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In Vivo Dehydroepiandrosterone Restores Age-Associated Defects in the Protein Kinase C Signal Transduction Pathway and Related Functional Responses

Emanuela Corsini,^{1*} Laura Lucchi,* Massimo Meroni,* Marco Racchi,[†] Bruno Solerte,[‡] Marisa Fioravanti,[‡] Barbara Viviani,* Marina Marinovich,* Stefano Govoni,[†] and Corrado L. Galli*

Elderly subjects are at increased risk of pneumonia, influenza, and tuberculosis. Besides the known age-related decrease in mechanisms for mechanical clearance of the lungs, impaired alveolar macrophage function contributes to the increased risk of illness in the elderly. We have previously shown that age-induced macrophage immunodeficiencies are associated with a defective system for anchoring protein kinase C. Castration of young male rats produces effects on alveolar macrophages similar to those of aging, suggesting a relationship between circulating sex hormones, particularly androgens, and the decreases in the receptor for activated C kinase (RACK-1) and macrophage function observed. The aging process in humans and rats is associated with a decline in the plasma concentrations of dehydroepiandrosterone (DHEA) and its sulfate, among other steroid hormones. We report here that in vitro and in vivo administration of DHEA to rats restores the age-decreased level of RACK-1 and the LPS-stimulated production of TNF- α in alveolar macrophages. DHEA in vivo also restores age-decreased spleen mitogenic responses and the level of RACK-1 expression. These findings suggest that the age-related loss in immunological responses, linked to defective pathways of signal transduction, are partially under hormonal control and can be restored by appropriate replacement therapy. *The Journal of Immunology*, 2002, 168: 1753–1758.

The aging immune system is less able to cope with infectious disease than earlier in life, with increased morbidity and mortality among the elderly (1, 2). Many factors contribute to immunosenescence including stem cell defects, thymus involution, aging of resting immune cells, replicative senescence of clonally expanding cells, defects in APC, and dysfunction in several signal transduction pathways (3, 4). Age-related alterations in the functional capacity of macrophages are likely to contribute significantly to the increased risk of illness in the elderly (5), because these cells directly affect natural immunity. The aging process depresses chemotaxis and phagocytosis (3), induces a deficient respiratory burst (4), and is associated with a decline in the ability of these cells to present Ags and to produce cytokines (6–10).

Alveolar macrophages (AM)² are resident airway cells, situated at the air-tissue interface in the alveoli and alveolar ducts, which are primarily responsible for the protection of the lungs against inhaled toxins, bacteria, viruses, parasites, and particles. It has recently been shown (11) that aging is associated with a significant decrease in accessory cell function and the release of TNF- α from human AM stimulated by LPS.

We have recently demonstrated (12) that the age-associated decline in macrophage function reflects an impaired protein kinase C (PKC) signal transduction pathway and in particular is correlated with a defective PKC-anchoring system. PKC is a family of phospholipid-dependent serine-threonine kinases involved in the signal transduction of hormones, neurotransmitters, and cytokines (13). Furthermore, we demonstrated in AM that many key mediators of the host response to infections, namely, hydrogen peroxide production, lysozyme release, and TNF- α production, are indeed dependent on PKC activation (14). Activation of PKC results in redistribution (translocation) of the enzyme from the cytosol to membrane compartments (13). A family of proteins that interact with PKC (for a review, see Ref. 15), which are receptors for activated C kinase (RACKs), are 30- to 36-kDa proteins located in various subcellular compartments. RACK-1, a 36-kDa protein cloned from rat brain, is the best characterized member of the RACK family; it preferentially interacts with PKC- β . We have shown that a deficit in RACK-1, in the absence of differences in the expression of LPS receptor or total PKC isoforms, contributes to the functional impairment in aged AM (12). In particular, the use of RACK-1 antisense oligonucleotide reduces the ability of macrophages to respond to LPS, indicating the importance of this protein in macrophage activation (12).

