Dehydroepiandrosterone Sulfate Is Neuroprotective in a Reversible Spinal Cord Ischemia Model
Possible Involvement of GABA\textsubscript{A} Receptors

Paul A. Lapchak, PhD; Deborah F. Chapman, MSc; Sonia Y. Nunez, BA; Justin A. Zivin, MD, PhD

Background and Purpose—Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) may function as neurotrophic or neuroprotective factors to protect central nervous system (CNS) neurons against a variety of insults, including excitotoxicity. The present study evaluated the pharmacological effects of DHEAS in a reversible spinal cord ischemia model.

Methods—DHEAS was administered (50 mg/kg IV) 5 or 30 minutes after the start of occlusion to groups of rabbits exposed to ischemia induced by temporary (15 to 60 minutes) occlusion of the infrarenal aorta. The group P\textsubscript{50} represents the duration of ischemia (in minutes) associated with 50% probability of resultant permanent paraplegia.

Results—The P\textsubscript{50} of the vehicle-treated control group, when behavioral analysis was assessed 18 hours after aortal occlusion, was 28.8\pm2.0 minutes. Neuroprotection was demonstrated if a drug significantly prolonged the P\textsubscript{50} compared with the vehicle-treated control group. Treatment with DHEAS at 5 minutes significantly (P<0.05) prolonged the P\textsubscript{50} of the group to 36.8\pm3.9 minutes. In addition, the DHEAS effect appeared durable, because a significant difference between the control and DHEAS-treated groups was still measurable at the 4-day time point. At 4 days, the P\textsubscript{50} of the control group was 26.1\pm2.2 minutes, whereas the P\textsubscript{50} for the DHEAS-treated group was 38.6\pm5.9 minutes. DHEAS was not neuroprotective if administered 30 minutes after occlusion. In addition, the GABA\textsubscript{A} antagonist bicuculline abolished the neuroprotective effect of DHEAS.

Conclusions—The present study suggests that neurosteroids may have substantial therapeutic benefit for the treatment of ischemic stroke. (Stroke. 2000;31:1953-1957.)

Key Words: GABA ■ ischemia ■ neuroprotection ■ steroids ■ rabbits

For some time, it has been known that glucocorticoids are effective at reducing the inflammatory response\textsuperscript{1} and also reduce neurological damage induced by spinal cord trauma.\textsuperscript{2} The glucocorticoid methylprednisolone is currently a standard of care for the acute management of spinal cord injury.\textsuperscript{1} Recently, a new family of central nervous system (CNS)—active steroids referred to as neurosteroids has been described.\textsuperscript{3} Neurosteroids including dehydroepiandrosterone (DHEA) and its sulfated derivative DHEAS are abundantly expressed in brain.\textsuperscript{4–6} Basic research has shown that both neurosteroids can interact with CNS neurotransmitter receptor systems to regulate fast neurotransmission and neuronal excitability.\textsuperscript{7–12} Neurosteroids may also have a variety of neurotrophic factor—like roles in the CNS, including enhancement of neuron and glial cell survival\textsuperscript{13,14} and neuroprotection against excitatory amino acid (EAA) toxicity.\textsuperscript{13,14} Thus, DHEAS may be of use in treating ischemic stroke where there is enhanced EAA neurotransmission.

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Steroids have been administered empirically to stroke victims for many years without proof of efficacy or safety. Various classes of steroids have been shown to reduce neurological damage. For example, the glucocorticoid methylprednisolone attenuates functional deficits induced by spinal cord trauma.\textsuperscript{2} In addition, the neurosteroid progesterone affords neuroprotection after cerebral ischemia induced by middle cerebral artery occlusion.\textsuperscript{15,16} Weaver et al\textsuperscript{17} also showed that steroidal inhibitors of EAA receptors (ie, NMDA) are protective in the middle cerebral artery occlusion model. Taken together, there is rationale for the belief that neurosteroids will be useful for other forms of neuroprotection. There have been relatively few tests of neurosteroids in neuroprotection from ischemia in studies that used in vivo models. This promising class of agents has not been evaluated previously in a reversible ischemia animal model. Thus, with this basis, we examined...
the pharmacological effects of DHEAS in the reversible spinal cord ischemia model (RSCIM) using clinical ratings as an end point.

