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# Dehydroepiandrosterone Inhibits Microglial Nitric Oxide Production in a Stimulus-Specific Manner

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Dehydroepiandrosterone (DHEA) is a steroid that circulates in abundance in the form of a sulfated reserve (DHEA-S). The levels of DHEA decline with age and further in age-related neuropathologies, including Alzheimer disease. Because of their reported anti-inflammatory effects, we tested the actions of these compounds on microglia. At concentrations of $3^{-9}$ to $1^{-6}$ M, DHEA and DHEA-S inhibited the production of nitrite and morphological changes stimulated by lipopolysaccharide. DHEA and DHEA-S also inhibited LPS induction of iNOS protein, but neither inhibited LPS-induced iNOS mRNA or the activation of NF-κB. These data suggest that the hormone regulates nitrite production through a post-transcriptional mechanism. Interestingly, microglial nitrite production in response to a secreted form of the β-amyloid precursor protein (sAPP) was unaffected by DHEA. Another Alzheimer-related factor, amyloid β-peptide, also stimulated microglial nitrite production but in a manner dependent on the co-stimulus interferon-γ. DHEA was found to inhibit only the interferon-γ component of the microglial response. These data add to a growing body of evidence for differences in the profiles of mononuclear phagocytes activated by distinct stimuli. J. Neurosci. Res. 62:503–509, 2000. © 2000 Wiley-Liss, Inc.

Dehydroepiandrosterone (DHEA) serves as a precursor of some sex hormones but also seems to have direct physiological effects on some cell populations (reviewed in Kroboth et al., 1999). DHEA circulates in a sulfated form (DHEA-S) at a high concentration relative to steroid hormones. Humoral DHEA/DHEA-S, however, peaks in young adulthood in humans and drops significantly during aging (Belanger et al., 1994; Thomas et al., 1994; Berr et al., 1996). Even greater declines have been noted in certain disease conditions, notably Alzheimer disease (Nasman et al., 1991). Supplementation of DHEA/-S levels in vivo can have an anti-inflammatory influence (Bellino, 1995). Together, these facts suggest that the common decline in DHEA/-S could play a role in age-related conditions that have an inflammatory component.

Considerable evidence points to a role for inflammatory mechanisms in Alzheimer disease. The senile plaques pathognomic for Alzheimer disease are inhabited by microglia bearing signs of inflammatory activation, including morphological transformation and elevated production of pro-inflammatory cytokines (Griffin et al., 1995; Mrak et al., 1995). Molecular species connected genetically and biochemically to the disease including amyloid β-peptide (Aβ) and the amyloid precursor protein (APP) are capable of activating inflammatory processes in cultured microglia (Meda et al., 1995; Barger and Harmon, 1997). Finally, non-steroidal anti-inflammatory drugs (NSAIDS) seem to delay the development of Alzheimer disease (Rogers et al., 1993; Breitner et al., 1995).

Microglia activated under inflammatory conditions are capable of producing large amounts of nitric oxide (NO), principally through increased activity of the enzyme inducible nitric oxide synthase (iNOS). First demonstrated in monocytes, iNOS is now known to be expressed in a variety of cells, including various CNS glia. Unlike the constitutive NOS isoforms, iNOS is independent of $Ca^{2+}$ /calmodulin and is regulated primarily by changes in expression. It is induced by proinflammatory cytokines and bacterial products such as lipopolysaccharide (LPS) (MacMicking et al., 1997). The NO produced by activated microglia is toxic to pathogens, but it is capable of also damaging neurons and oligodendrocytes (Chao et al., 1992; Merrill et al., 1993). Although this aspect of NO has been implicated in neurological disorders including Parkinson disease, Alzheimer disease, multiple sclerosis and AIDS encephalopathy (Snyder, 1996; Bo et al., 1994; Brosnan et al., 1994; Bukrinsky et al., 1995), its relative role in humans versus rodents is controversial (Colton et al., 1996). Regardless, NO production has practical utility as a rather sensitive marker of inflammatory responses in rodent microglia.

