Androgen-mediated modulation of macrophage function after trauma-hemorrhage: central role of 5α-dihydrotestosterone


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Androgen-mediated modulation of macrophage function after trauma-hemorrhage: central role of 5α-dihydrotestosterone. J Appl Physiol 95: 104–112, 2003. First published March 28, 2003; 10.1152/japplphysiol.00182.2003.—Androgens have been implicated as the causative factor for the postinjury immune dysfunction in males; however, it remains unknown whether androgens directly affect macrophages. To study this, male mice were sham operated or subjected to trauma (i.e., midline laparotomy) and hemorrhagic shock (mean arterial pressure, 30 ± 5 mmHg for 90 min and then resuscitated). The mice received the 5α-reductase inhibitor 4-hydroxyandrostenedione (4-OHA) before resuscitation. Plasma TNF-α, IL-6, and IL-10 levels were elevated after trauma-hemorrhage and normalized by 4-OHA. TNF-α and IL-6 production by splenic macrophages was decreased after injury, whereas Kupffer cell production of these mediators was enhanced. 4-OHA normalized cytokine production. Androgens suppressed cytokine production by splenic macrophages from hemorrhaged mice, whereas it enhanced TNF-α and IL-6 production by Kupffer cells. The addition of 4-OHA in vitro normalized cytokine production by cells treated with testosterone, but it had no effect on dihydrotestosterone-treated cells. These results indicate that androgens directly affect macrophage function in males after trauma and hemorrhagic shock and that the intracellular conversion of testosterone to dihydrotestosterone is of particular importance in mediating the androgen-induced effects.

Inflammation; immune dysfunction; gender; Kupffer cells; cytokine

Numerous clinical and epidemiological studies indicate that males are more susceptible to the lethal effects of sepsis compared with females (29, 31). In addition, experimental studies indicate that proestrus females maintain immune function after trauma-hemorrhage, whereas males do not (3). The maintained immune functional capacity of females under such conditions also correlates with a decreased mortality from subsequent sepsis (16). Studies also indicate that, after trauma-hemorrhage, the immunoprotected state in proestrus females is estrogen mediated, whereas the immunosuppressed state in males is androgen mediated (3). Thus gender dimorphism in the immune response to trauma, hemorrhage, and sepsis, which is supported by both clinical and experimental findings, appears to be mediated by sex steroids. Sex steroid-mediated regulation of immune function is also supported by other studies (38). In particular, the most potent androgen, 5α-dihydrotestosterone (DHT), has been shown to suppress cytokine release by murine T cells and alter their cytokine profile (4, 13, 24, 30, 52). Whereas several studies have demonstrated the presence of androgen receptors in human and murine immune cells (8, 13, 28), recent findings also indicate that immune cells have the ability to directly metabolize sex steroids (44, 51).

A number of steroidogenic enzymes, 5α-reductase, aromatase, 3β-hydroxysteroid dehydrogenase, and 17β-hydroxysteroid dehydrogenase, participate in the biosynthesis of testosterone, its active metabolite DHT, and 17β-estradiol in tissues other than the gonads and adrenal gland (23, 25, 26, 58). Studies have shown that 4-hydroxyandrostenedione (4-OHA) is a potent inhibitor of 5α-reductase activity, which is the enzyme responsible for the conversion of testosterone to its active metabolite, DHT (35, 59). Furthermore, recent studies by Samy et al. (42) indicate that trauma-hemorrhage increases 5α-reductase activity in the spleen and splenic T cells, which is immunosuppressive due to the increased intracrine synthesis of DHT. Macrophages represent the first line of defense in host resistance and play a central role in the regulation of the immune system. Macrophage functional capacity is significantly altered after traumatic injuries and hemorrhagic shock (49). Previous findings indicate that macrophage functions are regulated by male sex steroids (2, 12, 33), that these immune cells express androgen receptors (10, 27, 40) and can metabolize steroid hormones (45), and that gender dimorphism in their responsiveness exists after trauma-hemorrhage (3). The present study was designed to elucidate the mechanism(s) by which androgens modulate macrophage function after trauma-hemorrhage.
MATERIALS AND METHODS

Animals. Inbred male C3H/HeN mice (Charles River Laboratories, Wilmington, MA), 8–10 wk old (23–26 g body wt), were used for this study. 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. This study was conducted at Rhode Island Hospital and Brown University and approved by their Institutional Animals Care and Use Committee.

