

JEPonline
Journal of Exercise Physiologyonline

Official Journal of The American
Society of Exercise Physiologists (ASEP)

ISSN 1097-9751

An International Electronic Journal
Volume 7 Number 6 December 2004

Systems Physiology: Endocrinology And Metabolism

BIOLOGICAL ALTERATIONS AFTER FOOD RESTRICTION AND TRAINING IN RATS

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ABSTRACT

BIOLOGICAL ALTERATIONS AFTER FOOD RESTRICTION AND TRAINING IN RATS. **Filaire E, Degoutte F, Jouanel P, Dabonneville M, Lac G, Duchamp C, Pequignot JM.** JEPonline. 2004;7(1)37-44. The combined influence of exercise training and dietary restriction on metabolic, hormonal, muscle mass and performance parameters was evaluated by subjecting 36 Sprague-Dawley rats to different food and exercise interventions. The interventions were two food intake conditions (fed ad libitum (AL) or severely restricted (SR)) and two activity conditions (6 weeks of progressive force isometric resistance training (trained group; T) or no exercise program (untrained group; U). The dietary restriction took place throughout the 6th week of training. Blood concentrations of free fatty acids, glycerol, glucose, ammonia, insulin, leptin, IGF-1, testosterone, triiodothyronine and metabolites of oxypurine pathway were determined in plasma collected by cardiac puncture. The urinary excretion of methoxyamines was measured as an index of the metabolism of circulating catecholamines. Significant difference ($P < 0.05$) in body mass was observed between the trained rats and sedentary control rats after the fifth week (375.4 ± 8.4 vs 395.3 ± 11.7 , respectively). In the underfed group there was a significant reduction in sympathetic activity ($P < 0.05$) and decreases in testosterone (-25%; $P < 0.05$), insulin (-50%; $P < 0.001$) and T_3 concentrations (-30%; $P < 0.05$) were noted. Moreover, the whole oxypurine cascade from hypoxanthine to uric acid was activated. Plasma leptin levels became undetectable in the underfed group. A significant decrease in performance (-30%; $P < 0.01$), based on the maintenance of a climbing position as long as possible with a load corresponding to 80 % of the body weight was also noted. Dietary restriction induces a body weight loss affecting fat mass and fat free mass. Conversely when training was coupled to fasting, fat free mass was not affected. The effect observed for testosterone, T_3 and oxypurine plasma levels, with training or restriction alone, were more important when dietary restriction and training were associated.

Key words: Isometric exercise, Purine nucleotide cycle; Anabolic hormones; Fasting; Body composition

INTRODUCTION

Athletes may restrict their energy intake in order to reduce their body weight during the event or in order to reach a required weight class. Actually, most athletes in wrestling or boxing compete in a class 5-10 % below their usual weight (3). Such a body weight reduction may affect plasma and blood volume (1), endocrine functions (3), may induce a physiological and a psychological stress (8) and therefore may have immediate effects on sports performance during prolonged (aerobic) exercise

The effects of dietary restriction on anaerobic performance are more controversial (8). In a previous study (7), we noted that a 7-day food restriction (-30%) adversely affects the physiological (substrate solicitation) and psychological status of judo athletes and impairs physical performance. The modification of the lipidic and glucidic profiles induced by this restriction supposed a modification of hormonal regulation via IgF-1, catecholamines, leptin or cortisol. These alterations have been yet observed in studies combining at the same time prolonged exercise and food restriction that is rarely longer than 24 hrs in humans or animals (21). Although isometric contractions represent a significant component of muscle function during several sports (gymnastics, skiing, pistol-shooting, particularly in judo during the kumikata), information concerning the combined influence of isometric training and dietary restriction is not available.