The mechanism is unclear. An attractive hypothesis is that an age-associated change in the neuroendocrine system may affect macrophages. Aging is associated with several alterations in hormone production, secretion, and action. In particular, dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S), the most abundantly secreted adrenal steroids, are known to increase throughout childhood and puberty and then to decrease in old age. The average serum DHEA concentration in men 25–34 years old is 15.9 ± 6.1 nM, but this value falls to 5.4 ± 1.7 nM in those age 75–85 years

*Department of Pharmacological Sciences, University of Milan, Milan, Italy; and Departments of [†]Experimental and Applied Pharmacology and [‡]Internal Medicine, University of Pavia, Pavia, Italy

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¹ Address correspondence and reprint requests to Dr. Emanuela Corsini, Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan Italy. E-mail address: emanuela.corsini@unimi.it

² Abbreviations used in this paper: AM, alveolar macrophages; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; PKC, protein kinase C; RACK-1, receptor for activated C kinase.

(16). Reduced secretion of DHEA during aging has been related to a series of age-associated conditions, including atherosclerosis and cardiovascular disease, breast cancer, obesity, loss of muscle mass, and diabetes, and there is a view that supplementation with DHEA may have a number of significant clinical uses (17). Immunocompetence also declines with age (17, 18). We therefore investigated the role of DHEA in age-associated immunological dysfunction and in particular the effect of this hormone on RACK-1 expression and PKC-dependent functions in AM.

Materials and Methods

Animals

Young (3–4 mo) and old (>19 mo) Sprague Dawley male rats were purchased from Charles River (Calco, Italy), as were sham-operated and orchietomized young rats. Sham operated rats are rats anesthetized, incised, and sewed up but not orchietomized. All animal care procedures were in accordance with the Guidelines for Care and Use of Experimental Animals.

Chemicals

Steroid hormones, flutamide, and LPS from *Escherichia coli* were obtained from Sigma (St. Louis, MO), Con A from Boehringer (Mannheim, Germany), recombinant murine TNF- α from R&D Systems (Minneapolis, MN), Abs against murine CD14 and PKC- β II from Santa Cruz Biotechnology (Santa Cruz, CA), anti-rat RACK-1 from Transduction Laboratories (Affinity, Nottingham, U.K.), and anti-murine β -actin from Sigma. Electrophoresis reagents were from Bio-Rad (Richmond, CA).

Experimental procedures

For in vivo treatment with DHEA, rats were randomly assigned to the following groups ($n = 5$): 1) old rats given implants of vehicle (propylene glycol) alone; 2) old rats given DHEA (2.5 mg/kg) implants; and 3) young rats without implants. Alzet 2MLM2 osmotic minipumps (Alza, Palo Alto, CA) were implanted s.c. on the backs of the animals. DHEA in propylene glycol was delivered at 2 mg/day during 2 wk. In preliminary experiments, no significant differences in the parameters measured were noted in propylene glycol-treated and untreated rats.

Assay for DHEA-S

Circulating DHEA-S was determined by a commercially available RIA (Coat-A-count; DPC, Los Angeles, CA) for which the limit of detection was 0.11 ng/ml.

Cells

AM were collected by bronchoalveolar lavage as described previously (19). Recovery was $10\text{--}15 \times 10^6$ cells/animal, of which >98% were macrophages (Giemsa stain). Once washed and resuspended to 10^6 viable AM/ml, for functional assays cells were allowed to adhere to plastic plates in RPMI 1640 (Sigma) containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, and 50 ng/ml gentamicin (medium) for 1 h at 37°C in 5% CO₂. For TNF- α release $0.3\text{--}0.5 \times 10^6$ cells were plated in 24-well plates, whereas for Western blot analysis $4\text{--}5 \times 10^6$ cells were plated in 60-mm petri dishes. Cells were then exposed to medium with 10% FCS (Sigma) and incubated with or without LPS in the presence or absence of steroid hormones, or DMSO as vehicle control. In all experiments involving the in vitro use of hormones, RPMI without phenol red and charcoal-stripped calf serum were used.

