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Dehydroepiandrosterone Sulfate Is Neuroprotective when Administered Either before or after Injury in a Focal Cortical Cold Lesion Model

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Dehydroepiandrosterone and its sulfate (DHEAS) are sex hormone precursors that exert marked neurotrophic and/or neuroprotective activity in the central nervous system. The present study evaluated the effects of DHEAS and 17β-estradiol (E2) in a focal cortical cold lesion model, in which DHEAS (50 mg/kg, sc) and E2 (35 mg/kg, sc) were administered either as pretreatment (two subsequent injections 1 d and 1 h before lesion induction) or posttreatment (immediately after lesion induction). The focal cortical cold lesion was induced in the primary motor cortex by means of a cooled copper cylinder placed directly onto the cortical surface. One hour later, the animals were killed, the brains cut into 0.4-mm-thick slices, and the sections stained with 1% triphenyltetrazolium chloride. The volume of the hemispheric lesion was calculated for each animal. The results demonstrated that the lesion area was significantly attenuated in both the DHEAS- and E2- pre- and posttreated groups and that in the presence of letrozole, a nonsteroidal aromatase inhibitor, no neuroprotection was observed, suggesting that the beneficial effect of DHEAS on the cold injury might depend on the conversion of DHEAS to E2 within the brain. It is concluded that even a single posttraumatic administration of DHEAS may be of substantial therapeutic benefit in the treatment of focal brain injury with vasogenic edema. (*Endocrinology* 147: 683–686, 2006)

ALTHOUGH DEHYDROEPIANDROSTERONE (DHEA) and its sulfate (DHEAS) are the most abundant products of the human adrenal cortex, until recently they were regarded as relatively inert precursors of the sex steroids estradiol (E2) and testosterone (1). Since the discovery of neuroactive steroids (2), however, DHEA and DHEAS have also been referred to as neurosteroids. The members of this new family, which can be synthesized *de novo* in the nervous system and which interact with different neurotransmitter receptors, are primarily able to regulate glutamatergic and GABAergic neurotransmission (3–5). Both DHEA and DHEAS are abundant in the central nervous system (6) and have been implicated as potential signaling molecules in the neocortical organization during development, suggesting that they might have neurotrophic activity or could promote neuronal remodeling (3, 7).

Consistent with a neurotrophic role of these steroids, DHEAS has been revealed to protect certain neuronal populations against neurotoxic insults inflicted by the excitatory amino acid glutamate (3, 7, 8). Because both DHEA and DHEAS have widespread functions in the central nervous system (9, 10), their therapeutic application may potentially be beneficial in not only excitotoxicity-related diseases but also other types of focal traumatic brain injury (11). Neuroactive steroids (including E2 and its precursors DHEA and DHEAS) have been evaluated as potential therapeutic agents mostly in the form of pretreatment (12). Thus, mainly the neuroprotective efficacy of neurosteroid administration during the posttraumatic period remains to be elucidated, although these data would be extremely important in clinical practice (13). To address this issue, we used a well-established animal model of traumatic brain injury, a focal cold lesion (14), to examine the protective effects and potential mechanisms of both pre- and posttraumatic DHEAS and E2 treatment in a morphological experiment.

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Abbreviations: BBB, Blood-brain barrier; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; E2, 17β-estradiol; GABA, γ-aminobutyric acid; MI, primary motor cortex; TTC, 2,3,5-triphenyltetrazolium chloride.

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Materials and Methods

Animals

Young male Wistar rats (n = 55, 50–55 g, postnatal d 20–30) were kept on a 12-h light, 12-h dark cycle and provided with unlimited access to water and rat chow. All efforts were made to minimize the number of animals used and their suffering. The principles of laboratory animal care (National Institutes of Health publication no. 85–23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive of November 24, 1986 (86/609/EEC) were followed. The animals were anesthetized ip, using a mixture of Ketavet (100.0 mg/kg) and Rompun (xylazine, 8 mg/kg).