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In recognition of their inverse correlation with age-related conditions and inflammatory potential, DHEA and DHEA-S were tested for their influence on responses of microglia in multiple paradigms, including stimuli associated with Alzheimer disease. An inhibition of microglia-mediated inflammatory changes could have implications both for disease susceptibility and for therapeutic applications. iNOS and its product, NO, were the focus of the outcomes measured because of their associations with the mechanisms of inflammatory gene regulation in microglia. Together, the data indicate a surprising post-transcriptional influence of DHEA and DHEA-S on iNOS induction and a limitation of the inhibitory effects of these compounds to a subset of stimuli.

## MATERIALS AND METHODS

### Cell Cultures and Reagents

Primary microglia were cultured from neonatal Sprague-Dawley rats as described previously (Barger and Harmon, 1997). The N9 cell line is derived from myc-immortalized mouse microglia (Corridan et al., 1993) and was graciously provided by P. Ricciardi-Castagnoli (U. Milan, Italy). Cultures were treated with DHEA, DHEA-S, lipopolysaccharide (LPS) (Sigma, St. Louis MO), secreted APP (Barger and Mattson, 1996), $A\beta_{1-42}$ (Anaspec, San Jose CA), or rat interferon (IFN)-$\gamma$ (Peprotech, Rocky Hill NJ). DHEA was dissolved initially in DMSO and applied such that the final concentration of DMSO was $\leq$0.5%; controls for these conditions contained an equal concentration of DMSO. All other treatments were dissolved and diluted in aqueous buffers, including $A\beta_{1-42}$; the latter was applied without an "aging" protocol due to its rapid aggregation properties.

### Nitric Oxide Production and Cell Viability Assays

Nitrite levels were determined in the culture medium of cells grown in 96-well plates by Griess reaction as described previously (Barger and Harmon, 1997). Unless otherwise indicated, cultures were pretreated with DHEA or DHEA-S for 1 hr before application of inflammatory stimuli. A standard curve using $NaNO_2$ was generated for each experiment for quantitation. Cell viability was determined in the same cultures by MTT reduction assay as described previously (Chang et al., 1998).

### Western Blot Analysis

Cellular proteins were separated electrophoretically on 10% polyacrylamide gels containing SDS. Proteins then were transferred electrophoretically to nitrocellulose membranes (NitroBind, MSI, Westborough, MA) and then incubated overnight at 4°C with polyclonal rabbit anti-murine iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:3,000 dilution. Blots then were incubated for 45 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a 1:2,000 dilution. Visualization utilized ECL Western Blotting Detection Reagents as suggested by the manufacturer (Amersham Life Sciences, Arlington Heights, IL), followed by autoradiography.

### Northern Blot Analysis

RNA isolation and northern blot analysis were performed as described previously (Drew et al., 1993). Briefly, total RNA was isolated from cells using RNAzol™ (Tel-Test Inc., Friendswood, TX) as suggested by the manufacturer. RNA (40 $\mu$g) was separated on a 1.2% agarose gel containing 0.66 M formaldehyde in the presence of 1× MOPS buffer. RNA was transferred electrophoretically to nylon membranes (Micron Separations, Inc., Westboro, MA). cDNA probes were labeled with $^{32}P$ by random priming (Prime-It kit, Stratagene, La Jolla, CA) and used to hybridize northern blots at $10^6$cpm/mL. The experimental probe was a mouse macrophage iNOS cDNA (Alexis, San Diego, CA). Blots were also hybridized with human G3PDH cDNA (Clontech; Palo Alto, CA) to control for equal loading. After hybridization, blots were washed twice for 30 min in 2× SSC with 0.1% SDS at room temperature, and then twice in 0.1× SSC with 0.1% SDS at 65°C. Hybridization results were visualized by autoradiography.