In vitro lymphocyte proliferation assay

Spleens were aseptically removed and homogenized, and cells were resuspended at a concentration of 2×10^6 viable cells per ml of medium. Cultures were set up in triplicate with 0.1 ml cells and 0.1 ml medium alone or with LPS 5 μ g/ml and Con A 2 μ g/ml. Cell cultures were incubated for 72 h, pulsed during the last 18 h with 1 μ Ci/well [³H]thymidine (Amersham, Little Chalfont, U.K.), and harvested using a cell harvester (Dyna-tech; PBI, Milan, Italy). The uptake of [³H]thymidine was measured in a scintillation counter (Packard, Meriden, CT). Results are expressed as a stimulation index, i.e., cpm in mitogen-stimulated cells/cpm in cells treated with medium alone.

Assay for TNF

TNF content was assayed by determining the cytotoxicity of TNF against sensitive L929 cells, as previously described (20). The results are ex-

pressed in picograms per milliliter. TNF concentration was calculated against a standard curve with known amounts of recombinant murine TNF.

Western blot analysis

For CD14, PKC- β II, RACK-1, and β -actin, $\sim 4\text{--}10 \times 10^6$ cells were lysed in homogenization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA (pH 7.5), 0.5% Triton X-100, 50 μ M PMSF, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin) and denatured for 10 min at 100°C (21). The protein content of the cell lysate was measured using a commercial kit (Bio-Rad). The cell proteins (10 μ g) were electrophoresed into a 12% SDS-polyacrylamide gel under reducing conditions. The proteins were then transferred to PVDF membrane (Amersham). The different proteins were visualized using a CD14 antiserum (1/200), RACK-1 (1/2500), PCK- β II (1/5000) and β -actin (1/5000) as the primary Abs and developed using ECL (Amersham). The immunoblotting image was acquired with a Nikon CCD video camera module. The OD of the bands was calculated and analyzed by means of the Image 1.47 program for digital image processing (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

In vitro PKC- β II translocation assay

AM (5×10^6) obtained from young and old rats were treated for 24 h in the presence or absence of DHEA (1 nM) or DMSO as vehicle control in medium with 10% FCS in 15-ml polypropylene tubes. Then, LPS, 100 ng/ml, was added, after five min AM were recovered by centrifugation for 5 min at 1200 rpm at 4°C. The pellets were resuspended in 500 μ l homogenization buffer (see Western blot analysis) without Triton X-100 using a Teflon/glass potter. Cytosolic fractions were separated by centrifugation at $100,000 \times g$ for 60 min. The pellets were resuspended in the same volume of homogenization buffer by sonication at 2×15 s; this constituted the membrane fractions.

Statistical analysis

All experiments were performed at least twice; representative results are shown. Statistical significance was determined by Dunnett's multiple comparison test, after ANOVA.

Results

Diminished TNF- α production from LPS-stimulated AM from male castrated rats

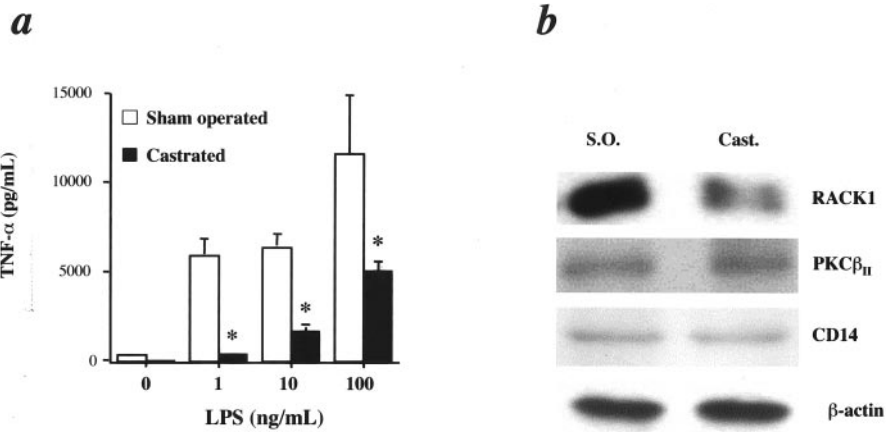
AM were obtained by bronchoalveolar lavage from sham operated and castrated male Sprague Dawley rats. Rats were orchietomized at 8 wk of age and were compared with sham operated rats 8 wk after operation. The samples, consisting of >98% macrophages, were tested for TNF- α production in response to increasing concentrations of LPS (0–100 ng/ml). Castration was associated with a drastic (>60%) reduction in TNF- α release after 24 h incubation at all concentrations of LPS tested (Fig. 1a).

