HOST RESPONSE TO INFECTION

Androstenediol and dehydroepiandrosterone protect mice against lethal bacterial infections and lipopolysaccharide toxicity

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The protective effects of the hormones androstenediol (androstene-3 β , 17 β -diol; AED) and dehydroepiandrosterone (5-androsten-3 β -ol-17-one; DHEA) on the pathophysiology of two lethal bacterial infections and endotoxin shock were examined. The infections included a gram-positive organism (*Enterococcus faecalis*) and a gram-negative organism (*Pseudomonas aeruginosa*). Both hormones protected mice from the lethal bacterial infections and from lipopolysaccharide (LPS) challenge. Treatment of animals lethally infected with *P. aeruginosa* with DHEA resulted in a 43% protection whereas treatment with AED gave a 67% protection. Both hormones also protected completely animals infected with an LD50 dose of *E. faecalis*. Similarly, the 88% mortality rate seen in LPS challenge was reduced to 17% and 8.5%, by treatment with DHEA and AED, respectively. The protective influences of both steroids were shown not to be directly antibacterial, but primarily an indirect antitoxin reaction. DHEA appears to mediate its protective effect by a mechanism that blocks the toxin-induced production of pathophysiological levels of tumour necrosis factor- α (TNF- α) and interleukin-1. AED usually had greater protective effects than DHEA; however, the AED effect was independent of TNF- α suppression, both *in vivo* and *in vitro*. The data suggest that both DHEA and AED may have a role in the neuro-endocrine regulation of antibacterial immune resistance.

Introduction

Androstenediol (androstene-3 β 17 β -diol; AED) and dehydroepiandrosterone (5-androsten-3 β -ol-17-one; DHEA) have been shown to exert an influence upon the immune system that results in a generalised and non-specific increase of host resistance against infections. Several models of acute virus infection have been used to determine the influence of both AED and DHEA on the pathophysiology of infection. The results of these studies have illustrated protection against six different human RNA and DNA viruses including West Nile, Sindbis and Semliki Forest [1–3], coxsackievirus B4, herpes simplex type 2 [4–6] and influenza A virus [7]. These studies led to the conclusion that administration of AED or DHEA up-regulated host immunity and reduced the virulence of

both RNA or DNA viruses that are lethal by widely different pathogenic mechanisms [8].

Because of their immunoregulatory influences, it was pertinent to examine the effects of these hormones on two models of lethal bacterial infection. In particular, the aim was to investigate the influence of AED and DHEA on both a gram-positive infection (*Enterococcus faecalis*) and a gram-negative infection (*Pseudomonas aeruginosa*). These two organisms are known for their resistance to antibiotics and high incidence of infection in diabetics and cancer patients [9, 10]. Therefore, the development of novel antibacterial strategies may provide new approaches to treating opportunist infections caused by resistant organisms.

Although gram-positive and gram-negative bacteria differ in their structural proteins and respective toxins, certain aspects of the pathophysiology of disease are comparable [10–13]. During the course of gram-negative infections, the release of endotoxin (lipopolysaccharide, LPS) can induce intense pathophysiological alterations [10, 14, 15]. One of the major

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responses to LPS *in vivo* is the rapid production and secretion of pro-inflammatory cytokines, including tumour necrosis factor- α (TNF- α) [16–18] and interleukin-1 α [19, 20]. Although gram-positive bacteria lack endotoxin, other constituents of their cell wall and exotoxins can induce TNF- α and IL-1 α production [21, 22]. The elevated production of these potent pro-inflammatory cytokines has profound influences on the physiology of the host and is responsible for many of the symptoms associated with bacterial infection, including the characteristic high fever and cachexia. Overactivation of this cytokine network can not only damage tissues of the host but can ultimately lead to death [18]. LPS toxicity can be reduced by administration of potent immunosuppressive glucocorticoids that inhibit the production of TNF and other cytokines if given before LPS challenge [17, 23]. However, the immunosuppressive influences of glucocorticoids leave the host susceptible to infection by other opportunist agents.

Materials and methods

Preparation of steroid solutions

DHEA was obtained from Steraloids, (Wilton, NH, USA). AED was purchased from Sigma [2, 6, 24]. Unless otherwise stated these steroids were dissolved in DMSO:EtOH (1:1) as the vehicle.

Mice

CD-1 female mice (Charles River, London) were 21 days old upon arrival and were maintained in the animal facility until the age of 4–6 months. ICR outbred male mice (Harland Sprague Dawley, Indianapolis, IN, USA) were 8 weeks old and underwent adaptation for no less than 1 week before being used. Animal facilities are routinely maintained at 24°C with water and food *ad libitum*.

