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Energy restriction restores the impaired immune response in overweight (cafeteria) rats

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Abstract

Impaired immune function linked to obesity has been shown in both human and animal studies. The purpose of this work was to evaluate the effects of a 4-week energy restriction (50% of total energy intake) on immune function in previously diet-induced (cafeteria) overweight rats. Flow cytometric analysis revealed that the number of spleen T helper cells were significantly ($P < 0.05$) elevated in control and overweight energy-restricted rats as compared with groups fed *ad libitum* groups. The proliferative response of splenocytes to phytohaemaglutinin and concanavalin A from overweight rats after energy restriction was significantly ($P < 0.05$) higher compared to overweight nonrestricted rats. The cytotoxic activity of natural killer cells tended to be lower in overweight rats compared to controls. Finally, control rats under the dietary deprivation period presented higher levels of uncoupling protein 2 mRNA and lower levels of leptin receptor mRNA compared with the reference control group. These results suggest that energy restriction is able to restore, at least in part, the impaired immune response commonly observed in overweight animals.

Keywords: Energy restriction; Lymphocyte subsets; Lymphoproliferation; Cytotoxic activity; Uncoupling protein 2; Leptin receptor

1. Introduction

Obesity has been linked to a wide variety of health problems including hypertension, dyslipidemia, cardiovascular diseases, diabetes mellitus, inflammation disorders, and cancer [1]. Furthermore, obese individuals have shown increased susceptibility to infections, bacteremia, and poor wound healing after surgical procedures [2–4]. Obesity has also been associated with a poor antibody response to hepatitis B plasma vaccine [5], with similar results found in animal models of excessive fat accumulation [6–8]. Despite these clear connections, the molecular mechanisms involved in the altered immune response in obesity are poorly known. In fact, it has been suggested that obesity could be an inflammatory condition in which different interleukins may be involved [9].

Diet-induced obese animals are considered a more comparable model for human obesity than genetically obese animals [10]. We have previously reported [11] a T helper lymphopenia and lower mitogenic response of splenocytes in diet-induced (cafeteria) rats. Long-term energy restriction has been shown to increase the survival of laboratory rodents by retarding ageing [12,13]. It has been postulated that energy restriction extends the life span of rodents by preventing early loss of T cell–mediated immunity [14]. Data regarding weight loss and immune function in human studies are very limited and often confusing. Several studies support the viewpoint that weight loss through a very low caloric diet is associated with decreases in several parameters of immune function [15,16].

Recently it has been proposed that leptin might act as a link between nutritional status and immune function [17,18]. The leptin receptor is expressed in a number of immune cell types [19] and leptin can affect proliferation and cytokine production in T lymphocytes and monocytes [20]. Restricted energy diets reduce plasma leptin levels, whereas in overweight situtations hyperleptinemia is commonly found [21]. Reduced body fat or nutritional deprivation, typically associated with hypoleptinemia, is a direct cause of secondary immunodeficiency and increased susceptibility to infection [22]. Another protein potentially involved in metabolism and immune function is the uncoupling protein 2 (UCP 2). A growing number of studies support the hypothesis that there is an inverse relationship

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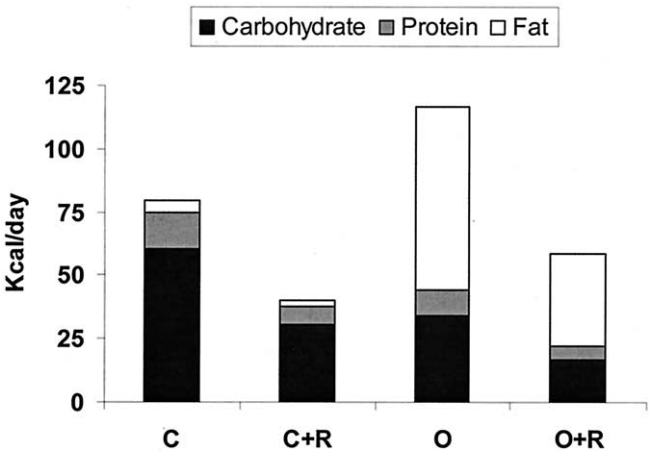

Fig. 1. Daily caloric intake of macronutrients in control and diet-induced overweight rats. C = control; C+R = energy-restricted control; O = diet-induced overweight; O+R = energy-restricted overweight.

between UCP 2 gene expression and production of free radicals in macrophages [23]. An enhanced production of free radicals was obtained in knockout mice for UCP 2 gene after *Toxoplasma gondii* infection, suggesting that lower UCP 2 expression may be beneficial in infective diseases [24].

