Biochemical Pharmacology, Vol. 59, pp. 753–762, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00385-8

Anti-stress Effects of Dehydroepiandrosterone

PROTECTION OF RATS AGAINST REPEATED IMMOBILIZATION STRESS-INDUCED WEIGHT LOSS, GLUCOCORTICOID RECEPTOR PRODUCTION, AND LIPID PEROXIDATION

*Yan Hu, Arturo Cardounel, Erdal Gursoy, Philip Anderson and Mohammed Kalimi**

Department of Physiology, and Clinical Chemistry Section, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0551, U.S.A.

ABSTRACT. In the present study, we have (i) examined the biological effects of repeated immobilization stress, and (ii) tested the hypothesis that the adrenal steroid hormone dehydroepiandrosterone (DHEA) is an anti-stress hormone, using male Sprague–Dawley rats. Rats (N = 6) were divided into the following four groups: (i) control, (ii) repeated immobilization stress (2 hr daily, for 60 days), (iii) repeated immobilization stress (2 hr daily, for 60 days) plus daily i.p. administration of 5 mg DHEA/0.1 mL DMSO, and (iv) daily i.p. administration of 5 mg DHEA/0.1 mL DMSO alone. Results obtained showed that repeated immobilization stress resulted in a significant (25%) inhibition in body weight gain, a significant increase in adrenal weight, an increase in glucocorticoid receptor (GR) in the liver, thymus, and spleen, decreased plasma triglyceride levels, and increased lipid peroxidation in the liver and heart as compared with control unstressed animals. Interestingly, DHEA administration resulted in a significant reversal in stress-induced inhibition in body weight gain, adrenal weight, GR levels in liver, thymus, and spleen, and lipid peroxidation levels in the liver and heart. In addition, animals treated with DHEA alone without stress showed a significant (15%) inhibition in body weight gain and an almost 60% decrease in plasma triglyceride levels as compared with control unstressed animals. It is concluded that DHEA acts as an anti-stress hormone in rats, as shown in its antagonizing the effects of repeated immobilization stress on total body weight, adrenal weight, GR levels, and free radical generation. BIOCHEM PHARMACOL **59**;7:753–762, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. dehydroepiandrosterone; immobilization stress; glucocorticoid receptor; lipid peroxidation; weight loss

Our laboratory has an ongoing interest in studying (i) the pathophysiology of stress, and (ii) glucocorticoid–DHEA† interactions. It is well known that glucocorticoids are produced in response to many common stressors such as immobilization, cold, swimming, and hypoglycemia. Glucocorticoids are essential for general adaptive responses to acute stress. On the other hand, chronic and excessive glucocorticoid secretion is reported to have deleterious effects including immunosuppression, hyperglycemia, hippocampal injury, hypertension, muscle degeneration, and osteoporosis [1]. However, it is obvious from the reviewed literature [2–6] that the biological effects of prolonged and repeated stress and the mechanisms through which glucocorticoids mediate various chronic stress-related pathophysiological responses remain largely unknown.

DHEA is an adrenal steroid secreted in large quantities in humans. DHEA dramatically declines with age in primates. It has been reported that a man in his eighties produces only 10–20% of the DHEA he made in his twenties. Except to serve as an intermediate in the synthesis of sex steroids such as estrogen and androgen, the precise physiological functions of DHEA remain largely unknown. Research carried out over the past decade has shown beneficial effects of DHEA in obesity, diabetes, cancer, atherosclerosis, enhancement of memory, and viral infection [7, 8]. However, the cellular and molecular mechanisms by which these various biological effects of DHEA are mediated are largely unknown. Other investigators and our group have shown that DHEA antagonizes many biological effects of glucocorticoids *in vivo* [8–13]. Since glucocorticoids are involved in the pathophysiology of chronic stress, in the present study we tested the hypothesis that treatment with DHEA should have beneficial effects in terms of prevention and treatment of experimental stress-induced responses, which produce pathophysiological changes in rats.

* Corresponding author: Dr. M. Kalimi, Box 551, MCV Station, Richmond, VA 23298-0551. Tel. (804) 828-9500; FAX (804) 828-7382; E-mail: Mkalimi@hsc.vcu.edu

† *Abbreviations*: DHEA, dehydroepiandroesterone; GR, glucocorticoid receptor; TBA, thiobarbituric acid; and HPA, hypothalamo-pituitary-adrenocortical.

Received 10 December 1998; accepted 19 August 1999.

MATERIALS AND METHODS

Chemicals

[1,2-^{3}H]Corticosterone (52 Ci/mmol) was purchased from the DuPont Co. Corticosterone antibody, unlabeled corticosterone, leupeptin, phenylmethylsulfonyl fluoride, and

aprotinin were obtained from the Sigma Chemical Co. DHEA was obtained from Searle Chemicals. GR antibody was purchased from Santa Cruz Biotechnology. All other chemicals used were of analytical grade.

Animals

All procedures involving animals were conducted in conformity with the guidelines of the 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 (CD strain) weighing 180–200 g were used. 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 steroid 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 7:00 a.m.) and temperature (22 ± 1°) for 1 week before and throughout the experiments. Animals were fed rodent chow and water *ad lib*.

Immobilization Stress

To prevent variation in plasma corticosterone levels from day to day, we carried out our immobilization stress experiments daily (7 days a week) between 9:00 and 11:00 a.m. The plasma corticosterone level is lowest during the morning in rats. Therefore, we chose morning hours to perform our experiments to get the maximum stress response. The immobilization stress was induced daily for 2 hr by putting the animals in plexiglass tubes. Preliminary experiments showed that the peak level of plasma corticosterone was reached within 60 min of immobilization stress and lasted as long as the experimental duration. Therefore, we used a 2-hr stress protocol in our experiments. The experiment lasted 2 months. All the animals were randomly divided into four groups. Six animals were used for each group. The following protocol was used: Group 1: unstressed animals (control), daily i.p. administration of 0.1 mL DMSO. Group 2: chronically stressed animals, daily i.p. administration of 0.1 mL DMSO followed by 2 hr of stress daily for 2 months. Group 3: daily i.p. administration of DHEA (5 mg/250 g body weight in 0.1 mL DMSO) followed by 2 hr of stress daily for 2 months. Group 4: treatment with DHEA alone (5 mg/250 g body weight in 0.1 mL DMSO) for 2 months.