Bacteria

P. aeruginosa. The virulence of the *P. aeruginosa* strain for mice had been determined previously [25]. A bacterial suspension containing 2×10^9 cfu/ml was kept frozen at -70°C in 1-ml tubes until use. Mice (6 months old) were inoculated intraperitoneally (i.p.) with 0.2 ml containing 2×10^7 cfu. AED or DHEA was injected subcutaneously (s.c.) 2 h before bacterial inoculation.

E. faecalis. *E. faecalis* $\times 1515$.OG1RF, a plasmid-bearing strain, which is virulent for mice was grown on blood agar. This strain was a generous gift from Dr H. Dalton (Medical College of Virginia, Richmond, VA, USA). The number of cfu was determined by spectrophotometry by reference to standards verified by subculture and plate counts. A dose of 1×10^{10} cfu/animal in 0.5 ml of saline was used i.p. for challenge experiments.

LPS challenge

A lethal dose (800 $\mu\text{g}/\text{mouse}$) of LPS (Lipopolysaccharide W, *Escherichia coli* O55:B5; Difco Labora-

tories, Detroit, MI, USA), was diluted in pyrogen-free saline and injected into CD-1 mice i.p. DHEA (80 mg) was dissolved in 0.8 ml of absolute alcohol and then diluted with inactivated rabbit serum (10%) in saline (RSSP) to give a final volume of 16 ml. Each mouse received 2 mg [24]. AED (30 mg) was dissolved in 1.2 ml of absolute alcohol and diluted to a final volume of 15 ml with RSSP. Each mouse was given 0.4 mg. Control mice were inoculated with the vehicle only at the same times. Mice were observed daily for mortality for 14 days.

TNF- α assay

To determine TNF- α production, mice were inoculated with LPS (100 $\mu\text{g}/\text{mouse}$) i.p. 20 min after the s.c. injection of DHEA or AED. Mice were bled 60 min after LPS treatment. Blood was collected into serum separator tubes (Becton-Dickinson). Serum was separated and frozen at -70°C until further processing.

In-vitro experiments

The procedure of Kruisbeek [26] was used for the proliferation of spleen cells from three of four BALB/c or C57BL/6J mice. Briefly, the mitogens concanavalin A (ConA) and *Escherichia coli* LPS were diluted in phosphate-buffered saline (PBS) with fetal calf serum 1% to a concentration of 1.0 mg/ml. The optimal working concentration of ConA was found to be 2.5 $\mu\text{g}/\text{ml}$ while that of LPS was 5.0 $\mu\text{g}/\text{ml}$. The viability of spleen cells was determined by exclusion of trypan blue 0.4% after a haemocytometer count had been made. Unfractionated lymphocytes were maintained in RPMI 1640 containing FCS 10%, 200 μM L-glutamine, 20 mM Hepes, penicillin 2.5 U/ml, streptomycin 2.5 $\mu\text{g}/\text{ml}$ and 5.0×10^{-5} M 2-mercaptoethanol. For proliferation studies, 0.1-ml volumes of the mixed cell suspension at a concentration of 5.0×10^6 cells/ml were cultured in 96-well tissue culture plates. For cytokine assays, 1.0-ml volumes of this same cell suspension were cultured in 24-well tissue culture plates. For each study unfractionated spleen cells were incubated at 37°C in a CO₂ 5% incubator. Murine macrophages RAW 264.7 (Ig and complement receptor positive), were obtained from the American Type Culture Collection (ATCC).

TNF- α bioactivity in serum was determined with A-9 cells (ATCC, Rockville, MO, USA). Briefly, 3×10^4 A-9 cells were seeded in wells of microtitration plates and allowed to adhere overnight. Wells were treated with serial dilutions of the sample serum followed by addition of cycloheximide 25 $\mu\text{g}/\text{ml}$. After incubation for 8–10 h, the cells were washed and the number of surviving cells was determined by uptake of neutral red. The mouse T-cell clone D10.G4.1 was used for measurement of soluble IL-1 and was maintained in complete Clicks-5 medium containing ConA 2.5 $\mu\text{g}/\text{ml}$. For a positive control, recombinant murine IL-1 was

purchased from R&D, Minneapolis, USA). In addition, representative results obtained from this assay were verified by repeat measurement with an IL-1-specific ELISA obtained from Genzyme (Cambridge, MA., USA).

Statistical analysis

Data are presented as means and SD and were analysed for significance by analysis of variance (ANOVA) or by Student's *t* test, as stated.

