Biological Effects of Long-Term Caloric Restriction: Adaptation with Simultaneous Administration of Caloric Stress Plus Repeated Immobilization Stress in Rats

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In the present study, we have established the biological effects during 8 weeks of (i) caloric restriction (Cal) and (ii) simultaneous administration of Cal plus 2 hr daily immobilization stress using male Sprague-Dawley rats. Animals were divided into three equal groups: (i) *ad libitum* fed, (ii) 30% restriction of food intake of the *ad libitum* diet, and (iii) 30% restriction of food intake plus 2 hr daily immobilization stress. Caloric-restricted animals gained only 30% of the total body weight of the unrestricted animals but received 70% of the food of those rats. Cal animals showed a significant loss in their relative liver and thymus weight and a significant gain in their relative adrenal and testis weight as compared to the control animals. Cal animals had almost 2-fold higher levels of plasma corticosterone levels with a dramatic decrease in the total glucocorticoid receptor (GR) levels in the liver, thymus, heart, and testis as compared to *ad libitum* fed control animals. Interestingly, Cal animals showed higher levels of lipid peroxidation in both the liver and heart, indicating increased oxidative activities in these tissues when compared with the control animals. In addition, Cal animals had increased heat shock protein 70 (HSP 70) content in the testis. Surprisingly, hardly any significant differences were observed in either total body weight gain, organ weights, plasma corticosterone levels, or lipid peroxidation between Cal animals and Cal plus immobilization-stressed animals. The results obtained suggest that (i) several stress-related responses such as inhibition of total body weight gain, increased adrenal weight, decreased thymus weight, increased plasma corticosterone, and lipid peroxidation levels in the liver and heart are associated with Cal, but (ii) no additional effects were observed on the parameters that were measured when two stress regimens were given simultaneously, suggesting that animals subjected to two stress regimens can protect themselves by controlling their stress-related thresholds of response through adaptation. [E.B.M. 2001, Vol 226:97–102]

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Studies using rodents have suggested that long-term caloric deprivation of 25–50% without deficiency in essential nutrients retards the pathophysiological changes associated with aging and also prolongs the life span of animals (1–5). Food restriction is also known to have protective effects against several types of cancers through the inhibition of cell proliferation and the induction of apoptosis (6, 7). Although the precise mechanisms involved in the beneficial effects of caloric restriction are not known at the present time, several hypotheses have been proposed for the caloric-restricted extension of the life span in rodents and primates (1, 4, 5). These include suppression of net metabolic rate (2), reduction in oxidative stress providing lower toxic by-products (4), decrease in body temperature (1), enhanced DNA repair capacity (8), and decrease in plasma insulin and glucose levels (9). Recently, Lee *et al.* (2) using mouse gastrocneminus muscles concluded that aging was associated with a marked stress response-related gene expression. Interestingly, they observed that these aging-related patterns of gene expression were partially or completely altered by the benefits of Cal (2).

As a part of our ongoing studies on the biological effects of stress, we have recently shown that repeated immobilization stress (2 hr daily, for 2 months) to male Sprague-Dawley rats resulted in 25% inhibition in total body weight gain and a significant increase in the glucocorticoid receptor and lipid peroxidation levels in the liver and heart (10). In regard to the above, it is reported that Cal is stressful to animals due to metabolic substrate deprivation (11, 12). Therefore, in the present study, we have examined the biological effects of long-term Cal and compared it to those previously obtained by us for repeated immobilization stress (10, 13). Furthermore, since hardly any data are available regarding the biological effects of simultaneous admin-

istration of two stress regimens, we have determined whether the biological effects of simultaneous administration of Cal plus repeated immobilization stress are synergistic, additive, or adaptive.

Materials and Methods

Materials. *Chemicals.* [1,2-^{3}H] Corticosterone (52 Ci/mmol) was purchased from Du Pont Company (Wilmington, DE). Corticosterone antibody, unlabeled corticosterone, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and aprotinin were obtained from Sigma Chemical Company (St. Louis, MO). Heat shock protein 70 antibody was obtained from StressGen Biotechnologies Corp (Victoria, Canada). Glucocorticoid receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and all other chemicals used were of analytical grade.

