Altered expression of adrenocorticotropic hormone in the epileptic gerbil hippocampus following spontaneous seizure

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INTRODUCTION

Individuals are continuously exposed to potential disturbances (stressors) to equilibrium of essential body functions. In general, stress is among the most frequently self-reported precipitant of seizures in patients with epilepsy (1-3). This leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis. The initial step in this process is elevation of corticotrophin-releasing factor (CRF) in response to input from extrahypothalamic sources. This release, in turn, causes the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the circulation, which subsequently leads to release of corticosteroid hormones (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Biochemically, there is evidence that cortisol plays a role in seizure control (4). In addition, a role of stress hormones in the control of epilepsy has shown predisposition to the development and/or progression of epilepsy (5, 6). Moreover, glucocorticoids, ACTH, and steroid manipulations have been shown to be effective in the management of pediatric epilepsies (7, 8).

Mongolian gerbils are good models for studies of genetic epilepsy because this animal offers genetic control, breeding proclivity, and an ease of behavioral testing (9, 10). The seizures exhibited by gerbils are consistent over time, so it is possible to correlate seizure intensity records with morphological observations (9, 11-15). Previous studies have focused on the role of CRF, the regulator of pituitary ACTH release, in epileptic mechanisms (16, 17). However, it has also been recently reported that adrenal glucocorticoid hormones have a potent effect on hyperelectratory neuronal damage of pyramidal neurons in CA1 (18). In addition, potent anticonvulsant effects of ACTH have been reported in Mongolian gerbils (19). Although the ACTH level increases in temporal lobe epilepsy patient, it is unknown whether expressions of ACTH are altered depending on the time course following spontaneous seizure onset in the hippocampus of epileptic Mongolian gerbils. Therefore, we investigated the temporal and spatial alterations of ACTH immunoreactivity in the gerbil hippocampus to characterize the possible changes and associations with different sequelae of spontaneous seizures in this animal model.

RESULTS AND DISCUSSION

Altered expression of ACTH in the epileptic gerbil hippocampus

As shown in Fig. 1, ACTH immunoreactivity was rarely observed in any hippocampal region in the SR gerbil hippocampus (Fig. 1A, 1E, 1F, 2A1, 3A1, and 4A). However, in the pre-seizure SS gerbil hippocampus, ACTH immunoreactivity was detected in all hippocampal regions (Fig. 1B, 1E, and 1F). Briefly, ACTH immunoreactive neurons, which were presumed by morphological and distributional patterns to be interneurons, were distinctly identified in the CA1, subiculum, and dentate hilum regions (Fig. 2A2, 3A2, and 4B). In addition, the immunoblot study for the ACTH levels in the hippocampus and quantitative analyses for the average cell numbers of ACTH-positive neurons revealed its expression in all hippocampal regions (Fig. 1E, 1F, 2B, 3B, and 4E).

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In this study, spatial distributions of ACTH immunoreactive neurons were observed chronologically in the pre-seizure SS gerbil hippocampus compared to the SR gerbil groups. This result indicates that ACTH may modulate seizure activity in the epileptic gerbil hippocampus. To be more specific, altered ACTH expression of the pre-seizure SS gerbil group may be indicative of the hyperexcitatory state of the hippocampus for seizure onset. Many research groups reported that ACTH possesses neurotrophic effects in the peripheral and central nervous system during both development and regeneration (20, 21). Thus, ACTH may influence the hyperexcitable state of the epileptic gerbil hippocampus. In addition, the significant enhancement of glucocorticoid immunoreactivities in the pre-seizure SS gerbil hippocampus compared to SR gerbils has been described (22). Glucocorticoids secreted from the adrenal glands are important in maintaining granule cell survival and structural integrity of the granule cell layer in the hippocampus, because the ACTH peptide may be the final common mediator to protect from hyperexcitatory neuronal death following glucocorticoid inhibition (21). Moreover, selective ACTH expression may play an important role in the neurodegenerative process, influencing microglia and neurons and injection of the ACTH analog to protect CA1 pyramidal neurons from neuronal damage (18). The previous and present results collectively support the notion that enhanced ACTH immunoreactivities in the pre-seizure SS gerbil hippocampus may be indicative of the hyperexcitatory neuronal state of epileptic hippocampus, showing a lower threshold for seizure initiation.

As shown in Fig. 2 and 3, the double immunofluorescence...
staining for ACTH-positive neurons showed enhanced expression of ACTH immunoreactivity in the CA1 and subiculum (Fig. 2D2 and 3D2) compared to the SR gerbil hippocampus (Fig. 2C1-4 and 3C1-4). Interestingly, ACTH-positive neurons were identified as GABA\(\alpha_1\)-positive interneurons (Fig. 2D4 and 3D4).