Characterising the influence of sustained dietary restriction on isometric force training would be very useful particularly to optimise the training program during the week of food restriction preceding the competition in judo. However, in man, the invasive method needed to measure the physiological parameters makes it difficult to obtain information about the biological alterations after food restriction and training. Several animal models of exercise have been developed to mimic the human response to resistance exercise training (5,11). Recently, our laboratory developed a resistance training protocol based on a series of isometric contractions by using a model of "climbing" rats with additive loads (10). The training program was based on the overload principle and required the successful completion of a number of sets and repetitions, in a style similar to that employed by humans engaged in resistance exercise training. Therefore, the primary aim of the present investigation was to examine the influence of two different dietary conditions (rats fed ad libitum or 50% dietary restriction) during the last week of a similar 6-week resistance exercise training program, on muscle mass, energy substrates and hormone responses involved in fuel metabolism and exercise performance.

METHODS

Animal care and experimental design

This experiment was carried out in agreement with the current legislation on animal experiments in the European Union. Thirty-six male Sprague-Dawley rats weighing 266 ± 30.4 g were randomly divided into two groups of 18 trained (T) or untrained (U) animals. They were housed in individual cages and were allowed food and water ad-libitum for 35 days after they were received in our laboratory. The daily light cycle extended from 7 a.m. to 7 p.m. and the room temperature was maintained at $21.6 \pm 0.5^\circ\text{C}$. Food contained 16% protein, 4.5 % fat and balance carbohydrate (UAR "A04", Villemoisson sur Orge, France).

Training

During the first week of the training protocol, the 18 trained rats underwent progressive acclimatization to a progressive isometric force, strength-training exercise program according to the previously described protocol (10). Untrained animals were excluded from this acclimatization but they were handled daily according to the same schedule. Then, during 4 weeks, rats were trained every morning, 5 days/wk, following a classical training program used in our laboratory (10). Briefly, each rat was set on the horizontal floor of a box and then the box was put toward the vertical position. Because the floor was made of wire netting, the animal gripped with claws and remained in the climbing position. This exercise was repeated 1-5 times during 4×30 s. Each animal was allowed to rest for 15 s between each 30 s exercise bout and for 2 min between each set. The intensity of the training program was progressively increased by adding a load to the tail from 0 g on the first day until 150 g during the fifth week. The daily food consumption of each rat was measured and each animal was weighed to the nearest gram every day. We did not use unnatural incentives such as cold water, forced air or electrical stimulation in order for the rats to perform the exercise.

At the end of the fifth week, rats were assigned to one of the following treatment groups in an ordered fashion:

- fed ad libitum and untrained (AL-U)
- fed ad libitum and trained (AL-T)
- Severely restrained (50% of their daily food intake) and untrained (SR-U)
- Severely restrained (50% of their daily food intake) and trained (SR-T)

In preliminary studies in our laboratory, we observed that seven days of restriction were able to induce a significant body weight loss, as it is the case in humans (12). For these reasons, we chose 7 days of dietary restriction. The food-restricted rats had free access to water throughout the study. The urine of each rat was collected over a 24-hr period on the last day (day 42) of the training session.

Performance

An endurance test was performed the same day. Briefly, after a short warm-up animals had to maintain the climbing position as long as possible with a load corresponding to 80 % of the body weight. Only trained rats were tested.

On day 44 (sacrifice), all rats were anesthetized with sodium pentobarbital (40 mg/kg). Blood was collected by cardiac puncture and centrifuged at 4°C. Plasma was frozen until analysis. A liver lobe and the left heart ventricle were dissected out, weighed, and frozen with aluminium block tongs cooled to liquid nitrogen temperature. The adipose tissue depots sampled included inter-scapular brown adipose tissue (BAT) and mesenteric depositions (MES). Next, the soleus and the extensor digitorum longus (EDL) muscles of the right leg were exposed, weighed and placed in liquid nitrogen. The reasons for choosing the EDL and soleus muscles was based on the facts that: (1) the climbing activity is predominantly a hind-limb activity which requires activation of all muscles within the upper and lower hind-limb compartments, including the EDL and soleus muscles; (2) the EDL and soleus represent relatively homogeneous fast-and slow-twitch muscles, respectively, enabling a direct comparison of the fibre type response to resistance training.

