by hypercortisosteronaemia and higher tissue catecholamines shown in the present study. Thus, it is possible that the result of this hormonal profile generates lipolysis. At the present moment, we do not know when those hormonal changes begin during development of the programmed offspring. With this purpose, further studies involving temporal evolution are being performed.

The hypoinsulinaemia can also be explained by an increase of catecholamines, since these hormones inhibit insulin secretion through their action on the  $\alpha 1$ -adrenoreceptors of the Langerhans  $\beta$ -cells<sup>40</sup>.

In models of programming by neonatal stress, a lower body weight and elevated catecholamine and glucocorticoid levels in adult animals have also been described<sup>32,41–43</sup>, but, contrary to our findings, hyperglycaemia and hyperinsulinaemia were present<sup>43–45</sup>.

In conclusion, maternal protein restriction during lactation programmes body composition and key hormones that control the intermediary metabolism of offspring in adulthood. Therefore, perhaps these programmed PR animals present a hypermetabolic state, which could explain their lower adiposity and body weight.

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