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ORIGINAL ARTICLE

Effect of aproteic diet and fasting on insulin, pancreatic noradrenaline and luteinizing hormone. Changes after 24-hour refeeding

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Abstract

OBJECTIVE AND DESIGN: the objective of this study performed in adult male rats was to determine the alteration in glycemic, insulin and gonadotrophin luteinizing hormone secretion, and noradrenaline pancreatic concentration caused by fasting (F) and aproteic diet (Ap) during 7 and 21 days respectively, as well as the recovery after 24-hour refeeding with control diet (Co).

RESULTS: a significant decrease in glycemic levels was only achieved through fasting (F: 86 ± 5.1 mg %), when compared with controls (Co: 107 ± 5 mg %). In spite of the high levels of carbohydrates (89%) present in the aproteic diet, the animals fed with this diet showed no differences in glycemic levels (Ap: 120.3 ± 12.2 mg %), compared with controls. As a result of fasting and aproteic diet, there was a significant decrease in insulin (F: 8.67 ± 1.36; Ap: 5.7 ± 0.67; Co: 31 ± 3.4 uU/ml) and LH levels (F: 10.175 ± 1.74; Ap: 13.7 ± 4; Co: 29.83 ± 4.91 ng/ml). The refed recovered insulin (FR: 50.57 ± 6.63; ApR: 43.5 ± 6.85 uU/ml), but not LH levels (FR: 14.25 ± 3.54; ApR: 13.03 ± 4.25 ng/ml). A significant increase was observed in the pancreatic noradrenaline concentration ($P<0.001$) of rats receiving aproteic diet (889.9 ± 34.65 ng/mg tissue) and fasting during 7 days (827.5 ± 55.7 ng/mg tissue), compared with controls (531.1 ± 48.6 ng/mg tissue).

CONCLUSIONS: fasting and aproteic diets altered gonadal and metabolic control. When returning to a normal nutritional condition, only the metabolic control, not the reproductive function, could be recovered in the first 24 hours of refeeding. Malnutrition-induced hypoinsulinemia would be caused by an increase in a specific noradrenergic tone.

Introduction

Malnutrition may alter the functions of the gonadal axis [1]. Normal development and function of the reproductive system require not only enough supply of calories, but also different nutrients such as lipids, minerals, vitamins, carbohydrates and proteins. Carbohydrates represent the most important energy metabolic substratum for the brain.

Insulin, a proteic hormone synthesized and secreted by the pancreas, is one of the chief metabolism regulators. This hormone, as well as its receptors, are present in the rat brain [2] and are highly concentrated in the hypothalamus, especially in the arcuate nucleus and median eminence [3, 4, 5], which are the main areas of GnRH liberation. It has been demonstrated that "in vitro" incubations with insulin of hypothalamic fragments containing these regulatory areas of GnRH liberation significantly increase the liberation of this hypothalamic factor [6]. Insulin secretion is regulated by the sympathetic system [7]. Catecholamine-induced hyperglycemia could be caused by direct and indirect mechanisms. The indirect actions result from alpha-adrenergic suppression of insulin secretion and augmentation of glucagon secretion [8].

The objectives of the present work were: to study the alteration in insulin and luteinizing hormone secretion under different malnutrition conditions, to determine whether animals refed for 24 hours recover normal hormonal levels, and to verify if both hormones present similar times of recovery. Since insulin secretion is regulated by the autonomic noradrenergic system, the effect of both malnutrition models on pancreatic noradrenaline secretion was further studied.

Materials and Methods

Experiment

Young male Wistar rats from the Department of Physiology at the School of Medicine of the University of Buenos Aires were used. Upon weaning time, the animals were fed with balanced commercial diet, beginning the experimental diets with a median body weight of 250 g. The animals were maintained under controlled temperature (22–24°C), humidity and light (light on from 7.00 AM to 7.00 PM) conditions. Animal body weight was recorded once a week. The animals were used in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals.