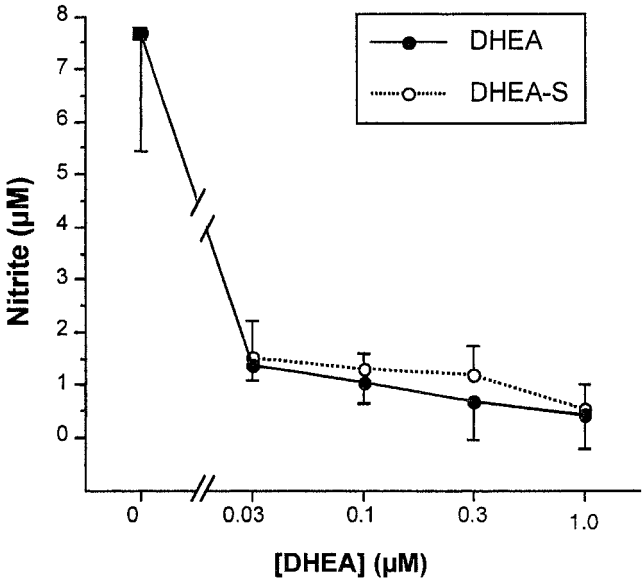


Fig. 1. DHEA and DHEA-S inhibit LPS-induced nitrite accumulations. N9 microglial cells were treated with 100 ng/mL LPS for 20 hr in the absence or presence of DHEA or DHEA-S at the indicated concentrations. Nitrite levels were measured by Griess reaction. Values represent the mean ± SEM for quadruplicate cultures.

### Electrophoretic Gel Mobility Shift Assays

Nuclear extracts were prepared from cells by the method of Dignam et al. (1983), with minor modifications (Drew et al., 1993). Oligonucleotide probes used in electrophoretic gel mobility shift assays were purchased from Geneset, and end-labeled using T4 polynucleotide kinase (Pharmacia; Piscataway, NJ). Double-stranded oligonucleotide probes used in the analyses were the proximal iNOS NF-$\kappa$B element (5′-AACTGGGGACTCTC-CCTTTG-3′) and the murine leukemia virus UCR element (5′-CTGCAGTAACGCCATTTTGCAAGGCATGAA-3′). The latter is bound by a ubiquitously expressed factor that generally is unaltered by experimental treatment and thus was used to control for equal loading and integrity of individual nuclear protein extracts. Binding assays were performed in a volume of 20 $\mu$L containing 20 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5% glyc-

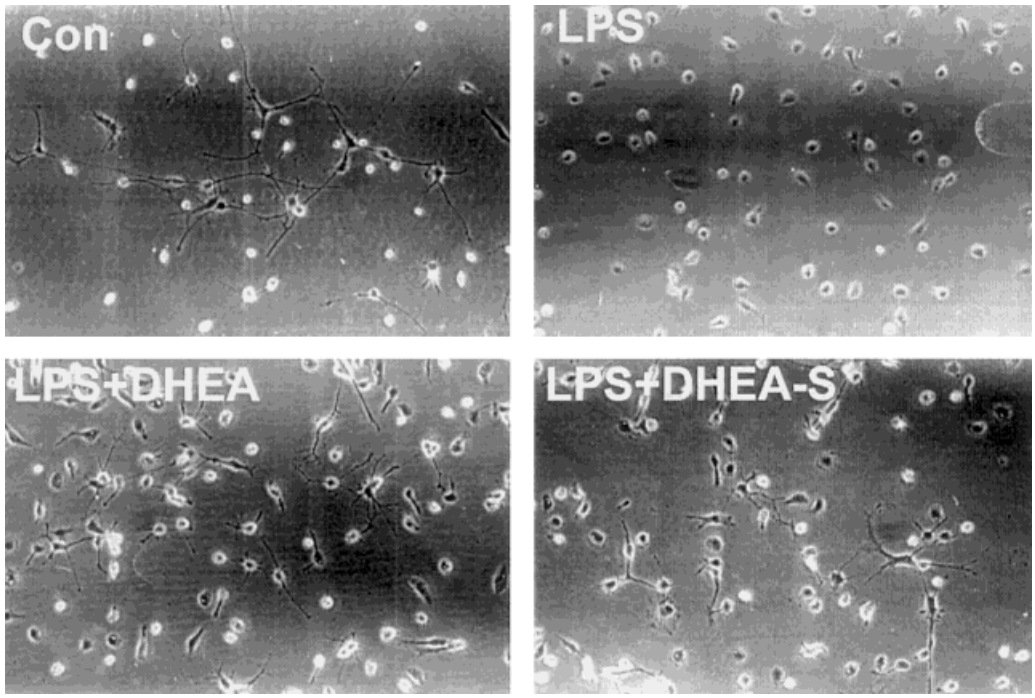


Fig. 2. DHEA and DHEA-S inhibit morphological activation by LPS. N9 cells were left untreated (Con) or were treated with 100 ng/mL LPS for 20 hr in the absence or presence of 100 nM DHEA or DHEA-S.

