Immunomodulatory effects of dehydroepiandrosterone in proestrus female mice after trauma-hemorrhage

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Knöferl, Markus W., Martin K. Angele, Robert A. Catania, Michael D. DiDato, Kirby I. Bland, and Irshad H. Chaudry. Immunomodulatory effects of dehydroepiandrosterone (DHEA) after trauma-hemorrhage in proestrus female mice after trauma-hemorrhage. Studies indicate that administration of DHEA in proestrus female mice after trauma-hemorrhage would deteriorate immune responses. The aim of our study, therefore, was to determine whether administration of DHEA in proestrus female mice after trauma-hemorrhage would improve immune responses. Proestrus female C3H/HeN mice (age 7–8 wk) were subjected to laparotomy (i.e., soft tissue trauma induced) and hemorrhagic shock (35 ± 5 mmHg for 90 min) or sham operation. The mice then received DHEA (100 μg/25 g body wt) or vehicle subcutaneously followed by fluid resuscitation (4x the shed blood volume). Plasma IL-6, splenocyte proliferation, splenocyte IL-2, IL-3, IFN-γ, IL-10 release, and splenic Mφ IL-1β, IL-6, IL-10, and IL-12 release were determined 24 h after TH. Plasma IL-6 levels were significantly increased in vehicle-treated females, and DHEA administration markedly attenuated this response. In vehicle-treated females, splenocyte proliferation, IL-2, IL-3, and IFN-γ release, and splenic Mφ IL-1β, IL-6, and IL-12 release were maintained or slightly enhanced after TH. In DHEA-treated females, however, these immune functional parameters were either unaltered compared with vehicle-treated animals or even further enhanced, but surprisingly were not depressed. Moreover, DHEA reduced splenocyte and splenic Mφ anti-inflammatory cytokine (i.e., IL-10) production after TH compared with vehicle-treated females. Because DHEA further enhances the immune responsiveness in proestrus females after TH, this hormone might be a useful adjunct even in females for further enhancing immune responses and decreasing the mortality rate after trauma and severe blood loss.

immunomodulation; cytokines; gender

IT IS WELL RECOGNIZED that the inflammatory response to trauma-hemorrhage exhibits a gender-specific pattern, and sex hormones have been identified to be responsible for this phenomenon. Although a profound depression of immune functional parameters is observed in males after trauma-hemorrhage, females in the proestrus state of the estrus cycle with elevated levels of female sex hormones have unaltered or enhanced immune functions under those conditions (28). Because the adverse effects of trauma-hemorrhage on immune functions are closely related to the hormonal environment in the host, the use of immunomodulatory hormones in males is considered a valuable therapeutic adjunct (28).

It is well known that dehydroepiandrosterone (DHEA), the most abundant steroid hormone in humans (27), is an intermediate in the pathway for the synthesis of both androgen and estrogen. In this regard, estrogen administration has been shown to have immunomodulatory effects under various physiological and pathological conditions (15). Recently, studies in animal models have shown that DHEA is effective in restoring immune functions after thermal injury as well as sepsis (4, 5, 22, 23). Furthermore, DHEA administration normalized splenocyte apoptosis and lymphocyte migration (23) after hemorrhagic shock, restored the depressed cell-mediated immune responses after trauma-hemorrhage, and significantly reduced the mortality rates from a subsequent septic challenge (3, 8). It is of interest to note that in those studies male animals were used to examine the effects of exogenous hormone administration. However, depending on the hormonal milieu, both androgenic and estrogenic effects of DHEA have been reported (9). Studies have also shown that administration of 17β-estradiol in male animals after trauma-hemorrhage had beneficial immunomodulatory effects (14). If DHEA is converted to estrogens in male animals, the increased concentrations of estrogen would be expected to further enhance/improve immune responses after trauma-hemorrhage. Conversely, in female animals, the conversion of DHEA to testosterone should have immunodepressive effects. This notion is supported by data from studies that showed that pretreatment of proestrus female animals with testosterone before the onset of trauma-
hemorrhage led to marked immune depression (1). In view of this, it could be postulated that the administration of DHEA in female animals after trauma-hemorrhage will have deleterious effects on immune responses since DHEA will be metabolized to testosterone, a hormone with immunosuppressive properties. The aim of the present study, therefore, was to determine whether administration of DHEA in female animals in the proestrus state of the estrus cycle has any deleterious effects on immune functional parameters after trauma-hemorrhage.

