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Social stress increases the susceptibility to endotoxic shock

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Abstract

The influence of social disruption stress (SDR) on the susceptibility to endotoxic shock was investigated. SDR was found to increase the mortality of mice when they were challenged with the bacterial endotoxin lipopolysaccharide (LPS). Histological examination of SDR animals after LPS injection revealed widespread disseminated intravascular coagulation in the brain and lung, extensive meningitis in the brain, severe hemorrhage in the lung, necrosis in the liver, and lymphoid hyperplasia in the spleen, indicating inflammatory organ damage. In situ hybridization histochemical analysis showed that the expression of the glucocorticoid receptor mRNA was down-regulated in the brain and spleen of SDR animals while the ratio of expression of AVP/CRH-the two adrenocorticotropic hormone secretagogue, increased. After LPS injection, the expression of pro-inflammatory cytokines, IL-1β and TNF-α, was found significantly higher in the lung, liver, spleen, and brain of the SDR mice as compared with the LPS-injected home cage control animals. Taken together, these results show that SDR stress increases the susceptibility to endotoxic shock and suggest that the development of glucocorticoid resistance and increased production of pro-inflammatory cytokines are the mechanisms for this behavior-induced susceptibility to endotoxic shock.

Keywords: Glucocorticoid resistance; Sepsis; Inflammatory cytokines; Social stress; Depression

Septic shock is one of the most common causes of death in intensive care units (Cheadle et al., 1996). It is generally accepted that this serious clinical condition is the result of excessive host inflammatory response to infection (Beishuizen et al., 1998). The most frequent trigger of septic shock is bacterial endotoxin, which drives the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) (Welbourn and Young, 1992). These cytokines then initiate a cascade of production of inflammatory mediators including other cytokines, complement fragments, eicosanoids, platelet-activating factor (PAF), nitric oxide (NO), endothelins, and adhesion molecules (Beishuizen et al., 1998). Consequently, a state of septic hemodynamic condition is developed that is characterized by hypotension, peripheral vasodilation, increased capillary permeability, aggregation of leukocytes, and diminished tissue perfusion. These changes ultimately lead to multi-organ failure (Purvis and Kirby, 1994).

One of the most important built-in mechanisms that limits tissue damage from hyper-inflammation is the integrated response to endotoxic shock by the nervous, immune, and endocrine systems (Sternberg et al., 1989). Via both neural and humoral pathways, peripheral IL-1 and TNFα activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in dramatic increases in plasma levels of glucocorticoids (Maier et al., 1998). Either adrenalectomy or hypophysectomy markedly sensitizes animals to endotoxin lethality, indicating a key role of the HPA axis in preventing endotoxic shock (Bertini et al., 1988). Injection

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of selective glucocorticoid receptor antagonists into animals also results in a heightened state of sensitivity to endotoxinemia (Hawes et al., 1992), whereas administration of glucocorticoids in animals challenged by endotoxin (Pitcairn et al., 1975), or in human with clinical sepsis, affords significant protection (Motsay et al., 1974). Furthermore, in rodent experiments, animal species that are relatively resistant to the effects of glucocorticoids are also more sensitive to endotoxic shock (Gadina et al., 1991). Thus, the actions of endogenous glucocorticoids seem essential for the survival of animals challenged by endotoxin. One may expect, therefore, that the susceptibility to septic shock might be reduced by psychosocial stress, which causes increased production of glucocorticoids (Sheridan et al., 1998).

Although many different types of stress induce the production of glucocorticoids and consequently immunosuppression (Sheridan et al., 1998), a surprising recent finding in our laboratory shows that social stress, unlike physical stress, triggers the development of a state of functional glucocorticoid resistance in spleen cells (Avitsur et al., 2001). In vitro analysis of spleen cells shows that the ability of glucocorticoids to suppress proliferation of mouse splenocytes in response to stimulation of bacterial endotoxin lipopolysaccharide (LPS) is significantly diminished if the animals were stressed by social disruption. It is possible that such SDR stress can alter the typical immune–neuroendocrine interaction such that the anti-inflammatory activities of glucocorticoids during endotoxic shock are rendered ineffective, thereby constituting a behavior-mediated susceptibility to endotoxinemia.

The objective of this study was to determine whether SDR stress increases the susceptibility of mice to endotoxic shock, and if so, what were the underlying neuroendocrine mechanisms for this enhanced susceptibility.

1. Methods and materials

1.1. Animals

Subjects were male C57BL/6 mice (Charles River, Wilmington, MA) aged 2–4 months and housed five per cage in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Mice were given free access to food and water and were maintained on a 12-h light/dark cycle (lights on at 6 AM). Intruders were singly housed male mice selected on the basis of pre-experimental screening of aggressive behavior. Screening entailed placing the intruder into the home cage of another mouse. Only intruders that initiated attacks within 5 min and showed consistent aggressive attacks towards the resident were used in these experiments. All animal care procedures were according to guidelines established by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care And Use Committee (ILACUC).