Animals. All procedures involving animals were conducted in accordance with the guidelines of Institutional Animal Care and Use Committee of Virginia Commonwealth University and the National Institute of Health (NIH) "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. [NIH] 80-23, Revised, Office of Science and Health Reports, Bethesda, MD 20205).

Male Sprague-Dawley rats weighing 200–240 g were used and housed singly. Sprague-Dawley rats show readily elevated basal and prolonged stress-induced plasma corticosterone levels; therefore, they are good experimental models to study stress related biological effects. Male animals were used to avoid variable steroids levels observed during the regular estrous cycle of female animals. Rats were kept one per cage in an animal room, separated from the laboratory under standard conditions of 12-hr light, 12-hr dark cycle (lights on at 7 AM) and temperature (22±1°C) for 1 week before and throughout the experiments. Animals were fed rodent chow and water *ad libitum*.

Methods. *Immobilization stress.* To prevent variation in plasma corticosterone levels from day to day, we carried out our immobilization stress experiments daily between 9 AM and 11AM. Because the plasma corticosterone level is lowest during the morning in rats, we chose morning hours to perform our experiments in order to get the maximum stress response. The immobilization stress was performed daily for 2 hr by putting the animals in Plexiglas tubes. Previously published results have showed that the peak level of plasma corticosterone was reached within 60 min of immobilization stress and lasted as long as the experimental duration (10, 13). Thus, we used a 2-hr stress protocol in our experiments. The experiments lasted for 8 weeks. All the animals were randomly divided into three groups. Six animals were used for each group and two cohorts of rats were used for this experiment (total 12 rats/group).

Protocol. Group I: Unstressed animals (control), fed *ad libitum*. Group II: 30% less food was given as consumed daily by the control group, the intake allowance was determined by the mean of the control group. Group III: 30% caloric restriction plus immobilization stress daily for two hours. Our studies represent the balanced reduction of the lipid, carbohydrate and protein content without reduction of its micronutrient content.

Following each stress session, animals were returned to their home cages and were given access to food and water for the remainder of the day. All animals were decapitated between 10 AM and 11 AM in the laboratory. After decapitation, about 3 ml of trunk blood was collected into tubes containing 100 μl of 0.3 *M* EDTA and centrifuged at 1500*g* for 20 min at 4°C using Sorvall RC-3 centrifuge. The supernatant plasma was collected and stored at –20°C until subsequent analysis. The liver, heart, thymus, kidneys, adrenals, and testes were rapidly removed, cleaned from connective tissues, etc., dried with paper towels, and weighed. The liver and heart were cut into small pieces, mixed thoroughly, and divided into two portions, one used for glucocorticoid receptor and HSP 70 determination and the other used for lipid peroxidation assay.

Preparation of tissue homogenate. The liver, testes, heart, and thymus were mixed with 3 ml of ice-cold 0.1 *M* phosphate buffer, pH 7.5, containing 5% SDS, 1% β-mercaptoethanol, a cocktail of protease inhibitors (0.1 m*M* PMSF, 1 μ*M* leupeptin, and 1 μ*M* aprotinin) and 10 m*M* sodium molybdate per gram of tissue. The mixtures were then homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged for 30 min at 14,000 revolutions per minute using an Eppendorf centrifuge 5415 C (Brinkmann Instruments, Westbury, NY). The supernatant was collected and stored at –75°C for future use. At the time of the analysis, samples were thawed and recentrifuged again with an Eppendorf centrifuge at 14,000 revolutions per minute for 30 min. Protein concentration was measured using standard Bio-Rad protein assay based on Bradford dye-binding procedure with bovine serum albumin as standard (14).