Following each stress session, animals were returned to their home cages and were able to access food and water freely for the remainder of the day. The body weight of the animals was determined at the beginning of the experiment, every 2 weeks during the experiment, and 24 hr prior to decapitation. All animals were decapitated between 10:00 and 11:00 a.m. 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° using a Sorvall RC-3 centrifuge. The supernatant plasma was collected and stored at -20° until subsequent analysis. The adrenal glands, thymus, liver, spleen, heart, and kidney were removed rapidly, cleaned of connective tissues, dried with paper towels, and weighed.

Preparation of Tissue Homogenate

The liver, thymus, and spleen each were mixed with 3 mL of ice-cold 0.1 M phosphate buffer, pH 7.5, containing 5% SDS, 1% β-mercaptoethanol, a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM aprotinin) and 10 mM sodium molybdate per g of tissue. Then the mixtures were homogenized using a Polytron tissue homogenizer (Brinkmann Instruments). The homogenate was centrifuged for 30 min at 14,000 rpm using an Eppendorf centrifuge 5415 C (Brinkmann). Protein concentration was measured using a standard Bio-Rad protein assay based on the Bradford dye-binding procedure with bovine serum albumin as the standard [14]. The supernatant was collected, used as homogenate, and stored at -75° for future use. At the time of analysis, samples were thawed and recentrifuged with an Eppendorf centrifuge at 14,000 rpm for 30 min.

Western Blotting

SDS–PAGE was performed by the method of Laemmli [15]. Blotting followed a modified protocol by Towbin *et al.* [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 mM dithiothreitol, and 0.005% bromophenol blue) and heated in a boiling water bath for 4 min. Seven-and-one-half percent 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 gels. Electrophoresis was done at 165 V using a Mini-Protean II slab gel apparatus. After electrophoresis, gels were allowed to equilibrate in a 20% methanol blotting buffer for 15 min. Immunoblotting was carried out by transferring proteins from slab gels to Immun-Lite membranes (No. 162-0170, Bio-Rad) using an electrophoretic transfer apparatus (Mini Transblot, Bio-Rad) at 100 V for 2 hr in a cold room (−20°). The membranes were blocked overnight in a cold room with 10% nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5). Then the membranes were incubated for 1 hr at room temperature with GR polyclonal antibody (M-20, No. sc-1004; Santa Cruz Biotechnology) diluted 1:200 in TTBS (TBS, 0.05% Tween-20). After they were washed with TTBS four times for 15 min, the membranes were incubated with conjugated secondary antibody [goat anti-rabbit

IgG (H+L)-alkaline phosphatase conjugate, No. 170-6518, Bio-Rad] diluted 1:3000 in TTBS containing 1% nonfat dry milk. After 4 × 15 min washes with TTBS, the membranes were detected with a chemiluminescent substrate kit (No. 170-6534, Bio-Rad); the membranes were incubated with chemiluminescent substrate for 5 min and exposed to film (BioMax MR film, Eastman Kodak) for 25 min. Quantitation of GR was performed by densitometric scanning of autoradiograms exposed within the linear range of the x-ray film using the Pharmacia LKB/Ultrascan XL, 1D analysis program. Optical density readings for the GR band were determined from samples run in at least three different blots.

In the present study, we have used a recently cloned GR antibody (specific to both GR α and β subtypes) to measure both cytosolic and nuclear GR. However, most previous studies on the regulation of GR have been done using radioligand binding assays [2, 3, 17, 18]. It is important to point out that binding assays are unable to measure steroid-bound GR as well as nuclear GR [19]. On the other hand, the antibody used by us measures steroid-unoccupied, molybdate-stabilized, activated, and DNA-bound forms of GR. Thus, the results presented here represent more accurately the levels of total GR proteins in the given tissue.

Lipid Peroxidation Determination

The liver and heart (~ 200 mg) were homogenized immediately in ice-cold 1.15% KCl using a Polytron homogenizer (Brinkmann Instruments) to make a 10% homogenate. The determination of the lipid peroxidation levels in the above tissues was performed by the TBA method [20]. Three milliliters of 1% phosphoric acid followed by 1 mL of 0.6% 2-TBA were added to 0.5 mL of the 10% homogenate. The mixture was heated in a boiling water bath for 45 min and then cooled. Four milliliters 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 a wavelength of 535 nm. The TBA values were expressed as nmol of malonaldehyde per 50 mg of wet tissue.

Plasma Corticosterone Measurements

Plasma samples were thawed and diluted 50-fold in diluent (0.1% BSA, 0.05 M Tris–HCl, 0.1 M NaCl, and 0.1% NaN_3, pH 8.0). The mixtures were heated in boiling water for 5 min to dissociate protein-bound corticosterone in plasma, and then were centrifuged at 1500 *g* for 15 min. The supernatant was further diluted 10-fold in diluent and used for corticosterone determination. Plasma corticosterone was measured by radioimmunoassay using an antiserum developed in rabbits against corticosterone-21-thyroglobulin (No. c-8784, Sigma). The working concentration of antiserum was diluted 10-fold.