Cold lesion induction

The rats were fixed in a stereotaxic frame and a small hole was drilled in the skull to access the primary motor cortex (MI), with its center 1.5 mm lateral and 1.5 mm rostral from the bregma. A copper cylinder (2 mm in diameter) cooled with a mixture of acetone and dry ice (~−78

C) was used to produce a lesion in the MI. The cooling mixture was continuously supplied for at least 15 min before application of the cylinder to attain the maximal level of cold. The thermod was placed onto the surface of the exposed cortex for 30 sec. Rectal temperature was monitored and maintained at 37.0 ± 0.5 C.

Histology

One hour after lesion induction, the animals were deeply anesthetized and killed by decapitation. Thereafter, 0.4-mm-thick slices were cut throughout the ischemic region with a vibratome (Campden Instruments, Sileby, UK). The injury was visualized by using a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, Munich, Germany) dissolved in artificial cerebrospinal fluid. When the reaction was complete, the slices were fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer on coverslips for 15 min and subsequently kept overnight in the same solution. Digital images of the stained slices were captured with a scanner (Hewlett-Packard, Palo Alto, CA) at a resolution of 1200 dpi (see Fig. 1B). The total surface area of the injured region on each section was measured with Adobe Photoshop software (version 8.0; San Jose, CA). Then for each brain, the surface areas of the injured regions from all sections were summed and multiplied by the slice thickness (0.4 mm) to determine the total injured brain volume in cubic millimeters. The data were further analyzed and displayed with Origin (version 7.0; OriginLab Corp., Northampton, MA) and SPSS12 software (Chicago, IL). The injured cortical volumes (mean ± SEM) within the treatment groups were compared statistically by using one-way ANOVA, followed by the Bonferroni test for the *post hoc* comparison of the individual group means. A criterion of $P < 0.05$ was adopted for the statistical confidence.

Evans blue labeling

In another experiment (n = 4 animals), Evans blue labeling was performed as an alternative approach to control the extent of the lesion extension and determine whether the cold lesion induced extravasation. Then 2% Evans blue (0.2 ml; Sigma, St. Louis, MO) was injected into the tail vein (iv) immediately after cold lesion induction. Thirty minutes later, the animals were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. The brains were removed and postfixed overnight in the same fixative. Coronal sections (50 μm thick) encompassing the Evans blue-stained area were prepared with a vibratome. The sections were then analyzed under an epifluorescent microscope (BX51; Olympus, Tokyo, Japan). Extravasated Evans blue was localized at an excitation wavelength of 530–550 nm and an emission wavelength of more than 590 nm.

Drug application

The control group (n = 6) received only the DHEAS vehicle, distilled water. DHEAS (50 mg/kg; Steraloids, Newport, RI) was administered in distilled water sc (DHEAS-pretreated group, n = 11) during the evening of the preceding day, and subsequently 1 h before lesion induction. The DHEAS-posttreated group (n = 14) received the steroid in the same concentration as a single sc injection immediately after lesion induction. A study was also made of the effects of letrozole, a nonsteroidal aromatase inhibitor, on the action of DHEAS. The letrozole+DHEAS group (n = 5) received DHEAS in the same manner as in the DHEAS-pretreated group, but letrozole (4 mg/kg dissolved in paraffin oil) was administered sc 1 h before the steroid treatment. In the E2-treated groups (Sigma, Munich, Germany), either pretreated (n = 7) or posttreated (n = 8), the steroid was applied in the same manner as described for the DHEAS animals. Then 35 mg/kg E2 dissolved in sesame oil was administered, *i.e.* an amount equimolar with the dose of DHEAS.

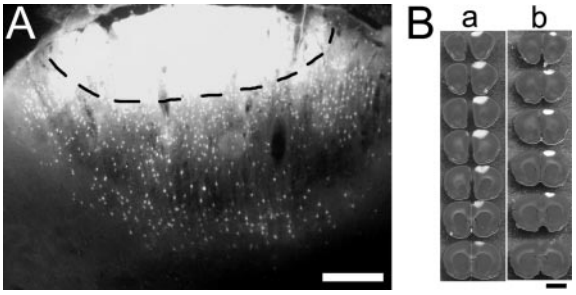

FIG. 1. Representative photomicrographs of coronal sections of rats with cold-induced lesions. A, Lesioned cortical area and affected cells in the perilesion rim are visualized by fluorescent microscopy after the iv injection of Evans blue. *Scale bar*, 0.5 mm. The *black line* denotes the border between the lesioned tissue and the perilesion rim. B, Lesioned area visualized with TTC. Photographic display of representative brain sections are presented from control (a) and DHEAS-posttreated animals (b). Note the decreased infarct size in the steroid-administered slices (b). *Scale bar*, 5 mm.