Results

The effects of AED and DHEA on P. aeruginosa infection

The protective effects of AED and DHEA towards a dose of 2×10^7 cfu of *P. aeruginosa* that causes 100% mortality in the strain of mouse used [25] is illustrated in Table 1. Experiments 1 and 2 show that DHEA treatment at a dose of 20 mg, 2 h before *P. aeruginosa* injection protected 50% and 38.5% of the animals, respectively, in two separate experiments.

A combination of the results of both experiments shows that 43% of the animals treated with DHEA

were protected from a lethal *P. aeruginosa* infection. Similarly, 2 mg of AED resulted in 71.5% and 62.5% protection in two separate experiments. A combination of the results of both experiments shows that AED protected 67% of animals infected with a lethal dose of *P. aeruginosa*.

The results show that treatment with either AED or DHEA *in vivo* significantly ($p < 0.05$) increased the survival of mice given a lethal dose. Furthermore, AED is more effective than DHEA against *P. aeruginosa* infection as is evident from the level of protection and the use of one-tenth the dose of DHEA.

The effects of AED and DHEA on E. faecalis infection

To establish that the effects of these hormones were not limited to gram-negative bacterial infections, the effects of both hormones were tested on a lethal challenge with *E. faecalis*. Treatment of mice infected with this bacterium with a single dose of either AED (8 mg) or DHEA (25 mg) 2 h before bacterial challenge protected all animals, whereas 57% of untreated animals died ($p < 0.05$) (Fig. 1). Thus, these data show that both AED and DHEA have a similar protective effect against a lethal *E. faecalis* infection.

Table 1. Protective effects of DHEA and AED against lethal infection in mice by *P. aeruginosa*

Treatment of infected mice	Mortality (%)		
	Experiment 1 (n = 14)	Experiment 2 (n = 16)	Cumulative data (n = 30)
None	14 (100)	15 (94)	29 (97)
DHEA	7 (50)	10 (62.5)	17* (57)
AED	4 (28.5)	6 (37.5)	10* (33)

Six-month-old CD-1 female mice were infected with 2×10^7 cfu of *P. aeruginosa*; DHEA 20 mg or AED 2 mg was injected s.c. 2 h before bacterial challenge.

* $p < 0.01$ compared with control group.

Fig. 1. The protective effects of DHEA and AED against a lethal *E. faecalis* infection. Mice were inoculated i.p. with $1 \times \text{LD}_{50}$ dose of the organism. Treatment with a single dose of either AED or DHEA (25 mg) 2 h before bacterial challenge afforded complete protection, whereas 57% of control animals died ($p < 0.05$); ■ *E. faecalis* + AED or DHEA, ● *E. faecalis* only.

The absence of direct antibacterial effects of AED or DHEA at concentrations ranging from 5×10^{-4} M to 5×10^{-8} M was determined in veal infusion broth cultures 24 h after inoculation. Changes in growth rate were determined by OD₃₄₀ measurements. Untreated *E. faecalis* control cultures had a mean OD₃₄₀ reading of 0.8565 (SD 0.01), while the averages of all DHEA- and AED-treated cultures were 0.8353 (SD 0.02) and 0.834 (SD 0.02) respectively (10 samples were used for each group). Thus, neither AED nor DHEA altered the growth rate of the bacterium at any of the concentrations tested.

Endotoxic challenge and TNF- α levels: protective effects of AED and DHEA

In all, 88% of untreated animals inoculated with 800 μ g of LPS per mouse died within 72 h (Fig. 2). However, pretreatment with DHEA (2 mg) given 1 h before the LPS reduced mortality from 88% to 17%. AED treatment (0.4 mg) reduced LPS-induced mortality from 88% to 8.5%. Even though both drugs were found to be effective in protecting from LPS toxicity, AED at one-fifth the dose of DHEA was again more protective.

Because LPS is known to mediate an increase in TNF- α levels, the study investigated whether the protective effects of AED or DHEA observed were caused by suppression of LPS-mediated TNF- α secretion *in vivo* was examined. Fig. 3 shows that 100 μ g of LPS induced high TNF- α levels *in vivo*, resulting in an increase from a mean of 1028.0 (SD 90) pg/ml in control mice to a mean of 7366.7 (SD 645.8) pg/ml. DHEA treatment

Fig. 2. Protective effects of DHEA and AED against LPS endotoxic shock. CD-1 female mice were each challenged with 800 μ g of LPS. The steroid, AED (0.4 mg/mouse) or DHEA (2 mg/mouse), was injected s.c. 1 h before LPS challenge. Each group included 14 animals with 42 animals per experiment. A total of 84 animals was tested. *Statistically significant at $p < 0.01$ by ANOVA.