The assay was carried out according to the protocol provided by Sigma: 0.1 mL of plasma sample or standard corticosterone sample (10, 5, 2.5, 1.25, and 0.625 ng/mL) and 0.5 mL of working antiserum were placed in polypropylene test tubes and incubated for 30 min at room temperature. Then 0.1 mL of a saturating concentration of [^{3}H]corticosterone was added to each test tube and incubated further for 1 hr at 37° in a water bath. Following cooling at 4° for 30 min, 0.2 mL of cold dextran-coated charcoal suspension (0.5% charcoal, 0.25% dextran in 0.05 M Tris–HCl buffer, pH 8.0, containing 0.1 M sodium chloride and 0.1% sodium azide) was added rapidly and incubated at 0° in ice water. Test tubes were centrifuged at

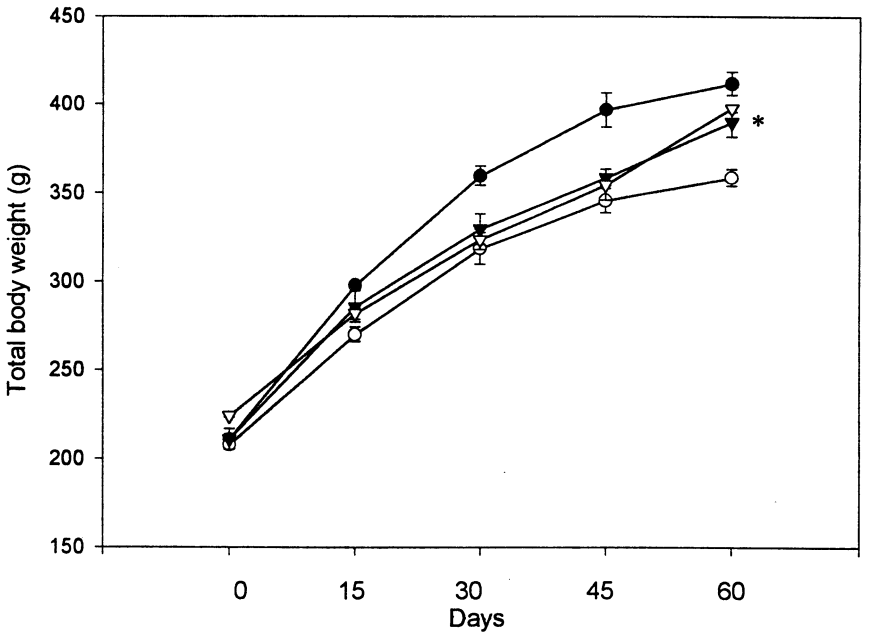

FIG. 1. Total body weight in control (C) (●), immobilization-stressed (I) (▼), immobilization-stressed plus DHEA (I+D) ([itri]), and DHEA only (D) [209] animals. Results are expressed as means ± SEM of two experiments (N = 12). Key: (*) significantly different from immobilization-stressed animals ($P < 0.05$).

TABLE 1. Total body weight gain changes

Days	Total body weight gain (g)/animal			
	C	I	I+D	D
15	87.2 ± 3.2	65.7 ± 6.2*	74.0 ± 4.3	58.5 ± 3.2*
30	150.2 ±3.4	115.8 ± 5.8*	123.8 ± 3.6	101.2 ± 5.4*
45	192.0 ± 6.0	133.3 ± 3.8*	148.0 ± 4.8†	136.2 ± 4.6*
60	201.7 ± 6.6	151.0 ± 5.5*	183.0 ± 3.0†	176.8 ± 4.9*

C, control animals; I, immobilization-stressed animals; I+D. immobilization-stressed plus DHEA-treated animals; and D, DHEA-alone treated animals. Values are means ± SEM, N = 12.

* Significantly different ($p < 0.05$) from control.

† Significant difference ($p < 0.05$) between I and I + D.

1500 *g* for 15 min at 4°. The supernatant was removed from each tube and put into new vials. Scintillation fluid (Budget-Solve, Research Products International Corp.) was added to each vial. A Beckman LS 100c counter (Beckman Instruments) determined the amount of radioactivity present in each vial with 65% efficiency for tritium. Triplicates were used for each sample. The plasma concentration of corticosterone was presented as μg per 100 mL of plasma.

Measurement of Plasma Cholesterol and Triglycerides

Total plasma cholesterol and glycerol-blanked triglycerides were measured with reagents from Boehringer Mannheim Diagnostics, Inc. All these assays were performed on a BMC-Hitachi 911 analyzer (Boehringer Mannheim Diagnostics, Inc.) according to the manufacturer's protocols.

Statistical Analysis

Results are presented as means ± SEM. ANOVA was used to determine differences among groups using a SigmaStat software package computer. A Student–Newman–Keuls test was used to compare groups. Statistical differences were considered significant if *P* was less than 0.05.

RESULTS

Results presented in Fig. 1 and Table 1 (C, I) show that 2 months of repeated immobilization stress administered to Sprague–Dawley rats caused a significant inhibition in total body weight gain as compared with controls (i.e. neither stressed nor DHEA-treated). Interestingly, daily administration of DHEA to the stressed animals for 2 months significantly prevented the inhibition of total body weight gain observed in chronically stressed animals (Fig. 1 and Table 1: I, I+D), and this effect was apparent between days 45 and 60 (Fig. 1). Of interest, DHEA administration to unstressed animals also resulted in significant inhibition of total body weight gain within 2 months as compared with control animals (Fig. 1 and Table 1: C, D), but this was much less than that seen in stressed animals.

Repeated immobilization stress caused a marked increase in adrenal weight (expressed as mg/100 g body weight) when compared with controls (30.5 ± 0.9 vs 22.5 ± 1.0). Administration of DHEA to the stressed animals significantly protected against the increase in relative adrenal

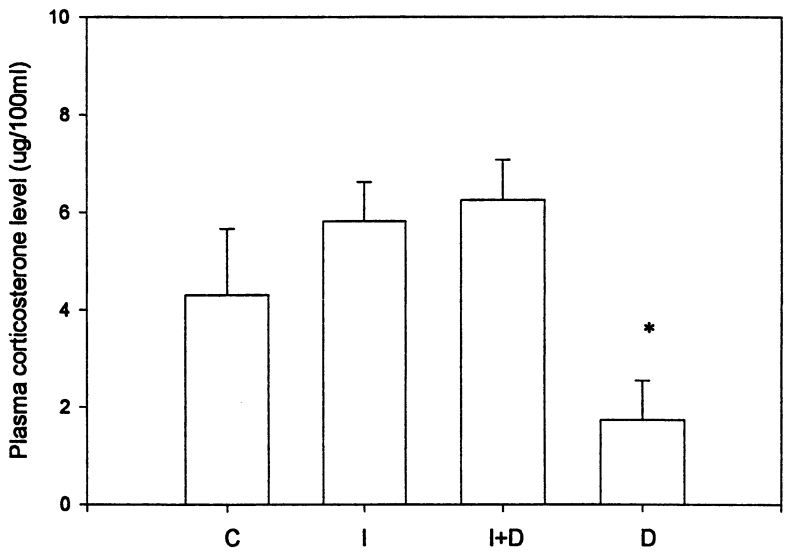

FIG. 2. Plasma corticosterone levels in control (C), immobilization-stressed (I), immobilization-stressed plus DHEA (I+D), and DHEA only (D) animals. The plasma corticosterone levels were measured as described in Materials and Methods. Each bar represents the mean ± SEM of two experiments (N = 6). Key: (*) significantly different from control levels ($P < 0.05$).