Western blotting. SDS–PAGE was performed by the method of Laemmli (15). Blotting followed a modified protocol by Towbin (16). Samples were mixed with an equal volume of SDS sample buffer (0.5 *M* Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 50 m*M* DTT, and 0.005% bromophenol blue) and heated in a boiling water bath for 4 min; 7.5% separating and 4% stacking SDS–polyacrylamide gels were prepared as described by Laemmli (15). Samples (adjusted to about 100 μg of protein) were loaded onto gel. Electrophoresis was done at 165 V using a Mini-Protean II slab gel apparatus. Immunoblotting was carried out by transferring proteins from slab gels to Immun-Lite membranes (no. 162-0170, Bio-Rad, Hercules, CA). The membranes were blocked overnight in cold room with 10% non-fat dry milk in Tris-buffered saline (TBS, 20 m*M* Tris, 500 m*M* NaCl, pH 7.5). The membranes were then incubated for 1 hr at room temperature with GR polyclonal antibody (M-20, no. sc-1004; Santa Cruz Biotechnology, Santa Cruz, CA) or

HSP 70 antibody (StressGen, Canada) diluted 1:200 in TTBS (TBS, 0.05% Tween-20). The membranes were incubated with conjugate secondary antibody (goat anti-rabbit IgG (H+L)–alkaline phosphatase conjugate, no. 170-6518, Bio-Rad) diluted 1:3000 and the membranes were detected with chemiluminescent substrate kit (no. 170-6534, Bio-Rad). Quantitation of GR and HSP 70 was performed by densitometric scanning of autoradiograms exposed within the linear range of the X-ray film using the Pharmacia LKB/Ultrasca XL 1D analysis program. Optical density (OD) reading for the GR and HSP 70 bands were determined from samples run in at least three different blots.

Lipid peroxidation determination. The liver and heart (~200 mg) were immediately homogenized in ice-cold 1.15% KCl using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) to make 10% homogenate. The determination of the lipid peroxidation levels in the above tissues was performed by the thiobarbituric acid (TBA) method (17); 3 ml of 1% phosphoric acid followed by 1 ml of 0.6% 2-TBA were added to 0.5 ml of 10% homogenate. The mixture was heated in a boiling water bath for 45 min and then cooled; 4 ml of n-butanol was added to the cooled mixture for extraction. After the extraction, the n-butanol layer was separated by centrifugation at 2000*g* for 10 min. The n-butanol layer was removed, and the optical density of the n-butanol layer was measured spectrophotometrically at 535-nm wavelength. The TBA values were expressed as nmol of malonaldehyde per 50 mg of wet tissue.

Statistical analysis. Results are presented as the mean ± SEM. Analysis of variance (ANOVA) was used to determine differences among groups using a SigmaStat software package. A Student–Newman–Keuls (SNK) test was used to compare groups. Statistical differences were considered significant if P was less than 0.05.

Results

Data presented in Fig. 1 show that animals maintained for eight weeks on a caloric-restricted diet gained only 30% of total body weight gained by control unstressed animals. Interestingly, only slight, but statistically insignificant differences in total body weight gain were observed between caloric-restricted and caloric-restricted plus repeated immobilization stressed animals (Fig. 1).

Caloric deprivation resulted in a significant decrease in relative liver and thymus weight, and an increase in testes weight when compared to the control *ad libitum* fed diet animals (Table I, C, Cal). Simultaneous administration of caloric stress plus repeated immobilization stress regimens had no observable effect on relative liver, thymus, testes, or adrenal weights (Table I, Cal+Imm) when compared to the caloric-restricted alone animals (Table I, Cal).

Figure 2 (C, Cal, Cal+Imm) demonstrates that long-term caloric stress alone or combined treatment of caloric restriction plus repeated immobilization stress resulted in a significant increase in plasma corticosterone levels as compared to the control animals.