Analytical Methods

Blood samples

Plasma glucose concentration was determined with the Boehringer kit (Meylan, France). Cholesterol and triglycerides (TG) were assayed by enzymatic techniques (HITACHI 911, Roche Diagnostics) according to the manufacturer's protocol. FFAs were determined by the Acyl-CoA synthetase-acyl CoA oxydase method with a kit (Wako Laboratories). Glycerol was phosphorylated by glycerolkinase and ADP. The ADP was reconverted in ATP by PEP with the aid of pyruvate kinase as detailed by Boehringer. Measurements of ammonia were performed using a test kit (Boehringer Mannheim). Xanthine and hypoxanthine were determined by HPLC, using a C18 column and detected at 220 nm by Terzuoli et al.'s (20) method. Intra-assay variability was < 5%.

RIA for plasma leptin concentration used a homologous assay incorporating anti-rat leptin antibody and rat leptin as the standard (Rat Leptin RIA kit, Linco Research Inc, St Charles, MO, USA). In our experimental conditions, the lowest limit of sensitivity was 0.5 ng/mL, and the intra- and inter-assay variations were 1.5 % and 2.5 %, respectively. Free testosterone and IGF-1 concentrations were measured in duplicate by radioimmunoassay using commercial kits (Nichols Institute Diagnostics, Paris, France). For IgF-1, the lowest limit of sensitivity was 0.06 ng/mL, the intra- and inter-assay variations were 2.4 and 5.2 %, respectively.

Plasma insulin was determined by an enzymatic-immunoassay (RatElit Plus, Eurobio, France). The intra- and inter-assay variations were 3.4 and 2.2 %, respectively. Serum concentrations of T₃ (triiodothyronine) were measured by sensitive and specific radioimmunoassays (Nichols Institute Diagnostics, Paris, France). The intra- and inter-assay variations were 2.5% and 2.8%, respectively.

Urinary Samples

The urine samples were collected in chilled tubes containing 1ml of 10 N HCl in order to prevent unspecific catecholamine degradation. From the urine sample, norepinephrine was isolated by means of a Dowex 1X4 (chloride form) column (0.5 x 10 cm) eluted with 0.02 M NaCl. Forty µl of the eluate were injected into the HPLC column. The mobile phase was a mixture of 30 mM citric acid, 50 mM sodium acetate, 1 mM disodic EDTA and 4% methanol. Methoxyamines, normetanephrine and metanephrine, were separately determined

after chemical hydrolysis in two urine aliquots (2 ml) using HPLC with electrochemical detection according to the procedure previously described for human plasma samples (17). The detection limits calculated by doubling the noise ratio and expressed in terms of pmoles of injected amounts were less than 0.03 pmoles for all compounds and intra-assay coefficient was 0.2 % for all these.

Statistical Analyses

Results are reported as means \pm SD. Data were analyzed using a two-way ANOVA to detect the effects of each dietary and exercise conditions and to determine whether there was interaction between the conditions. A level of $P < 0.05$ was accepted as significant after a Bonferoni correction. Correlation between leptin and fat mass was determined using the Pearson correlation coefficient.

RESULTS

All of the trained rats successfully completed the 6-week resistance-training program without injury and without the need for any form of artificial encouragement such as electric shock incentive. All animals were allowed food and water ad-libitum during the first five weeks. At the end of the fifth week, we observed a trend ($P = 0.09$) to lower food intake in trained (AL-T and SR-T) (-1.5%) compared with non-trained rats (AL-U and SR-U): (20.8 ± 0.4 g vs. 21.1 ± 0.6 g, respectively).