TABLE 2. Plasma cholesterol and triglyceride levels

Treatment	Cholesterol (mg/100 mL)	Triglyceride (mg/100 mL)
C	94.6 ± 3.3	75.0 ± 9.4
I	82.3 ± 1.6	55.0 ± 6.3*
I+D	85.7 ± 5.1	50.8 ± 3.4*
D	96.8 ± 4.8	37.0 ± 5.9*

C, controls animals; I, immobilization-stressed animals; I+D, immobilization-stressed plus, DHEA-treated animals; and D, DHEA-alone treated animals. Values are means ± SEM, N = 12.

* Significantly different ($P < 0.05$) from control.

weight induced by chronic stress (24.3 ± 1.4 vs 30.5 ± 0.9). On the other hand, repeated stress plus DHEA treatment, as well as DHEA treatment alone, resulted in no significant changes in liver, kidney, spleen, thymus, or heart weight as compared with unstressed control animals (data not shown).

Figure 2(C, I) shows that 2 months of repeated immobilization stress produced no statistically significant increase in plasma corticosterone levels as compared with controls. DHEA administration to the repeatedly stressed animals did not change the plasma baseline corticosterone concentration (Fig. 1: I, I+D). Interestingly, there was a significant decrease in the plasma corticosterone levels in DHEA-alone treated animals as compared with the other three experimental groups (Fig. 2: C, I, I+D, D).

Repeated immobilization stress seemed to decrease plasma cholesterol levels. However, the decrease was not statistically significant (Table 2). No significant changes in the plasma cholesterol levels were observed in either chronic stress-treated animals given DHEA or DHEA-alone treated animals (Table 2).

Data presented in Table 2 show an almost 50% reduction in plasma triglyceride levels in DHEA-alone treated animals as compared with control animals. An approximately 25% decrease in the plasma triglyceride levels was also observed in both repeated immobilization stress and repeated immobilization stress plus DHEA-treated animals as compared with control animals.

Data presented in Fig. 3A (C, I) show that prolonged repeated immobilization stress administered to rats resulted in a dramatic elevation in malonaldehyde levels (which represent lipid peroxidation levels) in the liver in comparison with controls. Interestingly, DHEA given to the stressed animals reversed the malonaldehyde levels in the liver almost to that observed for control unstressed animals (Fig. 3A: I, I+D). No changes in hepatic malonaldehyde levels were found in DHEA-alone treated animals (Fig. 3A: C, D).

Figure 3B (C, I) demonstrates that there was a significant increase in lipid peroxidation levels in the hearts of repeatedly stressed animals as compared with controls. Again, administration of DHEA to the stressed animals completely reversed this increase in lipid peroxidation levels observed in chronically stressed animals (Fig. 3B: I, I+D). DHEA treatment given alone to the unstressed control animals showed no changes in lipid peroxidation levels in the heart, and malonaldehyde levels remained at control unstressed levels (Fig. 3B: C, D).

Figure 4A (C, I) demonstrates that repeated immobilization stress increased total GR levels (both cytosolic and nuclear) in the liver by almost 60% when compared with the control levels. Interestingly, administration of DHEA to the stressed animals lowered the elevated GR levels in liver (Fig. 4A: I, I+D). However, repeated DHEA administration to the unstressed control animals did not influence the GR levels in the liver (Fig. 4A: C, D). As shown in Fig. 4B (C, I), repeated immobilization stress increased GR levels in the thymus by 25%. However, DHEA treatment of

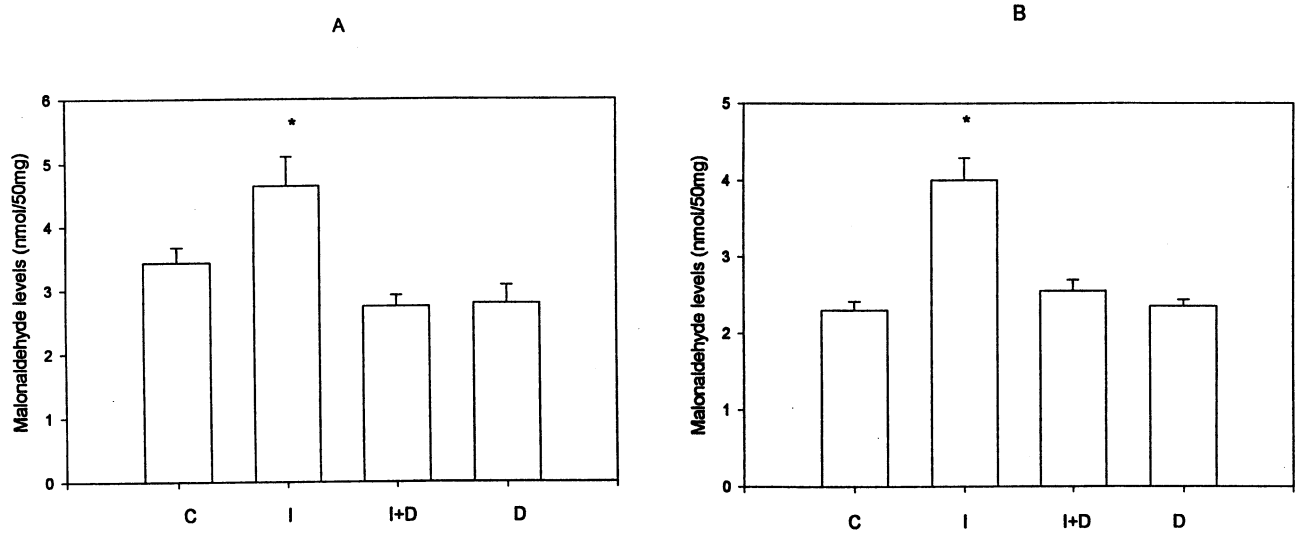

FIG. 3. Lipid peroxidation levels in control (C), immobilization-stressed (I), immobilization-stressed plus DHEA (I+D), and DHEA only (D) animals. (A) Liver; (B) heart. Lipid peroxidation was determined as described in Materials and Methods. Each bar represents the mean ± SEM of two experiments (N = 6). Key: (*) significantly different from control ($P < 0.05$).