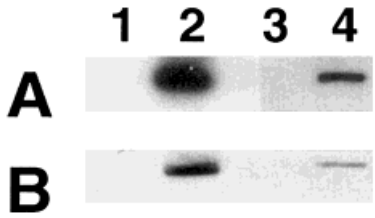


Fig. 3. DHEA and DHEA-S inhibit LPS induced iNOS protein levels. N9 cells were left untreated (**lane 1**) or were treated for 16 hr with 2.5 μg/mL LPS (**lanes 2**, **4**) or 50 μM DHEA (**A**, **lanes 3**, 4) or DHEA-S (**B**, lanes 3, 4). Total cellular protein was isolated, and iNOS protein levels were determined by western blot analysis. DHEA and DHEA-S also inhibited induction of iNOS protein levels at a concentration of 1 μM when LPS was applied at 100 ng/mL (data not shown).

erol, 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 4 μg poly dI-dC, 4 μg nuclear extract and $10^5$ cpm of $^{32}$P-labeled double-stranded oligonucleotide probe. DNA-protein complexes were resolved by electrophoresis through 4% polyacrylamide gels under non-denaturing conditions in the presence of 0.4% Tris-borate-EDTA for 1.5 hr at 150 V. Gels were dried and visualized by autoradiography.

## RESULTS

N9 microglial cells treated with LPS responded with elevated nitrite production. At concentrations of 3 nM–1 μM, DHEA inhibited the accumulation of nitrite in LPS-treated microglial cultures (Fig. 1). DHEA-S had a similar ability to suppress nitrite levels, although its potency showed an approximately 3-fold lower trend. MTT reduction assays demonstrated that the observed steroid inhibition of nitrite production was not due to steroid effects on cell viability (data not shown). In fact, DHEA and DHEA-S inhibited LPS induction of nitrite production in N9 cells at concentrations up to 50 μM without affecting cell viability (data not shown). Similar inhibition of nitrite production occurred whether cultures were pretreated with DHEA or the steroid was added simultaneously with the LPS, and similar responses to both LPS and DHEAs were observed in primary microglia from rat cerebral cortex.

Treatment with LPS altered the morphology of N9 microglial cells, converting them from a process-bearing to an amoeboid morphology characteristic of activated microglia. DHEA and DHEA-S partially prevented the morphological conversion of N9 cells without causing obvious increases in dead cells (Fig. 2).

Nitric oxide production by activated microglia results principally from the catalytic activity of iNOS. Coincident with nitrite accumulation in LPS-stimulated N9 cells, iNOS proteins levels were elevated as determined by western blot analyses (Fig. 3). DHEA and DHEA-S inhibited LPS induction of iNOS in these cells. Thus, hormone inhibition of nitrite production by N9 cells likely was consistent with decreased production of the iNOS enzyme.

Transcription of the iNOS gene is induced by pro-inflammatory stimuli. Many of these stimuli activate the NF-κB transcription factor, known to be a principal activator of iNOS gene expression. (Griscavage et al., 1996; Kleinart et al., 1996; Perrella et al., 1999). Several anti-inflammatory agents, including glucocorticoids and some