Fig. 3. Effects of DHEA and AED on levels of TNF- α induced *in vivo* by LPS. Female CD-1 mice (4 months old) were challenged with 100 μ g of LPS and inoculated i.p. with either AED (0.4 mg) or DHEA (2 mg) in inactivated rabbit serum 10% in saline containing penicillin, 20 min before LPS challenge. TNF- α levels were measured 1 h after LPS challenge. Six animals were used in each test group. *Statistically significant at $p < 0.01$ by ANOVA as compared with the LPS-treated group.

resulted in a marked reduction of TNF- α levels of around 50%. However, the lower dose of AED which protected mice *in vivo* from lethal challenge, was ineffective in reducing TNF- α levels in this system.

These observations demonstrate that the protective effect of AED and DHEA against bacterial and endotoxin challenge are distinct from each other and that this protective effect of AED is not mediated by the reduction of TNF- α .

Influence of AED and DHEA *in vitro*

Because of the ability of DHEA and AED to protect mice against LPS toxicity, the following experiments were designed to examine their roles *in vitro*. First, the ability of both steroids to modify proliferation of LPS-stimulated splenocyte cultures was assessed. C57BL/6 murine spleen cell cultures stimulated by LPS 5 μ g/ml were treated with concentrations of DHEA ranging from 5.0×10^{-6} M to 5.0×10^{-9} M. DHEA suppressed proliferation as determined by a reduced [³H] thymidine uptake after culture for 24 h (Table 2), e.g., at 5.0×10^{-6} M, DHEA suppressed proliferation by 21.5% compared with LPS-stimulated controls. In contrast, AED at the same concentrations had no effect on splenocyte proliferation.

Subsequent experiments were designed to determine whether the observed ability of DHEA to suppress TNF- α and IL-1 production *in vivo* could be modelled *in vitro* with an LPS-sensitive macrophage cell line.

Table 2. Effects of DHEA and AED on the proliferation of splenocytes from C57BL/6J mice induced by LPS

Concentration of steroid (moles/L)	Mean (SD) [³ H] thymidine uptake by splenocytes exposed to	
	DHEA	AED
5×10^{-9}	90.7 (3.6)	99.7 (3.5)
5×10^{-8}	87.0 (4.7)	96.7 (5.1)
5×10^{-7}	83.1 (6.5)*	100.3 (6.3)
5×10^{-6}	78.6 (6.8)*	97.4 (4.9)

Splenocytes (5.0×10^6 cells/ml) were treated with the indicated concentrations of steroid or control vehicle (DMSO 0.2%: EtOH). Cultures were stimulated with LPS $5.0 \mu\text{g/ml}$ for 24 h in 96-well culture plates and pulsed with $1.0 \mu\text{Ci}$ [³H]-thymidine for the final 6 h. Data represent six experiments and are expressed as the mean percentage of the control value for each experiment.

*Statistically different from control, $p < 0.05$.

Again, treatment of murine macrophages (RAW 264.7) stimulated by LPS 50 ng/ml with DHEA suppressed the production of both TNF- α and IL-1 (Tables 3 and 4). AED had no effect on the production of either pro-inflammatory cytokine. These in-vitro measures correlated with those from the in-vivo experiments and further revealed the dichotomy between the function of AED and DHEA even though each steroid hormone protected mice from the lethal effect of bacterial infection and LPS toxicity.

Table 3. Effects of DHEA and AED on the in-vitro production of TNF- α by RAW264.7 murine macrophages stimulated by LPS

Concentration of steroid (moles/L)	Mean (SD) TNF- α (pg/ml) secreted by macrophages exposed to	
	DHEA	AED
5×10^{-9}	461.93 (27.99)	496.09 (38.20)
5×10^{-8}	451.53 (14.90)	497.58 (25.87)
5×10^{-7}	451.53 (14.90)	521.84 (22.96)
5×10^{-6}	442.62 (19.03)*	490.64 (31.89)
0 (control)	495.00 (28.6)	

Cultures were stimulated with LPS 50.0 ng/ml for 24 h. TNF- α was measured with an L929 cytolytic bioassay and confirmed against a standard curve generated with dilutions of recombinant TNF- α . Data were compiled from three different experiments ($n = 6$ for each determination).

*Statistically different from control, $p < 0.05$.

Table 4. Effects of DHEA and AED on the in-vitro production of IL-1 by RAW264.7 murine macrophages stimulated by LPS

Concentration of steroid (moles/L)	Mean IL-1 (pg/ml) secreted by macrophages exposed to	
	DHEA	AED
5×10^{-9}	682.12*	756.13
5×10^{-8}	688.22*	804.20
5×10^{-7}	695.85*	766.81
5×10^{-6}	711.88	764.52
0 (control)	763.00	

Cultures were stimulated with LPS 5.00 ng/ml for 24 h. IL-1 was measured with a bioassay based on a T-cell clone (D10.G41).