The animals were divided into five nutritional groups: a) control group (Co) was fed with control diet consisting of 20% of casein protein, 6.5% lipids, 69% carbohydrate (dextrin), 3.5% mineral mixture and 1% vitamin mixture; b) aproteic group (Ap) received a diet consisting of 0% of protein and 89% of carbohydrates (dextrin), and the other components in the same proportion as control diet. Both groups were fed "ad libitum" for a 21-day period; c) fasting group (F) was fed with control diet during 14 days and fasted 7 days thereafter, receiving only water "ad libitum"; d) aproteic and refeeding group (ApR); and e) fasting and refeeding group (FR). The last two groups were refed on day 21 of treatment with control diet during 24 hours. All the animals except the fasting group received food up to the moment of sacrifice. Both diets (control and aproteic) were isocaloric and prepared in our laboratory.

Exactly at noon of day 22 all animals were sacrificed by decapitation, trunk blood collected, and their glucose plasmatic levels measured immediately. The remaining blood was centrifuged at 2500 rpm during 10 minutes, and serum was separated and kept at –20°C until quantitation of insulin and gonadotrophin luteinizing hormone (LH) in all groups. Pancreatic tissue was dissected immediately after decapitation and was frozen until noradrenaline extraction process.

Biochemical Assays

Glucose levels were calculated using glucose analyzer (Glucometer, Bayer). Insulin was measured with the method of Herbert et al. [9]. Rat insulin was used as standard (Novo Nordisk, Gentofe, Denmark), and pork insulin I^{125} as tracer [*Comisión Nacional de Energía Atómica*, *CNEA* (National Commission of Atomic Energy), Argentina]. Pork antiinsulin serum was also used. LH levels were determined by a double antibody radioimmunoassay technique [10]. The material for these assays was kindly provided by the NIAMDD rat pituitary program (Bethesda). Plasma LH was determined in duplicate. LH results are expressed as nanograms per ml in terms of the reference preparation.

Catecholamine extraction and assay

Pancreatic samples were weighed and homogenized in 1 ml 0.3 M perchloric acid, using a Vibra Cell (Sonics and Material INC, Donbury, CN U.S.A.) refrigerated with ice. The homogenate was centrifuged at 13000 rpm for 2 min. Tris HCl (2 M) buffer (pH 8.6), 3,4- dihydroxybenzilamine (Sigma, St. Louis, MO) (100 ng/ml) as internal standard, and previously dehydrated alumine were added to the supernatant obtained. After 15 min shaking, samples were centrifuged at 13,000 rpm and pellets were washed twice. Then, samples were centrifuged and

100 uL of acetic-phosphoric acid solution (80:20) was added. Finally, after 15 min shaking, samples were centrifuged. Twenty uL of supernatant was injected in an analytic column (LKB) (Spherisob C-18, ODS: 2.3; 4´100 mm) to separate catecholamines, which were identified with an electrochemical detector and recorded with an integrator.

Standard curves were prepared with noradrenaline bitartrate, adrenaline bitartrate and dopamine chlorhydrate (Sigma, S. Louis, MO) as catecholamine standards and 3,4- dihydroxybenzilamine as internal standard. These curves were processed simultaneously. Recovery was in the range of 75 to 90%.

The mobile phase was prepared with sodium phosphate monohydrate (13.78 g/L), octanesulfonicacid (35 mg/L), EDTA (100 mg/L), and acetonitrile (15 ml/L), pH 3.0.

The catecholamine final concentration was related to tissue weight and values obtained with a standard curve that were not processed.

Statistical Analysis

The data were expressed as mean ± SEM. These data were analyzed using ANOVA and Bonferroni test (Multiple Bonferroni T-test, San Diego, Instant Program GraphPad). Differences between means were considered significant if $P < 0.05$.

Results

The animals submitted to a free protein diet lost 25% of body weight by the end of treatment, and those fasted for 7 days showed 15% of body weight loss by that time. The animals refed with control diet during 24 hours did not recover the body weight loss.

Glucose plasmatic levels decreased significantly ($P<0.02$) in animals submitted to absolute fasting for 7 days (F: 86 ± 5.1 mg%) compared with controls (Co: 107 ± 5 mg%). In spite of the high levels of carbohydrates (89%) present in the aproteic diet, the animals fed with this diet did not show significant differences in the glycemic levels compared with controls (Ap: 120.3 ± 12.2 mg%). When the animals were refed with the control diet, glucose plasmatic levels did not vary in the rats that had received aproteic diet (ApR: 115.9 ± 12 mg%). However, these levels increased significantly ($P<0.005$) in the previously fasted animals (FR: 122 ± 8.6 mg%).