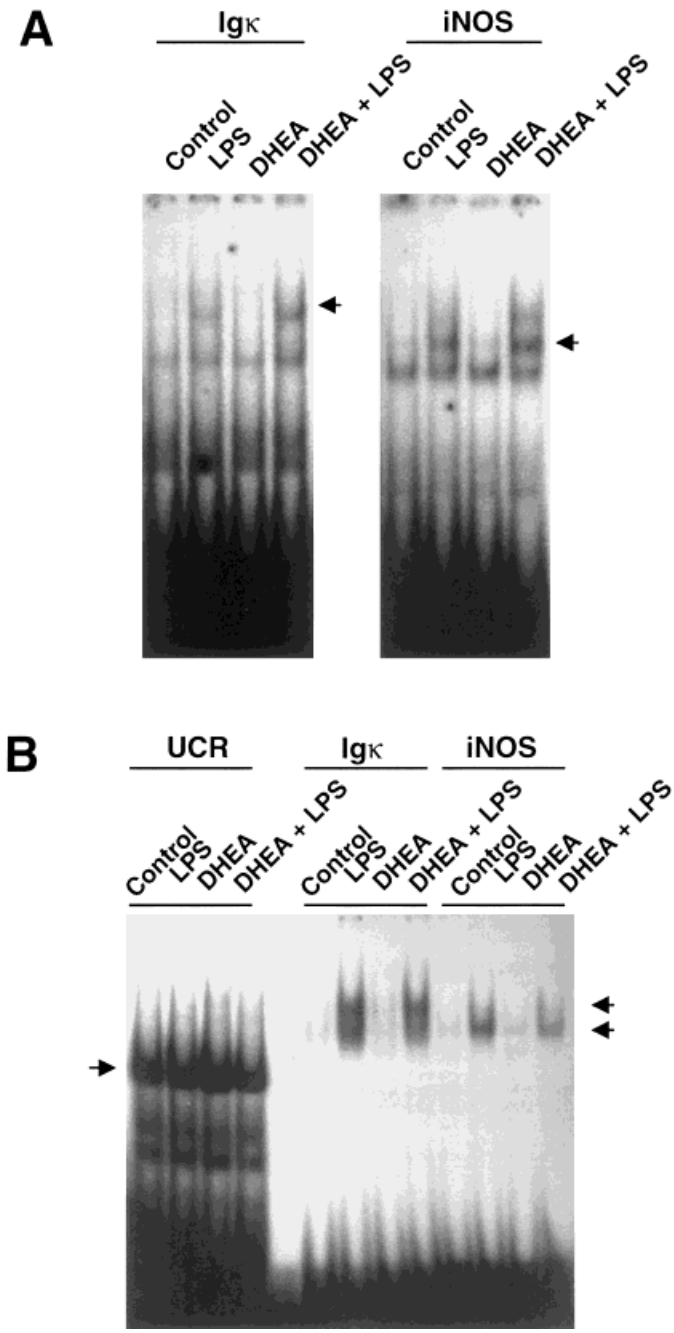


Fig. 4. DHEA does not influence NF-κB binding to the iNOS gene promoter element. Nuclear extracts were prepared from N9 cells that were either untreated (Control) or treated for 4 hr with LPS (2.5 μg/mL) or DHEA (**A**) or DHEA-S (**B**) (each at 50 μM). DNA-binding activity was assessed by EMSA analysis with two different NF-κB elements: that from the immunoglobulin κ light chain or the sequence present in the iNOS promoter (as indicated above the groups of four treatment conditions). Specific, inducible κB-binding activities are noted by the arrows on the right. Normalization of loading was confirmed by analysis of an unmodulated nuclear factor binding the UCR element, as illustrated in B (arrow on left).

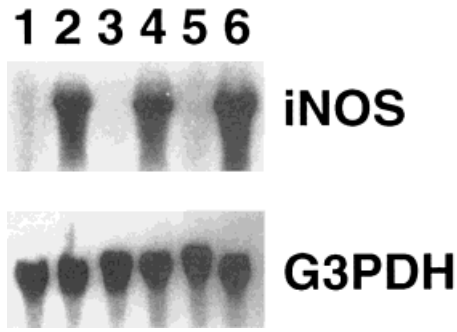


Fig. 5. DHEA does not influence expression of iNOS RNA. N9 cells were treated for 6 hr as follows; **lane 1:** no treatment, **lane 2:** LPS alone (2.5 μg/mL), **lane 3:** DHEA alone (50 μM), **lane 4:** DHEA + LPS, **lane 5:** DHEA-S alone (50 μM), **lane 6:** DHEA-S + LPS. Total RNA was isolated, and iNOS mRNA levels were determined by northern blot analysis. Hybridization with G3PDH also was performed to confirm RNA integrity and equalization of loading.