Male mice were randomized into four groups (7–8 mice/group). Group 1 and 2 mice underwent sham operation, whereas group 3 and 4 mice were subjected to trauma-hemorrhage. Groups 1 and 3 received a 0.1-ml subcutaneous corn oil injection (hereafter referred to as vehicle) after the experimental procedure. Groups 2 and 4 received a subcutaneous injection of 4-OHA (4-androsten-4-ol-3, 17-dione; Steraloids, Newport, RI) dissolved in corn oil (5 mg/kg body wt) just before resuscitation. The dosage regime of 4-OHA employed was based on previous in vitro studies, the treatment regime for patients with prostatic and breast cancer, and previous work by our laboratory (17, 32, 46). No deleterious side effects of subcutaneous 4-OHA administrations at this dose were observed in the present study.

Trauma-hemorrhage procedure. Mice were lightly anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL) and restrained in a supine position. A 2.5-cm midline laparotomy (i.e., induction of soft tissue trauma) was performed, and the abdominal incision was closed aseptically in two layers by using 6-0 sutures (Ethilon, Ethicon, Somerville, NJ). Both femoral arteries were then aseptically cannulated with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ) by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique. Heparin (porcine intestine heparin; Elkins-Sinn, Cherry Hill, NJ), 2 mg/kg body wt, was administered by using a minimal dissection technique.

In vivo treatment with testosterone, DHT, and 4-OHA. Testosterone and DHT were purchased from Sigma Chemical, and 4-OHA was purchased from Steraloids. A stock solution (1 mM) of testosterone and DHT was prepared in ethanol and diluted to appropriate molarity in complete phenol red-free RPMI-1640 media containing 10% charcoal-dextran-treated FCS, 1 μg/ml gentamycin, 50 U/ml penicillin-G, and 50 μg/ml streptomycin (GIBCO, Grand Island, NY) and placed at 107 cells/ml onto 12-well plates. After incubation for 2 h (37°C, 95% humidity, and 5% CO2), nonadherent cells were removed by washing with warm PBS.

Kupffer cells were obtained as previously described by Ayala et al. (5). In brief, the peritoneal cavity was opened aseptically, the portal vein was exposed and catheterized with a 27-gauge needle attached, and the vena cava inferior was nicked. The liver was then blanched to remove blood by perfusion of 20-ml ice cold HBSS without Ca2+/Mg2+ (GIBCO). This was immediately followed by perfusion of 10 ml of 0.075% collagenase class IV (Sigma Chemical, St. Louis, MO) in HBSS without Ca2+/Mg2+ at 37°C. The liver was removed (ed blue) and transferred to a petri dish containing ice-cold 0.075% collagenase in HBSS. After incubation at 37°C for 10 min, cell suspension was then passed through a sterile 150-mesh stainless steel screen into a 150-ml beaker containing 10-ml ice cold HBSS, and the cell suspension was washed twice by centrifugation at 400 g for 15 min with HBSS. The cells were then resuspended in Click's medium containing 10% FBS, 1 μg/ml gentamycin, 50 U/ml penicillin-G, and 50 μg/ml streptomycin (GIBCO), layered over 16% metrizamide (Accurate Chemical, Westbury, NY) in HBSS, and centrifuged at 1,500 g for 45 min. After removal of the nonparenchymal cells from the interface, the cells were washed twice again by centrifugation at 400 g for 15 min with complete Click's and plated in a 12-well plate at a cell density of 106 cells/ml. After 40-h incubation (37°C, 95% humidity, and 5% CO2), nonadherent cells were removed by gentle washing with complete Click's medium.

Viability of splenic macrophages and Kupffer cells was consistently >95%, as determined by Trypan blue exclusion, regardless of whether or not the cells were derived from hemorrhage or sham animals. Previous studies from our laboratory demonstrated that these protocols provide adherent cell populations that were >95% positive by nonspecific esterase staining and exhibit typical macrophage morphology (6).