We then explored the possibility that this diminished capacity of AM from castrated rats to produce TNF- α might be due to decreased levels of LPS receptor (CD14) or a defect in the activation. In particular, we focused our attention on RACK-1 expression given that we previously demonstrated that RACK-1 plays an important role in LPS-induced TNF- α release in AM (12). As shown in Fig. 1b, the diminished production of TNF- α by AM of castrated rats can be explained by a reduction in RACK-1 expression, whereas there was no difference in CD14 or PKC- β II expression (Fig. 1b). β -Actin immunoreactivity reveals that equivalent amounts of proteins have been loaded onto gels (Fig. 1b). The 60% lower RACK-1 expression in castrated rats (densitometric analysis of immunoreactivity showed 1465 ± 441 vs 4050 ± 470 U for sham operated; mean \pm SD of four independent samples; $p < 0.01$) corresponds to the reduction in TNF- α release after LPS stimulation, which suggests a role for androgen in immunosenescence.

DHEA and DHEA-S restore age-associated decrease in RACK-1 level in vitro

It has been shown that following castration in the rats, plasma levels of testosterone and DHEA become undetectable (22). To

FIGURE 1. Diminished TNF- α production and RACK-1 expression in AM obtained from castrated male rats. *a*, TNF- α release after LPS stimulation. AM produced from sham operated vs castrated rats: 5,908 \pm 1,025 vs 423 \pm 142 pg/ml TNF- α at 1 ng/ml LPS; 6,331 \pm 862 vs 1713 \pm 404 at 10 ng/ml; and 11,606 \pm 3,166 vs 5,026 \pm 695 at 100 ng/ml. Each value represents the mean \pm SD of three to four independent samples. *, $p < 0.01$ vs sham operated AM, Dunnett's *t* test. *b*, Representative Western blot analysis of RACK-1, PKC- β II, CD14 and, as control for protein loading, β -actin immunoreactivity in AM obtained from sham operated (S.O.) and castrated (cast.) rats.



test the hypothesis that the hormonal change in the cell environment associated with aging might be responsible for the loss in RACK-1 expression and macrophage function, AM obtained from old rats were treated in vitro for 24 h with a physiologically relevant concentration (1 nM) of DHEA, and with equimolar concentrations of DHEA-S, testosterone, and β_2 -estradiol or with

DMSO as vehicle control (-) and RACK-1 expression was compared with that from young rats by Western blot analysis. DHEA and its sulfate to a large extent restored the levels of RACK-1 to those of young animals, whereas testosterone treatment brought about only a slight not statistically significant increase and β_2 -estradiol caused no increase (Fig. 2*a*). Under the same experimen-