Materials and Methods

Reversible Spinal Cord Ischemia Model

Spinal cord ischemia was produced by occluding the aorta for up to 60 minutes as described previously. Briefly, the aorta of male New Zealand White rabbits weighing 3 to 3.5 kg was exposed at the level of the renal arteries through a midline abdominal incision, and a small-diameter Tygon tube was placed around the aorta just distal to the left (caudal) renal artery. The ends of this tube were threaded through a small plastic button and then a larger-diameter tube to form a snare, and the incision was closed around the tubing so that the free ends were accessible externally. Rabbits were allowed to recover from anesthesia for at least 2 hours, at which time it was possible to establish whether sensations and motor activities were normal. Pulling on and clamping the small tube occluded the aorta, and release of the tubing after a predetermined period allowed restoration of blood flow, after which all tubing was removed and the small hole in the abdominal wall was closed with surgical clips. The durations of ischemia were selected to span all grades of damage ranging from full recovery to permanent paraplegia. Zivin et al previously showed that there was a good correlation between the length of occlusion and the extent of spinal cord damage. Short periods of occlusion produced no apparent neurological deficit. Intermediate durations of occlusion produced partial neurological deficits involving central and medial gray matter, sparing long white matter tracts, gray horns, and dorsal root ganglia. Long durations of occlusion produced paraplegia and extensive lesions throughout the gray matter from the upper lumbar region to the end of the sacral segments.

Animals were observed while the aorta was occluded and for 1 hour after release of the snare, followed by daily examinations for the next 4 days. The degree of neurological function at 24 hours was graded on a binary scoring system. Rabbits were classified as normal if they ambulated normally, responded normally to noxious stimuli, and had normal bowel and bladder function. This group also included abnormal animals that did not hop normally, were less responsive than normal to pinching of the hindlimbs, and exhibited variable bowel and bladder function. Animals that showed any degree of motor impairment (from barely detectable to severe) were included in this grade and were considered normal for quantal analysis. Paraplegia animals were completely unresponsive to noxious stimuli in the hindquarters and were incontinent. The graders were blinded as to the treatments the animals had received. Animals that died within 4 days of the insult were excluded from the analysis. Paraplegia animals were completely unresponsive to noxious stimuli, and had normal bowel and bladder function. Animals that showed any degree of motor impairment (from barely detectable to severe) were included in this grade and were considered normal for quantal analysis. Paraplegia animals were completely unresponsive to noxious stimuli in the hindquarters and were incontinent. The graders were blinded as to the treatments the animals had received. Animals that died within 4 days of the insult were excluded from the analysis.

We administered the neurosteroid DHEAS (Sigma) intravenously at a dose of 50 mg/kg either 5 or 30 minutes after initiation of aortic occlusion. Control animals received the vehicle required to solubilize the steroid (25% β-hydroxypropyl cyclodextrin in 0.9% saline). In the drug combination study, DHEAS and the GABA A antagonist bicuculline (0.1 mg/kg IV) were administered concomitantly. Bicuculline was prepared as described previously. After 4 days, animals were killed with Beuthanasia-D (Scherer-Ploug Animal Health Care Corporation). All animal-use procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the San Diego Veterans Administration Medical Center.

The duration of occlusion for individual animals was varied from 15 minutes up to 60 minutes, which provided a wide range of ischemia for each experimental group. The group P50 represents the duration of ischemia (in minutes) associated with 50% probability of resultant permanent paraplegia. The therapeutic benefit was statistically analyzed and graphically demonstrated by computer construction of an ischemic duration dose-response curve for each group (similar to the LD50 curves of pharmacological studies). The computer calculated an ET50, which represented the duration of ischemia that produced permanent paraplegia in 50% of the animals in a group. Statistical significance was assessed with the group t test and adjusted for multiple comparisons with the Bonferroni correction (P<0.05). Neuroprotection was demonstrated if a drug significantly prolonged the P50 compared with the control group. The quantal dose-response analysis method used in the present study has been published previously.