In this study, ACTH immunoreactive interneurons observed in hippocampal formations on the basis of their localization and morphology were identified as GABAergic interneurons, which are known to have an inhibitory function with respect to glutamatergic granular and pyramidal neurons of the hippocampal CA1 and the dentate hilar region (23), especially GABA\(\alpha_1\) receptor containing neurons. In fact, GABAergic interneurons inhibit the excitatory effect of pyramidal neurons on hippocampus and GABA\(\alpha_1\) subunit containing interneurons involved in spontaneous seizure activity in the gerbil hippocampus as a compensatory response (24). In addition, various GABA functions are related to the assembly of multiple GABA\(\alpha_1\) receptor subunits (25) and its heterogeneity plays an important role in the regulation of GABA\(\alpha_1\) receptor-mediated inhibition within the hippocampal circuit (26). Interestingly, ACTH expression following ischemia was shown to be enhanced in GABAergic interneurons; thus, its increased expression in the CA1 after ischemic insult was involved in the reduction of cellular damage and was capable of preventing neuronal death induced by hyperexcitatory neuronal damage (18). Therefore, our findings support the hypothesis that the increase of ACTH immunoreactivity in

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**Fig. 3.** High magnification views of ACTH immunoreactivity in the subiculum of SR and SS gerbil hippocampi. In SR gerbil, the immunoreactivity of ACTH is rarely observed in all of the subiculum (A1). The arrows indicate the obvious expression of ACTH expression in pre-seizure SS gerbil groups in some interneurons (A2). At the 3 hr postictal SS gerbil, the ACTH-positive interneurons are markedly increased in the subiculum region (arrows in panel A3). Nevertheless, in the 12 h postictal SS gerbil groups, immunoreactivities of ACTH in the interneurons are down-regulated to the level of the pre-seizure SS gerbil groups (A4). Bar = 50 \(\mu\)m (panels A1-A4). Quantitative analyses of ACTH immunoreactive interneurons in the SR and SS gerbil hippocampi (B, mean ± S.E.M). Significant differences from SR gerbil hippocampus, *P < 0.05, **P < 0.01. Double immunofluorescent staining for ACTH (red) and GABA\(\alpha_1\) (green) in the subiculum region (C and D). In the SR gerbil, ACTH immunoreactivity in the GABA\(\alpha_1\)-positive interneurons is rarely observed in the subiculum (C1-C4). However, ACTH expression within GABA\(\alpha_1\)-positive interneurons in the pre-seizure SS gerbil hippocampus is obviously detected in the pyramidal cell layer of the subiculum (D1-D4). Blue is DAPI counter staining. Bar = 18.8 \(\mu\)m (panels C1-C4 and D1-D4).

**Fig. 4.** High magnification views of ACTH immunoreactivities in the dentate gyrus (DG) of SR and SS gerbil hippocampi. In SR gerbil, ACTH expression is rarely observed in the DG (A). However, the immunoreactivities of ACTH within the DG in the pre-seizure SS gerbil are obviously detected in some interneurons (B, arrows). In the 3 hr postictal SS gerbil, the ACTH-positive interneurons are increased near the granule cell layer of the DG (C, arrows). In the 12 h postictal SS gerbil, however, the expression of ACTH in the interneurons is down-regulated (D), similar to pre-seizure SS gerbil groups. Bar = 50 \(\mu\)m (panels A, B, C, and D). Quantitative analyses of ACTH immunoreactive interneurons in the SR and SS gerbil hippocampi (E, mean ± S.E.M). The average cell numbers of ACTH-positive interneurons in the epileptic gerbil hippocampus show that ACTH expression is changed depending on the time course following spontaneous seizure compared to SR gerbil hippocampus. Significant differences from SR gerbil hippocampus, *P < 0.05, **P < 0.01.
the pre-seizure gerbil hippocampus may be indicative of an abnormal hyperactive neuronal state, which presumably results in spontaneous seizure activity in this animal model.

Up-regulated ACTH immunoreactivity in the SS gerbil hippocampus following spontaneous seizure

As shown in Fig. 1C and 1D, ACTH immunoreactivities after spontaneous seizure onset were changed in the hippocampus, depending on the time course of the post-ictal stage. At 3 hr following spontaneous seizure, ACTH immunoreactive interneurons were significantly enhanced in the CA1, subiculum, and dentate gyrus (Fig. 2A3, 3A3, and 4C). Indeed, its expression was remarkable in the somata and processes of ACTH-positive interneurons in the CA1 and subiculum. Immunoblot and quantitative analyses for ACTH levels of the hippocampus and ACTH-positive interneurons also showed the same results as the immunohistochemistry, respectively (Fig. 1E, 1F, 2B, 3B, and 4E). However, ACTH-positive interneurons were down-regulated in all hippocampal regions at the 12 hr postictal stage (Fig. 1D).

In general, corticosteroid hormones, which are subsequently exposed and prepare the organism for future challenges (27). Interestingly, in this study, an increase of ACTH immunoreactivity within the hippocampus proper was observed from 30 min to 3 hr post-ictal. This result may indicate that ACTH localized in the hippocampus is utilized in lowering intracellular glucocorticoids to prevent alterations of intracellular functions. CF administration in animals produces a number of behavioral and physiological responses, including generalized motor convulsions (28, 29). However, ACTH and ACTH fragments injected into the brain or systemic circulation have been reported to have an effect in motivation, learning, and memory processes (30).