Splenic macrophages and Kupffer cells were isolated from mice treated in vivo with either 4-OHA or vehicle 2 h after trauma-hemorrhage and resuscitation or sham operation. Cells were resuspended in complete RPMI-1640 and stimulated with 10 μg/ml LPS (Sigma Chemical) for 24 h, and supernatants were collected as described above.

In vitro treatment with testosterone, DHT, and 4-OHA. Testosterone and DHT were purchased from Sigma Chemical, and 4-OHA was purchased from Steraloids. A stock solution (1 mM) of testosterone and DHT was prepared in ethanol and diluted to appropriate molarity in complete phenol red-free RPMI-1640 media containing 10% charcoal-dextran-treated FCS, 1 μg/ml gentamycin, 50 U/ml penicillin-G, and 50 μg/ml streptomycin (GIBCO). The 1 mM stock solution of 4-OHA was prepared by dissolving 4-OHA in DMSO and diluting to appropriate molarity in complete phenol red-free RPMI-1640 media. The carried over ethanol and DMSO did not exceed 0.025% in the media. Splenic macrophages and Kupffer cells were isolated from untreated animals directly after trauma-hemorrhage and resuscitation or sham operation, resuspended in complete phenol red-free RPMI-1640 media, and incubated with either 250 nM testos-
terone or DHT with or without 4-OHA (100 nM) for 2 h (37°C, 95% humidity, and 5% CO₂). A concentration of 250 nM testosterone or DHT was chosen, based on previously reported plasma testosterone and DHT levels in the literature (28). Two hours thereafter, 10 μg/ml LPS (Sigma Chemical) were added to the cultures, and all cell cultures were incubated for a further 24-h period (37°C, 95% humidity, and 5% CO₂). At the end of the incubation, cell-free supernatants were harvested and stored at −80°C until assayed.

Assessment of macrophage cytokine productive capacity. TNF-α, IL-6, and IL-10 levels in cell-free splenic macrophage and Kupffer cell supernatants were determined by ELISA technique, according to the manufacturer’s recommendation (Pharmingen). In certain experiments, IL-6 levels in the cell-free supernatants prepared from macrophages treated with vehicle or 4-OHA in vivo were determined by assessing the proliferation of the IL-6-dependent murine hybridoma 7TD1, as previously described (54). In brief, 7TD1 cells were cultured with serial dilutions of plasma for 72 h. During the 4 h of culture, 20 μl of a 3-(4,5-dimethylthiazol-2-L)/2,5-diphenyltetrazolium-bromide solution (5 mg/ml in RPMI-1640, Sigma Chemical) were added to each well. The amount of dark blue formazan crystal formation was then measured spectrophotometrically. The units of IL-6 activity were determined by comparison of curves produced from dilutions of a recombinant mouse IL-6 standard (200 U/ml, Genzyme, Cambridge, MA).

RT-PCR analysis of cytokine gene expression. Total RNA was prepared from splenic macrophages and Kupffer cells by using denaturing solution (Clonetech, Palo Alto, CA) followed by three rounds of phenol-chloroform and chloroform-isooamyl alcohol (Sigma Chemical) extraction (9). RNA was precipitated with isopropanol and washed with 80% ethanol. After air drying, the pellet was resuspended in diethyl pyrocarbonate-treated water and stored at −80°C before undergoing PCR.

