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24-hour rhythms of splenic mitogenic responses, lymphocyte subset populations and interferon γ release after calorie restriction or social isolation of rats

PILAR CANO[1], DANIEL P. CARDINALI[2], PILAR FERNÁNDEZ[1], CARLOS F. REYES TOSO[2], & ANA I. ESQUIFINO[2]

[1]*Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense de Madrid, 28040 Madrid, Spain and* [2]*Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, 1121 Buenos Aires, Argentina*

Abstract
To assess the effect of calorie restriction equivalent to a 66% food restriction or social isolation on splenic immune responses, calorie-restricted five week-old male Wistar rats, pair fed controls individually caged and pair fed controls caged in groups, were studied for four weeks. Calorie restricted and isolated rats showed increased splenic concanavalin A response with peak activity during the activity span. Mean 24 h values of splenic lipopolysaccharide response decreased in isolated rats compared to grouped rats. The highest values of T cells occurred in calorie restricted rats and those of B cells in isolated rats. Mean values of splenic $CD4^+$ and $CD8^+$ cells augmented in isolated or calorie restricted groups. The highest in vitro interferon-γ production occurred in the isolated group and the lowest in the grouped rats, the differences among groups being significant. Both experimental procedures generally disrupted 24-h rhythmicity of splenic immune parameters. Mean values of plasma corticosterone were higher in calorie restricted rats than in isolated rats and both differed significantly from grouped rats. The results indicate that either calorie restriction or social isolation augmented cell-mediated immunity in rat spleen.

Keywords: *Calorie restriction, isolation, stress, spleen, lymphocyte subsets, interferon-γ, mitogenic response*

Introduction

It has been known for more than a century that stress alters physiopathological processes often via an altered immune function (reviewed by Biondi & Zannino 1997; Habib et al. 2001; Tsigos & Chrousos 2002; Grammatopoulos & Chrousos 2002). Although the general concept is that stress is immunosuppressive, under certain conditions a stress response may enhance immune function. Recently, we examined in independent experiments whether a calorie restriction equivalent to a 66% of food restriction for four weeks (Esquifino et al. 2004b) or the individual caging of rats for 4 weeks (Esquifino et al. 2004a) were effective manipulations to change immune parameters in the submaxillary lymph nodes. After caloric restriction, mean values of concanavalin (Con) A response, lymph node T and $CD4^+$ cell number and $CD4^+/CD8^+$ ratio augmented, whereas those of B cell number and IFN-γ release decreased

Correspondence: Daniel P. Cardinali, Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires. Argentina. Tel/Fax: 54 11 5950 9611. E-mail: dcardinali@fmed.uba.ar

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(Esquifino et al. 2004b). In contrast, after social isolation, lymph node mitogenic responses to Con A and LPS (lipopolysaccharide) decreased and interferon (IFN)-γ release decreased, while T, B, non T-non B, $CD8^+$ and $CD4^+$-$CD8^+$ lymphocyte subsets augmented (Esquifino et al. 2004a). Calorie restriction or social isolation disrupted 24 h rhythmicity of most submaxillary lymph node parameters examined.

Since both in calorie restricted and social isolated animals plasma corticosterone levels increased (Chacon et al. 2005; Perelló et al. 2006), we considered it worthwhile to examine in the same experiment the effect of both experimental manipulations on cellular and immune responses in another immune tissue, i.e. the spleen. Plasma levels of corticosterone were also measured.

Materials and methods

Animals and experimental design

Five week-old male Wistar rats were kept under standard conditions of controlled light (fluorescent cool white bulbs, 100 lux intensity at the level of cages) (12:12 h light/dark schedule, lights on at 0800 h) and temperature ($22 \pm 2^{\circ}$C). Three groups of rats were studied, i.e. calorie restricted rats, pair fed controls individually caged and pair fed controls caged in groups. Caloric restricted rats had daily access to 7g of an unbalanced AIN-93G diet enriched in proteins and low in fat and carbohydrates (Reeves et al. 1993) and water *ad libitum* for 30 days. This calorie restriction was equivalent to a 66% of food restriction, calculated by considering the average of food consumption of the control group. Presentation of the restricted calorie diet was at 0900–1000 h daily, and food was consumed over a short time. Calorie restricted rats must be caged individually to avoid cannibalism (Goonewardene & Murasko 1995; Pugh et al. 1999; Pahlavani & Vargas 2001). The other two experimental groups examined included rats individually caged (isolation) or group-housed (eight rats per cage, grouped) that were left undisturbed, having free access to an equilibrated diet (AIN-93G, Diets Inc., Pennsylvania, USA) and water for four weeks.