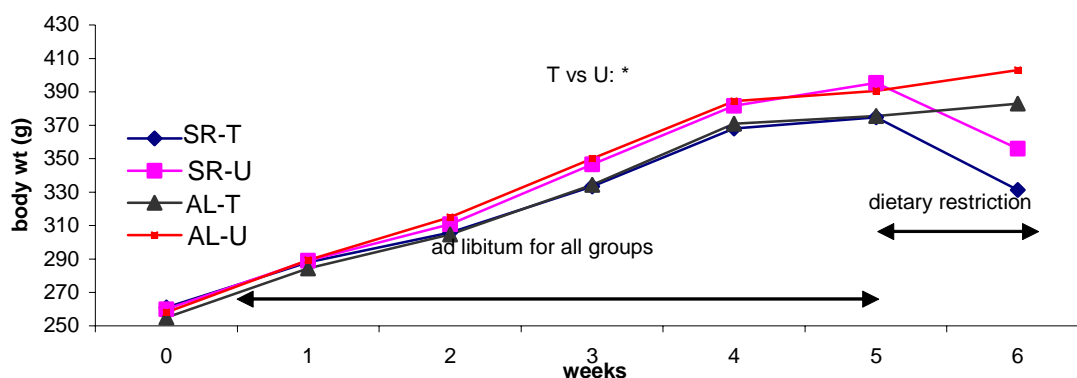


Figure 1. Mean body mass for each group over the 6-wk study: ad-libitum and untrained (AL-U); ad-libitum and trained (AL-T); severe restriction and trained (SR-T); severe restriction and untrained (SR-U). Significant difference between Trained (T) and untrained Rats (U): * $P < 0.05$

The dietary restriction took place the sixth week. The mean daily food intake of the restricted rats (10 ± 1.4 g) was half of the food amount ingested by rats fed ad-libitum during the sixth week. The weekly changes in body mass for each group are shown in Figure 1. By the end of the fifth week, the body weight was significantly lower ($P < 0.05$) in trained than in resting rats.

Effect of Training

Training alone decreased body mass 6 % ($P < 0.05$) (Figure 1) and fat body mass (Table 1). In fact, exercise-training ($P < 0.01$) effects were obtained for inter-scapular brown adipose tissue (-8 %) and mesenteric white adipose tissue (-15 %) with lower values in the trained animals.

No significant differences were found between the groups with respect to the soleus and EDL muscle mass. When expressed relative to body mass, the mass of the EDL and soleus was similar in trained rats than control rats. Absolute and relative left heart ventricle mass of the trained rats was similar than that of control rats. A significant relationship between leptin and fat mass was noted in the trained rats ($r = 0.8$; $P < 0.05$).

A training effect ($P < 0.05$) was obtained for ammonia (18%), hypoxanthine (21%), xanthine (32%) and uric acid (38%), with higher values in trained animals. Training alone also induced a significant decrease in IgF-1 (-12%), testosterone (-10%) and T_3 (-18%) concentrations ($P < 0.05$). Leptin levels were significantly lower in trained rats compared to control rats ($P < 0.05$) (-7%).

Table 1. Mass of selected body parts the day of sacrifice (mean±SD).

	<i>AL-U</i>	<i>AL-T</i>	<i>SR-U</i>	<i>SR-T</i>	<i>Effect</i>
<i>Body mass (g)</i>	409.6 ± 18.1	378.7 ± 15.4	359.0 ± 23.4	335.8 ± 18.5	D,T, I
<i>BAT (g)</i>	0.32 ± 0.07	0.23 ± 0.07	0.17 ± 0.02	0.10 ± 0.01	D,T, I
<i>MES (g)</i>	1.47 ± 0.3	1.25 ± 0.3	0.76 ± 0.3	0.41 ± 0.07	D,T,I
<i>Soleus (g)</i>	0.14 ± 0.01	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	
<i>EDL (g)</i>	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.02	0.16 ± 0.01	D
<i>Liver (g)</i>	12.9 ± 0.9	12.7 ± 1.3	8.7 ± 0.9	8.4 ± 0.09	D
<i>LV (g)</i>	1.17 ± 0.8	1.14 ± 0.06	1.0 ± 0.05	1.0 ± 0.04	D

EDL = Extensor digitorum longus; BAT = brown adipose tissue; MES = mesenteric depot; LV = Left ventricle
Significant main effects as determined by ANOVA. D = diet, T = training;
Significant interaction between training and diet = I

Effect of Diet

After one week of dietary restriction, the SR-U and SR-T rats weighed 88 % and 83 % of AL-U respectively (Figure 1). Food restriction ($P < 0.001$) effects were also obtained for MES and BAT with lower values in the restricted animals (Table 1). The combination of exercise training and caloric restriction (Diet x Training interaction) resulted in greater alterations ($P < 0.05$) in body weight and body fat mass (Table 1). On the other hand, fat free mass was not affected.