There was a significant decrease ($P<0.05$) in plasmatic insulin levels (Fig.1) in rats fed with aproteic diet (Ap: 5.7 ± 0.67 uU/ml) and those who fasted (F: 8.67 ± 1.36 uU/ml), compared with controls (Co: 31 ± 3.4 uU/ml). Nevertheless, when both Ap and F groups were refed with the control diet, a significant increase

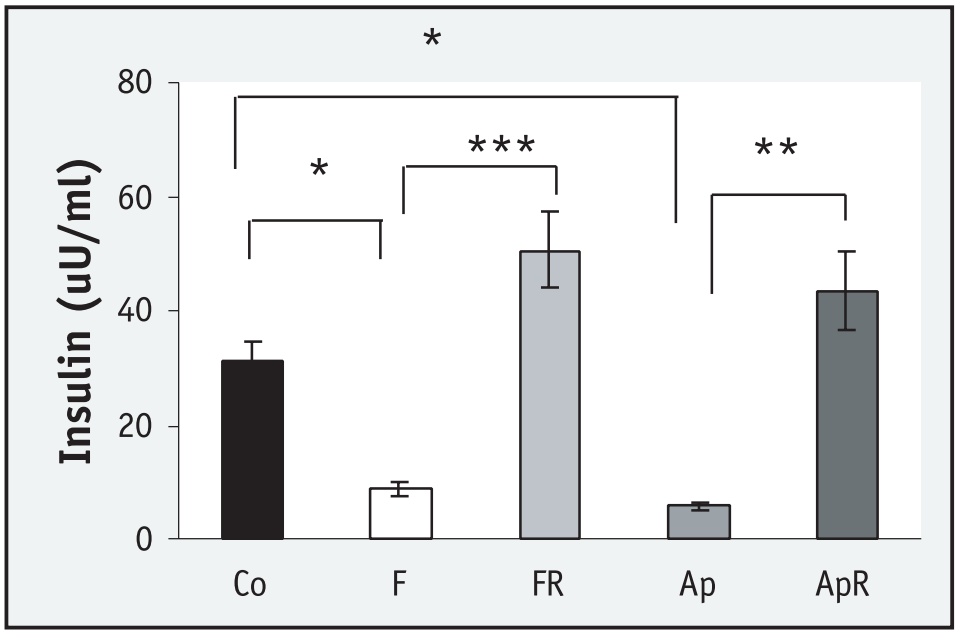

Fig. 1. Effects of 21-day aproteic diet (Ap), 7-day fasting (F), and refeeding with control diet for 24 hours (ApR, FR) on plasmatic insulin in adult male rats. Co: n=7, Ap: n=7, F:

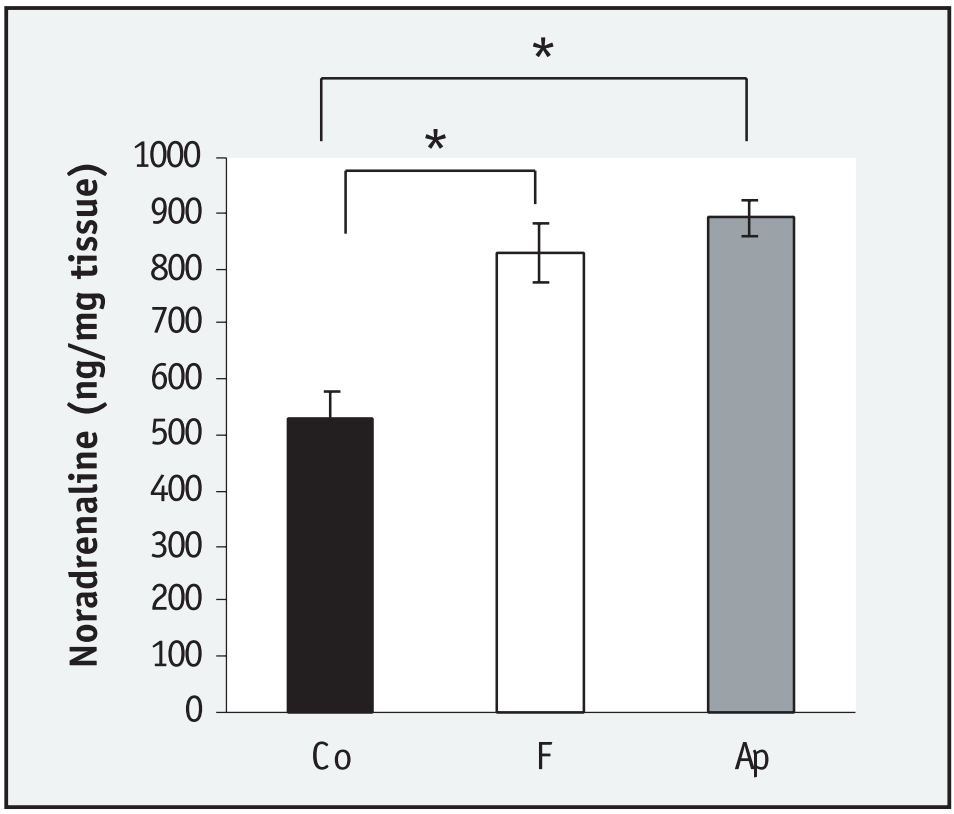

Fig. 2. Effects of 21-day aproteic diet (Ap) and 7-day fasting (F) on pancreatic tissue noradrenaline concentration in adult male rats. Co: n=9, Ap: n=10, F: n=9. Values are mean

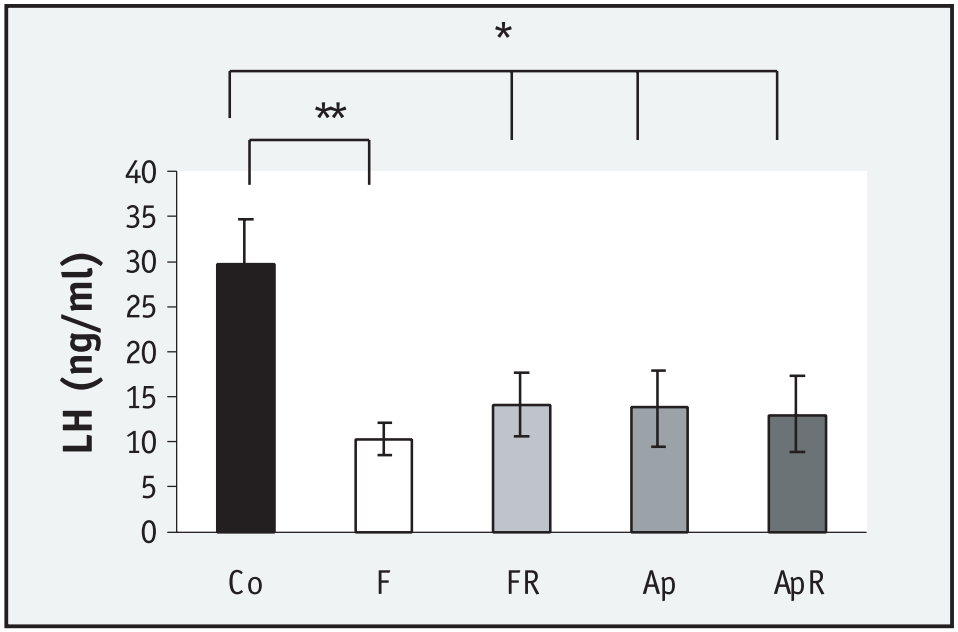

Fig. 3. Effects of 21-day aproteic diet (Ap), 7-day fasting (F), and refeeding with control diet for 24 hours (ApR, FR) on plasmatic luteinizing hormone in adult male rats. Co: n=7,

in plasmatic insulin levels was observed in those previously fasted (FR: 50.57 ± 6.63 uU/ml) ($P<0.001$), and in those fed with the aproteic diet (ApR: 43.5 ± 6.85 uU/ml) ($P<0.01$).

Pancreatic noradrenaline concentration (Fig.2) significantly ($P<0.001$) increased in rats receiving the aproteic diet (889.9 ± 34.65 ng/mg tissue) and fasting during 7 days (827.5 ± 55.7 ng/mg tissue), compared with controls (531.1 ± 48.6 ng/mg tissue).