The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The study was in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996).

Groups of 6–8 rats were killed by decapitation at six different time intervals, every 4 h, throughout a 24-h cycle starting at Zeitgeber time (ZT) 1 (ZT 0 = lights on). At night intervals animals were killed under red dim light. Trunk blood was collected and plasma samples were obtained by centrifugation of blood at $1500 \times g$ for 15 min and were stored at -20°C until further analysis. The spleen was removed aseptically, weighed and placed in Petri disks containing balanced salt solution. Tissue was gently teased apart. After removing the clumps by centrifugation, harvested cells were suspended in sterile supplemented medium (RPMI 1640), containing 10% heat-inactivated, fetal bovine serum, 20 mg/ml L-glutamine, 0.02 mg/ml 2-mercaptoethanol and gentamicin (50 mg/ml), and were counted.

Mitogen assays

Mitogen assays were performed as described in detail elsewhere (Esquifino et al. 1996). Splenic cells were used at a final number of cells/well (0.1 ml) of 5×10^5. Control and experimental cultures were run in triplicate. Mitogens were added to the cultures at final

supramaximal concentrations of 5μg/ml. The cultures were incubated in a humidified 37°C incubator in an atmosphere of 5% CO_2. After 48 h incubation, ^{3}H-thymidine (0.2 μCi) was added to each well in a volume of 0.02 ml. Cells were harvested 5 h later using an automated sample harvester, and the filters were counted in a liquid scintillation spectrometer. The proliferation index was estimated as the ratio between stimulation in the presence of mitogens/controls. Results were expressed as proliferation index/number of cells.

Lymphocyte subsets

Lymphocyte subsets were determined by FACS analysis, as previously described (Castrillon et al. 2000b). For these studies, we used the following monoclonal antibodies: Anti-rat LCA (OX-33) for B lymphocytes (Serotec, Oxford, UK), Anti-rat TCR alpha/beta (R7.3) for T lymphocytes (Serotec, Oxford, UK), Anti-rat CD4 (OX-35) which recognise a rat T helper cell differentiation antigen (Pharmingen, San Diego, CA, USA), and Anti-rat CD8a (OX-8) which recognise the reactive antigen expressed on rat T cytotoxic/suppressor cells (Pharmingen, San Diego, CA, USA). Splenic lymphocytes were washed in cold PBS with 0.02% sodium azide and then incubated (3×10^5 cells/tube) with appropriate primary antibodies for 30 min at 4°C. Following two washes, the cells were incubated with 1ml of PBS-BSA 1%, during 5 min at 4°C, washed three times, resuspended in 1% paraformaldehyde in PBS. Fluorescence intensity was analysed by fluorescence activated cell sorting (FACStarplus; Beckton Dickinson, Mountain View, CA). Dead cells were excluded by gating with propidium iodide.

IFN-γ release

Splenic cells (10^5/100 μl) were incubated for 24 h, their media removed, and after adding fresh media including all the components they were incubated for 24 h or more. Both media were collected and pooled for IFN-γ measurement. The incubations were performed in triplicate. Microscopic examination of cell preparation used indicated that $>95\%$ was lymph node cells. Neither treatment affected the viability of the cells. IFN-γ concentration in media culture was measured after centrifugation to remove adherent cells. An ELISA commercial kit from Endogen (Woburn, MA, USA) previously validated in our laboratory was employed (Castrillon et al. 2000a). The assay was as follows: 100 μl of standards or unknown samples were added to each antibody-coated well, and the plates were incubated overnight at room temperature. The reaction was stopped by washing three times with wash buffer (2% Tween 20 in 50 mM Tris, pH 3.6). The wells were incubated with 100 μl of biotynilated detecting antibody at the titer previously tested. After 1 h at room temperature the reaction was stopped by washing three times with wash buffer. One-hundred μl of streptavidin-HRP solution (in Dulbecco's phosphate-buffered saline, pH 7.4) was then added and the samples were incubated for 30 min. The reaction was stopped by adding 100 μl of 0.18 M sulfuric acid. The plates were read within 30 min in an ELISA reader set at 450 nm and 550 nm. Values were obtained by subtracting the reading at 550 nm from the reading at 450 nm, to correct for any optical defect of microtiter plate. IFN-γ release was expressed as pg/mL/48 h incubation. Sensitivity of the assay was 100 pg/mL.