METHODS AND MATERIALS

Animals. Inbred female C3H/HeN mice (Charles River Laboratories, Wilmington, MA) 7–8 wk of age were used in this study (n = 6–7 mice/group). All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The experiments described in this study were conducted while the authors were at Rhode Island Hospital. The Institutional Animal Care and Use Committee of Rhode Island Hospital and Brown University approved the project.

Experimental groups. The state of the estrus cycle was determined daily in each female animal by light microscopic examination of vaginal smear cytology for at least 1 day before the experiment. In the morning of the experiment, female mice were selected in the proestrus state of the estrus cycle by typical cytology and then randomly assigned to the treatment or vehicle groups. Proestrus mice underwent trauma-hemorrhage or sham operation. After sham operation, the mice received a subcutaneous injection of vehicle (propylene glycol, Sigma Chemical, St. Louis, MO). Immediately before fluid resuscitation, the mice in the trauma-hemorrhage group received a subcutaneous injection of DHEA (4 mg/kg body wt, Sigma Chemical) or vehicle. This dose and route of DHEA administration have been previously found to be effective in restoring immune functional parameters after trauma-hemorrhage (3, 8).

Trauma-hemorrhage procedure. Mice in the trauma-hemorrhage groups were lightly anesthetized with methoxyflurane (Metofane, Pitman Moore, Mundelein, IL) and restrained in a supine position, and a 2.5-cm midline laparotomy (i.e., soft tissue trauma induced) was performed, which was then closed aseptically in two layers by using 6-0 Ethilon sutures (Ethicon, Somerville, NJ). Both femoral arteries were then aseptically cannulated with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ) by using a minimal dissection technique, and the animals were allowed to awaken. Blood pressure was constantly monitored by attaching one of the catheters to a blood pressure analyzer (Micro-Med, Louisville, KY). Lidocaine was applied to the incision sites to provide analgesia during the study period. On awakening, the animals were bled rapidly through the other catheter to a mean arterial blood pressure of 35 ± 5 mmHg (mean arterial blood pressure prehemorrhage was 95 ± 5 mmHg), which was maintained for 90 min. At the end of that procedure, the animals were resuscitated with four times the shed blood volume in the form of lactated Ringer solution. The catheters were then removed, the vessels ligated, and the groin incisions closed. Sham-operated animals underwent the same surgical procedure, which included ligation of both femoral arteries, but neither hemorrhage nor fluid resuscitation was carried out. There was no mortality observed in this model of trauma-hemorrhage (26).

Blood, tissue, and cell harvesting procedure. The animals were killed by methoxyflurane overdose at 24 h after trauma-hemorrhage to obtain the spleen and whole blood.

Plasma collection and storage. Whole blood was obtained by cardiac puncture and placed in microcentrifuge tubes (Microtainer, Becton Dickinson, Rutherford, NJ). The tubes were then centrifuged at 16,000 g for 15 min at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored at −80°C until assayed.

Cell line maintenance. The IL-2-dependent CTLL-2 cells and the IFN-γ-dependent RAW 264.7 cells were obtained from the American Type Culture Collection and maintained according to their directions. The IL-3-dependent FDC-P1 cells (a gift from Dr. Charles Janeway, Yale University, New Haven, CN) were maintained as previously described (10). The IL-6-sensitive murine B-cell hybridoma (7TD1) (a gift from Dr. Jacques Van Snick, The Ludwig Institute for Cancer Research, Brussels, Belgium) was maintained as previously described (10).

Preparation of splenocyte culture. At 24 h after trauma-hemorrhage or sham operation, the spleens were removed aseptically, and splenocytes were isolated as previously described in detail (29). In brief, the spleens were gently ground between frosted microscope slides to produce a single-cell suspension. This suspension was centrifuged at 300 g for 15 min. After resuspension, the erythrocytes were lysed hypotonically, and the remaining cells were washed with PBS by centrifugation (300 g for 15 min). Viability was tested by using trypan blue exclusion and found to be ~95% irrespective of the group assessed. The splenocytes were then resuspended in RPMI1640 (GIBCO-BRL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO-BRL) to yield a final concentration of 1 × 10^6 cells/ml. The ability of the splenocyte cultures to produce lymphokines in response to a mitogenic challenge, was assessed by incubation for 48 h (at 37°C, 5% CO₂, and 90% humidity) in the presence of 2.5 μg/ml Concanavalin A (Pharmacia/LKB Biotech, Piscataway, NJ). After incubation, the cell suspension was centrifuged at 300 g for 15 min, and the supernatants were harvested and stored at −80°C until assayed.