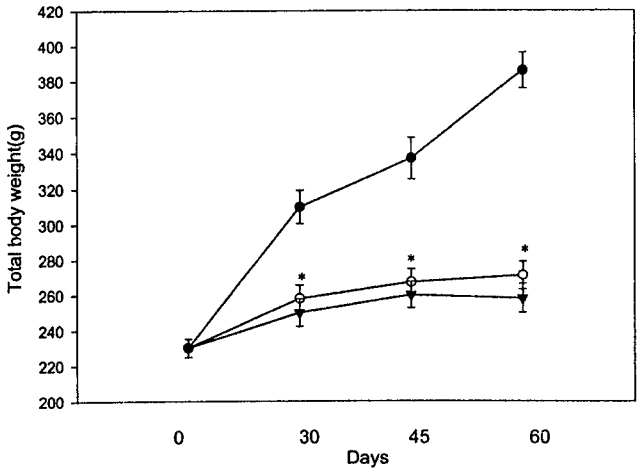

Figure 1. Body weight gain in control (●), caloric-restricted (○), and caloric-restricted plus immobilization stressed (▼) rats. Each bar represents mean ± SEM of two experiments (total $n = 12$). *Significantly different from control levels ($P < 0.05$).

Figure 3A and B (Cal, Cal+Imm) shows that caloric restriction or caloric restriction plus repeated immobilization stressed resulted in significant elevation in malonaldehyde levels (that represents lipid peroxidation levels) in the liver (Fig. 3A) and heart (Fig. 3B) in comparison to the control animals.

Figure 4 (C, Cal, Cal+Imm) demonstrates that both caloric-restricted and caloric-restricted plus immobilization stressed animals had almost a 30–40% decrease in total GR levels (both cytosolic and nuclear) in thymus when compared to the control unstressed animals. Almost 20–40% (statistically significant) decreased GR levels were also observed in the liver and heart (data not shown) of caloric-stressed alone or in caloric-stressed plus repeated immobilization-stressed animals compared to the control unstressed animals.

Data presented in Fig. 5 (C, Cal, Cal+Imm) shows that both caloric-restricted and caloric-restricted plus repeated immobilization-stressed animals had increased HSP 70 levels in testes when compared to *ad libitum* fed control animals.

Discussion

Results obtained suggest that in two months of Cal animals had almost a 2-fold elevated plasma corticosterone levels and exhibited a classical glucocorticoid excess physiological profile in terms of decrease in thymus weight and increase in adrenal weight when compared to *ad libitum* fed control animals. We have also observed a significant decrease in the liver weight, increase in testes weight, and decrease in total GR in thymus, the liver, and heart of caloric-stressed animals. Thus, results obtained by us are consistent with the prior studies using mice (5), Fischer rats (18), and primates (1) showing elevation in plasma corticosterone and inhibition in total body weight gain as two key

Table I. Relative Tissue Weights

	C	Cal	Cal+Imm
Relative thymus weight, mg/100 g b.w.	92 ± 5.4	83 ± 4.4*	81 ± 3.9*
Relative adrenal weight, mg/100 g b.w.	58 ± 2.4	83 ± 6.3*	84 ± 5.5*
Relative liver weight, g/100 g b.w.	3.39 ± 0.3	2.46 ± 0.09*	2.48 ± 0.1*
Relative spleen weight, g/100 g b.w.	0.196 ± 0.008	0.186 ± 0.011	0.185 ± 0.009
Relative testes weight, g/100 g b.w	0.97 ± 0.02	1.25 ± 0.03*	1.32 ± 0.02*
Relative heart weight, g/100 g b.w.	0.33 ± 0.01	0.30 ± 0.007	0.31 ± 0.02
Relative kidney weight, g/100 g b.w.	0.69 ± 0.01	0.64 ± 0.02	0.66 ± 0.02

Note. Relative tissue weight in control (C), caloric-restricted (Cal), and caloric-restricted + immobilization-stressed (Cal + Imm) animals. Results are expressed as mean + SEM of two experiments (total animals $n = 12$). Groups labeled with * show significant differences ($P < 0.05$.)