One microgram of RNA was reverse transcribed in 20-μl reaction volume containing 1× RT reaction buffer (50 mM Tris·HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂), 0.5 mM dNTP, 20 units RNase inhibitor, 20 μM oligo(dT)18 primer, and 200 units RT (Clonetech). The RT reaction was incubated at 42°C, 95% humidity, and 5% CO₂). A concentration of 250 nM testosterone or DHT with or without 4-OHA (100 nM) for 2 h formed with the use of “Robocycler” (Stratagene, Torrey Pines, CA) in thin-walled 0.2-ml PCR tubes (Stratagene), according to the following protocol: first cycle of 95°C (3 min), 60°C (2 min), and 72°C (3 min) followed by 35 cycles of 95°C (40 s), 60°C (1 min), and 72°C (1 min). A final 5-min incubation was used to “polish” the DNA termini. Resulting amplicons (TNF-α 239 bp, IL-6 638 bp, IL-10 455 bp, and G3PDH 983 bp) were resolved on ethidium bromide-stained (0.5 μg/ml) 1.8% Tris-boric acid-EDTA-agarose gels and photographed with the use of a gel documentation system (Chemilmager, Alpha Inotech, San Leandro, CA). The amount of PCR products generated by the target gene and the control gene (G3PDH) was measured using by a Chemi-Imager System (Alpha Inotech, Palo Alto, CA), and the amount of the target in percentage of the control gene was calculated. The results of typical experiments, repeated a minimum of three times, are presented.

Statistical analysis. Data are presented as means ± SE of seven to eight animals in each group. One-way ANOVA and Tukey’s test were employed to determine the significance of the differences between experimental means. P < 0.05 was considered significant for all statistical analysis.

RESULTS

Effect of 4-OHA treatment on plasma steroid and cytokine levels. Trauma-hemorrhage induced a significant increase in the plasma levels of the androgen precursor androstenedione, whereas testosterone and 17β-estradiol were significantly decreased in the vehicle group compared with sham animals (Table 1). Plasma levels of DHT were not altered under those conditions. In vivo treatment with 4-OHA before resuscitation significantly increased plasma androstenedione level in sham animals, whereas no change occurred in the plasma levels of testosterone, DHT, and 17β-estradiol. Animals subjected to trauma-hemorrhage and treated with 4-OHA had significantly increased plasma androstenedione levels compared with hemorrhaged mice receiving no treatment. Plasma levels of testosterone and DHT remained unchanged in hemorrhaged mice treated with 4-OHA compared with untreated hemorrhaged mice.

At 2 h after trauma-hemorrhage, plasma levels for TNF-α, IL-6, and IL-10 were significantly elevated compared with those for sham-vehicle animals (Fig. 1, A–C, respectively). Treatment with 4-OHA did not alter cytokine plasma levels in sham animals, but it

| Table 1. Plasma steroid levels at 2 h after trauma-hemorrhage or sham operation

| Plasma Steroid, pg/10 mg plasma protein |
|---|---|---|---|
| Androstenedione | Testosterone | DHT | 17β-Estradiol |
| Sham | 10.60 ± 1.60 | 38.40 ± 10.50 | 1.10 ± 0.50 | 2.20 ± 0.30 |
| Hemorrhage | 40.30 ± 11.70 | 5.40 ± 5.90 | 0.70 ± 0.20 | 0.04 ± 0.01 |
| Sham + 4-OHA | 102.90 ± 19.60 | 41.50 ± 9.90 | 1.80 ± 0.10 | 3.20 ± 0.60 |
| Hemorrhage + 4-OHA | 73.80 ± 10.80 | 31.00 ± 8.80 | 1.00 ± 0.30 | 0.09 ± 0.04 |

Values are means ± SE; n = 8/group. Plasma levels are shown of androstenedione, testosterone, 5α-dihydrotestosterone (DHT), and 17β-estradiol at 2 h after trauma-hemorrhage and resuscitation (Hemorrhage) or sham operation and treated with either vehicle or 4-hydroxyandrostenedione (4-OHA) (5 mg/kg body weight) at the time of resuscitation. Steroid levels were determined by radioimmunoassay procedure. ANOVA, P < 0.05 vs. *Sham, †Hemorrhage, and ‡Sham + 4-OHA.
normalized plasma TNF-α levels and significantly attenuated the increase in plasma IL-6 in animals subjected to trauma-hemorrhage (Fig. 1, A and B, respectively). Conversely, administration of 4-OHA at the time of resuscitation further increased the plasma IL-10 level compared with that in untreated animals (Fig. 1C).