1.2. Social Disruption Stress (SDR)

Cages of five mice were randomly assigned into either control or SDR groups. Control mice remained undisturbed in their home cage (HC). HC and SDR mice within each experiment were the same age, arrived from the vendor at the same time and were housed under the same conditions. In each SDR cycle, an aggressive intruder was introduced into the subjects' home cage. The aggressor normally started to attack the cage residents within 5–10 min from the beginning of the session and the residents exhibited submissive responses to these attacks. If one or more of the residents attacked the intruder, the intruder was replaced with a new intruder. Typically, attacks lasted 20–30 s, after which the intruder rested for 1–2 min before attacking again. All SDR cycles began at 5 PM. Typically animals underwent 6 SDR cycles over a week: three nightly cycles, one night off, and three more cycles. The duration of 1 SDR cycle was 2 h. Different intruders were used on consecutive nights. In all the experiments presented here the subjects were the defeated residents. The response of the intruders to SDR was not studied.

1.2.1. Restraint stress (RST)

To compare the effects of SDR with another stressor, mice were also restraint stressed in well-ventilated 50 ml conical polypropylene tubes as described previously (Sheridan et al., 1991). They were restrained each day for a week during their active period each day (from 17:00 to 09:00). They did not have access to food and water during this time period. Therefore, similarly food- and water-deprived (FWD), but not restrained, mice were used as control animals.

1.2.2. LPS injection

To assess the survival of these animals to endotoxic shock, HC, SDR, FWD, and RST animals were injected with 40 mg/kg of LPS (*Escherichia coli*, serotype 055:B5) intraperitoneally (i.p.) at 9:00 am after the last day of the SDR or RST stress. They were monitored for 7 days in their home cage after the LPS injection. To compare histopathology, stressed and control animals received the LPS injection and were sacrificed at 12 h post-injection. Brain, spleen, lung, and liver were collected, fixed, and processed for the conventional H&E staining. To study the expression of pro-inflammatory cytokines, animals were sacrificed 3 h after they were injected with 40 mg/kg of LPS. Tissue samples from these animals were collected for analysis of mRNA and protein levels of IL-1 and TNFα.

1.2.3. Corticosterone sensitivity assay

On the morning after the last SDR cycle, spleens from 18 HC and 16 SDR animals were harvested to assess the responsiveness of the splenocytes to glucocorticoids. These animals did not receive LPS injection. Spleens from animals in the same group were pooled and harvested into ice-cold Hanks' balanced salt solution (HBSS) and mashed between glass slides to obtain single cell suspensions. Red blood cells were eliminated by adding 2 ml of lysis buffer (0.16M NH_4Cl, 10mM $KHCO_3$, 0.13 mM EDTA) for 2 min followed by one wash with HBSS/10% heat-inactivated fetal bovine serum (FBS). Each pellet was resuspended in HBSS, filtered through a sterile 70 μm nylon cell strainer to remove debris, and washed a final time in HBSS. Viable mononuclear cells were counted using trypan blue dye exclusion and samples were resuspended (2.5×10^6 cells/ml) in supplemented RPMI medium (10% heat-inactivated FBS, 0.075% sodium bicarbonate, 10 mM Hepes buffer, 100 U/ml penicillin G, 100 μg streptomycin sulfate, 1.5 mM L-glutamine, 0.00035% 2-mercaptoethanol). Lipopolysaccharide (LPS; Sigma L-2630) was added at a concentration of 1 μg/ml for mitogen stimulation. Aliquots from each cell suspension were treated with corticosterone (Sigma, C2505, 0–5 μM) diluted in a buffer of 0.2% ethanol in supplemented RPMI. Cell suspensions were added in triplicate to flat-bottom 96-well plates at a volume of 100 μl/well, and plates were incubated at 37°C and 5% CO_2. After 48 h the proliferation assay was performed.

1.2.4. Proliferation assay

The CellTiter 96 Aqueous non-radioactive proliferation assay kit was purchased from Promega (Madison, WI). The tetrazolium substrate solution was prepared according to the instructions, and 20 μl were added to each well of the 96-well plates. Living cells convert this substrate to formazan, producing a brown precipitate. The plates were incubated at 37°C and 5% CO_2 for 3 h and the resulting color changes were quantified by obtaining optical density readings at 490 nm on an ELISA plate reader. To account for differences in background activity of cells, the mean optical density (O.D.) of the three RPMI/FBS wells for a given treatment was subtracted from each of the corresponding LPS-stimulated values.

1.2.5. Assessment of corticosterone

Serum corticosterone was analyzed to compare the levels of corticosterone produced in HC and SDR animals in response to LPS injection. Blood was drawn from the retro-orbital capillary plexus 3 h after the LPS injection (40 mg/kg); serum was collected and stored at −70°C until assayed. Serum levels of corticosterone were quantified using the DA Rat Corticosterone radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA) according to manufacturer's instructions.