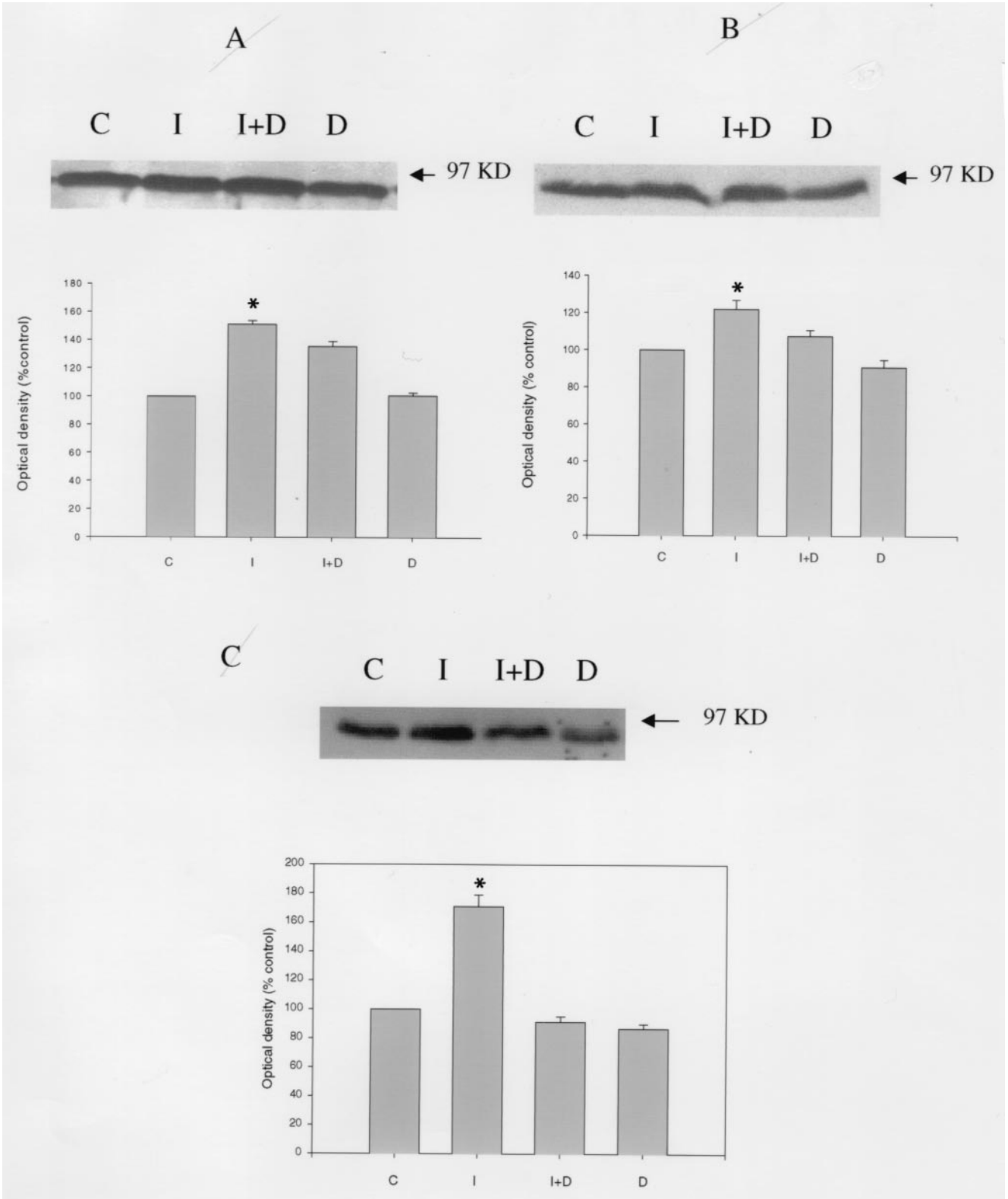

FIG. 4. Immunoblot studies of glucocorticoid receptor in control (C), immobilization-stressed (I), immobilization-stressed plus DHEA (I+D), and DHEA only (D) animals. (A) Liver; (B) thymus; and (C) spleen. The homogenates were analyzed by western blot as described in Materials and Methods. Each bar represents the mean ± SEM of 4–6 samples. Key: (*) significantly different ($P < 0.05$) from control.

these animals lowered the elevated GR levels observed in chronically stressed animals (Fig. 4B: I, I+D). DHEA treatment alone showed no changes in GR levels; they remained at the same level as those observed in the control animals (Fig. 4B: C, D). Panel C of Fig. 4 shows that chronic immobilization stress caused an increase in GR levels in the spleen by almost 50–60% as compared with controls (C, I). Following treatment of the stressed animals with DHEA, the increased GR levels in the spleen were attenuated (I, I+D). No changes in GR levels in the spleen were noted in DHEA-alone treated animals (C, D).