Effect of 4-OHA in vivo treatment on macrophage cytokine production and gene expression. The ability of splenic macrophages to release TNF-α and IL-6 was significantly reduced, whereas the release of IL-10 increased in vehicle-treated animals after trauma-hemorrhage (Fig. 2, A, C, and E, respectively). In contrast, Kupffer cell capability to produce all three cytokines was significantly upregulated under those conditions (Fig. 2, B, D, and F, respectively). Treatment of mice with 4-OHA after trauma-hemorrhage normalized in vitro cytokine productive capacity in both macrophage populations, and the levels for all three cytokines were comparable to those seen in sham-operated animals.

The results presented in Fig. 3 show that TNF-α, IL-6, and IL-10 mRNA expression in vehicle-treated splenic macrophages and Kupffer cells after trauma-hemorrhage were significantly increased compared with that in cells from sham mice. In vivo treatment with 4-OHA before resuscitation normalized TNF-α and IL-6 mRNA expression to sham levels. IL-10 mRNA expression by both macrophage populations increased after treatment with 4-OHA in sham animals. Conversely, accumulation of IL-10 mRNA decreased in splenic macrophages and further increased in Kupffer cells from trauma-hemorrhaged mice treated with 4-OHA compared with that of their respective sham groups.

Effect of in vitro 4-OHA on macrophage cytokine productive capacity. The addition of testosterone to the macrophage cultures had no effect on cytokine production in either sham cell population; however, cells from hemorrhaged mice had suppressed IL-10 production under such culture conditions (Fig. 4). In contrast to testosterone, the addition of DHT to the in vitro cultures suppressed TNF-α production by splenic macrophages from sham mice (Fig. 4A) and increased TNF-α and IL-6 production by Kupffer cells from sham mice (Fig. 4, B and D, respectively). Similar to testosterone, DHT suppressed splenic macrophage and Kupffer cell IL-10 release in the hemorrhage group (Fig. 4, E and F, respectively).

To determine whether the intracellular conversion of testosterone to DHT by 5α-reductase was responsible for the effects of testosterone on cytokine production in the hemorrhage group, 4-OHA was added to the macrophage cultures along with testosterone. The in vitro addition of 4-OHA was able to restore the cytokine productive capacity for all three cytokines by splenic macrophages after trauma-hemorrhage, but it had no effect on Kupffer cell cytokine production under those conditions (Fig. 4). 4-OHA, added together with testosterone, was able to reverse the trauma-induced changes in cytokine releases to sham levels in both macrophage populations. In contrast, the addition of 4-OHA, together with DHT, did not affect trauma-induced alterations in the cytokine by either splenic macrophages or Kupffer cells.

DISCUSSION

Clinical studies showed that males are at an increased risk for developing septic complications after traumatic injury and have increased mortality compared with females (37, 47). The host’s response and outcome after sepsis is, in large part, immune mediated. In this regard, a sexual dimorphism in the immune system of females and males is well established, and the differences between female and male immune responses under normal as well as pathological condi-

Fig. 1. Plasma levels of TNF-α (A), IL-6 (B), and IL-10 (C). Mice were treated with either vehicle or 4-hydroxyandrostenedione (4-OHA; 5 mg/kg body wt) after trauma-hemorrhage (hemorrhage) or sham operation, and plasma was collected 2 h after resuscitation. TNF-α, IL-6, and IL-10 levels were determined by ELISA. Values are means ± SE; n = 8/group. ANOVA, *P < 0.05 vs. Sham and †Hemorrhage/Vehicle.
tions are generally attributed to the influence of estrogens, progestins, and androgens (3, 14, 38, 53, 55). Nonetheless, limited information has been reported on the specific mechanisms of action of steroids on immune cells. The suppressive effects of male sex hormones on immune functions have been observed in a wide variety of disease processes and appear to be testosterone mediated (48). With regard to trauma-hemorrhage, testosterone depletion, by precastration or administration of the androgen receptor antagonist flutamide, after trauma-hemorrhage, has been shown to prevent the depression in cell-mediated immunity observed in males (3). Furthermore, our laboratory has recently demonstrated that treatment with 4-OHA after trauma-hemorrhage restored T-cell function and decreased susceptibility to a subsequent septic challenge (46). 4-OHA, which was classically considered an aromatase inhibitor, has been shown to prevent testosterone-induced thymic atrophy in males and mature female rats (20, 21). The observed effect of 4-OHA under such conditions is due to the ability of 4-OHA to also inhibit 5α-reductase activity, the enzyme responsible for the conversion of testosterone to DHT (15, 45).