1.2.6. In situ hybridization analysis of HPA activity

To determine the effects of SDR and RST stress on the activity of HPA axis, stressed and control animals were sacrificed without receiving LPS injection. Spleen and brain were extracted and frozen by immersion in 2-methyl butane at −30°C, and stored at −70°C prior to sectioning. They were then cryostat-cut to 15-μm-thick sections and thaw-mounted onto pretreated adhesive slides (Superfrost Slides, Fisher Scientific), dried, and stored at −80°C until further processing. The in situ hybridization protocols were performed as described previously for ribonucleotide (cRNA) probes (Whitfield et al., 1990; Quan et al., 1997). First, tissue sections were processed by fixation with 4% formaldehyde solution, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine–HCl, pH 8.0 solution, dehydration with ethanol, and delipidation with chloroform. Second, the antisense probes directed against the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) were transcribed with appropriate RNA polymerase and α-^{35}S-UTP (specific activity >1000 Ci/mmol; New England Nuclear, Boston, MA). To control for the specificity of the probe, sense probes from these cDNAs were also generated. Radiolabeled probes were diluted in the riboprobe hybridization buffer and applied to brain and spleen sections (500,000 CPM/section). After overnight incubation at 55°C in a humidified chamber, slides containing brain sections were washed first in 20 μg/ml RNase solution and then in $2\times$ SSC and $0.2\times$ SSC (55°C and 60°C) solutions to reduce nonspecific binding of the probe. The slides were then dehydrated with ethanol and air-dried for autoradiography.

1.2.7. Autoradiography

Slides and ^{14}C plastic standards containing known amounts of radioactivity (American Radiochemicals, St. Louis, MO) were placed in X-ray cassettes, apposed to film (BioMax MR, Kodak, Rochester, NY) for 2–8 days (slides from the same experiment were exposed for the same amount of time) and developed in an automatic film developer (X-OMAT, Kodak).

1.2.8. Data analysis

Autoradiographic film images of brain sections and standards were digitized on a Macintosh computer-based image analysis system with IMAGE software (Wayne Rasband, Research Services Branch, National Institute of Mental Health). Light transmittance through the film was measured by outlining the structure on the TV monitor. A density-slice function was applied to each structure to select densities greater than film background and thus measured transmittance confined to the cellular sources of the radioactivity. The density so obtained was used to represent the relative amount of mRNA expression.

1.2.9. Realtime PCR analysis of IL-1β and TNFα mRNA expression

To measure the amounts of mRNA expression in the brain, spleen, lung, and liver, these tissues were removed from RST, FWD, SDR and HC animals at 3 h after they were injected with 1 mg/mouse of LPS. This time point was chosen because previous studies have shown that elevated expression of IL-1β and TNFα can both be observed after this dose of LPS injection (Givalois et al., 1994). These samples were processed with TRIzol (Gibco BRL) to extract both total RNA and protein from the tissue. The cDNAs were generated from total RNA by reverse transcription using random hexamer as primers. Realtime PCR was run for IL-1β and TNFα as we described previously using the 7700 Sequence Detector (PE/Applied Biosystems, Foster City, CA) (Fehniger et al., 1999). 18S Ribosomal RNA was run in the same PCR reaction to provide an internal control. The amount of mRNA was calculated using the conventional C_T method.

1.2.10. Elisa analysis of IL-1α and TNFα

Protein samples were analyzed by the conventional cytokine ELISA assay. Briefly, monoclonal coating antibodies for IL-1α (2 μg/ml, R&D) and TNFα (6 μg/ml, Pharmingen, San Diego, CA) were bound to enhanced protein binding 96-well plates (Corning). After overnight incubation at 4°C, excess antibody was removed and the wells were blocked with 10% FBS/PBS at room temperature for 2 h. Standards and samples were added and the plates were incubated overnight at 4°C. For detection of IL-1α, 100 μl of polyclonal rabbit anti-mouse IL-1α (Genzyme) was added at 1:500 and the plates were incubated for 1 h. This antibody was then detected with 1:500 goat anti-rabbit antibody conjugated with horse radish peroxidase (HRP). For TNFα detection, biotinylated rat anti-mouse TNFα monoclonal antibody was added at 4 μg/ml and the plates were further incubated for 1 h. After washing, avidin–horseradish peroxidase (Vector Laboratories) diluted 1:500 was added to the plates and incubated for 30 min. For both IL-1α and TNFα, ABTS substrate was added. Color was allowed to develop at room temperature. Optical density was read at 405 nm on a Cambridge Technology Model 7520 Microplate Reader.

2. Results

Fig. 1 shows the percent survival from two experiments. In the first experiment, the survival of FWD and RST animals were compared (Fig. 1, top). Ten percent of the FWD animals and 5% of the RST animals died within 24 h after injection of 40 mg/kg of LPS. Within 48 h, 30% of the FWD animals and 35% of RST animals died. The difference in survival between these two groups was not statistically different ($N = 20$ per group, $p > 0.05$, Chi-square test). All the animals survived if they did not die within the first 48 h after challenge. In the second experiment, HC and SDR animals were also injected with 40 mg/kg of LPS (Fig. 1, bottom). Ten percent of HC animals died within 24 h after the LPS injection and 35% of them died by 48 h. In the SDR group, there was 40% mortality within 24 h and 75% of the animals died by 48 h after the LPS injection. In both groups, animals that survived for 48 h after the LPS injection survived the LPS-induced shock. The differences in the mortality between SDR and HC groups at both 24 and 48 h after the LPS injection were statistically significant ($N = 20$ per group, $p < 0.05$, Chi-square test).