The EDL mass in the food-restricted rats was significantly less (-8 %) than in the rats fed ad-libitum ($P < 0.01$). Left heart ventricle was affected by food restriction ($P < 0.001$), weighing significantly less in the food-restricted rats than in the animals fed ad-libitum (-13 %). Regarding the liver, a decrease of the weight ($P < 0.001$) was noted for the restricted animals (33 %). The interactions between diet and training (Diet x Training interaction) induced no additional effect for all these parameters.

The food-restricted group showed a trend for higher blood FFA concentrations ($P < 0.1$) (Table 2) while triglycerides were lowered (-50 %; $P < 0.001$) compared to the group fed ad-libitum. Glucose concentrations were lower ($P < 0.01$) in restricted than in non-restricted animals.

Table 2. Metabolic and hormonal concentrations (Mean±SD) for the four groups of rats.

	<i>AL-U</i>	<i>AL-T</i>	<i>SR-U</i>	<i>SR-T</i>	<i>Effect</i>
<i>FFA (mmol/l)</i>	0.55 ± 0.08	0.61 ± 0.09	0.62 ± 0.1	0.68 ± 0.07	D
<i>Glycerol (mmol/l)</i>	0.14 ± 0.03	0.13 ± 0.06	0.15 ± 0.06	0.19 ± 0.10	
<i>TG (mmol/l)</i>	0.79 ± 0.1	1.01 ± 0.4	0.3 ± 0.07	0.24 ± 0.12	D
<i>Glucose (mmol/l)</i>	10.9 ± 0.6	10.1 ± 0.9	8.53 ± 0.8	8.88 ± 0.9	D
<i>Ammonia (µmol/l)</i>	49.3 ± 15.2	60.6 ± 10.4	66.8 ± 9.2	79.6 ± 11.3	T, D, I
<i>Inosine (µmol/l)</i>	100.7 ± 23.3	115.6 ± 24.2	107.0 ± 31.5	114.6 ± 9.8	
<i>Hypoxanthine (µmol/l)</i>	13.07 ± 1.1	14.4 ± 1.2	15.8 ± 4.2	20.43 ± 3.6	D,T, I
<i>Xanthine (µmol/l)</i>	122.1 ± 25.6	148.3 ± 33.9	144.0 ± 31.5	209.6 ± 21.3	D,T, I
<i>Testosterone (mmol/l)</i>	3.33 ± 0.9	2.54 ± 0.7	1.90 ± 0.7	1.36 ± 0.9	D, T, I
<i>IgF-1 (ng/ml)</i>	1034.9 ± 256.7	636.7 ± 231.2	794.5 ± 108.8	776.2 ± 49.5	T
<i>NMN (ng)</i>	2171.7 ± 151.1	2145.6 ± 99.1	2109.8 ± 59.5	1563.9 ± 349.6	D
<i>MN (ng)</i>	690.3 ± 81.4	675.9 ± 82.4	692.7 ± 100.8	641.7 ± 189.4	
<i>Leptin (ng/ml)</i>	2.00 ± 0.3	1.78 ± 0.5	ND	ND	D, T
<i>Insulin (µg/l)</i>	0.74 ± 0.4	0.75 ± 0.5	0.19 ± 0.08	0.21 ± 0.09	D
<i>T₃ (nm/l)</i>	0.95 ± 0.06	0.70 ± 0.09	0.61 ± 0.06	0.55 ± 0.09	D, T, I

NMN = normetanephrine; MN = metanephrine; T₃ = triiodothyronine; TG = triglycerides
Significant main effects as determined by ANOVA. D = diet, T = training
Significant interaction between training and diet = I

A food restriction ($P < 0.01$) effect was obtained for ammonia (23 %), hypoxanthine (32 %), xanthine (31 %) and uric acid (50 %) with higher values for restricted animals. The effect observed for these parameters with training or restriction alone. There was an effect of combination of exercise training and caloric restriction (Diet x Training interaction, $P < 0.05$) resulted in significant greater alterations for all these parameters (Table 2 and Figure 2).