Plasma corticosterone assay

Plasma concentration of corticosterone was evaluated by a specific radioimmunoassay (Spinedi et al. 1991) with a standard curve between 1 and 250 μg/dL and intra- and inter-assay coefficients of variation of 4–6 and 8–10%, respectively.

Statistical analysis

Statistical analysis of results was performed by a univariate analysis of variance (ANOVA) or by a one-way ANOVA by using SPSS software, version 13 (SPSS Inc., Chicago, IL, USA), as stated. Generally, the univariate ANOVA included assessment of treatment effect (i.e. the occurrence of differences in mean values between calorie restricted, isolated and grouped animals), of time-of-day effects (the occurrence of daily changes) and of the interaction between treatment and time, from which inference about differences in timing and amplitude could be obtained). Post-hoc Bonferroni's multiple comparisons tests in a one-way ANOVA were then employed to show which time points were significantly different within each experimental group to define the existence of peaks. *P*-values lower than 0.05 were considered evidence for statistical significance.

Results

Figure 1 (upper panel) shows the 24-h changes in mitogenic responses to Con A and LPS of splenic cells from grouped, isolated and calorie restricted rats. A univariate ANOVA indicated a significant increase of Con A response in calorie restricted and isolated rats as compared to grouped animals, as well as the occurrence of significant time of day changes ($p < 0.05$, Bonferroni's test). Peak activity of splenic Con A response was at ZT 17 in the three groups of animals.

LPS mitogenic responses of splenic cells changed significantly with time in grouped rats only (Figure 1, lower panel). Mean 24 h values of LPS response was significantly lower in isolated than in grouped rats ($p < 0.05$, Bonferroni's test). Figure 2 depicts the changes in splenic T and B cells, T/B ratio, $CD4^+$ and $CD8^+$ cells and $CD4^+/CD8^+$ ratio, along the 24 h span. Mean values of T and B cells differed significantly among the three groups with the highest values of T cells found in calorie restricted rats and of B cells in isolated rats ($p < 0.05$, Bonferroni's test). The T/B ratio in calorie restricted rats was significantly higher than in the other two groups ($p < 0.05$, Bonferroni's test) (Figure 2, upper right panel). Daily changes in T and B cells were non-significant in isolated rats. In calorie restricted animals, B cells and the T/B ratio attained maxima during the rest span of the daily cycle while maxima in T, B and the T/B ratio occurred during the activity span for grouped rats.

Mean values of splenic $CD4^+$ and $CD8^+$ cells augmented significantly in isolated or calorie restricted groups; in the case of $CD8^+$ cells, mean values were significantly higher in calorie restricted than in isolated rats ($p < 0.05$, Bonferroni's test). The $CD4^+/CD8^+$ ratio was lower in isolated rats than in grouped animals ($p < 0.05$, Bonferroni's test). Daily changes $CD4^+$ cells were significant in grouped rats only, with maxima during the activity span. In the case of $CD8^+$ cells and the $CD4^+/CD8^+$ ratio, maxima in isolated or calorie restricted occurred during the rest or activity span, respectively, while a biphasic pattern was apparent for grouped rats.

In vitro IFN-γ production by splenic cells in the three experimental groups is shown in Figure 3. The highest mean value observed occurred in the isolated group and the lowest in the grouped rats, differences among groups being significant (Bonferroni's test, $p < 0.05$). Peak values in the three groups were observed during the activity span (Figure 3).

Figure 4 shows the plasma corticosterone concentration. The mean value of plasma corticosterone was higher in calorie restricted rats than in isolated rats and both differed from grouped rats ($p < 0.05$, Bonferroni's test). Calorie restricted and isolated rats exhibited abolition of the daily rhythm observed in grouped rats.