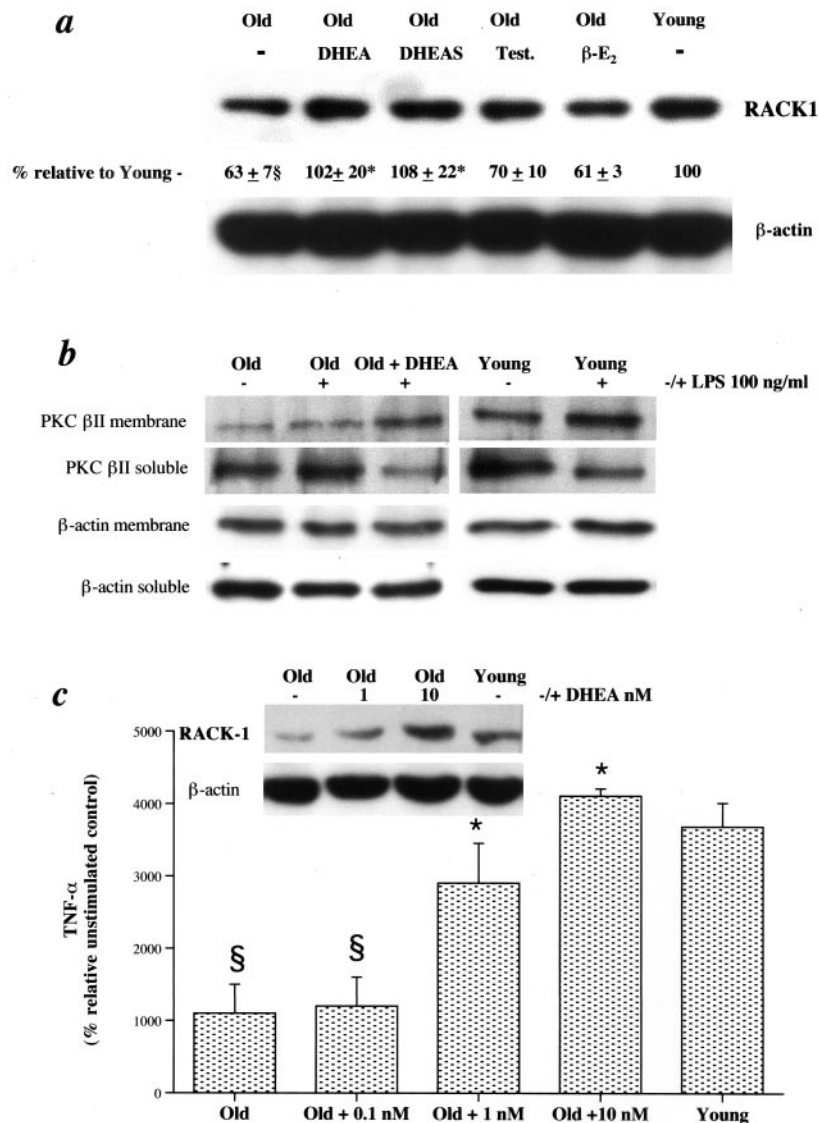


FIGURE 2. DHEA restores in vitro age-associated decline in RACK-1 expression, LPS-induced PKC translocation, and LPS-stimulated TNF- α release. *a*, Representative Western blot analysis of RACK-1 immunoreactivity. Differences in RACK-1 expression from old AM-treated rats for 24 h with 1 nM concentrations of different hormones were calculated by densitometric analysis and expressed as percent of young AM. Each value represents the mean \pm SD of three independent experiments. β -Actin was used as control for protein loading. *b*, Representative Western blot analysis of PKC- β II translocation in old and young AM after 24 h treatment with DHEA (1 nM) and 5 min LPS (100 ng/ml) treatment. β -Actin was used as control for protein loading. *c*, TNF- α release. AM were treated for 24 h with increasing concentrations of DHEA (0–10 nM), and then LPS (100 ng/ml) was added for 24 h. Each value represents the mean \pm SD of three to four independent determinations. *, $p < 0.05$ vs old AM treated with LPS; §, $p < 0.01$ vs young and old + DHEA AM treated with LPS, Dunnett's *t* test. *Inset*, Representative Western blot analysis of RACK-1 immunoreactivity after 24 h treatment with increasing concentrations of DHEA.