The concentrations of leptin in the restricted groups could not be detected in the blood samples (Table 2). Food restriction induced a significant decrease in insulin (-50%; $P < 0.001$), T_3 (-30%; $P < 0.05$) and testosterone concentrations (-25%; $P < 0.05$). No significant difference concerning the metanephrine concentrations between the different conditions was observed. However, food restriction effects ($P < 0.05$) were measured for the normetanephrine concentrations, with values being lower in restricted rats (-13%) than in non-restricted group. The combination of exercise training and caloric restriction (Diet x Training interaction) resulted in significant greater alterations ($P < 0.05$) for testosterone and T_3 .

Exercise Performance

Measurement of exercise performance noted in the SR-T was significantly lower ($P < 0.01$) than those reported in AL-T group (39.2 ± 3.9 vs. 59.3 ± 7.1 s, respectively).

DISCUSSION

The main objective of this investigation was to study the effects of food restriction and isometric resistance training on muscle mass, adipose tissue and substrate and hormone responses involved in energy metabolism. The training protocol used in this study was based on the progressive overload principle of resistance exercise, commonly employed by humans involved in resistance training.

The results revealed that 6 weeks static exercise training in rats reduced body weight and fat body mass (Figure 1 and Table 1). This observation, which had been reported by Lac (10), can be explained by the energetic demand of the training. Moreover, in agreement with the data of Mathey (12), which showed that trained rats spontaneously reduce daily food intake, we observed a trend to lower food intake (about 1.5 %) in the trained compared to the non-trained rats during the first five weeks. We also observed that the soleus and EDL muscles did not appear to be affected by the training and such findings are supported by Lee (11). However, Duncan (5) using a similar resistance training protocol reported hypertrophy of these muscles. The reason for the discrepancy might be explained by the training duration. The rats in Duncan's model were trained for 26 weeks (5). Thus, it might be possible that we could induce hypertrophy if we increase the length of training protocol from 6 weeks.

It is well accepted that chronic exercise resulting in weight reduction corresponds to a decline in leptin concentrations (14). It has also been shown that the metabolic state of the adipocyte is also reflected by the level of leptin, since the expression of leptin is regulated mainly by the flux of energy in the adipocyte (6). In this study, leptin decreased as we have a supposed increased energy influx out of the adipocyte. Moreover, as in previous studies, there was a positive correlation between the leptin concentrations and the fat mass in the trained rats (12). We also noted a significant decrease in T_3 concentrations during the training program (Table 2).

Only a few studies have investigated the relationship between leptin and the thyroid hormones. Smisch (18) assumed exercise induces a signal to save energy and, in turn, decreases leptin and the involvement of the hypothalamic-pituitary-thyroid-axis. Thus, because of the down-regulation of the thyroid axis, a shortage of T_3 is developed which saves energy. However, this point needs to be investigated by future studies.

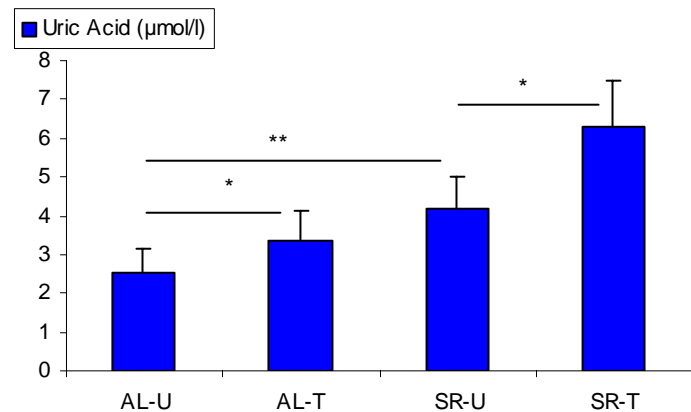


Figure 2. Mean uric acid concentrations for each group. ad-libitum and untrained (AL-U); ad-libitum and trained (AL-T); severe restriction and trained (SR-T); severe restriction and untrained (SR-U).