Results

Cortical lesion

Although a highly standardized cortical lesion was induced within the MI region in the cortex, it did not result in any significant change in the rectal temperature of the animals. Cold injury-induced brain trauma, a classical model of vasogenic edema, is characterized by increased blood-brain barrier (BBB) permeability, and this was clearly revealed by the Evans blue labeling (Fig. 1A). Immediately after its iv injection, Evans blue binds to serum albumin (15). In the present study, the intravascular Evans blue was washed out by the perfusion. Accordingly, the Evans blue fluorescence observed in the brain parenchyma reflected the extravasated and retained serum albumin, indicating the cortical area that underwent severe damage during the cold injury induction. Besides the primarily lesioned cortical tissue (above the *dashed line* in Fig. 1A, this region appearing as the *white area* after TTC labeling in Fig. 1B), additional Evans blue-positive cells were detected in the perilesion rim. These cells fluoresced bright red.

DHEAS and E2 protect against cold lesion-induced injury in vivo

Both DHEAS and E2, administered either before or after cold lesion induction, led to a reduction in the volume of the damaged cortical region ($F_{5,45} = 6.145$; $P < 0.001$). Pretreatment with DHEAS caused a significant decrease in the extent of the infarct relative to the vehicle-treated controls (Fig. 2). This protective effect of DHEAS pretreatment was completely blocked when the neurosteroid was administered after letrozole. Surprisingly, DHEAS was even more effective in diminishing the injured area when it was administered just after cold injury induction (Fig. 2). E2 treatment either before or just after cold lesion induction resulted in a similar level of protection to that observed with DHEAS (Fig. 2).

Discussion

In this study, we investigated the neuroprotective ability of DHEAS applied before or after cold lesion injury induction and compared it with that of E2 treatment. The results clearly demonstrate that both pre- and posttreatment with DHEAS are effective in attenuating the brain injury. Our morphometric analysis revealed that both pre- and posttreatment with DHEAS result in a reduction in the infarcted area, whereas the aromatase inhibitor letrozole abolish this phenomenon.

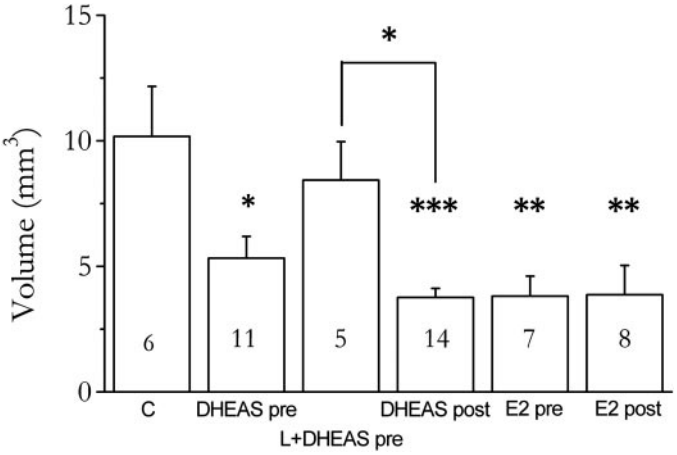

FIG. 2. Diagrams summarizing the effects of DHEAS and E2 as pre- and posttreatments. The averaged lesioned brain volumes within the groups were calculated and compared. Both pre- and posttreatment with DHEAS or E2 significantly reduced the volume of the lesion as compared with the control group, whereas the volume in the letrozole+DHEAS-pretreated group was not significantly different from that in the control group but did differ significantly from that in the DHEAS-posttreated group. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, for comparisons with the controls by ANOVA. C, Controls (n = 6); DHEAS pre (n = 11); L+DHEAS pre, letrozole and DHEAS-pretreated group (n = 5); DHEAS post (n = 14); E2 pre (n = 7); E2 post (n = 8).