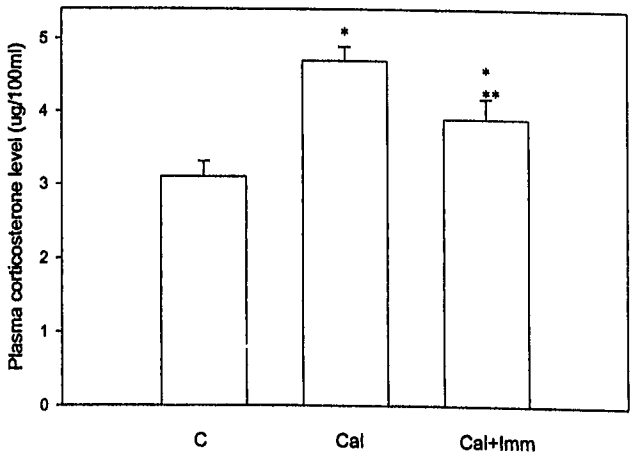

Figure 2. Plasma corticosterone levels in control (C), caloric-restricted (Cal), and caloric-restricted plus immobilization stressed (Cal+Imm) rats. The plasma corticosterone levels were measured as described in Methods. Each point represents the mean ± SEM of two experiments (total $n = 12$). *Significantly different from control levels ($P < 0.05$) and **significantly different between Cal and Cal+Imm animals.

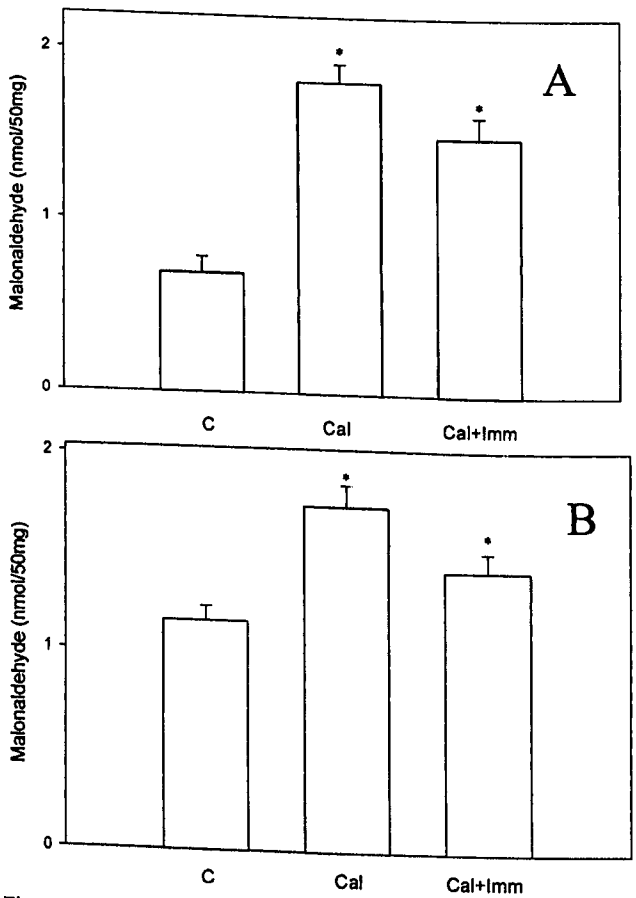

Figure 3. Lipid peroxidation levels in (A) liver, and (B) heart of control (C), caloric-restricted (Cal), and caloric-restricted plus immobilization-stressed (Cal+Imm) rats. Lipid peroxidation was determined as described in Methods. Each bar represents the mean ± SEM of two experiments (total $n = 12$). *Significantly different from control levels ($P < 0.05$).