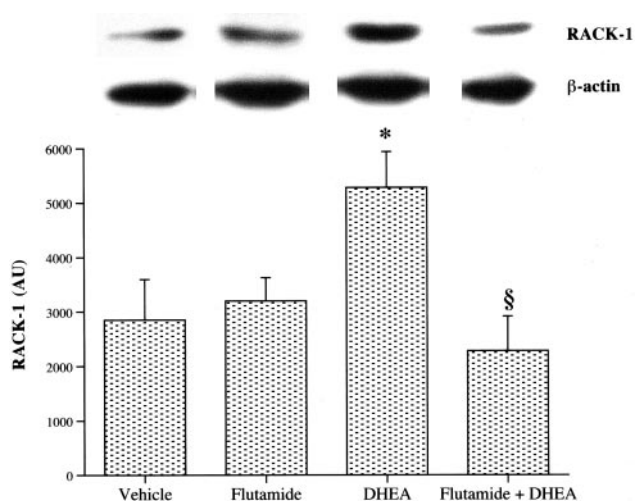


FIGURE 3. Flutamide blocks DHEA-induced RACK-1 expression. AM obtained from old rats were treated for 1 h with flutamide (10 μ M) or DMSO as vehicle control, and then DHEA (1 nM) was added for 24 h. Representative Western blot analysis of RACK-1 immunoreactivity. The histogram shows the quantification of RACK-1 immunoreactivity by densitometric analysis. Each value represents the mean \pm SD of three independent experiments. *, $p < 0.01$ vs all other groups; §, $p < 0.01$ vs DHEA-treated AM, Dunnett's t test. β -Actin was used as control for protein loading.

tal conditions, no changes in PKC- β II expression were found (data not shown). Furthermore, DHEA (1 nM) restored both the age-associated deficit in PKC translocation and in a dose-dependent manner TNF- α production after LPS (100 ng/ml) stimulation (Fig. 2, *b* and *c*), which paralleled a dose-dependent increase in RACK-1 expression (Fig. 2*c*, *inset*). With flutamide, an androgen receptor antagonist (23), DHEA-induced RACK-1 expression was blocked, indicating that this hormone functions through the androgen receptor (Fig. 3).

DHEA restores age-associated decrease in RACK-1 level and LPS-induced TNF- α production in vivo

The *in vitro* evidence of the ability of DHEA to oppose the age-associated decrease in RACK-1 expression and TNF- α production

Table I. Plasma level of DHEA-S^a

Group	DHEA-S (ng/ml)
Young (3 mo)	24.78 \pm 3.91
Old (21 mo)	13.78 \pm 4.90 [§]
Old + DHEA, 2.5 mg/kg	123.06 \pm 62.53*

^a Each value represents the mean \pm SD of four to five animals. *, $p < 0.01$ vs young group; §, $p < 0.01$ vs young and old plus DHEA groups.

led us to investigate its ability to restore RACK-1 expression and macrophage functions *in vivo*. We implanted ALZET 2ML2 osmotic pumps *s.c.* in 21-month-old male Sprague Dawley rats. We used osmotic pumps to reduce animal handling and stress and to maintain constant plasma DHEA concentration, and the *s.c.* route was chosen because it has been reported (24) to allow 100% bioavailability. DHEA (2.5 mg/kg) was delivered at a rate of 2 mg/day during 2 wk, the control group receiving propylene glycol as vehicle control. Animals were killed 2 wk after implantation, AM were obtained by bronchoalveolar lavage, and RACK-1 expression and TNF- α production in response to LPS at 24 h were compared with the values obtained from 3-mo-old rats.

The plasma concentrations of DHEA-S, as a stable marker of circulating DHEA, are shown in Table I, showing that 21-mo-old rats had only about one-half the plasma DHEA-S of 3-mo-old rats, but DHEA administration increased this significantly. Furthermore, the plasma concentrations of DHEA-S reached after delivery indicates that the dose of 2.5 mg/kg is a pharmacological dose. Fig. 4 shows that AM from the old rats produced at least 50% less TNF- α than AM from young rats at all concentrations of LPS tested, confirming our previous results (12); the 2-wk treatment with DHEA restored the ability of old AM to produce TNF- α in response to LPS (Fig. 4*a*), and this recovery was associated with a corresponding increase in the level of RACK-1 as assessed by Western blot analysis and relative densitometric analysis (Fig. 4*b*).