Treatment of injured mice with 4-OHA after hemorrhage prevented the increase in plasma TNF-α and markedly attenuated the increase in IL-6 levels observed in vehicle-treated mice after trauma-hemorrhage. Previous findings indicate that suppression of TNF-α levels after hemorrhagic shock has beneficial effects on immune function (49). Moreover, Remick and coworkers (41) have shown that elevated IL-6 levels after injury correlate with poor outcome. Thus the salutary effects of 4-OHA may be related to prevention of the systemic proinflammatory response after trauma-hemorrhage. Our findings here are also consistent with previous findings that 4-OHA treatment improved survival in a two-hit model of trauma-hemorrhage and sepsis (46). The increased systemic IL-10 levels observed after trauma-hemorrhage were unaffected by 4-OHA, suggesting that the regulation of this cytokine may be androgen independent. Studies have shown that traumatic injury alters circulating concentrations of gonadal steroids (3, 18, 22). Consistent with these findings, we observed in vehicle-treated mice a significant decrease in plasma testosterone after trauma-hemorrhage, possibly due to increased 5α-reduction.

![Fig. 2. LPS-stimulated production of TNF-α (A and B), IL-6 (C and D), and IL-10 (E and F) by splenic macrophages (A, C, and E) and Kupffer cells (B, D, and F). Mice were treated with either vehicle or 4-OHA (5 mg/kg body wt) after trauma-hemorrhage or sham operation, and splenic macrophages and Kupffer cells were isolated 2 h after resuscitation. The cells were cultured in complete media of 10 μg/ml LPS for 24 h. TNF-α and IL-10 levels were determined by ELISA. In this series of experiments, IL-6 levels were measured by specific bioassay. Values are means ± SE; n = 6–8/group. ANOVA, *P < 0.05 vs. Sham.](image-url)
ductase activity (42). Treatment with 4-OHA prevented this decrease in systemic testosterone levels under such conditions, indicating inhibition of 5α-reductase activity leading to accumulation of substrate (i.e., testosterone). The testosterone precursor androstendione increased significantly in the plasma after 4-OHA administrations in the sham and the trauma-hemorrhage groups. This is due to the fact that 4-OHA is an androstendione derivative. Interestingly, extraction of plasma DHT, which represents <5% of the total circulating testosterone, was not altered after trauma-hemorrhage. The fact that reciprocal systemic changes in the 5α-reductase product DHT were not observed may be due to tissue uptake and degradation of this potent androgen. Whereas both DHT and testosterone are established ligands for the androgen receptor, the receptor binding affinity of DHT is sixfold higher than that of testosterone, and DHT transcriptional activity is also greater and more prolonged than that from testosterone (42, 58).

The monocyte/macrophage system represents the first line of defense in the host resistance and plays a central role in the regulation of the immune response. The existence of this macrophage heterogeneity has been demonstrated after trauma-hemorrhage, as decreased proinflammatory cytokine productive capacity by splenic macrophages and increased productive capacity by Kupffer cells have been observed (5, 49). Previous findings have demonstrated a depressed capacity of splenic, peritoneal, and alveolar macrophages to release IL-1, IL-6, and TNF-α after hemorrhage (36, 57). Furthermore, the depression of IL-6 release by splenic and peritoneal macrophages persists for up to 5 days after trauma and hemorrhage (57). Interestingly, in contrast to splenic and peritoneal macrophages, Kupffer cells have been shown to have an enhanced
capacity to produce proinflammatory cytokines, (i.e., IL-1, IL-6, and TNF-α) during the first 24 h after hemorrhage (5). Several studies emphasize the importance of Kupffer cells in the proinflammatory response after hemorrhagic shock (1, 5, 6, 39). Kupffer cell depletion significantly reduced plasma IL-6 levels after hemorrhage, thereby implicating these cells as the major source of in vivo IL-6 after hemorrhage (39). It appears that gut-derived mediators contribute to the enhanced proinflammatory cytokine release by Kupffer cells after hemorrhage. This concept is supported by findings that splenic immune responses were maintained in animals in which a portocaval shunt (which diverts blood flow from the gut to the lungs, thus bypassing the liver) had been established before hemorrhage (7). In vivo treatment with 4-OHA after trauma-hemorrhage normalized both splenic and Kupffer cell TNF-α, IL-6, and IL-10 productive capacity. Furthermore, this effect of 4-OHA appeared to be genomic, because mRNA expression for these cytokines was not upregulated in 4-OHA-treated animals after trauma-hemorrhage. IL-10 represents a potent downregulator of proinflammatory cytokines that acts via receptor-dependent activation of the STAT3/SOCS3 signal transduction pathway (34, 54). It is possible that the upregulation of IL-10 expression in macrophages from vehicle treated after trauma-hemorrhage represents an autocrine mechanism by which the proinflammatory response is dampened.