Results

The first series of experiments investigated the effects of DHEAS when administered 5 minutes after the initiation of occlusion. The P50 of the vehicle-treated control group, when behavioral analysis was assessed 18 hours after aorta occlusion, was 28.8±2.0 minutes (mean±SE). Treatment with intravenous DHEAS at 50 mg/kg significantly (P<0.05) prolonged the P50 of the group to 36.8±3.9 minutes. The results of these experiments are shown in the Figure and presented in Tables 1 and 2. In addition, the DHEAS effect appeared durable, because there was a significant difference between the vehicle-treated control and DHEAS-treated groups when behavioral analysis was assessed 4 days after occlusion. The P50 of the control group was 26.1±2.2 minutes, whereas the P50 for the DHEAS-treated group was 38.6±5.9 minutes (P<0.05). DHEAS administration improved the mobility, tactile sensation, and use of the hindlimbs of ischemic rabbits.

The second series of experiments evaluated the neuroprotective effect of DHEAS when the drug was administered 30 minutes after the start of occlusion of the aorta. The P50 of the DHEAS-treated group, when behavioral analysis was assessed 18 hours and 4 days after aortal occlusion, was respectively 27.3±4.9 minutes and 16.7±8.4 minutes. This
TABLE 1. Comparison of the Effects of Vehicle or DHEAS on Clinical Outcome of Rabbits, Measured 18 Hours After Aortic Occlusion

<table>
<thead>
<tr>
<th>Duration of Ischemia, min</th>
<th>Behavioral Outcome for Treatment Groups</th>
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<th>30 Minutes after start of occlusion</th>
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The results above are behavioral readings for each group of rabbits. They are given as the number of rabbits either normal or paraplegic for each treatment group. Rabbits were treated intravenously with either vehicle (n=15) or DHEAS (n=28) administered 5 minutes after the start of occlusion. The ellipses indicate that an animal was not included in that particular group for that specific duration of ischemia.

Absence of a neuroprotective effect suggests that the DHEAS must be administered early after an ischemic event.

The third series of experiments determined whether the neuroprotective effect of DHEAS was still observed when the GABA\(_\alpha\) antagonist bicuculline was administered concomitantly with DHEAS. Bicuculline, which has previously been shown to decrease the P\(_{50}\) when the RSCIM is used, 23 abolished the neuroprotective effective of DHEAS (Figure and Table 2) in the RSCIM. The P\(_{50}\) of the DHEAS-and-bicuculline–treated group was 24.0±1.5 minutes versus 36.8±3.9 minutes for DHEAS alone when measured 18 hours after administration and 17.2±5.7 minutes versus 38.6±5.9 minutes when measured 4 days after administration.

**Discussion**

We investigated the effects of DHEAS on clinical outcome using an RSCIM. The results of our study suggest that DHEAS exerts a prominent neuroprotective effect, because DHEAS significantly shifted the P\(_{50}\) to the right by 28% (increased tolerance to ischemia) when administered 5 minutes after the start of ischemia. Moreover, the neuroprotective effect of DHEAS was not observed if bicuculline was administered concomitantly with DHEAS, which suggests that GABA\(_\alpha\) receptors may mediate the effects of DHEAS.

Recently, there has been some intriguing information concerning the neurosteroid family that includes DHEAS, a steroid that is abundant in the brain of many species. 4 DHEAS, which is synthesized and metabolized in the brain, 5,6 is a multifunctional steroid in the CNS, including neuroprotection and reduction of neurodegeneration. 23 DHEAS is also a potential signaling molecule for neocortical organization and remodeling during development, which suggests it has neurotrophic factor–like activity. 7 DHEAS has also been linked to neuroprotection via a nuclear transcription factor, NF\(_\kappa\)B, activation mechanisms 13 by which it effectively protected against glutamate toxicity. 13 Additionally, DHEAS protects hippocampal neurons against EAA-induced neurotoxicity. 14 Taken together, these findings suggest that DHEAS may be useful in treating neurodegenerative diseases, in particular ischemia or stroke, in which there is an excessive release of EAA. 24–26