The aim of the present work is to analyze the effects of energy restriction on the immune response in control and previously diet-induced (cafeteria) overweight rats when consuming standard chow laboratory diet

2. Methods and materials

2.1. Animals and diets

A total of 30 male Wistar rats weighing approximately 140 g (4–5 weeks old) were obtained from the Center of Applied Pharmacology (Pamplona, Spain). Animals were housed (three per cage) in polycarbonate cages in temperature controlled rooms (22±2°C) with a 12-hour light/dark cycle, fed a pelleted chow diet (Rodent Toxicology Diet, B&K Universal, Grimston, Aldbrough, England), and given water *ad libitum* for an adaptation period of 1 week. Rats were then assigned to two dietary groups for 5 weeks. The control group ($n = 15$) was fed with the standard pelleted chow laboratory diet (362.0 kcal/100 g) containing 18% of energy as protein, 76% of energy as carbohydrate and 6% of energy as lipid by dry weight (Fig. 1). The overweight group ($n = 15$) was fed a fat-rich hypercaloric "cafeteria" diet (hypercaloric diet, 467.0 kcal/100 g) composed of paté, potato chips, chocolate, bacon, biscuits, and pelleted diet in a proportion of 2:1:1:1:1:1 as previously described [11,25]. The composition was 9% of energy as protein, 29% of energy as carbohydrate and 62% of energy as lipid by dry weight (Fig. 1). All materials were weighed before feeding and presented in excess. After 5 weeks of feeding the respective diets, both groups were distributed into two new subgroups and were fed for additional 4 weeks. Control (C) and Overweight (O) groups were fed with the standard pelleted chow laboratory diet in excess (both groups $n = 8$). Control + Restricted (C+R) and Overweight + Restricted (O +R) groups were fed with 50% of total energy intake (both groups $n = 7$) with respect to their appropriate control groups. Body weights were recorded daily. At the end of the experimental period, rats were killed by decapitation and blood, fat depots, and tissue samples were carefully collected, weighed, and immediately frozen in liquid nitrogen and stored at −80°C before analysis. All procedures were conducted according to the University Ethics Committee on the use of laboratory animals.

2.2. Serum measurements

Serum glucose, proteins, cholesterol, glycerol, triglycerides (TG), and free fatty acids (FFA) were analyzed using an Autoanalyzer (Cobas MIRAS Roche Diagnostic, Basel, Switzerland) by routine procedures. Serum leptin was assayed by a radioimmunoassay for rat leptin (Linco Research, St. Charles, MO), as previously described [26].

2.3. Measurement of splenic lymphocyte subsets and in vitro blastogenic response of splenocytes to mitogens

The spleens were aseptically removed, gently expressed between glass slides, and resuspended in RPMI 1640 (Invitrogen, Life Technologies, Barcelona, Spain) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Complete medium) as previously described [27,28]. Cell suspensions were centrifuged and washed twice with RPMI 1640. Erythrocytes were lysed with 155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA. The viability of mononuclear cells was > 95% assayed by trypan blue dye exclusion. Viable splenocytes (1×10^5/mL) were incubated with 5 μL of mouse anti-rat PE-conjugated $CD4^+$ (Clon W32, Labgen, Barcelona, Spain) and 5 μL of mouse anti-rat FITC-conjugated $CD8^+$ (Clon MRC OX-8, Labgen) for 20 minutes on ice in the dark. Samples were then centrifuged for 10 minutes at 3000 × *g* and 4°C. The supernatant was discarded and cells were resuspended in 500 μL of PBS. Cells stained with monoclonal antibodies were analyzed by flow cytometry with a FACScan Cell Sorter (Becton Dickinson, Mountain View, CA) as previously described [11,29]. For each experiment 5,000 cells with the forward and 90° light scattering characteristics of mononuclear cells were analyzed. The relative proportion of T cell subsets were obtained as a percentage of the total cells and the count of each subset was calculated.