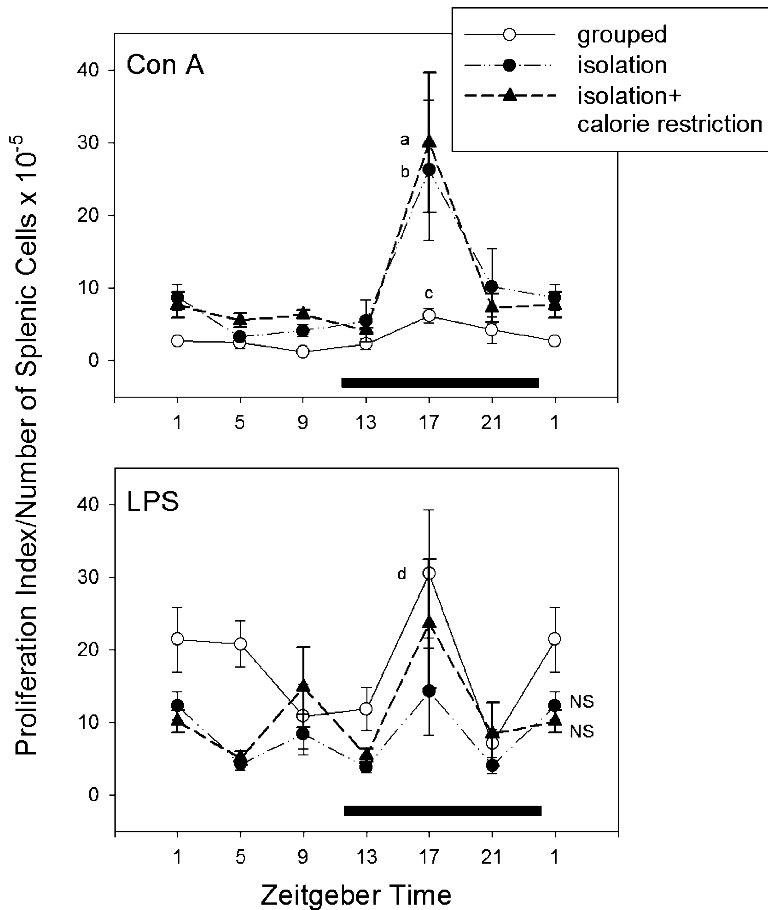

Figure 1. Effect of a 66% calorie restriction or social isolation of rats on 24-h changes in splenic mitogenic responses to Con A and LPS. Groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at ZT 1 are repeated on the 'second' day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Bonferroni's multiple comparison test, as follows: $^{a}p < 0.01$ vs. all time points; $^{b}p < 0.01$ vs. ZT 5 and 9; $^{c}p < 0.05$ vs. ZT 9; $^{d}p < 0.05$ vs. ZT 21. NS: non-significant one-way ANOVA. For further statistical analysis, see text.

Discussion

The response of an organism to a stressor will depend on a variety of factors, including the chronicity of the stressor; the nature and intensity of the stressor; the species, strain, and sex of the animal; the life history of the animal; the timing of the stressor in relation to the immune response; and the immune parameter or effector function examined. Relevant to the direction of the effect is the definition of what chronic versus acute stressors are. Generally, acute defines a single exposure to stressor, albeit for differing lengths of time. Chronic stress will refer to multiple sessions of stressor after longer periods of time (days to weeks). In addition, following stressor administration, lymphocyte responses may differ depending upon the site being examined, e.g. peripheral blood or immune organs (Lysle et al. 1987).

Previous studies in rats subjected to calorie restriction or social isolation for four weeks indicated augmented or decreased lymph node mitogenic response to Con A, respectively.