Splenocyte proliferation. The cells’ ability to proliferate in response to mitogenic stimulation with 0 (negative control) or 2.5 μg/ml Concanavalin A was measured by3[H]thymidine incorporation technique as previously described (19, 26).

Preparation of splenocyte macrophage culture. Splenic macrophage cultures were established as previously described in detail (30). The monolayers of splenic macrophages (1 × 10^6 cells/ml) were stimulated with 10 μg/ml Concanavalin A (Pharmacia/LKB Biotech) to yield a final concentration of 1 × 10^6 cells/ml. The capacity of the mixed splenocyte culture to produce IL-2 (CTLL-2), IL-3 (FDC-P1), and IFN-γ (RAW 264.7) was assessed by determining the amount of respective lymphokines in the collected culture supernatant by using specific bioassays as previously described in detail (16, 20). IL-6 activity was determined by assessing the 72-h proliferation of the IL-6-dependent murine hybridoma 7TD1 cells stimulated by serial dilutions of plasma or supernatants as described in detail elsewhere (6, 21). Concentrations of IL-18 (Duoset, Genzyme, Cambridge,
MA), IL-10, and IL-12 (BD OptEIA ELISA Set, BD Phar-mingen, San Diego, CA) in macrophage and splenocyte supernatants were determined by using a sandwich-enzyme-linked immunoabsorbent assay technique (ELISA) according to the manufacturer’s recommendations.

**Determination of plasma hormone concentrations.** Concentrations of 17ß-estradiol, testosterone, and DHEA were determined by using commercially available radioimmunoassays (ICN Biomedicals, Costa Mesa, CA), as described by the manufacturer.

**Statistical analysis.** The results are presented as means ± SE. One-way ANOVA followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was used to determine the significance of the differences between experimental means. A P value of <0.05 was considered significant.

**RESULTS**

**Plasma concentrations of IL-6.** At 24 h after trauma-hemorrhage, plasma concentrations of IL-6 were markedly increased in vehicle-treated proestrus females compared with the corresponding shams. Administration of DHEA significantly attenuated the increase in plasma IL-6 concentrations (P < 0.05; Fig. 1).

**Plasma hormone levels.** At 24 h after administration of DHEA, no significant effects on circulating concentrations of DHEA were detectable (Fig. 2A). Plasma concentrations of 17ß-estradiol decreased after trauma-hemorrhage, and administration of DHEA had no significant effects on 17ß-estradiol levels (Fig. 2B). Likewise, plasma testosterone concentrations were decreased in animals that underwent trauma-hemorrhage, and administration of DHEA did not increase plasma levels of testosterone (Fig. 2C).

**Splenocyte proliferation.** At 24 h after trauma-hemorrhage, splenocyte proliferative capacity was unaltered in proestrus females, irrespective of whether the animals received vehicle (64.964 ± 16.431 counts/min) or DHEA (59.195 ± 16.339 counts/min) during resuscitation (sham group: 65.754 ± 12.868 counts/min).

**Splenocyte T helper 1 and 2 cytokine release.** Although splenocyte IL-2 release was slightly elevated in vehicle-treated female mice after trauma-hemorrhage, DHEA administration led to a further increase in IL-2 release that did not reach statistical significance (Fig. 3A). Comparable to IL-2, the release of IL-3 was increased (P > 0.05) in vehicle-treated female mice after trauma-hemorrhage, and treatment with DHEA led to a significantly increased splenocyte IL-3 release in proestrus female mice (P < 0.05; Fig. 3B). In proestrus female mice, trauma-hemorrhage as well as DHEA administration had no effect on IFN-γ release. DHEA administration significantly reduced anti-inflamma-
tory, i.e., IL-10, release in proestrus female mice after trauma-hemorrhage ($P < 0.05$; Fig. 4A).