features of caloric-deprived mammals (5, 18). Although lack of weight gain in the Cal animals is primarily due to inadequate caloric availability, it is suggested that (11, 12) resulting metabolic substrate deprivation is stressful to animals. Increased plasma corticosterone levels observed in Cal animals support this assumption. On the other hand, we observed that Cal elevated lipid peroxidation levels in the liver and heart. Thus the results obtained are in contrast to those previously reported by several investigators showing that caloric restriction, by decreasing lipid peroxidation in various tissues, protects the animals from free-radical damage (19–22). Furthermore, it has been proposed (4, 21, 22) that as an organism ages, the amount of oxidative damage increases with accumulation in reactive oxygen species due to normal metabolic turnover in lipids, proteins, and DNA. On the other hand, caloric restriction, by lowering oxidative stress and decreasing free-radical production, is supposed to retard age-associated pathologies and prolong the life of mammals. However, it is possible that increased lipid peroxidation levels in the liver and heart in our Cal animals that we observed may be due to species differences, age of animals, duration of Cal, or the percentage of caloric deprivation employed. However, in this regard, Davis *et al.* (23) found the highest lipid peroxidation levels in hepatic microsomes from young (3.5-month-old) on a caloric intake of 75% of *ad libitum* mice, while very low levels of lipid peroxidation were found in old (27-month-old) animals.

Finally, we observed that caloric-restricted animals had a significant increase in HSP 70 levels in their testes. There is little available literature on the biological effects of caloric restriction on male sexual function (18). Since heat shock proteins are known to play a critical role in protecting cells from hyperthermia and various other stress-related damages (24), this observation may be clinically important.

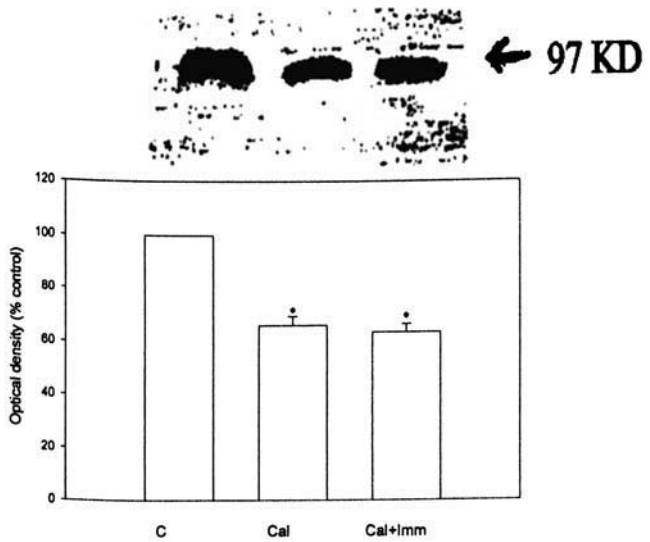

Figure 4. Immunoblots studies of glucocorticoid receptor in thymus of control (C), caloric-restricted (Cal), and caloric-restricted plus immobilization stressed (Cal+Imm) rats. The homogenates were analyzed by Western blot as described in Methods. Each bar represents the mean ± SEM of 4–6 samples.

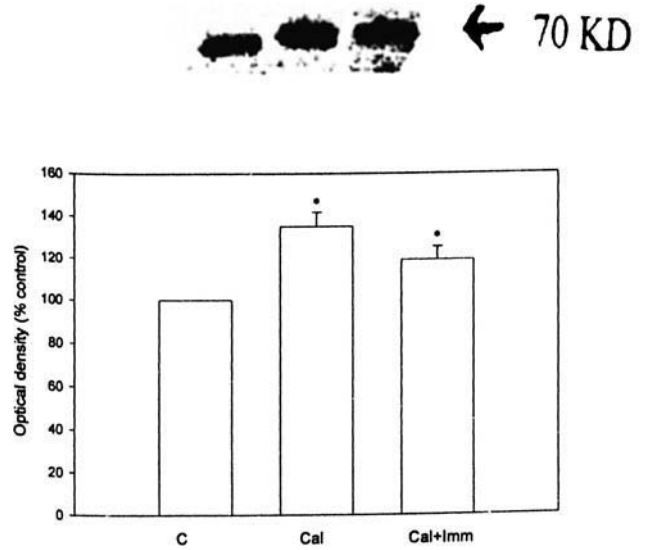

Figure 5. Immunoblots studies of HSP 70 in testes of control (C), caloric-restricted (Cal), and caloric-restricted plus immobilization stressed (Cal±Imm) rats. The homogenates were analyzed by Western blot as described in Methods. Each bar represents the mean ± SEM of 4–6 samples.