For *in vitro* blastogenic response experiments, viable cells (1×10^5/mL) were placed in a tissue culture plate with or without Concanavalin A (Con A; Sigma Chemical Co.,

St. Louis, MO) and phytohaemaglutinin (PHA; Sigma Chemical Co.) at 5μg/mL and 50μg/mL, respectively, as described elsewhere [27,28]. Lipopolysaccharide (LPS; Sigma Chemical Co.) was added to B-lymphocyte cultures at 100 μg/mL and preliminary experiments confirmed that these concentrations of lectins were mitogenic for both T and B-lymphocytes from both overweight and control rats. The plates were cultured as published elsewhere [11] for 72 hours, after which [^{3}H]-thymidine was added at a concentration of 1μCi/well. Finally, [^{3}H]-thymidine incorporation was determined [11]. Data are expressed as stimulation index (SI) calculated as follows: cpm culture stimulated with mitogen/cpm culture not stimulated).

2.4. Cytotoxic activity of natural killer cells

To determine the cytotoxic activity of natural killer cells (NK) a kit purchased from Orpegen Pharma (Heidelberg, Germany) was used according to the supplier's instructions. NK cells were isolated from peripheral blood as previously published [29]. NK cells (Effector cells; E) at a concentration of 5×10^6 cells/mL were mixed with target cells (K562) with concentration of 1×10^5 cells/mL at the final E:T ratio of 25:1. Effector and target cells were incubated in a final volume of 200 μL on ice in the dark. Next, samples were vortexed and centrifuged 3 minutes at 120 $\times$ g. Samples were incubated for 2 hours in a 5% CO_2-humidified air incubator (Haereus, Bilbao, Spain). At the end of the incubation, samples were placed on ice until flow cytometric analysis and 50 μL of DNA staining solution were added per sample, vortexed, and incubated 5 minutes on ice in the dark. Cells were analyzed with the flow cytometer immediately after the incubation with DNA staining solution, using the blue-green excitation light (488 nm) with a FACScan Cell Sorter and LYSIS-II software (Becton Dickinson, USA). The percentage of killed target cells or specific cytotoxicity (%) was determined by subtracting the dead cells in incubated target cells alone from the percentage of killed target cells in the test samples [29,30].

2.5. Extraction of total RNA and semiquantitation by reverse transcriptase–polymerase chain reaction

Total RNA was isolated by the Ultraspec-II RNA Isolation System (Bioteck, Houston, TX, USA) from 100 mg of spleen. After 60 minutes at 37°C treatment with RNase free DNase I (Boehringer Mannheim GmbH, Germany), 1 μ of RNA was used to synthesize first-strand cDNA. Reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out as previously published [31]. Primers used to amplify UCP 2 cDNA (GenBank AF039033) were 5′-CAAACAGTTCTACACCAAGGG-3′ (sense, 308-329) and 5′-CGAAGGCAGAAGTGAAGTGG-3′ (antisense, 648-667); Ob-Rb (GenBank U60151) were 5′-CTCCGCACTCACAGGCAACA-3′ (sense, 297-316) and 5′-GGGGGCAGAG-GCAAATCATC-3′ (antisense, 508-527); and β-actin (GenBank J00691) were 5′-TCTACAATGAGCTGCGTGTG-3′ (sense, 1599-1618) and 5′-GGTCAGGATCTTCATGAGGT-3′ (antisense, 2357-2376). Primers were designed using the Oligo, 6.0 Primer Analysis Software for Windows (National Biosciences, USA). Amplifications were linear under these conditions and carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). cDNA was amplified for 25 cycles (UCP 2), 33 cycles (Ob-Rb), and 28 cycles (β-actin), using the following parameters: 95°C for 30 seconds 62°C for 30 seconds (UCP 2 and Ob-Rb), and 95°C for 30 seconds 59°C for 30 seconds (β-actin) with a final extension step at 72°C for 7 minutes. Our preliminary experiments demonstrated that those cycle numbers were well below the plateau phase for PCR products. Linearity of the PCR reactions were tested by amplification of 200 ng of first strand cDNA per reaction from 20 to 40 cycles. The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. PCR band intensity was semiquantified by scanning densitometric analysis using the Gel Doc 1000 UV fluorescent gel documentation system and Molecular Analyst software for quantification of images (Bio-Rad, USA). Levels of mRNA were expressed as the ratio of signal intensity for UCP 2 or Ob-Rb relative to that for β-actin.