NSAIDS, inhibit NF-κB activation (Grilli et al., 1996; De Bosscher et al., 1997). We tested the influence of DHEA on NF-κB activity in LPS-treated N9 cells by EMSA analysis of DNA-binding activity. Surprisingly, exposure of the cells to DHEA or DHEA-S had no significant influence on the ability of LPS to activate NF-κB (Fig. 4). Consistent with this finding, DHEA did not significantly inhibit the elevation of iNOS mRNA stimulated by LPS (Fig. 5). As a control, nitrite assays were performed in parallel with northern and EMSA analyses, that confirmed that DHEA and DHEA-S inhibited nitrite production in these cells, although the hormones did not affect iNOS mRNA levels or NF-κB binding to the iNOS promoter. Collectively, these studies suggest that the inhibition of nitrite production by DHEA/-S depends on events that occur downstream of iNOS RNA induction.

It has become clear from recent studies that inflammatory responses of microglia and macrophages can be variable with respect to both outcome measures and activating stimuli (Gebicke-Haerter et al., 1996). To explore the latter in our model, we tested the influence of DHEA/-S on responses of microglia to stimuli relevant to Alzheimer disease. Primary microglia exposed to sAPP produced a substantial increase in nitrite accumulation that was completely refractory to DHEA or DHEA-S (Fig. 6). Simultaneous treatment of sister cultures with LPS, however, showed responses that were inhibited quite effectively with either DHEA or DHEA-S. Comparisons also were made between the responses of microglia to sAPP and Aβ. In some models, the ability of Aβ to activate microglial NO or cytokine production has been shown to depend upon a co-stimulus. Some studies have documented a synergistic cooperation between Aβ and several cytokines (Murphy et al., 1998), but the pro-inflammatory activity of Aβ was reported originally to depend on interferon (IFN)-γ priming (Meda et al., 1995). Therefore, we divided cultures into a set that received priming with IFN-γ and another that remained unprimed. To each set,

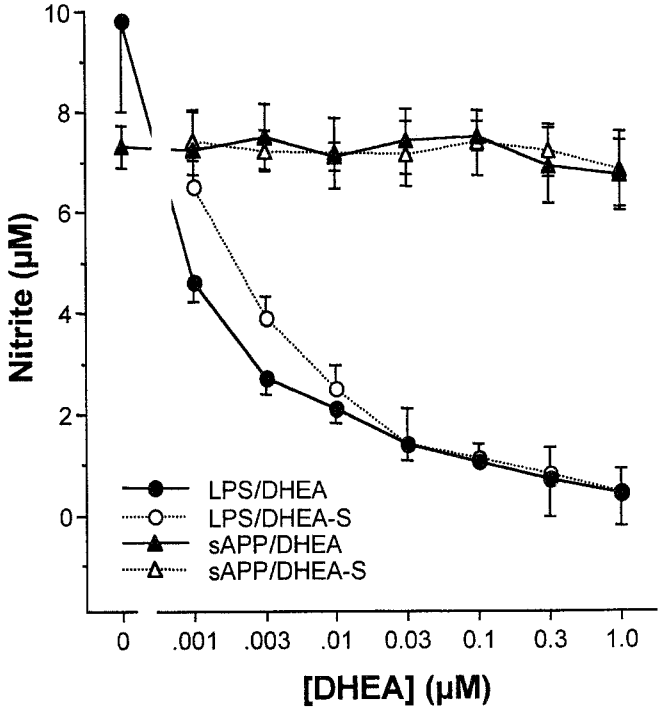


Fig. 6. DHEA and DHEA-S do not influence nitrite accumulations evoked by sAPP. Rat primary microglial cultures were treated for 24 hr with 100 ng/mL LPS (circles), 10 nM sAPP (triangles), or these treatments in the presence of various concentrations of DHEA (filled symbols) and DHEA-S (open symbols). Nitrite levels in the medium were assayed by Griess reaction. Values reflect mean ± SEM from quadruplicate cultures.