Our in vitro results clearly indicated that DHT represents the androgen with the most potent action on macrophage cytokine productive capacity. In vitro treatment of both splenic macrophages and Kupffer cells with 4-OHA was able to modulate the effects of testosterone in the culture media, but not the effects of DHT. This finding indicates that the immunomodulatory effects of testosterone on macrophage function after trauma-hemorrhage are mediated via the 5α-reductase-dependent conversion of testosterone to DHT. In this regard, Cutolo et al. (11) made a similar observation with primary cultures of human synovial macrophages, showing an increased formation of DHT after short-term exposure with 3H-testosterone. In addition, Schmidt et al. (45) showed that macrophages are capable of converting dehydroepiandrosterone to downstream steroid hormones, providing evidence of intrinsic sex steroid synthesis by these immune cells. With regard to trauma-hemorrhage, Samy et al. (42) have demonstrated that splenic lymphocytes are capable of intrinsic sex steroid synthesis and specifically that 5α-reductase activity in cells from hemorrhaged male animals is significantly increased. Our observa-

Fig. 4. LPS-stimulated production of TNF-α (A and B), IL-6 (C and D), and IL-10 (E and F) by splenic macrophages (A, C, and E) and Kupffer cells (B, D, and F) isolated immediately after trauma-hemorrhage and resuscitation or sham operation. Cells were cultured in phenol red-free RPMI-1640 complete media containing 10% charcoal-dextran-treated FCS in the presence or absence of 250 nM testosterone (Testos), 250 nM 5α-dihydrotestosterone (DHT), 100 nM 4-OHA, and 10 μg/ml LPS for 24 h. Values are means ± SE; n = 6–8/group. ANOVA, P < 0.05 vs. *Sham and †control.
tions here are consistent with these and indicate that macrophage sex steroid synthesis is also significantly altered after trauma-hemorrhage. Studies have shown that the 5α-reductase gene is differentially regulated by testosterone in androgen-responsive vs. nonresponsive tissue. Moreover, the response of the 5α-reductase genes to DHT represents a feed-forward regulation in which the product of the enzyme was positively affecting the expression of its gene (19).

A potential limitation of the present study is that only the early response after trauma-hemorrhage was determined (i.e., 0–2 h after resuscitation). However, previous findings by Stephan et al. (50) have shown that the immune dysfunction observed after hemorrhage persists for at least 5 days. Xu et al. (56) have also shown that splenic macrophage functions are suppressed at 7–10 days after trauma-hemorrhage, similar to that in the present study. Based on these previous findings, it is likely that the effects of 4-OHA on macrophage function would be similar at later times (i.e., 5–10 days) after hemorrhagic shock.

In summary, the results of this study provide evidence to support the concept that androgens directly regulate macrophage functions after trauma and hemorrhagic shock. Both Kupffer cells and splenic macrophages possess intrinsic sex steroid synthesis capacity, because 4-OHA directly inhibited the effects of testosterone, but not DHT, on cytokine production. Moreover, inhibition of 5α-reductase enzyme activity, which converts testosterone to DHT, restored in vitro as well as in vivo macrophage immune responses after traumatic injury, indicating that DHT represents the crucial androgen that regulates macrophage cytokine release after traumatic injury.

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