2.6. Statistical analysis

Results are given as mean $\pm$ SE. Statistical differences and interactions were evaluated through a factorial two-way analysis of variance (diet, D $\times$ energy restriction R). When statistically significant differences resulted at the interaction level, the Mann-Whitney U test was carried out to compare the effects of each treatment. Differences were considered as statistically significant at $P < 0.05$. Pearson correlation and regression coefficients were performed to verify the relationships between final body weight and serum leptin levels as appropriate. The calculations were performed using the SPSS/Windows version 7.5 statistical package (SPSS Inc., Chicago, IL).

3. Results

3.1. Dietary effects on body, organ weights, and serum measurements

The energy-restricted rats showed a significant decrease (C+R, O+R *vs* C, O) in final body weight (−34 %, $P < 0.001$) when compared to rats fed *ad libitum* (Fig. 2). Retroperitoneal and epididymal white adipose tissue (WAT) weights were also significantly lower ($P < 0.001$) in energy-restricted rats, but no change was found in interscapular brown adipose tissue (BAT) weight (Table 1). Diet-induced overweight rats showed a significantly higher ($P < 0.05$) epididymal WAT weight. A statistically significant interaction ($P < 0.05$) between diet and energy restriction on

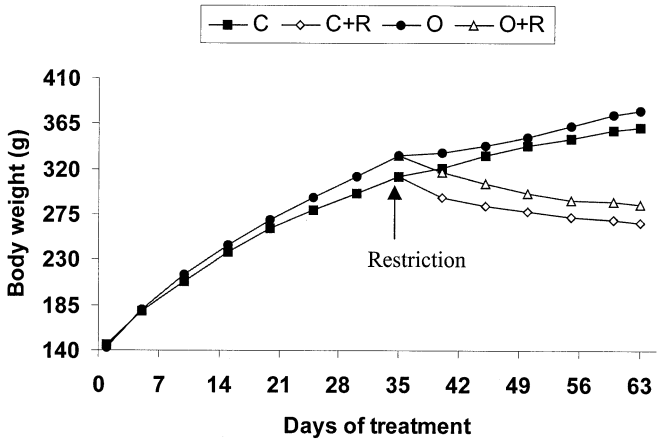

Fig. 2. Effects of energy restriction on the growth curve in control and diet-induced overweight (cafeteria diet) rats. Body weight was recorded daily. Control (C) and overweight (O) groups contain eight animals each, whereas control + restricted (C+R) and overweight + restricted groups (O+R) contain seven animals each.

spleen weight was found. Energy-restricted rats had lower spleen weight ($P < 0.01$) compared to nonrestricted rats. Serum glucose and triglyceride levels were diminished ($P < 0.01$) in energy-restricted rats, although no significant changes were induced by diet (Table 1). However, serum glycerol was found to be higher ($P < 0.05$) in diet-induced overweight rats. No differences were found in serum FFA, total cholesterol (data not shown) and total protein (Table 1). As expected, serum leptin was significantly diminished ($P < 0.001$) in energy-restricted rats and showed a positive correlation ($r = 0.82$; $P < 0.001$) with the final body weight (data not shown).

3.2. Dietary effects on immune response

Splenic T helper $CD4^+$ cell levels were significantly elevated ($P < 0.05$) in energy-restricted rats, although no change was induced by the diet (Table 2). No differences were found in T cytotoxic $CD8^+$ cells. Likewise, the proliferative response of B lymphocytes stimulated with LPS was unaffected by energy restriction or diet. Two-way analysis of variance indicated an interaction on splenocyte proliferation in response to T cell mitogens. Energy-restricted control rats had a diminished splenocyte proliferative response against PHA and Con A ($P < 0.01$) when compared to control nonrestricted rats. On the other hand, energy-restricted diet-induced overweight rats had significantly elevated ($P < 0.01$) splenocyte proliferation upon stimulation with PHA or Con A (Table 2) as compared with diet-induced overweight rats. Moreover, a statistically significant interaction ($P < 0.01$) was found between diet and energy restriction on NK cytotoxic activity. Diet-induced overweight rats had a significantly lower ($P < 0.01$) NK cytotoxic activity compared to energy-restricted overweight rats (Table 2). No differences were found between energy-restricted control rats and control nonrestricted rats in NK cytotoxic activity.