In support of this, previously, it has been reported that HSP 70 participates in synaptonomal complex function during meiosis in male germ cell and germ cells apoptosis. A lack of HSP 70 has resulted in failed meiosis and male infertility (25). Thus, results obtained by us showing an increase in HSP 70 levels afforded by caloric restriction in testes should have important beneficial implications regarding male optimum testicular function and male reproduction. From the results obtained, it is fair to postulate that glucocorticoids play an important role in the overall metabolic profile of caloric deprivation and in the mechanisms involved in the suppression of cell proliferation and the prolongation of life span in mammals (18, 27). In this regard, it is well known that biological effects of glucocorticoids are perplexing and at times paradoxical (18, 26). For example, glucocorticoids are needed in order to maintain proper cellular homeostasis by having anti-inflammatory activity and providing trophic factors during acute stress. On the other hand, prolonged and excessive glucocorticoid secretion disrupts the HPA axis (27), manifested by the pathophysiological symptoms of hypercortisolism as seen in hypertension, immune dysfunctions, osteoporosis, cardiac malfunctions, neuronal degeneration, insulin resistance, muscle wasting, etc. Thus, it is fair to conclude from the results presented here that moderate food deprivation by down regulation of glucocorticoid receptor enhances metabolic turnover in lipids, proteins, and DNA. However, paradoxically in this process that generates free radicals, curtails body weight gain, and cell proliferation, it also prolongs the life span. These observations are supported by the facts that (i) adrenalectomy inhibits the protection offered by caloric restriction and (28) is consistent with the hypothesis of Nelson *et al.* (11) suggesting that a daily period of mild hyperadrenocorticism induced by Cal may potentiate cellular and organismic homeostasis throughout the life span of rats similar to that observed during controlled periods of acute stress.

Although there are some distinct differences between the biological effects of caloric deprivation and repeated immobilization stress, overall, caloric stressed animals share many common biological effects with repeated immobilization stress animals previously reported by us (10, 13). Previously we have shown (10) that rats adapt to repeated 2 hr of daily immobilization stress delivered over 2 months with both stabilizing the rapid inhibition of total body weight gain as well as lowering the plasma corticosterone to the levels of almost control unstressed animals noted during the first 30 days of repeated immobilization stress regimen (13). We observed that 2 months of daily repeated immobilization stress to rats resulted in about 25% inhibition in body weight gain, increase in plasma corticosterone at set point higher, 60% increase in total (both cytosolic and nuclear) glucocorticoid receptor in liver, and almost 50% and 90% increase in lipid peroxidation levels in the liver and heart, respectively, compared with control unstressed animals. Therefore, we have asked the question as to whether the combined biological effects of these two stress regimens are synergistic, additive, or adaptive.

We hypothesized that the overall effects of prolonged administration of these two stressors should have additive effects on the inhibition of body weight gain, plasma corticosterone levels, and lipid peroxidation levels. To our surprise, the results obtained by us suggest that instead of additive effects, the combination of two stress regimens showed enhanced adaptation. In fact, combined stress regimen animals significantly maintained the characteristic biological effects observed in caloric stress animals alone. Thus it seems that once a certain threshold is reached, animals may adapt to a second stress regimen with universal toler-

ance to any subsequent stress. It is also possible that our observed lack of additional inhibition in weight gain, despite added stress, with elevation of plasma corticosterone levels, lipid peroxidation levels, etc. may not be due to enhanced adaptation to an additional stress but may yet act as another beneficial effect to caloric stress. In other words, caloric stress helped animals to adapt to their experience with repeated immobilization stress. Thus, the failure to see an additive effect with simultaneous administration of two stressors could be due to the possibility that (i) animals adapt more rapidly to simultaneous administration of two stressors as opposed to a single stress regimen or that (ii) both repeated immobilization treatment and Cal act through the same pathway. Additional studies using various combinations of other stressors are needed to answer this important question with obvious implications to our all understanding of how humans respond when confronted with multiple stress situations.

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