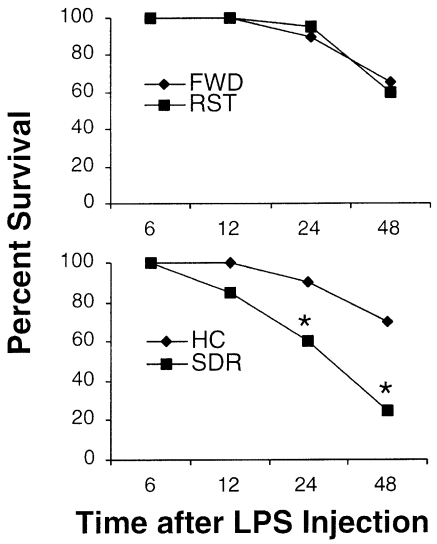

Fig. 1. Percent survival in C57BL/6 mice (FWD vs. RST, top; HC vs. SDR bottom) after they received intraperitoneal (i.p.) injection of 40 mg/kg of LPS.

In Fig. 2, H&E staining patterns of brain, lung, liver, and spleen of HC and SDR animals sacrificed at 12 h after the LPS injection are shown. In HC animals, no significant pathology was found in the brain (Fig. 2A) and liver (Fig. 2E) except occasional appearance of microthrombi indicating sparse disseminated intravascular coagulation (DIC). In the lung, there was widespread DIC (arrow in Fig. 2C) in HC animals, but most of the alveolar space was not obstructed. In the spleen, apparent proliferation of lymphoid cells characterized by lighter stained large cellular nuclei occurred around the blood vessels (within dashed oval in Fig. 2G). These histopathological patterns were also found in FWD and RST animals after LPS injection (data not shown). In contrast, more intense inflammatory pathology was found in SDR animals after LPS injection. In SDR brain, there was widespread DIC (arrow, Fig. 2B) together with infiltration of the meninges by leukocytes (arrowhead, Fig. 2B) indicating meningitis. In SDR lung (Fig. 2D), widespread DIC (arrow) was accompanied by hemorrhage (indicated by *) and sanguineous edema that blocked out at least 1/3 of the alveolar space. Apparent