3.3. Dietary effects on spleen UCP 2 and Ob-Rb mRNA expression

Energy restriction significantly elevated (+146 %, $P < 0.01$) spleen UCP 2 mRNA expression in energy restricted control rats as compared to control nonrestricted rats. On the other hand, energy restriction did not change spleen UCP 2 mRNA levels (Fig. 3) in diet-induced overweight rats, but a statistically significant interaction ($P < 0.01$) between diet and energy restriction on UCP 2 mRNA levels was found (Fig. 3). Energy-restricted control rats also presented lower spleen Ob-Rb mRNA levels (−55 %, $P < 0.01$) compared to control nonrestricted rats. Although no differences were found between diet-induced overweight rats in Ob-Rb mRNA expression (Fig. 4), a two-way analysis of variance

Table 1
Effects of energy restriction on body fat weights, spleen weight, and serum measurements in control and diet-induced overweight rats

	C	C+R	O	O + R	ANOVA 2 × 2		
					D	R	D×R
Retroperitoneal WAT (%)	1.51 ± 0.2	0.41 ± 0.1	2.28 ± 0.2	0.83 ± 0.1	*	†	ns
Epididymal WAT (%)	1.85 ± 0.1	1.07 ± 0.1	2.71 ± 0.3	1.53 ± 0.2	‡	†	ns
Interscapular BAT (%)	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	ns	ns	ns
Spleen weight (g)	0.64 ± 0.04	0.56 ± 0.02	0.72 ± 0.04	0.53 ± 0.03	*	ns	ns
Serum glucose (mmol/L)	7.3 ± 0.2	5.9 ± 0.3	7.5 ± 0.2	6.5 ± 0.1	ns	†	ns
Serum triglycerides (mmol/L)	1.64 ± 0.36	0.81 ± 0.01	1.58 ± 0.23	0.96 ± 0.10	ns	*	ns
Serum glycerol (μmol/L)	148.2 ± 12.2	157.9 ± 14.5	189.6 ± 14.2	193.2 ± 18.8	‡	ns	ns
Serum total proteins (g/dL)	6.4 ± 0.1	6.3 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	ns	ns	ns
Serum leptin (ng/mL)	9.0 ± 1.9	0.8 ± 0.3	15.6 ± 3.7	1.4 ± 0.2	ns	†	ns

Data are mean ± SE. An ANOVA 2 × 2 factorial design was used to study the effects of diet, energy restriction, and potential interaction between main effects. C = control; C+R = energy-restricted control; O = diet-induced overweight; O+R, energy-restricted overweight. Organ weights are expressed as % of total body weight.

† $P < 0.001$ main effect of energy restriction (when interaction was not statistically significant).

* $P < 0.01$ main effect of energy restriction (when interaction was not statistically significant).

‡ $P < 0.001$.

Table 2
Effects of energy restriction on immune parameters in control and diet-induced overweight rats

	C	C+R	O	O+R	ANOVA 2 × 2		
					D	R	D×R
% T helper (CD4)	29.97 ± 1.76	37.07 ± 1.38	31.99 ± 1.68	34.52 ± 2.23	ns	*	ns
% T cytotoxic	12.17 ± 1.05	12.15 ± 0.96	11.52 ± 1.41	11.77 ± 1.18	ns	ns	ns
LPS-stimulated proliferation (100 μg/mL)	2.39 ± 0.16	1.96 ± 0.09	2.48 ± 0.08	2.42 ± 0.26	ns	ns	ns
PHA-stimulated proliferation (50 μg/mL)	2.27 ± 0.13[a]	1.77 ± 0.10[b]	2.48 ± 0.11[a]	3.62 ± 0.27[c]			†
Con A–stimulated proliferation (5 μg/mL)	2.24 ± 0.11[a]	1.80 ± 0.08[b]	2.13 ± 0.11[a,b]	3.91 ± 0.23[c]			†
NK cytotoxic activity (%)	28.44 ± 0.77[a]	28.77 ± 1.93[a]	14.33 ± 1.18[b]	26.73 ± 2.29[a]			†

Data are mean ± SE. An ANOVA 2 × 2 factorial design was used to study the effects of diet, energy restriction and potential interaction between main effects:

* $P < 0.05$ main effect of energy restriction (when interaction was not statistically significant).

[a,b,c] Different superscripts indicate significant differences ($P < 0.05$) after two tailed Mann-Whitney *U* test to compare single groups (when interaction was statistically significant).