DISCUSSION

DHEA is known to possess numerous biologically beneficial effects including anticancer, antiobesity, antiaging, antiviral, and antidiabetogenic effects [7]. However, few studies have been done on the anti-stress effect of DHEA [8]. It was obvious from our results that DHEA antagonized most stress-induced effects, e.g. inhibition in total body weight gain, adrenal weight, total cellular GR levels in the liver, thymus, and spleen, and lipid peroxidation in the liver and heart. Thus, it is fair to postulate from the results obtained that DHEA administration to chronically stressed animals exerts anti-stress effects. Interestingly, we observed two distinct and apparently opposite biological effects of DHEA administration in rats, depending on whether DHEA was given to chronically stressed animals or to unstressed control animals. Whereas DHEA administered to chronically stressed animals resulted in (i) reversal of weight loss, (ii) increase in adrenal weight, (iii) increase in GR, and (iv) increase in lipid peroxidation compared with chronically stressed animals, DHEA treatment of chronically stressed animals had no effect on plasma corticosterone, cholesterol, or triglyceride levels, which remained almost similar to those of chronically stressed animals. In contrast, DHEA given to control unstressed animals resulted in reduction in body weight, with a dramatic decrease in plasma corticosterone and triglyceride levels when compared with control untreated animals. Finally, DHEA administration, *in vivo*, had no effect on GR or lipid peroxidation. When administered to chronically stressed animals, DHEA was capable of reversing stress-induced GR and lipid peroxidation. These data show that DHEA antagonizes chronic stress effects at the level of lipid peroxidation and GR production.

To the best of our knowledge, this is the first long-term (8 weeks) repeated immobilization stress study in relation to DHEA. Therefore, it is important to highlight some data obtained by us and compare them with those obtained by others using various chronic stress protocols. In our hands, 2 months of repeated stress resulted in a significant inhibition of body weight gain of animals when compared with control unstressed animals. In addition, repeatedly stressed animals showed a significant increase in their adrenal weight. On the other hand, liver, kidney, heart, spleen and thymus weights remained unchanged when compared with control unstressed animals. Following the use of various paradigms varying the durations of repeated stress (5–18 days) in rats, it is generally reported [2–4] that chronic stress results in a decrease in body weight. In regard to tissue weight, several investigators [2, 3, 18] have reported increased adrenal weights, while changes in thymus weight in rats remain inconclusive [2, 4, 17]. Lowy [17] and Herman *et al.* [3] reported no change in thymus weight, whereas Alexandrova and Farkas [2] found involution of the thymus gland in rats exposed for 18 days to various stressors. It is possible that stressed animals do not gain weight as rapidly as the controls, either due to a decrease in their food intake, or because stressed animals exercised more when relieved of stress, and this greater activity could account for inhibition in weight gain in the stressed animals. To reverse weight loss, DHEA administration could either increase food intake or diminish hyperactivity associated with our repeated stress paradigm.

Like other investigators [21–23], we also observed that single (2 hr) acute immobilization stress administered to Sprague–Dawley rats dramatically increased plasma corticosterone levels, as much as 3- to 4-fold [24]. However, in our chronically stressed animals, although statistically insignificant, the plasma corticosterone levels remained at a set point higher than in control unstressed animals.

Results obtained by us suggest that animals adapt to repeated stress within 2 months by both lowering plasma corticosterone levels and stabilizing the rapid inhibition of weight gain observed during the first 30 days of repeated immobilization stress [24]. Therefore, in the present study, we employed 2 months of a repeated stress protocol to study the cumulative biological effects of repeated stress and to test the hypothesis that DHEA is an anti-stress hormone.

Using the western blot technique, we observed that 2 months of repeated immobilization stress significantly increased total GR in liver, spleen, and thymus. However, most reports using binding assays have shown that 5–30 days of repeated stress down-regulates GR in several tissues [2, 3, 21]. Since binding assays can measure only cytosolic GR, it has been concluded that on repeated stress, there is a depletion of cytosolic GR due to translocation of cytosolic GR into the nucleus. Thus, results obtained by us showing up-regulation of total GR during 2 months of repeated immobilization stress differ from those reported earlier by others [2–4] showing decreased levels of GR under various stress conditions, but are consistent with those of Kitayama *et al.* [25], Biagini *et al.* [6], and Curtis and Rarey [26], who, using immunological approaches, observed a significant increase in immunoreactivity in hippocampal and spiral ligament tissues of chronically stressed rats.

Changes in lipid profiles in response to acute stress have been shown in several articles. However, little is known regarding the effects of chronic stress on the lipid profile. Some reports noted that stress affects plasma cholesterol and triglyceride levels, and others found no changes [27–31]. Ours is the first report showing that 2 months of

repeated immobilization stress resulted in significant decreases in plasma triglyceride levels without any significant changes in plasma cholesterol levels.

Finally, chronic stress resulted in a significant increase in lipid peroxidation levels in the liver and heart. Therefore, the results obtained by us are consistent with the observation that besides activation of the HPA axis, chronic stress stimulates free radical production due to accumulation of excessive amounts of free fatty acids and reduced activity of antioxidant enzymes [32, 33].

It is reasonable to assume from the results obtained that elevated plasma glucocorticoids, by up-regulating GR and by increasing reactive oxygen species, may enhance degradation of proteins and fats to provide much-needed carbohydrates to overcome the profound disturbances of glucose homeostasis during chronic stress.

In spite of the many and varied biological effects of DHEA, the cellular and molecular mechanism of the action of DHEA remains largely unknown [7, 8]. We have reported previously [9] that s.c. administration of 7.5 mg DHEA along with 1.5 mg dexamethasone to male Sprague–Dawley rats prevents increased systolic blood pressure induced by administration of 1.5 mg dexamethasone alone. DHEA is able to reverse the involution of lymphoid organs in stressed mice [34] and can block sound stress-induced increased activity of the enzyme tryptophan hydroxylase in the rat brain [13]. DHEA was able to antagonize the effects of corticoid injection on thymus involution [35, 36]. In addition, Blauer *et al.* [11] reported that mice pretreated *in vivo* for 3 days with 60 mg/kg/day of DHEA reduced the dexamethasone-induced thymic and splenic atrophy caused by a single injection of 60 mg/kg of dexamethasone.

The above studies suggest that DHEA is an antiglucocorticoid hormone [9–11, 13, 34–36]. Thus, it is possible that DHEA, by direct displacement of corticosterone from GR, may antagonize the observed antiglucocorticoid effects. The direct competition of DHEA with corticosterone for GR should increase plasma corticosterone levels. However, since we did not observe any changes in plasma corticosterone levels in DHEA administered to chronically stressed animals when compared with control stressed animals, this possibility is highly unlikely.