**Splenic macrophage responses.** In vehicle-treated proestrus female mice, there was a trend toward increased splenic macrophage release of IL-18, IL-6, and IL-12, which was even more pronounced in DHEA-treated female mice ($P > 0.05$; Fig. 5). In contrast to proinflammatory cytokine release, splenic macrophage anti-inflammatory cytokine (i.e., IL-10) release decreased in female mice that received DHEA during resuscitation. This decrease, however, did not reach statistical significance (Fig. 4B).

**DISCUSSION**

Studies demonstrate that DHEA treatment in male mice after trauma-hemorrhage improved immune responses (3, 8). DHEA is an intermediate in the pathway for the synthesis of testosterone, 5α-dihydrotestosterone, and estrogen, and, depending on the hormonal milieu, both androgenic and estrogenic effects of DHEA have been reported (9). In this respect, testosterone has been shown to exhibit immunosuppressive properties in male and female mice (1, 2). Thus we postulated that the prevailing hormonal milieu in female mice might lead to conversion of DHEA into testosterone, thereby exerting deleterious effects on immune responses. To test this hypothesis, female mice in the proestrus state of the estrus cycle were treated with DHEA after trauma-hemorrhage. Because our laboratory’s previous studies (28) have shown that female mice in this state exhibit maintained/enhanced immune responses after trauma-hemorrhage, DHEA could be immunosuppressive under those conditions.

Our results indicate that plasma concentrations of IL-6 significantly increased in vehicle-treated proestrus female mice after trauma-hemorrhage. Adminis-
DHEA administration significantly reduced the increase in circulating IL-6, suggesting an attenuation of the inflammatory response to trauma-hemorrhage. However, in view of the fact that stimulated in vitro IL-6 production by splenic macrophages was further increased in DHEA-treated female mice, it appears that cell populations other than splenic macrophages are susceptible to functional modulation by DHEA. In this regard, the effects of DHEA on Kupffer cell function should be further investigated, because these liver tissue-fixed macrophages have been previously shown to play a pivotal role in the elaboration of systemic cytokine levels after trauma-hemorrhage (24).

The data further indicate that vehicle-treated proestrus female mice showed no depression in splenocyte immune functions after trauma-hemorrhage. Interestingly, administration of DHEA further enhanced splenocyte responses after trauma-hemorrhage. These findings are in sharp contrast to our hypothesis, which was that in female mice DHEA would be converted to androgenic intermediates and cause deleterious effects on immune responses. Comparable to splenocytes, splenic macrophage function was unaltered in vehicle-treated proestrus female mice after trauma-hemorrhage, and administration of DHEA had no adverse effects on splenic macrophage function. In both splenocytes and splenic macrophages, DHEA administration led to decreased anti-inflammatory cytokine (i.e., IL-10) production after trauma-hemorrhage. In this regard, an increased IL-10 release in male mice has been shown to contribute to the immunosuppression after trauma-hemorrhage (7). The decreased release of anti-inflammatory cytokines in DHEA-treated mice after trauma-hemorrhage might thus contribute to the enhanced immune responses in those animals. Whether the improved immune response in DHEA-treated female mice after trauma-hemorrhage is associated with an increased survival rate after the induction of subsequent sepsis remains to be determined.

The analysis of circulating hormone concentrations at 24 h after trauma-hemorrhage or sham operation revealed no significant changes in DHEA plasma levels between animals receiving vehicle or DHEA. Furthermore, plasma concentrations of 17β-estradiol and testosterone were decreased in female mice that underwent trauma-hemorrhage compared with sham-operated female mice, and DHEA administration had no significant effect on hormonal status at that time point studied. It could therefore be suggested that dilutionary effects due to resuscitation with Ringer lactate after trauma-hemorrhage masked the increase in circulating DHEA in proestrus female mice. It is also possible that DHEA concentrations were significantly elevated at an earlier time after trauma-hemorrhage. The findings that neither 17β-estradiol nor testosterone levels were significantly increased in DHEA-treated mice compared with animals receiving vehicle are in line with the results of Jarrar et al. (11). In that study, a higher dose of DHEA (30 mg/kg body wt) was used compared with the dose used in this study, and the authors found an ~17- to 19-fold increase in the plasma levels of DHEA at 24 h after its administration with no effect on 17β-estradiol or testosterone concentrations. In view of this, it could be postulated that DHEA modulates immune functions primarily by direct effects. Support for the notion that DHEA has actions independent of the conversion to androgens comes from studies that have shown that DHEA produces salutary effects on immune functions in androgen-unresponsive mice (27). However, because DHEA can be metabolized at the cellular level in peripheral tissues (18), one cannot rule out alterations in the