In the animals fed with the aproteic diet a significant ($P<0.05$) decrease in LH levels (Fig.3) was observed (Ap: 13.7 ± 4 ng/ml), compared with controls (Co: 29.83 ± 4.91 ng/ml). Fasting animals also showed a significant ($P<0.01$) decrease in LH plasmatic levels (F: 10.175 ± 1.74 ng/ml). Following 24-hour refeeding, LH levels were not recovered either by the aproteic group (ApR= 13.03 ± 4.25 ng/ml) or by the previously fasting group (FR: 14.25 ± 3.54 ng/ml).

Discussion

Low insulin levels are associated with an alteration in the hypothalamic-pituitary-gonadal axis function. Under normal conditions glucose is the main normal substratum of the central nervous system. GnRH "in vitro" liberation from hypothalamic fragments is lowered under conditions of diminished glucose levels in the incubation medium [11]. Although the action of insulin on the CNS is not clear, this hormone has receptors located chiefly in the hypothalamus [2], which is the area intimately related to the control of two basic body functions: feeding and reproduction. It has been demonstrated that there is a change in the hypothalamic electric activity after local administration of insulin [12]. However, both glucose and insulin are needed to produce an increment in hypothalamic GnRH liberation (in the absence of glucose in the incubation medium, insulin is unable to modify GnRH secretion). Moreover, in the presence of insulin, the higher the levels of glucose, the higher the increments in GnRH liberation. Therefore, the presence of insulin and glucose is necessary as a metabolic signal [6]. Incidentally, glucose is not the only sign, since under amino acid deficiency conditions with normal glycemic levels, as those herein described, gonadotrophin and insulin secretion are also altered.

Glucose intolerance usually occurs in cases of proteic-caloric malnutrition, especially in children with severe deficiency of proteins [13, 14, 15]. Glucose-stimulated insulin secretion is highly diminished in animals with caloric restriction and 5% protein, while in animals with 35% nutritional caloric restriction but 15% protein, insulin-stimulated secretion drops only moderately. The basal levels of insulin only diminish in the group with proteic-caloric restriction [16].

The effect on insulin and luteinizing hormone levels was studied through two different malnutrition experimental models: the absolute fasting and the free protein and isocaloric diet, which were compared with the control diet. Plasmatic insulin levels decreased by 20% in the two models herein studied, compared with control values. Simultaneously, the luteinizing hormone dropped approximately 50%. However, when both groups were refed for 24 hours with the control diet containing 20% of protein, only basal plasmatic insulin recovered control levels, showing even higher values. LH plasmatic values still remained low.

Adequate nutritional conditions are necessary for an organism to maintain its reproductive capacity. Therefore, following a chronic malnutrition condition, the gonadal function will normalize once the metabolic balance is recovered. This work has demonstrated that in the same animals refed after malnutrition, (a) the metabolic balance is recovered within 24 hours according to plasmatic glucose and insulin normalization, while (b) the gonadal function remains inhibited after the same period.

It has been postulated that the decrease in plasmatic insulin levels could be due to damage of the pancreatic islets and a reduction in the total mass of beta cells [17, 18, 19].

Furthermore, sympathetic noradrenergic forms inhibited pancreatic insulin secretion, acting on receptors of the alpha type [8]. We found that both aproteic and fasting groups showed a significant increment in pancreatic noradrenaline levels. This effect is not unspecific, since we have recently demonstrated that this amine is reduced in hypothalamic areas related to GnRH secretion, where it has a stimulatory effect [20]. The noradrenaline increase would result in malnutrition-induced insulin decrease. Furthermore, this noradrenaline increase would not only be a response to hypoglycemia, since it is also present in the aproteic group, in which animals had normal glycemic levels.

The level of proteins in the diet has dramatic effects on the plasmatic concentration of several hormones [21]. We have recently shown that free protein diets lower not only LH but also prolactin [22]. It is also known that absolute fasting may produce a decrease in the aforementioned hormones, as well as in insulin. Therefore, malnutrition conditions like fasting and free protein diet produce a decrease in insulin plasmatic levels, which would act as a metabolic sign in the hypothalamus lowering gonadal axis function. These

alterations are rapidly reversed in the case of insulin, while the gonadal axis needs more time to recover its functions. Noradrenergic ways would be involved in malnutrition-induced decreased insulin secretion.

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