*Statistically different from control, $p < 0.05$.

Discussion

This study demonstrated the potential of the naturally occurring steroid hormones AED and DHEA to protect against lethal bacterial infections in a rodent model. Both hormones were effective in preventing death from a lethal challenge by either the gram-negative organism *P. aeruginosa*, or the gram-positive *E. faecalis*. Although a precise mechanism of action has not been defined for AED and DHEA, neither steroid appeared to have direct antibacterial effects, because the growth characteristics of *P. aeruginosa*, and *E. faecalis* were not altered in an in-vitro assay. Furthermore, because both *P. aeruginosa* and *E. faecalis* exert their virulence by their toxigenic actions [9, 10, 13], the protective effects of these hormones may be mediated, at least in part, by their ability to modulate the actions of virulence factors associated with each organism.

Injection of LPS i.p. has been reported to stimulate the release of many cytokines, including TNF- α and IL-1 α , within 45 min. Zuckerman *et al.* [27] reported that maximal serum TNF- α was detected within 1 h of injection; elevated levels returned to baseline with 3–4 h. The peripheral inflammatory response elicited as a result of the increase in these cytokines results in activation of a central response involving the hypothalamic-pituitary-adrenal (HPA) axis which among others leads to changes in thermo-regulation, and an overactivation of the immune response [28–30]. Danenberg *et al.* [24] reported that following inoculation of CD-1 mice with $100 \mu\text{g}$ of LPS, plasma corticosterone levels increased almost eight-fold from 94 ng/ml to 740 ng/ml . Activation of the HPA axis may function as feedback control over the production of pro-inflammatory cytokines because the principal product of HPA activation, namely adrenal glucocorticoids, suppresses production of TNF- α and IL-1 α by macrophages [31, 32].

This was supported by the observation that the decrease in TNF- α 3–4 h after LPS injection correlated with the appearance of significant amounts of endogenous serum corticosterone [27]. Furthermore, treatment of LPS-stimulated mice with the glucocorticoid receptor antagonist RU486 blocked corticosterone function and led to augmented production of serum TNF- α [33]. TNF- α is considered to be a major proximal mediator of septic shock [16, 23, 34–37], a claim substantiated by the finding that passive immunisation against TNF- α protected mice against the lethal effects of LPS [16]. However, TNF is not the sole mediator of LPS-induced phenomena [20], but rather acts in conjunction with other cytokines, augmenting their activity [27, 37].

The results reported here support the premise that DHEA protects mice from bacterial infection and LPS toxicity by blocking the toxin-induced production of TNF- α in view of the data showing suppression of

serum TNF- α levels *in vivo* and suppression of TNF- α production from cultured macrophages *in vitro*. The results of this study also show that significantly lower doses of AED had a greater protective effect than DHEA against *in-vivo* challenge with *P. aeruginosa*, *E. faecalis* or LPS. However, AED did not reduce the levels of TNF- α either *in vivo* or *in vitro*. On the basis of these observations, it is apparent that the protective actions of these two hormones operate by different mechanisms, with that of AED being independent of TNF- α production. DHEA has been reported to function as a weak glucocorticoid because it suppressed splenocyte proliferation, T-cell secretion of IL-2 and IL-3, [38] and pro-inflammatory monokine production (Tables 3 and 4). Indeed this appears to be similar to the action of glucocorticosteroids after LPS challenge [24, 27, 33, 39]. On the other hand, AED did not suppress any of these aspects of immune function. Furthermore, Padgett *et al.* [7] reported that AED suppressed the activation of the HPA induced by influenza virus. The suppression of corticosterone levels mediated by AED during the influenza infection correlated with increased production of IFN- γ from antigen-specific T cells and increased survival. In contrast, Dobbs *et al.* [40] showed that stress-induced elevation of serum corticosterone during influenza infection suppressed IFN- γ production and increased morbidity associated with infection.

Thus the influences of AED clearly differ from those attributed to glucocorticoids and, by inference, to DHEA, because the protective effects of DHEA against bacterial or LPS challenge are mediated in part by its glucocorticoid-like properties which result in suppression of TNF- α . However, AED exerted its protective effect by a different as yet unknown mechanism that did not result in suppression of TNF- α production. Further investigations into the possible use of AED in providing protection against antibiotic-resistant opportunist infections may be warranted.

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