C = control; C+R = energy-restricted control; O = diet-induced overweight and O+R; energy-restricted overweight. Lymphocyte proliferation was measured as a stimulation index.

indicated that there was an interaction between diet and energy restriction ($P < 0.01$) on spleen Ob-Rb mRNA expression (Fig. 4).

4. Discussion

Obesity is a major health problem, and its prevalence is increasing worldwide [32]. Obese individuals have been shown to have a higher incidence of infections and infection-related mortality [3,33] and several types of cancer occur more frequently in obese population. In a previous study, we found that diet-induced (cafeteria) overweight rats present an altered immune response [11]. In the current work, we analyzed the effect of weight loss (energy restriction) in control and diet-induced overweight rats on immune function markers to test the hypothesis whether weight loss has a beneficial effect on immunity. Although it is well established that chronic, severe energy and nutrient deprivation result in compromised immunocompetence [34,35], the rats in our design were restricted to 50% of their total energy intake [23,36,37] for a duration of 1 month, which was previously shown to achieve a marked weight loss [37]. A major finding of this work is that weight loss after caloric restriction may restore immune function in rats with similar body weight but different body composition when consuming standard chow laboratory diet.

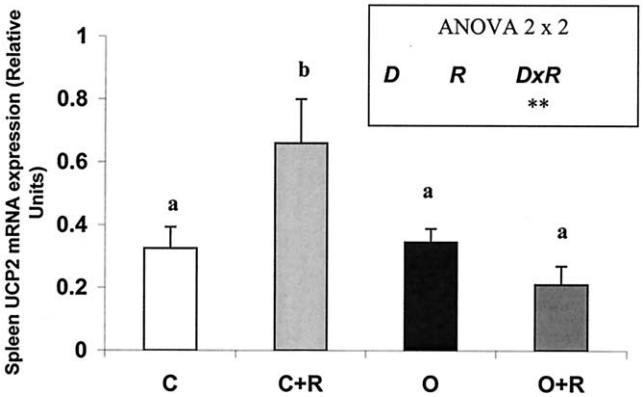

Fig. 3. Uncoupling protein 2 mRNA expression levels in spleen of control rats (C), control rats with energy restriction (C+R), diet-induced overweight rats (O), and diet-induced overweight rats with energy restriction (O+R). D = diet; R = restriction; DxR = diet plus restriction. Statistical analysis by two-way analysis of variance. Different letters indicates significant differences ($P < 0.05$) after Mann-Whitney *U* test to compare single groups (when interaction was statistically significant). Data are expressed as means ± SE.

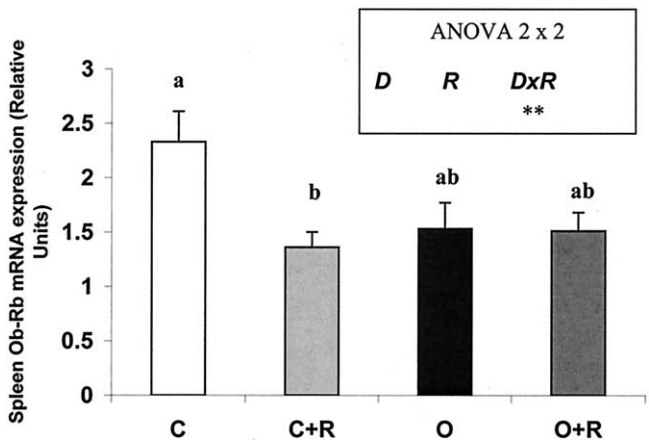

Fig. 4. Leptin receptor long isoform mRNA expression levels in spleen of control rats (C), control rats with energy restriction (C+R), diet-induced overweight rats (O), and diet-induced overweight rats with energy restriction (O+R). D = diet; R = restriction; DxR = diet plus restriction. Statistical analysis is by two-way analysis of variance. Different letters indicates significant differences ($P < 0.05$) after Mann-Whitney *U* test to compare single groups (when interaction was statistically significant). Data are expressed as means ± SE.