Significant difference between Trained (T) and untrained Rats (U): * $P < 0.05$.

Significant difference between ad libitum (AL) and severe restricted Rats (ST): ** $P < 0.01$.

Concerning muscle metabolism, one could observe that training reduces body weight and body fat without significant influence on muscle mass (Table 1). However, muscle mass is a poor index of muscle protein metabolism. Sutton (19) suggested that increased activity of the purine nucleotide cycle is demonstrated by increased oxypurine plasma levels. In the present study, the whole oxypurine cascade from hypoxanthine to uric acid was activated (Table 2). In the same time, decreased concentrations of anabolic hormones (i.e., lowered testosterone and IgF-1 levels) were noted, which suggest an increase of the catabolic process. These results support previous observations in human studies, particularly in judo athletes. In fact, Degoutte (4) reported that a single judo match (which lasted no more than 5 min) induces mobilization of both protein and lipid metabolism.

Concerning hormonal parameters, we noted that the fall of T_3 induced by food restriction alone is exaggerated by the combination of food restriction and exercise, with these higher alterations being in agreement with those reported for endurance exercise (9). We also reported that food restriction induced decreases in the T_3 , normetanephrine and insulin concentrations, without alterations in metanephrine values. Rosenbaum (16) and Weisner (21) reported that the fasting-induced T_3 decrease is controlled by the fall of sympathetic nerve activity. In our study, we chose to measure the urinary output of methoxyamines, normetanephrine and metanephrine, which are metabolites of the circulating norepinephrine and epinephrine, respectively. Plasma catecholamines whose half-life is short (< 1 min) can be used as an index of instantaneous sympatho-adrenal activity. In contrast, the methoxyamines, which are end-catabolites produced in the liver by the catechol-O-methyl transferase enzyme, are a more reliable integrated index of the overall sympatho-adrenal activity over long time periods (17). The present finding of a reduced sympathetic activity following food-restriction confirms previous data obtained in fasting animals (8,22). The decreased sympathetic activity may be a mechanism whereby the body attempts to spare energy expenditure (22). The lack of alterations noted in metanephrine values are in accordance with the results of McKnight (13) in animals, who noted that the adrenal medulla activity is stimulated only after 10 days of dietary restriction, thus showing that the adrenergic adaptation presents a certain inertia.

Concerning the leptin response to the food restriction, noticeably the kit used to assay the leptin failed to detect this hormone in our blood samples, the lowest limit of sensitivity being 0.5 ng/mL. Several studies have shown in humans as well as in animals a decrease in plasma leptin concentrations in fasting humans or animals. Boden (2) and Pratley (15) suggested that this decrease may be a potent stimulus of the food intake, an important homeostatic response to maintain the body energy stores. Essig (6) commented that as leptin appears to regulate the energy balance by an action on appetite, leptin may play a role in the recovery from repeated exercise. In our study, the lack of detectable leptin levels following the dietary restriction both in untrained and trained rats may due to a marked drop in leptin secretion, which may thus facilitate the restoration of body fuel homeostasis (6).

Conclusion

Although data in the literature relating to the effects of food restriction and endurance exercise on biological and hormonal parameters are available, our study is original because it associates at the same time a food restriction and a force-resistance training program in rats. Our findings indicated that dietary restriction associated with training significantly reduces exercise performance. Dietary restriction alone induces a body weight loss affecting fat mass and fat free mass (lower EDL and left heart ventricle mass). Conversely when training is coupled to fasting, fat free mass is not affected. Despite modifications in several biological parameters, this training model did not induce chronic training adaptations in skeletal muscle. The effect observed for testosterone, T_3 and oxypurine plasma levels, with training or restriction alone, are more important when dietary restriction and training are associated.

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