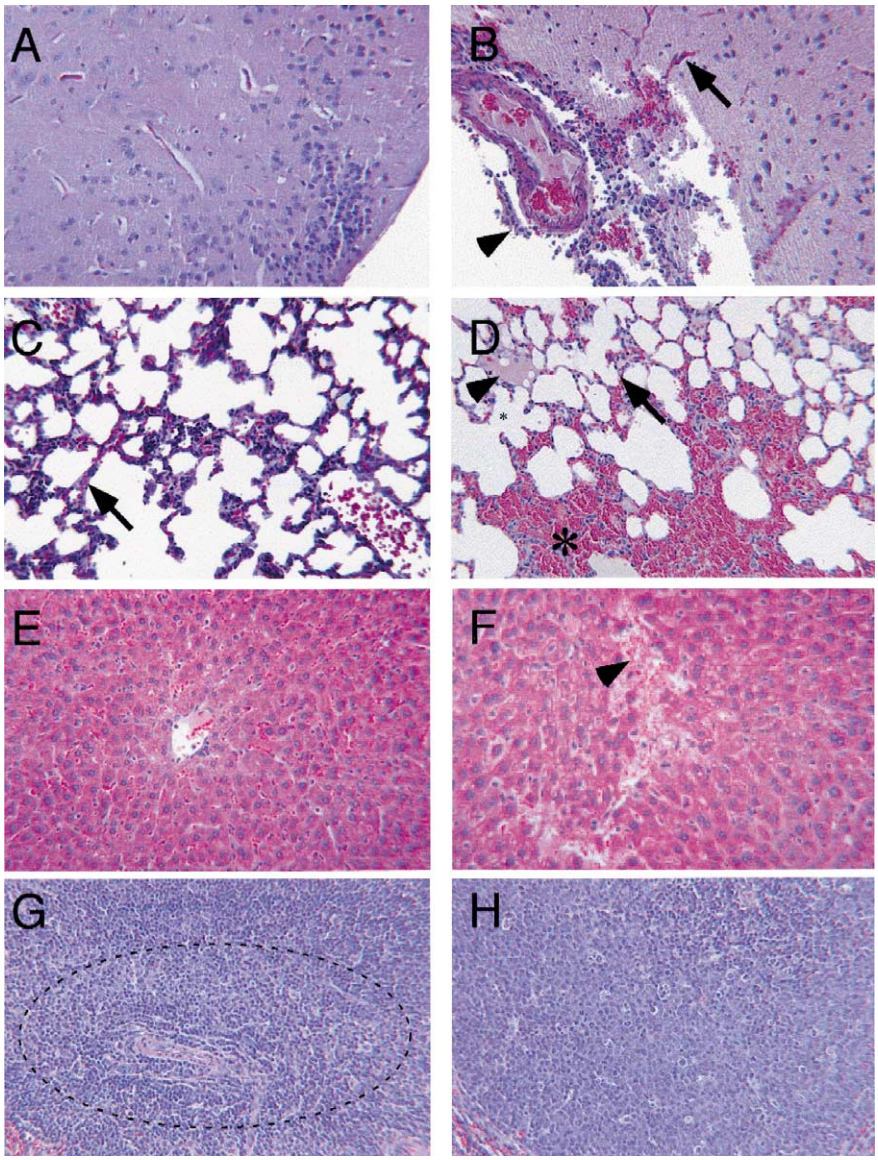

Fig. 2. Representative microphotographs show H&E staining of brain (A and B), lung (C and D), liver (E and F), and spleen (G and H) from HC (A, C, E, G) and SDR (B, D, F, H) animals at 12 h after the LPS injection. Arrows in (B–D) points to examples of disseminated intravascular coagulation (DIC). Arrowhead in (B) points to the site of meningitis. In (D), widespread DIC (arrow) was accompanied by hemorrhage (indicated by *) and sanguineous edema (arrowhead). Arrowhead in (F) points to necrosis in the liver. In (G), the area of apparent proliferation of lymphoid cells characterized by lighter stained large cellular nuclei around the blood vessels was encircled by the dashed oval.

hepatic necrosis was found in SDR animals (arrowhead, Fig. 2F) and proliferating spleen cells were evident throughout the entire spleen (Fig. 2H).

Fig. 3 shows the responsiveness of splenocytes from HC and SDR animals to glucocorticoids in vitro. LPS-induced proliferation of splenocytes from HC animals (Control) was dose-dependently suppressed by corticosterone whereas the proliferation of splenocytes from SDR animals resisted the effects of corticosterone.

Serum levels of corticosterone measured from HC and SDR animals after LPS injection are shown in Fig. 4. High levels of corticosterone were detected in both HC (620 ± 33 ng/ml) and SDR (756 ± 71 ng/ml) animals. These levels were not statistically different.

The expression of glucocorticoid receptor mRNA in the spleen and brain is shown in Fig. 5. HC, SDR, FWD, and RST animals without receiving LPS injection were studied. Because there was no difference between HC and FWD control animals, only the results from HC group are shown. Expression of GR was detectable in both the spleen and brain of HC animals (Fig. 5A–B). GR expression was found throughout the brain with the highest expression levels in the CA1 and CA2 regions of the hippocampus and in dentate gyrus. Very little GR expression was found

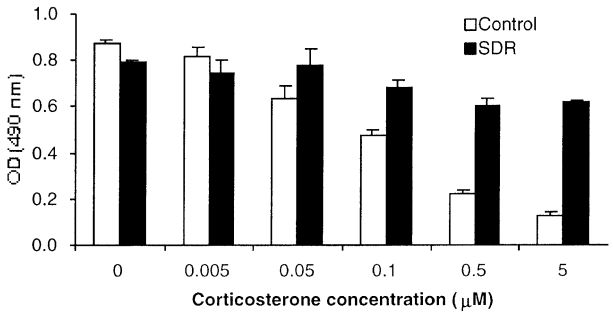

Fig. 3. Effects of SDR on the responsiveness of splenocytes to corticosterone. Optical densities (O.D. measurement of cell viability) of LPS-stimulated splenocytes treated with increasing concentrations of corticosterone are shown. Data is presented as mean ± SD from triplicate cultures.

in the CA3 region of the hippocampus. In the spleen, the highest expression of GR was found in the cell-rich white pulp. RST stress reduced the size of the spleen by about 40%, resulting in a smaller size of the cross-section (Fig. 5C) when compared to the spleen from HC animals (Fig. 5A). No significant change in GR expression was found in either spleen (Fig. 5C) or brain (Fig. 5D) of RST animals. In contrast, significant reduction of GR mRNA expression was found in the spleen (Fig. 5) and brain (Fig. 5F) of SDR animals. Quantitative analysis showed that there was a $70 \pm 14\%$ ($N = 5$, $p < 0.05$ by t-test) reduction in GR mRNA expression in the spleen (A vs. E) and a $42 \pm 7\%$ ($N = 6$, $p < 0.05$ by t-test) reduction of GR mRNA expression in the hippocampus of SDR animals (B vs. F).

Fig. 6 shows the levels of IL-1β and TNFα mRNA expression in brain, lung, liver, and spleen at 3 h after LPS injection from HC (control) and SDR animals. The relative quantities of mRNAs for TNF-α and IL-1β were determined by the signal intensity obtained at a given cycle of Realtime PCR where the PCR amplification was logarithmically linear. Significantly higher levels of TNF-α and IL-1β were found in all of the organs tested in SDR animals after the LPS injection. The largest increases of IL-1β and TNF-α mRNA levels were found in the lung and spleen. IL-1β and TNFα mRNA levels obtained from

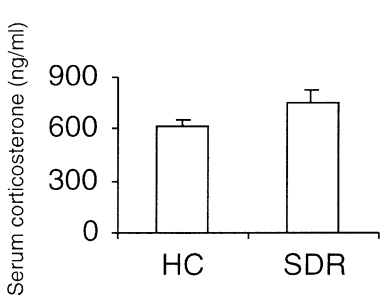

Fig. 4. Levels of serum corticosterone 3 h after LPS (40 mg/kg) injection from HC and SDR animals. Means and standard errors are presented ($N = 10$).