No differences were found in final body weight between nonrestricted groups, although energy restriction induced important changes in body composition. Thus, energy restriction led to white fat mass depletion in several adipose depots, although no differences were found in interscapular BAT. This contrast with a previous report [11], in which it has been found that diet-induced obese animals have increased BAT levels as a result of an active diet-induced thermogenesis. Serum glucose levels diminished with energy restriction, which is consistent with results found in the literature [38,39]. Although caloric restriction improves skeletal muscle insulin sensivity leading to a decrease in glycemia, an improvement in glucose homeostasis may also be attributed to a decrease in adipose cells and their products [40]. As expected, energy restriction diminished serum triglyceride and plasma leptin levels. The decreased leptin levels are included within the neuroendocrine adaptations to starvation and energy restriction [41,42]. Additionally, overweight rats presented higher levels of glycerol, possibly suggesting a higher lipolytic activity accompanying a higher lipogenesis. Also, glycerol is an indicator of lipolysis and may increase in food deprivation situations [43].

Spleen T helper $CD4^+$ cells were elevated with energy restriction, but no differences were found in the T cytotoxic $CD8^+$ lymphocyte subset. A previous report [8] with genetically obese diabetic Wistar rats showed that T cell subsets were increased after an adequate weight loss program. The same research group has shown that human obese patients presented increased T cell function after weight reduction [44]. Recently, malnourished mice were shown to have an overabundance of T cells expressing CD45RA among $CD4^+$ subset [45]. The $CD4^+$ $CD45RA^+$ (naive-phenotype) T cell is a quiescent relative to its CD45RA(memory/effector) counterpart in terms of proliferative and cytokine response capacity. Thus, an imbalance favoring quiescence within the $CD4^+$ T cell subset might contribute to depressed T-dependent immunocompetence. Although this finding could explain the lower proliferative response upon Con A stimulation of energy-restricted control rats, this hypothesis does not fit with our results in energy restricted overweight rats upon stimulation with PHA (a T cell specific mitogen) and Con A (a T helper $CD4^+$ specific mitogen). It is possible that the control animals had a higher number of native-phenotype T cell or quiescent cells, whereas obese animals had a higher number of memory/effector or proliferative T cells. This would support both the decrease PHA and Con A with the controls and the increase PHA and Con A with the obese. However, it is more likely that this increase in proliferative response is associated with the observation that energy restriction stimulated mitogen-induced lymphocyte proliferation correlated with the induction of IL-2 expression [46]. Increased expression of IL-2 is associated with an increase in binding activity of the transcription factor NFAT, which plays a predominant role in IL-2 transcription [46]. Several studies [47,48,49] have shown that PHA and Con A activated NFAT through the stimulation of the mitogen-activated protein kinase (MAPK) cascade. Moreover, energy restriction was found to increase MAPK activity suggesting a molecular mechanism for a higher proliferative response [46].

We also analyzed NK cell cytotoxic activity in energy-restricted control and overweight rats. As found in earlier reports, our diet-induced overweight animals present lower NK cytotoxic activity [50,51]; however, the energy restriction on overweight rats restored to normal values the NK cytotoxic activity. In this context, energy restriction seems to enhance the immune system in diet-induced obese animals. Although a negative correlation has been reported between body fat content and NK cytotoxic activity, the molecular mechanisms involved are not fully understood although diets high in polyunsaturated fatty acids inhibit NK-cell activity [51].

Energy-restricted control rats showed higher spleen UCP 2 mRNA and lower Ob-Rb mRNA expression compared with control untreated rats. The UCP 2 gene has been previously reported [21] to be differently affected by dietary intake as well as other physiological and nutritional circumstances. A negative correlation exists between UCP 2 gene expression in macrophages and ROS production. Furthermore, energy restriction has been found to decrease free radical production in mitochondria [24,52]. In this context, higher levels of UCP 2 in spleen may contribute to a poor innate immune response. Moreover, it is known that leptin exerts a positive regulation on immune function [18], especially on inflammation. The reduced Ob-Rb mRNA expression may have negative effects on immune cells and could help to explain, at least in part, why T cells from energy-restricted control rats have lower proliferation upon stimulation with Con A, although T helper $CD4^+$ subset is elevated. Taken together, these data suggest a reduced immune response in energy-restricted control animals compared to nonrestricted control animals.

In summary, we have shown that energy restriction can be useful to restore a previously impaired immune function in diet-induced overweight rats. In this context, energy restriction led to higher $CD4^+$T cell subset, increased splenocyte proliferation upon stimulation with PHA and Con A, and enhanced NK cytotoxic activity, with no differences in UCP 2 and Ob-Rb mRNA expression in the spleen [30].

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