DHEA partially restores age-associated decline in splenocyte proliferation

In an investigation of systemic immunity, we evaluated the proliferative response to mitogens in splenocytes obtained from young rats, old rats, and from old rats treated with DHEA. Old rats

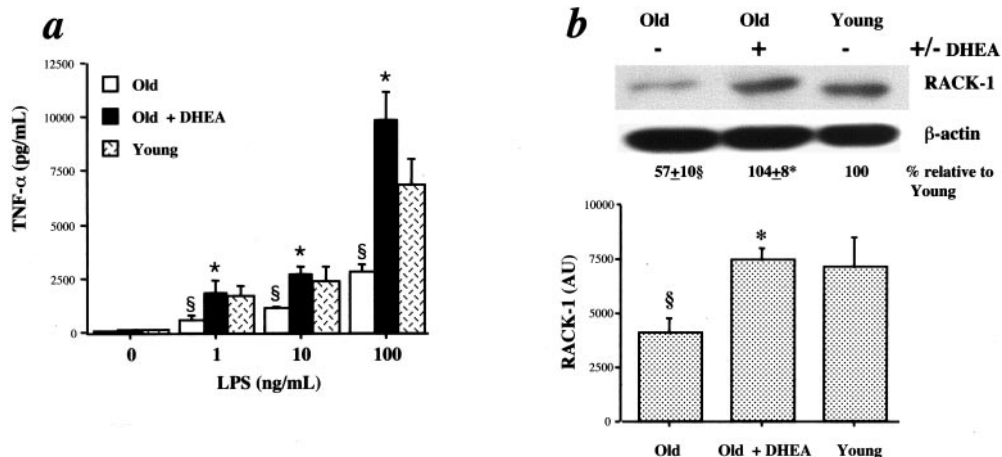


FIGURE 4. *In vivo* administration of DHEA to rats restores the age-associated decline in TNF- α production and RACK-1 expression by AM. *a*, TNF- α release. Each value is the mean \pm SD of four to five independent samples. *, $p < 0.01$ vs AM from old vehicle-treated rats; §, $p < 0.01$ vs young AM and DHEA-treated old AM, Dunnett's t test. *b*, Representative Western blot analysis of RACK-1 immunoreactivity in homogenates from AM of old rats treated with vehicle (-) or with DHEA (+) and of young rats, with percent of reactivity relative to young level below. β -Actin was used as control for protein loading. The histogram shows the quantification of RACK-1 immunoreactivity by densitometric analysis. Each value represents the mean \pm SD of five animals. *, $p < 0.01$ vs AM vehicle-treated old rats; §, $p < 0.01$ vs young AM and AM from DHEA-treated old rats, Dunnett's t test.

Table II. DHEA partially restores age-associated decline in splenocyte proliferation^a

Group	Stimulation Index	
	LPS (2.5 µg/ml)	Con A (1 µg/ml)
Young (3 mo)	31.9 ± 3.3	40.1 ± 8.9
Old (21 mo)	9.2 ± 2.9§	3.2 ± 2.4§
Old + DHEA, 2.5 mg/kg	22.5 ± 8.2	10.2 ± 3.5*

^a Each value represents the mean ± SD of four to five animals. *, $p < 0.05$ vs young group; §, $p < 0.05$ vs young and old plus DHEA groups.

showed a significantly lower mitogenic response to LPS and Con A, which DHEA treatment significantly increased (Table II). At the molecular level, the spleen cell homogenates from older rats again showed $69 \pm 21\%$ (mean ± SD of three independent samples; $p < 0.01$) decrease in RACK-1 immunoreactivity, whereas DHEA restored these levels to young values (Fig. 5).

Discussion

These results elucidate the mechanism by which the naturally occurring adrenal steroid hormone DHEA corrects age-related dysfunctions in rat alveolar macrophages, with implications for the role of this hormone in immunosenescence. Age-related immunological changes result in part from a decrease in the functional capacity of macrophages. We have shown recently that the age-associated decline in macrophage functions reflects a defective PKC signal transduction pathway that specifically affects the ability of PKC to translocate to the physiological anchoring sites (12). We demonstrate here the role of DHEA in the age-associated decrease in RACK-1 expression and macrophage function and the ability of DHEA supplementation to boost immunological function in the aging rat.

Castration of young rats produces effects on AM similar to those of aging. Castration was associated with decreased expression of RACK-1 and release of TNF- α in response to LPS stimulation despite there being no differences in CD14, the LPS receptor, or in total PKC- β II expression, which suggested a relationship between circulating sex hormones, particularly androgens, and the decreases in RACK-1 and macrophage function observed. It has been demonstrated that after castration in the rats the plasma concentrations of both testosterone and DHEA become undetectable (22), indicating a possible role of these hormones in age-associated decrease in RACK-1 expression and PKC-dependent functions.