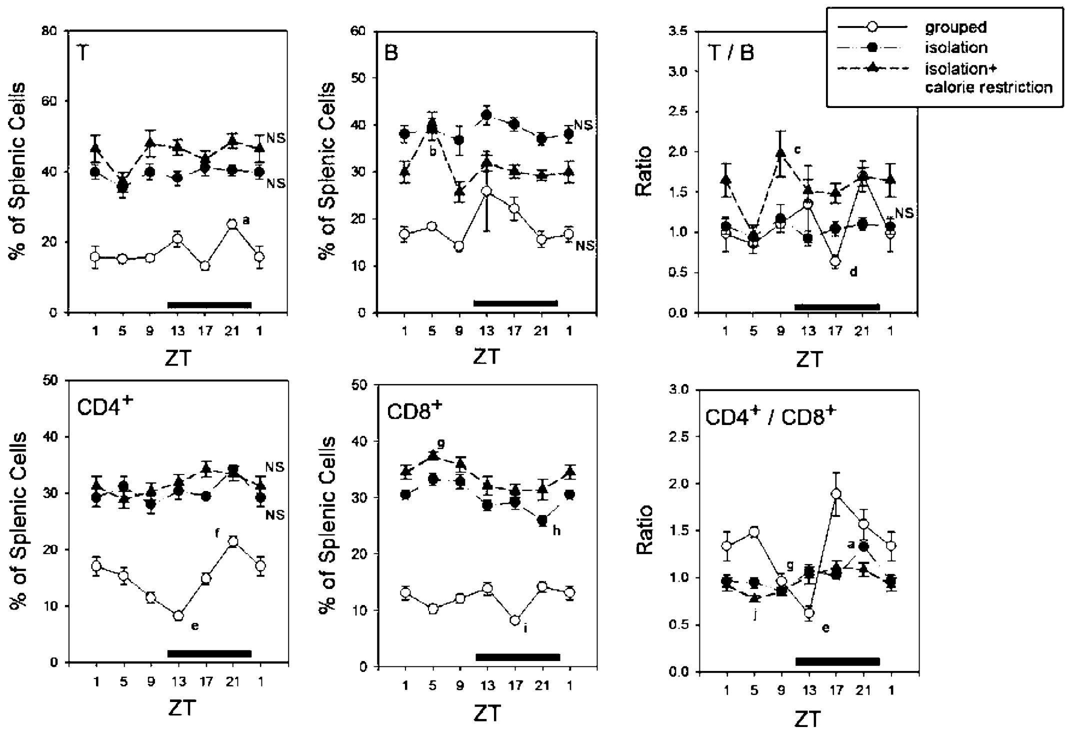

Figure 2. Effect of a 66% calorie restriction or social isolation of rats on 24-h changes in splenic T and B lymphocytes, T/B ratio, $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ ratio. Groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at ZT 1 are repeated on the 'second' day. Bar indicates scotophase duration. Shown are the means $\pm$ SEM. Letters indicate the existence of significant differences between time points within each group after a Bonferroni's multiple comparison test, as follows: $^{a}p < 0.01$ vs. ZT 1, 5, 6, 17; $^{b}p < 0.01$ vs. all time points; $^{c}p < 0.05$ vs. ZT 5; $^{d}p < 0.05$ vs. ZT 21; $^{e}p < 0.01$ vs. ZT 1, 5, 17 and 21; $^{f}p < 0.01$ vs. ZT 5, 9, 13 and 17; $^{g}p < 0.05$ vs. ZT 17: $^{h}p < 0.05$ vs. ZT 5 and 9; $^{i}p < 0.01$ vs. ZT 1, 13 and 21; $^{j}p < 0.05$ vs. ZT 17. NS: non significant one-way ANOVA. For further statistical analysis, see text.

Most lymphocyte populations increased in submaxillary lymph nodes after calorie restriction or social isolation and their 24-h rhythmicity became disrupted while IFN-γ production decreased.

The foregoing results derived from a combined study in which the effect of both experimental manipulations, i.e. calorie restriction or social isolation, on splenic immune response was assessed, indicated that the spleen of calorie restricted or isolated rats shows an increased Con A response. Mean 24 h values of splenic LPS response were lower in isolated than in grouped rats. The highest values of T cells occurred in calorie restricted rats and those of B cells in isolated rats. Mean values of splenic $CD4^+$ and $CD8^+$ cells augmented in isolated or calorie restricted groups. The highest in vitro IFN-γ production observed occurred in the isolated group and the lowest in the grouped rats, the differences among groups being significant. A disrupted 24-h rhythmicity of splenic immune parameters followed either experimental procedure.

As an index of stress, plasma corticosterone levels were measured in the experimental groups examined. Plasma corticosterone attained significantly higher values in calorie restricted rats than in isolated rats and both were higher than in grouped rats. The results indicate that calorie restriction was the strongest stressor examined and that both experimental manipulations, either calorie restriction or social isolation, augmented cell-mediated immunity in rat spleen.