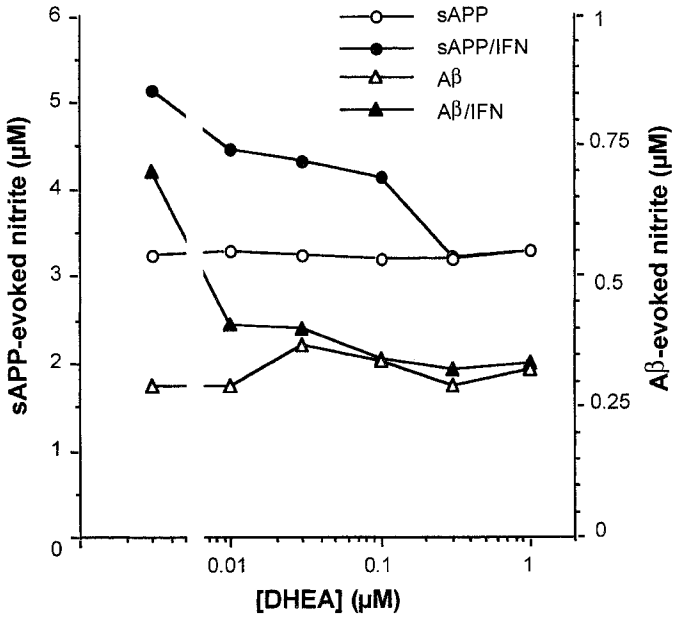


Fig. 7. DHEA inhibits IFN-γ priming. Rat primary microglial cultures were treated with 3 ng/mL IFN-γ or 10 nM sAPP, with or without the indicated concentrations of DHEA. A set of sister cultures was treated identically except that 10 μM $A\beta_{1-42}$ was substituted for sAPP. After 20 hr, nitrite was measured by Griess reaction. Values represent the means of quadruplicate cultures.

sAPP or Aβ was added, and these sets were further divided into co-treatments with various concentrations of DHEA. The elevation of nitrite accumulation by Aβ was completely dependent upon IFN-γ priming (Fig. 7). IFN-γ also elevated the effects of sAPP slightly. In this scenario, DHEA inhibited NO accumulation triggered by Aβ/IFN-γ in a dose-dependent manner. DHEA, however, inhibited the effects of sAPP/IFN-γ only by the degree to which IFN-γ elevated the nitrite levels above that of sAPP alone. Similar results were obtained with DHEA-S. These data suggest that DHEA blocked pathways or mechanisms activated by IFN-γ without influencing those stimulated directly by sAPP. Collectively these studies indicate that DHEA and DHEA-S inhibit nitrite production in microglia in a stimulus-specific manner. The hormones repressed induction of nitrite by LPS and IFN-γ but not sAPP.

## DISCUSSION

Alzheimer disease and several other neurodegenerative conditions are associated with inflammatory responses in microglia. Therefore, we have begun to test the influences of anti-inflammatory factors on disease-relevant stimuli. In two types of microglial cultures, DHEA and DHEA-S were able to suppress biochemical and morphological responses to lipopolysaccharide or to a combination of Aβ and IFN-γ. Surprisingly, the steroids had no measurable effect on responses to sAPP. Analyses of the molecular chain of expression suggested that DHEA and DHEA-S suppressed iNOS expression at the level of translation or protein stability.

Production of NO has mixed effects in the nervous system, as it can contribute to either neurotoxicity or neuroprotection (Lipton et al., 1993). iNOS expression and NO production, however, provide at least one index of a proinflammatory response in microglia. The expression of the iNOS gene is believed to be regulated by several promoter elements bound by inflammation-related transcription factors, including NF-κB (Griscavage et al., 1996; Kleinart et al., 1996; Parrella et al., 1999). Activation of NF-κB can result from a variety of stimuli, including LPS, viruses, and cytokines. In addition, NF-κB activates genes implicated in Alzheimer disease, such as cytokines and proteins found in association with amyloid plaques, and protein subunits of NF-κB are elevated in plaques (Kaldschmidt et al., 1997). Inhibition of NF-κB activity can be achieved by a variety of agents capable of monocyte suppression, including glucocorticoids and NSAIDS (Grilli et al., 1996; DeBosscher et al., 1997; Rossi et al., 2000). For these reasons, we investigated the effect of DHEA on NF-κB activation and were surprised to find that DHEA had no significant effect on NF-κB binding to an iNOS promoter element. This absence of DHEA effect on iNOS promoter activity correlated with