In the present study, DHEAS was also shown to have neuroprotective activity against ischemic stroke. However, the neuroprotection was dependent on the timing of administration of the neurosteroid, such that the drug did not exert a neuroprotective effect if there was a prolonged interval between the initiation of the ischemic event and the administration of DHEAS. The failure at 30 minutes may be caused by other variables such as dose or duration of therapy. These possibilities remain to be examined in future studies. The observation that DHEAS is effective if administered early after the start of ischemia suggests that DHEAS may be attenuating a rapid process that is activated after the start of ischemia, such as turnover of a neurotransmitter. It has been postulated that one of the initial events that becomes activated after ischemia is an accumulation of glutamate followed by activation of metabotropic and NMDA receptors. 27 Our findings are consistent with the hypothesis that DHEAS may regulate a rapid neurotransmis-
sion, because DHEAS has previously been shown to protect against EAA-induced neurotoxicity,14 and it also directly regulates GABA-gated chloride currents.11

Naturally occurring neurosteroids like DHEA and DHEAS are potent allosteric modulators of GABA<sub>A</sub> receptor function.5,22–26 Moreover, DHEAS binds to the picrotoxin site of the GABA<sub>A</sub> receptor.10,11 Because it has been suggested that GABA<sub>A</sub> receptors may be modulated by DHEAS or may mediate the pharmacological effects of DHEAS,20 we determined whether the effects of DHEAS observed in the RSCIM were mediated by the GABA<sub>A</sub> receptor. For this, we administered the GABA<sub>A</sub> receptor antagonist bicuculline intravenously concomitantly with DHEAS 5 minutes after the start of occlusion. The neuroprotective effect of DHEAS in our ischemia model was not observed if the DHEAS-treated animals were also given bicuculline. This observation is quite interesting, because a previous study20 showed that the GABA agonist muscimol significantly increased and an antagonist bicuculline significantly decreased the P<sub>90</sub>. Our results with the combination of DHEAS and bicuculline suggest that DHEAS may interact with GABAergic neurons at some level.

A recent clinical trial with DHEAS20 determined the effect of DHEAS in patients with multi-infarct dementia.30 DHEAS improved infarct-induced decrease of daily activity and emotional disturbances and also normalized the EEG.30

Conclusions

We have demonstrated that the neurosteroid DHEAS is neuroprotective in a reversible ischemia model if administered early during the ischemic event, which suggests that DHEAS may modulate the function of a neurotransmitter. Our results showing that bicuculline attenuates the neuroprotective effect of DHEAS suggest that the neurotransmitter may be GABA and that DHEAS may be enhancing GABAergic transmission to be neuroprotective. The idea that enhancement of GABAergic neurotransmission is neuroprotective in a reversible ischemia model is consistent with the findings of Madden.20 Because steroids have been shown to be safe and effective for the treatment of spinal cord injury2 and considering their ease of administration, our results suggest that neurosteroids like DHEAS may have therapeutic benefit for the treatment of spinal cord and cerebral ischemia.

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Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are neuroactive steroids secreted by the adrenal gland and also expressed in the brain. DHEA and DHEAS levels decline with aging. The over-the-counter availability of DHEA has increased its use as a supplement to delay aging.1 In the preceding article by Lapchak and associates, DHEAS was noted to reduce functional impairment after spinal cord ischemia in a rabbit model. There are a number of in vitro studies supporting the contention that DHEA and DHEAS may be neuroprotective. These findings have been succinctly summarized by the authors. The present study demonstrated a modest neuroprotective effect of DHEAS in vivo. The therapeutic effect of DHEAS was noted only when it was given within 5 minutes, but not 30 minutes, after ischemia. This finding suggests that DHEAS is more likely to act on acute injury processes, such as those involving neurotransmitter mechanism.2 This contention is supported by a very interesting finding in the present study in which the protective role of DHEAS was abolished by bicuculline, a GABA_A receptor antagonist. GABA_A receptor agonists such as muscimol have previously been shown in the same model to protect spinal cord against ischemic insult.3 In view of the well-documented DHEA and DHEAS interactions with the GABA_A receptor, results from the present study collectively suggests a plausible mechanism of DHEAS neuroprotection via this receptor. The authors briefly address a parallel neuroprotective role of glucocorticoids in traumatic spinal cord injury. It remains to be determined whether glucocorticoids and DHEAS share the same mechanism of action in CNS injury models. The observation that DHEAS actions may be independent of glucocorticoid-mediated mechanisms4 and that glucocorticoids exacerbate ischemic brain injury5 suggest that these 2 groups of steroid are likely to act on different injury cascades triggered by CNS ischemia.

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