Fig. 5. IL-1β (A), IL-6 (B), and IL-12 (B) release of splenic macrophages harvested from proestrus female C3H/HeN mice at 24 h after trauma-hemorrhage or sham operation. Animals received DHEA (4 mg/kg body wt) or vehicle at the beginning of resuscitation. Splenic macrophages were cultured in the presence of 10 µg/ml lipopolysaccharide W for 48 h, and IL-1β and IL-12 concentrations in supernatants were determined by using sandwich-enzyme-linked immunosorbent assay technique. IL-6 levels were measured by using a specific bioassay (7TD1). Values are means ± SE of 6–7 animals/group.
site-specific hormonal micro-milieu under those conditions. It appears, however, that, in female mice, the conversion of DHEA to androgenic steroids plays a minor role in regulating posttrauma immune functions. Alternatively, the female sex hormones present in the proestrus state of the estrus cycle might be sufficient to preserve immune responses despite newly developed testosterone. In addition, it is possible that in proestrus females DHEA acts synergistically with other hormones to further stimulate/prevent immune responses after trauma-hemorrhage. In this regard, female rodents in the proestrus state of the estrus cycle have elevated concentrations of the immunomodulatory hormones such as prolactin, estrogen, and progesterone (25). If DHEA acts via estrogen receptors, it is possible that upregulation of estrogen receptors because of elevated estrogen levels during the proestrus phase may also contribute to the immunomodulatory effects of DHEA. In this regard, recent studies have suggested that DHEA can also directly bind to and activate the estrogen receptor (8, 11). Support for an important role of estrogen receptors comes from studies by Catania et al. (8). The in vitro stimulatory effects of DHEA on splenocyte proliferative capacity were blocked in the presence of the estrogen-receptor antagonist tamoxifen; however, addition of the androgen-receptor antagonist flutamide had no inhibitory effects (8). Furthermore, it has been shown that the salutary effects of DHEA administration on cardiovascular and hepatocellular function after trauma-hemorrhage were abolished in the presence of the estrogen receptor-antagonist ICI-182,780 (11). Thus it appears that the beneficial effects of DHEA on immune, cardiac, and hepatocellular functions in male rodents after trauma-hemorrhage are, in part, mediated via the estrogen receptor. Support for the pivotal role of estrogen receptors for immunoprotection after trauma-hemorrhage comes from studies that indicate that in vivo administration of a selective estrogen-receptor antagonist in proestrus female mice significantly depressed immune responsiveness (17). Additional studies are required to further elucidate the precise mechanisms involved in mediating the beneficial immunomodulatory effects of DHEA in proestrus female mice after trauma-hemorrhage.

Although our findings and previous data indicate that immune responses are not compromised in young proestrus female mice, it has been shown that ovariec-tomized female mice as well as aged female mice with decreased circulating levels of female sex steroids have depressed immune functional parameters after trauma-hemorrhage (12, 13). However, administration of 17β-estradiol after trauma-hemorrhage normalized cell-mediated immune responses in ovariec-tomized female mice (15). Because the adverse immune response to trauma is closely related to the hormonal environment in the host, the use of immunomodulatory hormones in female mice with lowered hormone levels or hormonal deficiencies appears a valuable therapeutic strategy. In view of the known salutary immunomodulatory properties of DHEA in male animals and of our findings that exogenous DHEA had stimulatory effects on immune functions even in proestrus female mice after trauma-hemorrhage, it could be proposed that this hormone should be considered as a potential therapeutic adjunct for use in patients with known hormonal deficiency, such as postmenopausal or postovariectomy patients.

In summary, the data presented indicate that administration of DHEA in proestrus female mice after trauma-hemorrhage further improved splenocyte and splenic macrophage function compared with vehicle-treated animals. Therefore, it appears that DHEA has beneficial immunomodulatory effects in female mice despite the possibility of its potential metabolism toward immunosuppressive androgen. Thus DHEA represents a safe steroid hormone in males and females for improving the depressed immune responses after trauma-hemorrhage and for decreasing the mortality rates from subsequent sepsis.

REFERENCES