Alternatively, it is tenable that DHEA is converted to various androgenic metabolites in male rats when administered in conditions of chronic stress. DHEA is a precursor of various sex steroids including testosterone, Δ^5-androstenediol, and other metabolites. This hypothesis is supported by the fact that the magnitude of ACTH and corticosterone responses to restraint was negatively correlated with the physiological level of testosterone in gonadectomized animals, suggesting that testosterone has an inhibitory effect on the HPA axis response to stress [37]. Also, the fall in testosterone secretion is implicated in the decreased levels of lipogenesis in chronically stressed animals [38]. Thus, DHEA, by being metabolized to testosterone and/or other bioactive metabolites in chronically stressed animals, may replenish the levels of testosterone in these animals. Anabolic androgenic metabolites, in turn, may directly or indirectly modulate GR, through modulation of other growth factors such as insulin and insulin-like growth factor-I, and/or through cytokines such as interleukin-2 or interleukin-4 [39, 40], which may antagonize inhibition of weight loss, GR in the liver, thymus, and spleen, and lipid peroxidation in the liver and heart without changing plasma corticosterone levels. Finally, it is possible that besides conversion to its bioactive metabolites or receptor-mediated processes, DHEA may directly act as an antioxidant and neutralize the increase in free radicals induced by chronic stress. In contrast, DHEA administered to control unstressed animals, in the presence of the basal physiological levels of testosterone, may not be converted to androgenic metabolites. In this situation, DHEA appears to directly suppress weight gain and lower plasma triglyceride levels, as observed by others [8, 41–43]. In addition, this exogenously administered DHEA, by increasing the plasma DHEA/corticosterone ratio, should suppress corticosterone levels by directly acting on the HPA axis.

Further work is needed to test this hypothesis by examining whether (i) DHEA is differentially metabolized, and (ii) varied hormones, growth factors, and cytokines are differentially regulated depending on administration of DHEA to chronically stressed versus unstressed animals. DHEA is known to possess immune modulating effects: Riley [35] found that in mice DHEA prevents corticosteroid stress-induced thymic involution. DHEA also upregulates resistance against viral and bacterial infection in the mouse [44] and significantly protects dexamethasone-induced thymic and splenic involution [11, 36]. Therefore, the observed suppression of stress-induced GR levels in immunologically important tissues such as the thymus and spleen in the present study is of considerable interest. In addition, our results suggest that besides anti-stress effects, DHEA also has immunoenhancing effects.

It is important to emphasize that previous studies to determine the metabolic effects of DHEA have used 0.4 to 0.6% DHEA in the diet or given it i.p. or orally at a dose as high as 50–300 mg/kg body weight [8]. Since we have used a low dosage of DHEA in the present study, we postulate that the observed anti-stress effects of DHEA in our study may have physiological relevance.

Finally, stress is believed to cause an imbalance between free radical-mediated pathology and its protective system, which favors the activation of free radicals. Free radicals are known to produce numerous disorders, including aging, cardiac diseases, cancer, immune dysfunction, and degeneration of neuronal cells. For example, chronic stress has been associated with heart disease [45], and oxidative modification of low density lipoprotein has been proposed to account for foam cell formation and the initiation of the atherosclerotic process [46]. We feel that the most important novel finding of our present study is that DHEA used in stress protection prevented the increased lipid peroxidation induced by chronic stress at a low, physiologically relevant dosage. Therefore, it is important to examine

whether DHEA administration in humans would have clinical antioxidant effects similar to those observed by us in rats.