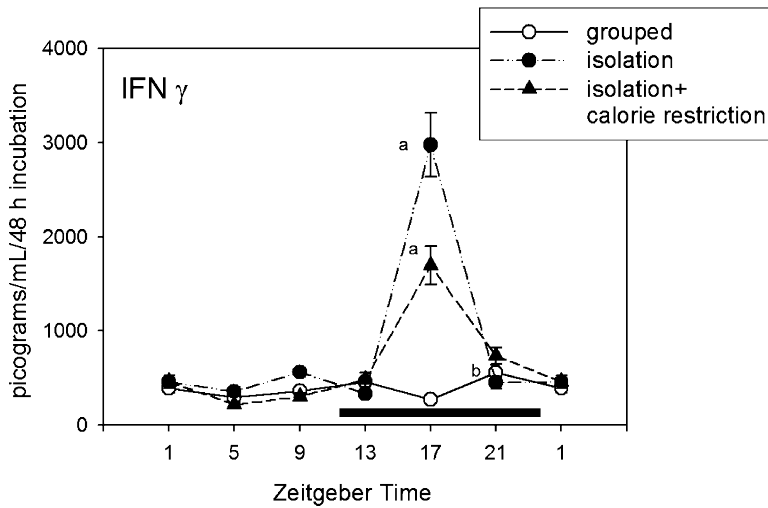

Figure 3. Effect of a 66% calorie restriction or social isolation of rats on 24-h changes in splenic IFN-γ release in vitro. Groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at ZT 1 are repeated on the 'second' day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Bonferroni's multiple comparison test, as follows: $^{a}p < 0.01$ vs. all time points; $^{b}p < 0.05$ vs. ZT 5 and 17. NS: non significant one-way ANOVA. For further statistical analysis, see text.

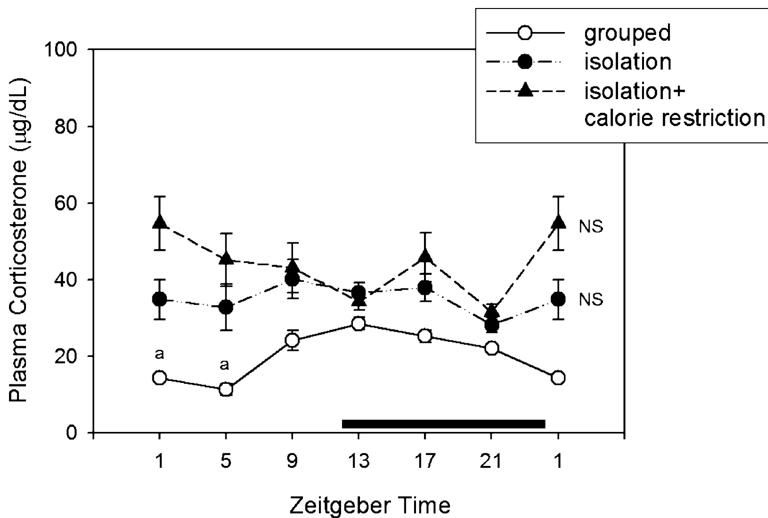

Figure 4. Effect of a 66% calorie restriction or social isolation of rats on 24-h changes in plasma corticosterone. Groups of 6–8 rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Values at ZT 1 are repeated on the 'second' day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points of grouped rats each group after a Bonferroni's multiple comparison test, as follows: $^{a}p < 0.01$ vs. ZT 9, 13, 17 and 21. NS: non significant one-way ANOVA. For further statistical analysis, see text.

A calorie restriction of adult rats ranging from 25 to 50% reduction of caloric intake (without deficiency in essential nutrients) has been often employed in immunomodulation studies. Under these conditions calorie restriction augments stress resistance and

immunocompetence (Tian et al. 1995; Yu & Chung 2001; Pahlavani 2004). Calorie restriction inhibits age-related dysregulation of cytokines (Spaulding et al. 1997) and prevents, by enhancement of T cell apoptosis, accumulation of non-replicative, non-functional, senescent T cells (Spaulding et al. 1997). The present observations on an increased T-cell mediated response i.e. Con A mitogenic activity, as well as in the T/B ratio in the spleen of calorie restricted rats are in line with the prior data.

The most profound change that occurs with individual housing is an increase in aggression of males seen in rodents following even relatively brief periods of isolation (Brain 1975; Baurmel et al. 1978). This indicates that isolation is a mild stress for rats. Individually housed animals are also hyper-responsive to stressors. For example, after eights weeks of isolation, single housed rats continued to spend more time apparently attempting to escape (sniffing and chewing at the bars and suddenly dashing around their cage) while those housed in groups spent more time sleeping and feeding (Hurst et al. 1999).

The present results of a disrupted circadian rhythmicity of splenic immune response after calorie restriction or social isolation agreed with prior data on lymph nodes. Indeed, stress is capable of perturbing temporal organisation by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself. In particular, stress in rodents has been found to cause disruptions of circadian rhythms in body temperature, heart rate and locomotor activity (Greco et al. 1989; Sgoifo et al. 2002; Spani et al. 2003).

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