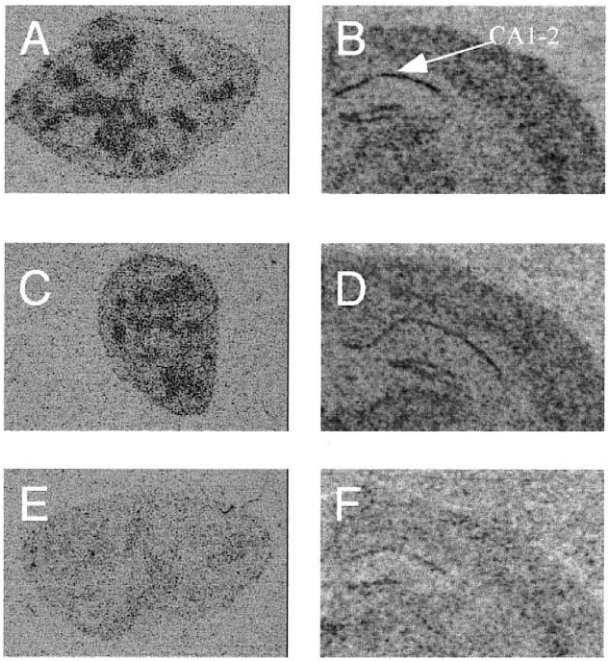

Fig. 5. Representative autoradiographs show GR mRNA labeling in the spleen (A, C, E) and brain (B, D, F) from HC (A and B), RST (C and D), and SDR (E and F) animals.

FWD and RST animals were not different from those obtained from HC animals (data not shown).

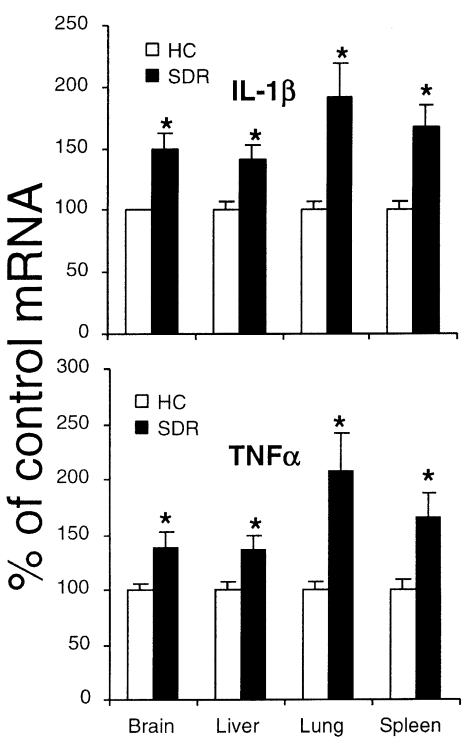

Fig. 6. Levels of IL-1β (top) and TNFα (bottom) mRNA in the brain, liver, lung, and spleen of HC and SDR animals at 3 h after the LPS injection. Means and standard errors are shown. * $p < 0.05$.

Compared with HC animals (control), significantly increased protein expression of IL-1α (Fig. 7, top) and TNF-α (Fig. 7, bottom), expressed as nanograms (ng) of cytokine per gram (g) of tissue sample, was also detected in spleen and liver.

Expression patterns of AVP and CRF in PVN are shown in Fig. 8. Expression of CRF was found in the PVN whereas AVP expression was found in both supraoptic nuclei (SON) and PVN. Again, the HC and FWD animals were not different, so only the data from HC group is shown (AVP: Fig. 8A; CRF: Fig. 8B). Both restraint (Fig. 8C–D) and SDR stress (Fig. 8E–F) induced increased expression of AVP and CRF in the PVN. AVP expression was also apparent in the SON. Densitometrical analysis revealed that there was a $87 \pm 12\%$ ($n = 5$, $p < 0.05$) and a $73 \pm 11\%$ ($n = 5$, $p < 0.05$) increase in the AVP mRNA expression over the control value in restraint and SDR stressed animals, respectively. There was a $114 \pm 12\%$ ($p < 0.05$) increase in the CRF expression in restraint-stressed animals, but only $50 \pm 7\%$ ($p < 0.05$) increase in SDR animals. Therefore, SDR stress appears to have caused a unique alteration in the production of the ACTH secretagogue, favoring the production of AVP. This is further substantiated after we exposed AVP and CRH hybridization slides to the same autoradiograph film and analyzed the change in the ratio of AVP/CRH. The ratios of AVP/CRH in HC, RST, and SDR animals were 4.2 ± 0.4, 3.8 ± 0.3, and 6.9 ± 0.6, respectively. The increase in the ratio of AVP/CRH mRNA labeling in the SDR animals over those in HC and RST animals was statistically significant ($p < 0.05$, *t*-test).