Recent studies (9, 10) demonstrated the neuroprotective effect of DHEAS in different models, although cellular and molecular mechanisms of this effect remain unclear. Lapchak *et al.* (9) found that the γ-aminobutyric acid $(GABA)_A$ receptor antagonist bicuculline abolishes the neuroprotective effect of DHEAS. This may be connected with the disturbed calcium trafficking of injured neurons, which is an important factor in the neurodegenerative process (16). The recent results of Kurata *et al.* (17) suggest that the inhibitory effects of E2, DHEA, and DHEAS on the GABA-induced increases in intracellular calcium concentrations differ and that DHEA and DHEAS may act primarily at $GABA_A$ receptors rather than at voltage-gated calcium channels. Rupprecht and Holsboer (18) also concluded that DHEA and DHEAS are potent allosteric modulators of the $GABA_A$ receptor function. Racchi *et al.* (19) demonstrated that DHEA and other neurosteroids can regulate the neuronal function through their concurrent influence on transmitter-gated ion channels and gene expression, and they postulated that DHEA can directly modulate the age-associated impairment of protein kinase C signal transduction. Another possible mechanism has been proposed by Mao and Barger (7), who found that DHEAS (but not DHEA) is able to elevate κB-dependent transcription factor activity, a phenomenon previously associated with neuroprotection. Furthermore, steroids are known to exert antiinflammatory effects by interaction with the immune system, a possibility that cannot be ruled out in the present experiments (20). Steroids also play a crucial role in the development of the nervous system (21). As concerns our own findings, it might be of particular importance that the experiments were performed on young (2 to 3 wk old) animals.

In contrast with the above-mentioned data, our results demonstrate that letrozole, an aromatase inhibitor, is able to block the neuroprotective effect of DHEAS. This raises the question of whether DHEAS-triggered mechanisms are implicated in the neuroprotective events in our model.

Our results with letrozole indicate that the administered DHEAS was converted, partially or totally, to E2, which seems to mediate the neuroprotective effect: E2 is also neuroprotective in the brain (22–25). The mechanisms underlying this effect are not clear at present. However, a number of authors have suggested that, among other actions, E2 exerts its protective effect via mechanisms that attenuate apoptosis but do not influence necrosis (26–28). Moreover, evidence is accumulating that suggests that estrogen receptor-α, rather than estrogen receptor-β, is the critical factor mediating the protective effect (29, 30). Our results, together with the observations of Hajszan *et al.* (31), suggest that aromatic conversion of DHEAS and DHEA occurs within the brain, which opens up the possibility of its use in hormone replacement therapy without incurring the known side effects associated with systemic estrogen administration (32, 33). Maclusky *et al.* (34) found that letrozole blocks DHEA-induced hippocampal synaptogenesis only in females, whereas we observed letrozole-dependent DHEAS neuroprotection against focal cortical cold lesion induction in males. These results suggest that the synaptogenetic and neuroprotective effects of DHEA/DHEAS are regulated in different ways in the two sexes, this phenomenon demanding further examination.

It seems particularly important that even a single posttraumatic administration of DHEAS proved to be neuroprotective in our investigations. This is in good accordance with the results of Lapchak *et al.* on an ischemic model (9). The injury-induced vasogenic edema observed in our experiments clearly indicates the malfunction of the BBB. However, Evans blue not only leaks through the BBB but may also enter cells in the perilesion rim, the membrane of which may be damaged in consequence of the cold lesion. It is important that E2 induces endothelial nitrogen monoxide synthase-3 expression, which is essential for the protection of endothelial cells (35). Hence, DHEAS treatment may contribute to the maintenance of the BBB integrity (25) and probably the cell membrane integrity, thereby protecting them from further damage in the posttraumatic period. Another important issue is the therapeutic window of DHEAS treatment. In our experiments we used a postlesion time of 1 h, although this time window might be longer: Yang *et al.* (13) reported that E2 posttreatment is effective within 3 h after focal ischemia.

Despite the fact that the mechanisms have not yet been elucidated, our observations clearly show that DHEAS is neuroprotective when administered either before or after trauma induction in our animal model of brain injury, the extent of this protection being similar to that achieved with E2. Nevertheless, it remains to be established whether a similar effect can be detected in humans. These results suggest that DHEAS may be of substantial therapeutic benefit for the treatment of traumatic brain injury.

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