the inability of the hormone to inhibit LPS induction of iNOS RNA levels. Collectively, these data suggest that DHEA affects NO production in microglia through a post-transcriptional mechanism. The differential sensitivity of various stimuli to an event so far down the chain of events in iNOS production is surprising, as well. The simplest explanation may be that sAPP utilizes mechanisms less dependent on post-transcriptional events for its induction of iNOS. An alternative explanation is that sAPP activates a parallel process that serves to override the post-transcriptional effect of DHEA.

The exact physiological functions of DHEA are not known. DHEA is a precursor of sex steroids and a portion of its effects are mediated indirectly through the production of these hormones. Indeed, determinations of the roles of DHEA are hampered by the fact that DHEA is readily metabolized to other hormones that have variable physiological effects. Studies indicate, however, that DHEA also functions directly, possibly through stimulation of peroxisome-proliferator activated receptor (PPAR)-α (Peters et al., 1996). Clinical trials involving DHEA supplementation have produced variable results, with some studies indicating that DHEA has effects on immune and cardiovascular function, maintenance of muscle mass, cognition, and cancer, although other studies have demonstrated no clinical effects of DHEA supplementation (reviewed in Kroboth et al., 1999).

A variety of agents including NSAIDS, ApoE, cytokines, cyclic nucleotides, and steroids have been demonstrated to exert anti-inflammatory effects in microglia (reviewed in Wood, 1997). DHEA is a metabolite of cholesterol and a precursor of sex steroids. DHEA is commonly believed to suppress immune activity (Rom and Harkin, 1991; Mohan and Jacobson, 1993; DiSanto et al., 1996; Padgett and Loria, 1998); although some studies have indicated that DHEA may activate immune cells, including macrophages (McLachlan et al., 1996; Delpedro et al., 1998). DHEA is of particular interest in Alzheimer disease because the levels of this steroid hormone decrease dramatically with age (Belanger et al., 1994; Thomas et al., 1994; Berr et al., 1996). In addition, DHEA levels are lower in Alzheimer patients than age-matched individuals, suggesting that DHEA may play a role in inhibiting the development of this disease (Nasman et al., 1991). In addition to suppressing immune activation, DHEA or DHEA-S has been demonstrated to protect neurons from excitotoxic cell death (Mao and Barger, 1998; Kimonides et al., 1998). Another class of steroid hormones, the glucocorticoids, have a strong anti-inflammatory effect, but they also compromise other aspects of immunity and have been implicated in neurotoxicity to the hippocampus. Therefore, DHEA would seem a more desirable choice for therapeutic intervention if it possessed effective anti-inflammatory activity against Alzheimer-relevant stimuli. Promising effects of DHEA-S on hypersensitive natural killer cells in Alzheimer have been reported (Solerte et al., 1999). A role for lymphocytes in Alzheimer disease has not been established, however. The fact that DHEA suppressed the effects of Aβ/IFN-γ suggests that this hormone would be effective in abating Alzheimer disease if this stimulus combination is involved in the disease pathogenesis. IFN-γ is not, however, widely accepted as a relevant factor in the Alzheimer brain, and factors other than IFN-γ have been shown to potentiate the proinflammatory activity of Aβ (Murphy et al., 1998; Rossi and Bianchini, 1996). Further, it should be noted that our studies relied heavily on microglia derived from neonatal rats and it is possible that the requirement for a costimulus is species- or age-specific, as Aβ is capable of autonomously stimulating responses in adult human microglia (Joseph Rogers, personal communication). Finally, the ineffectiveness of DHEA against microglial responses to sAPP suggests that the steroid would be futile if sAPP contributes significantly to Alzheimer-related inflammation.

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