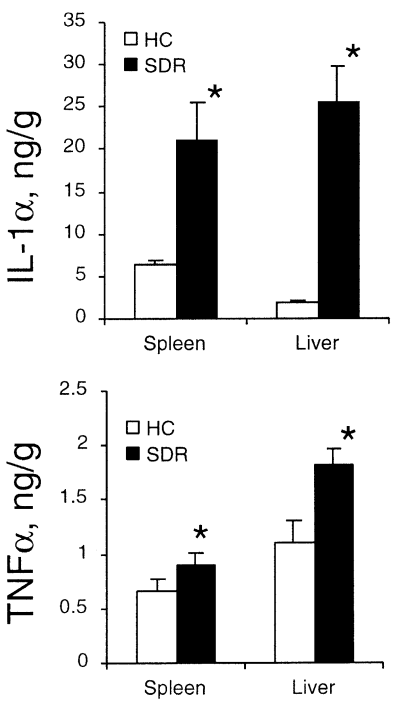

Fig. 7. Levels of IL-1α (top) and TNFα (bottom) protein in the spleen and liver of HC and SDR animals at 3 h after the LPS injection. Means and standard errors are shown. * $p < 0.05$.

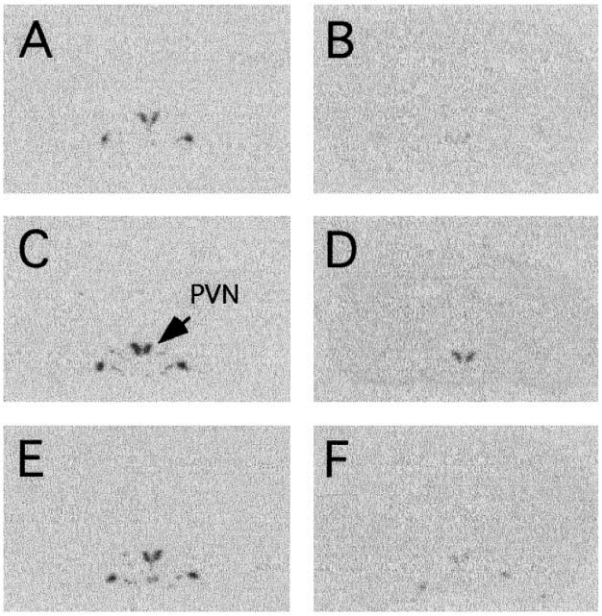

Fig. 8. Representative autoradiographs show AVP (A, C, E) and CRH (B, D, F) mRNA labeling in the PVN of HC (A and B), RST (C and D), and SDR (E and F) animals.

3. Discussion

The results of the present study show that stress caused by social disruption (SDR), but not by physical restraint, increased the mortality in mice when they were subsequently challenged by a septic dose of LPS. The increased susceptibility to endotoxinemia was associated with increased inflammatory histopathology found in brain, lung, liver, and spleen. SDR stress resulted in reduced expression of GR mRNA in the spleen and brain and induced functional glucocorticoid resistance in the splenocytes. The ability of SDR animals to produce glucocorticoids after LPS challenge, on the other hand, was not attenuated. In addition, SDR stress caused a shift in the mRNA expression of AVP and CRH in the brain, resulting in an increase in the ratio of AVP/CRH. Injection of LPS into SDR animals caused elevated IL-1β and TNFα mRNA expression in all of the above-mentioned organs compared with LPS-injected control animals. Significantly increased protein expression of IL-1α and TNFα was also detected in SDR animals. Taken together, these findings suggest that SDR stress causes reduced function of glucocorticoids. This in turn caused increased production and activity of pro-inflammatory cytokines, when SDR animals were challenged by LPS, rendering them susceptible to endotoxic shock.

In a previous study, Capitanio et al. (1998) reported that social stress shortened the survival of SIV-infected mon-

keys. They postulated the altered glucocorticoid regulation in these monkeys may have significantly contributed to their mortality. It is possible, therefore, that the development of glucocorticoid resistance in socially stressed animals can also worsen the health outcome after viral infection.

The dose of injected LPS, 40 mg/kg, did not cause high mortality in FWD (30%) and HC (35%) groups of animals. Previous reports have shown that i.p. injections of 10–30 mg/kg of LPS can induce 50–90% mortality in mice(Smith et al., 1993) (Roth et al., 1998). It is likely that the serotype of LPS used in the present study had relatively weaker bioactivity compare to the other serotypes of LPS used in the previous studies. We chose this dose to examine whether the effect of this threshold dose of LPS may be augmented by prior stress.

Significantly increased mortality was found in SDR animals after the LPS injection. This increased mortality was not likely due to increased production of the stress hormone corticosterone. Studies in our laboratory have shown that SDR and restraint stress induced comparable levels of corticosterone in the plasma (R. Avitsur, and J.F. Sheridan, personal communication). In the present study, the mortality of restraint stressed animals after LPS injection was not found to be different from the FWD controls. In addition, HC and SDR animals produced the similar amounts of corticosterone after the LPS injection.