The aging process in humans and rats is associated with a decline in the plasma concentrations of DHEA and its sulfate, among other steroid hormones. In old male Sprague Dawley rats, we

found ~50% lower DHEA-S levels, a stable marker of circulating DHEA, than in young ones. Using a physiological concentration of DHEA, which in the young adult rat is 0.3 ng/ml (equivalent to 1 nM), we were able to restore age-associated decreases in RACK-1 expression, LPS-induced PKC translocation, and AM functions in vitro. We tested other steroid hormones at equimolar concentrations and found that whereas DHEA-S, and testosterone to a lesser extent, stimulated RACK-1 expression in vitro, β_2 -estradiol was ineffective. The latter result is in agreement with data (not shown) we obtained in ovariectomized rats, where no change in RACK-1 expression in AM was observed, which suggests that estrogens are not involved in maintaining RACK-1 expression.

Although a receptor for DHEA in both monocytes (25) and T cells (26) has been described, the physiological effects of DHEA are probably brought about by its derivatives. With the use of flutamide, an androgen receptor antagonist, DHEA-induced RACK-1 expression was prevented, suggesting that DHEA acts through the androgen receptor.

The observed association between DHEA/DHEA-S and macrophage function gives further support to the positive role of these steroids in the normal functioning of the immune system. The ability of DHEA to potentiate LPS-induced TNF- α production is consistent with data obtained using LPS-stimulated human monocytes (27). DHEA increased the monocyte response to LPS, whereas dihydrotestosterone and 17 β -estradiol did not, indicating the specificity of DHEA.

In vivo, using a pharmacological dose of DHEA in old rats that restored the plasma DHEA-S concentration to that in young rats, we also restored the levels of RACK-1 expression, of LPS-induced TNF- α production by AM, and of LPS-induced splenocyte proliferation. Our results confirm the immunostimulatory properties of DHEA (reviewed in Refs. 18 and 28) and, more importantly, highlight its mechanism of action: DHEA restores the age-associated impairment in PKC signal transduction by increasing RACK-1 expression, which is essential for PKC β translocation, thus restoring PKC-dependent functions, such as B and T lymphocyte proliferation, respiratory burst, phagocytosis, etc. (12, 29–33).

At present, we are not able to establish whether DHEA or its metabolites increase directly the transcription of RACK-1 through interaction with the androgen receptor or by interacting with other downstream transcription factors. To our knowledge, the sequence of the promoter region of RACK-1 is not available. Furthermore, specific DHEA-responsive elements have yet to be described.

Overall, the present findings are consistent with the possibility that age-related loss in functional responses linked to signal transduction-defective pathways may be at least partially under hormonal control and restored by appropriate replacement therapy. Our results further support the concept that DHEA serves important regulatory functions. By its ability to restore RACK-1 expression and PKC functional impairment, supplementation of DHEA may be beneficial for such factors as hormones, neurotransmitters, and cytokines; those require correctly functional PKC machinery for their physiological activities.

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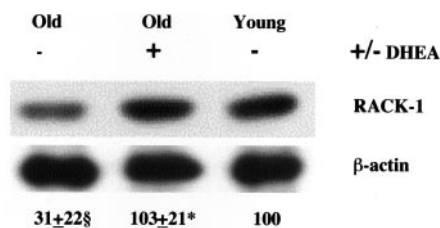


FIGURE 5. DHEA restores the age-associated decline in rat spleen RACK-1 immunoreactivity. Splenocytes obtained from rats treated in vivo were analyzed by Western blot analysis for immunoreactivity in cell homogenates. β -Actin was used as control for protein loading. The RACK-1 immunoreactivities relative to young AM (100%) are reported. Each value represents the mean ± SD of three animals. *, $p < 0.01$ vs AM vehicle-treated old rats; §, $p < 0.01$ vs young AM and AM from DHEA-treated old rats, Dunnett's t test.

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