References

1. Herman JP and Cullinan WE, Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* **20**: 78–84, 1997.
2. Alexandrova M and Farkas P, Stress-induced changes of glucocorticoid receptor in rat liver. *J Steroid Biochem Mol Biol* **42**: 493–498, 1992.
3. Herman JP, Adams D and Prewitt C, Regulatory change in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology* **61**: 180–190, 1995.
4. Sapolsky RM, Krey LC and McEwen BS, Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* **114**: 287–292, 1984.
5. Gomez F, Lahmame A, de Kloet ER and Armario A, Hypothalamic-pituitary-adrenal response to chronic stress in five inbred rat strains: Differential responses are mainly located at the adrenocortical level. *Neuroendocrinology* **63**: 327–337, 1996.
6. Biagini G, Pich EM, Carani C, Marrama P, Gustafsson J-A, Fuxe K and Agnati LF, Indole-pyruvic acid, a tryptophan ketoanalogue, antagonizes the endocrine but not the behavioral effects of repeated stress in a model of depression. *Biol Psychiatry* **33**: 712–719, 1993.
7. Kalimi M and Regelson M, *The Biologic Role of Dehydroepiandrosterone (DHEA)*, Walter de Gruyter, New York, 1990.
8. Svec F and Porter J, The actions of exogenous dehydroepiandrosterone in experimental animals and humans. *Proc Soc Exp Biol Med* **218**: 174–191, 1998.
9. Shafagoj Y, Opoku J, Qureshi D, Regelson W and Kalimi M, DHEA prevents dexamethasone-induced hypertension in rats. *Am J Physiol* **263**: E210–E213, 1992.
10. Browne ES, Wright BE, Porter JR and Svec F, Dehydroepiandrosterone: Antiglucocorticoid action in mice. *Am J Med Sci* **303**: 366–371, 1992.
11. Blauer KL, Poth M, Rogers WM and Bernton EW, Dehydroepiandrosterone antagonizes the suppressive effects of dexamethasone on lymphocyte proliferation. *Endocrinology* **129**: 3174–3179, 1991.
12. Daynes RA, Dudley DJ and Araneo BA, Regulation of murine lymphokine production *in vivo*. II. Dehydroepiandrosterone is a natural enhancer of interleukin 2 synthesis by helper T cells. *Eur J Immunol* **20**: 793–802, 1990.
13. Singh VB, Kalimi M, Phan TH and Boadle-Biber MC, Intracranial dehydroepiandrosterone blocks the activation of tryptophan hydroxylase in response to acute sound stress. *Mol Cell Neurosci* **5**: 176–181, 1994.
14. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
15. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
16. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
17. Lowy MT, Corticosterone regulation of brain and lymphoid corticosteroid receptors. *J Steroid Biochem Mol Biol* **39**: 147–154, 1991.
18. Gagnon J, Ho-Kim MA, Boissonneault G, Dube JY, Rogers PA and Tremblay RR, The influence of immobilization stress on cardiac protein synthesis. A possible regulatory role for glucocorticoids. *J Recept Res* **7**: 639–652, 1987.
19. O'Donnell D, Francis D, Weaver S and Meaney MJ, Effects of adrenalectomy and corticosterone replacement on glucocorticoid receptor levels in rat brain tissue: A comparison between western blotting and receptor binding assays. *Brain Res* **687**: 133–142, 1995.
20. Zhang CY and Tan BK, Hypotensive activity of aqueous extract of *Andrographis paniculata* in rats. *Clin Exp Pharmacol Physiol* **23**: 675–678, 1996.
21. Lemberger T, Saladin R, Vazquez M, Assimacopoulos F, Staels B, Desvergne B, Wahli W and Auwerx J, Expression of the peroxisome proliferator-activated receptor α gene is stimulated by stress and follows a diurnal rhythm. *J Biol Chem* **271**: 1764–1769, 1996.
22. Spencer RL, Miller AH, Moday H, Stein M and McEwen BS, Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues. *Endocrinology* **133**: 1941–1950, 1993.
23. Alexandrova M, Stress induced tyrosine aminotransferase activity via glucocorticoid receptor. *Horm Metab Res* **26**: 97–99, 1994.
24. Al-Mohaisen M, Cardounel A, and Kalimi M, Repeated immobilization stress increases total cytosolic glucocorticoid receptor in rat liver. *Steroids*, in press.
25. Kitayama I, Cintra A, Janson AM, Fuxe K, Agnati LF, Eneroth P, Aronsson M, Härfstrand A, Steinbush HWM, Visser TJ, Goldstein M, Vale W and Gustafsson J-Å, Chronic immobilization stress: Evidence for decreases of 5-hydroxytryptamine immunoreactivity and for increases of glucocorticoid receptor immunoreactivity in various brain regions of the male rat. *J Neural Transm* **77**: 93–130, 1989.
26. Curtis LM and Rarey KE, Effect of stress on cochlear glucocorticoid protein. II. Restraint. *Hear Res* **92**: 120–125, 1995.
27. Ruiz de Gordoa JC, Santafe J, Segarra Domenech J and Santisteban A, Modification of rat plasma lipoproteins induced by acute immobilization stress. *Psychosom Med* **56**: 486–492, 1994.
28. Hershock D and Vogel WH, The effects of immobilization stress on serum triglycerides, nonesterified fatty acids, and total cholesterol in male rats after dietary modifications. *Life Sci* **45**: 157–165, 1989.
29. Abd el Mohsen MM, Fahim AT, Motawi TM and Ismail NA, Nicotine and stress: Effect on sex hormones and lipid profile in female rats. *Pharmacol Res* **35**: 181–187, 1997.
30. Tsopanakis C and Tesserommatis C, Cold swimming stress: Effects on serum lipids, lipoproteins and LCAT activity in male and female rats. *Pharmacol Biochem Behav* **38**: 813–816, 1991.
31. Tsopanakis C, Kotsarellis D, Dontas I, Karayannacos P and Skalkeas G, Effects of cold stress on serum lipids, lipoproteins, and the activity of lecithin: Cholesterol acyltransferase in rabbits. *Biochem Med Metab Biol* **39**: 148–157, 1988.
32. Zezerov A, Ivanova S and Ushakov A, Lipid peroxidation in tissues of rats exposed to head-down tilt, exercise or immobilization stress. *Kosm Biol Aviakosm Med* **21**: 39–43, 1987.
33. Seckin S, Alptekin N, Doogra-Abbasoglu S, Kocak-Tokiv N, Toker G and Uysal M, The effect of chronic stress on hepatic and gastric lipid peroxidation in long-term depletion of glutathione in rats. *Pharmacol Res* **36**: 55–57, 1997.
34. Ben-Nathan D, Lustig S, Kobiler D, Danenberg HD, Lupu E and Feuerstein G, Dehydroepiandrosterone protects mice inoculated with West Nile virus and exposed to cold stress. *J Med Virol* **38**: 159–166, 1992.
35. Riley V, Psychoneuroendocrine influences on immunocompetence and neoplasia. *Science* **212**: 1100–1109, 1981.
36. May M, Holmes E, Rogers W and Poth M, Protection from

glucocorticoid induced thymic involution by dehydroepiandrosterone. *Life Sci* **46**: 1627–1631, 1990.
37. Viau V and Meaney M, The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. *J Neurosci* **16**: 1866–1876, 1996.
38. Tsuchiya T and Horii I, Immobilization-induced stress decreases lipogenesis in sebaceous glands as well as plasma testosterone levels in male Syrian hamsters. *Psychoendocrinology* **20**: 221–230, 1995.
39. Daynes RA and Araneo BA, Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4. *Eur J Immunol* **19**: 2319–2325, 1989.
40. Suzuki T, Suzuki N, Daynes R and Engleman E, Dehydroepiandrosterone enhances IL-2 production and cytotoxic effector function of human T cells. *Clin Immunol Immunopathol* **61**: 202–221, 1991.
41. Tagliaferro AR, Davis JR, Truchon S and Van Hamont N, Effects of dehydroepiandrosterone acetate on metabolism, body weight and composition of male and female rats. *J Nutr* **116**: 1977–1983, 1986.
42. Cleary M, Shepherd A, Zisk J and Schwartz A, Effect of dehydroepiandrosterone on body weight and food intake in rats. *Nutr Behav* **1**: 127–136, 1983.
43. McIntosh M, Bao H and Lee C, Opposing actions of dehydroepiandrosterone and corticosterone in rats. *Proc Soc Exp Biol Med* **221**: 198–206, 1999.
44. Loria R, Inge T, Cook S, Szakal A and Regelson W, Protection against lethal viral infections with the native steroid dehydroepiandrosterone (DHEA). *J Med Virol* **26**: 301–314, 1988.
45. Frankenhauser M, *Women, Work and Health*. Plenum Press, New York, 1991.
46. Steinberg D, Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* **272**: 20963–20969, 1997.