The production of glucocorticoids may actually be beneficial against endotoxic shock. Thus, previous studies have shown that endotoxic shock invariably induces the production of glucocorticoids which serve to limit inflammatory tissue injury by suppressing the synthesis and activity of pro-inflammatory cytokines (Bertini et al., 1988). Treatment with glucocorticoids or their synthetic agonists have also been shown to reduce the mortality rate in mice challenged with septic doses of LPS (Ferluga et al., 1979). One would expect that a stressful event that leads to increased glucocorticoids should improve the likelihood of animals surviving LPS challenge. Increased mortality in LPS-injected SDR animals, therefore, was unexpected.

A possible mechanism for this unexpected finding is that, unique to SDR stress, the glucocorticoid responsiveness has been altered. We have found in the present study that splenocytes isolated from SDR animals exhibited a state of functional glucocorticoid resistance in culture, i.e., LPS-stimulated proliferation in these cells is not suppressed by the addition of corticosterone. In addition, we found that in the spleens of SDR animals there was a reduced expression of GR mRNA in situ and increased lymphoid hyperplasia after LPS injection, suggesting that a state of glucocorticoid resistance existed in SDR animals in vivo.

GR mRNA expression was also reduced in the hippocampus which is one of the most important neural structures responsible for the negative feedback action of glucocorticoids (De Kloet et al., 1998). It is not clear, however, to what extent the feedback activity of glucocorticoids was impaired in the brain of SDR animals because the expression of MR was not altered (data not shown). MR is the high affinity glucocorticoid receptor that is also involved in the glucocorticoid feedback action. Another change observed in the central nervous system of SDR animals was the shift from the balanced AVP and CRH production towards a more dominant AVP production. It is known that the ACTH stimulatory activity of AVP is less sensitive to the glucocorticoid feedback inhibition than that of CRH and that AVP is more sensitive to chronic stress (Bilezikjian et al., 1987). The increased ratio of AVP/CRH production in SDR animals, therefore, suggests a reduced sensitivity of the HPA axis to glucocorticoid regulation, but an enhanced sensitivity to chronic stress. The decreased GR expression and increased ratio of AVP/CRH expression resembles the conditions for some human patients with major depression (Scott and Dinan, 1998). Interestingly, these patients are also likely to exhibit profound glucocorticoid insensitivity in their peripheral immune cells (Miller et al., 1999).

One of the functional consequences of glucocorticoid insensitivity in peripheral immune cells is that the ability of glucocorticoids to inhibit the production and activity of pro-inflammatory cytokines will be diminished. In the present study, we found that LPS-induced mRNA expression of IL-1β and TNFα was increased in spleen, liver, lung, and brain of SDR animals compared with control animals. In addition, LPS-induced protein expression of IL-1α and TNFα was also enhanced in the spleen and liver of SDR animals. These results, therefore, demonstrate that the production of pro-inflammatory cytokines in the SDR animals after LPS challenge was indeed exaggerated.

Histopathological changes observed in the present study suggest that increased production and activity of pro-inflammatory cytokines is likely to be a major cause for the increased tissue damage in SDR animals after LPS challenge. Thus, in SDR animals, increased intravascular coagulation was observed in the lung and brain, and increased necrosis was found in the liver. There was also increased hemorrhage in the lung in these animals. These changes resemble those produced in animals that received injections of septic doses of TNFα (Tracey et al., 1986; Ohnishi et al., 1989). Most critical in the multiple organ failure induced by endotoxic shock is the lung. Bleeding and intravascular coagulation in the lung can significantly impair the organism's oxygen intake which will result in poor oxygen perfusion in other organs, thereby setting forth a chain reaction, together with local inflammation, that leads to the failure of other organs. The activity of pro-inflammatory cytokines in the brain has been implicated in the recruitment of leukocytes into the brain during bacterial meningitis (Glimaker et al., 1993). In the present study, meningitis was only found in the LPS-injected SDR animals. It is likely that increased production and activity

of pro-inflammatory cytokines detected in the brain of SDR animals significantly contributed to the development of meningitis in this model. Because increased inflammatory histopathology was observed in all of the organs examined in SDR animals, increased mortality of these animals may be the net result of enhanced inflammation in all of these organs.

The state of glucocorticoid resistance in SDR animals could have been further exacerbated by the LPS challenge. Injection of LPS has been found to reduce GR binding affinity in vivo (Liu et al., 1993) and treatment with pro-inflammatory cytokines inhibit the binding of GR to its ligand in vitro (Verheggen et al., 1996). A recent report by Pariante et al. demonstrated that IL-1α reduces the function of GR by preventing the translocation of GR from cytoplasm to the nucleus (Pariante et al., 1999). Whether LPS injection and SDR stress synergistically decrease the function of GR remains to be elucidated.

The findings of the present study may have important implications for stress-induced glucocorticoid resistance in humans. A relevant situation may be seen in patients with major depression. These patients often exhibit high levels of plasma glucocorticoids without apparent Cushing's syndrome (clinical signs of glucocorticoid excess) (Miller et al., 1999). Glucocorticoid resistance has also been found in many of these patients. It is intriguing to speculate that chronic social stress in these patients might have contributed to their development of glucocorticoid resistance and these patients may have increased susceptibility to inflammatory diseases including septic shock.

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