In conclusion, the present study collectively indicate that the increased ACTH immunoreactivities following spontaneous seizure in the SS gerbil hippocampus may be a delayed response to seizure activity. These results suggest that ACTH plays an important role in the inhibition of excitototoxicity in all hippocampal regions by hyperactivation of inhibitory interneurons, which suppress excitatory neurotransmissions of the hippocampal circuit.

MATERIALS AND METHODS

Experimental animals

These studies utilized the progeny of Mongolian gerbils (Meriones unguiculatus) obtained from the Experimental Animal Center of Soonchunhyang University, Cheonan, South Korea. Animals were provided with a commercial diet and water ad libitum under controlled temperature, humidity and lighting conditions (22 ± 2°C, 55 ± 5%, with an alternating 12 hr light/dark cycle). Procedures involving animals conformed to our institutional guide lines, which fully comply with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 80-23, 1996). All efforts were made to minimize the number of animals used and the amount of animal suffering.

Seizure induction

Each animal was tested a minimum of five times, as described by Paul et al. (9) and other previous studies (14, 15). Only animals with a consistent stage 4 or 5 seizure score according to the seizure severity rating scale of Loskota et al. (34) were included in the present study, and were described as seizure sensitive (SS, n = 10) gerbils. Seizure resistant (SR, n = 10) gerbils never demonstrated seizure activity.

Tissue processing and immunohistochemistry

At the designated time courses (30 min, 3, 6, 12, 24 and 48 h after spontaneous seizure, n = 10 respectively), experimental animals (about 8-months-old) were anesthetized (urethane, 1.5 g/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS. After this, tissue processing and immunohistochemistry processes were performed according to pervious described studies (14, 15). The sections were firstly incubated in mouse anti-ACTH (1:2000) and then processed for ACTH immunoreactivity using the streptavidin-biotin-peroxidase complex (Dako). Anti-ACTH antibody was not cross-reacted with CRF, ACTH, melanotropin hormone (α-MSH) or β-lipotropin hormone.
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(LPH). The sections were washed three times for 10 min each time with PBS, incubated sequentially in biotinylated goat anti-mouse IgG (Vector, USA) and avidin/biotinylated enzyme complex (ABC; Vector) and diluted 1:200 in the same solution as the primary antiserum.

Double immunofluorescence staining
Based on the results of the immunohistochemical study, we performed double immunofluorescent staining for both mouse anti-ACTH IgG (diluted 1:200; Peninsula) and rabbit anti-GABAα2, α1 receptor (1:2,500; Chemicon, USA) to confirm the cell type according to previous studies (14, 15). In this immunofluorescence staining, we used secondary antibodies as Cy2 conjugated donkey anti-goat IgG (1:200; Amersham, USA) and Cy3 conjugated donkey anti-mouse IgG (1:200; Amersham) for 1 h at room temperature.

Western blot
Based on the immunohistochemical results, we performed immunoblot analysis for identifying ACTH levels of hippocampus as in previous study (14, 15). Five animals in each experimental group (SR, pre-seizure SS, and 3 h groups following spontaneous seizure activity) were used in the immunoblot study. For tissue preparation, the animals were decapitated; the hippocampi were removed, then each tissue was homogenized in 10 mM PB containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF. After centrifugation, the protein concentrations in the supernatants were determined using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 30 μg of total protein were boiled with an equal volume of 2 × SDS sample buffer and boiled for 3 min, and then each mixture was loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, USA). To reduce background staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, sequentially incubated with primary anti-sera (1:200), peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO, USA) and then with an ECL kit (Amersham, USA). Optical densities were measured using NIH Image 1.59 software.

Cell count
Cell counts were carried out with a microscope connected via a CCD camera to a PC monitor. At a magnification of 25-50 ×, the hippocampal regions were outlined and the surface areas measured. ACTH-positive cells were counted by clicking on the monitor at a magnification of 100 ×. All ACTH-positive cells were counted regardless of the intensity of labeling. Based on the localization and the morphology, ACTH-positive neurons were identified as interneurons [CA1, dentate gyrus (DG), and subiculum]. Cell counts were performed by two different investigators who were blinded to the classification of tissues. The estimated cell number (n) was the average of values from three adjacent sections. Since the nucleus size measurement was used to correct the potential sampling bias, the diameter for each nucleus in the sample population was also measured at a magnification of 200 × and reduced to a mean diameter (D). The true estimate of cell number was then calculated using the Abercrombie correction method: N (per 250 × 250 μm2) = n (T/T + D/A) where N is the true cell number, T is the section thickness, and A is the measured area (per 250 × 250 μm2) of each hippocampal region (13-15).

Statistical analysis
All data obtained from the quantitative measurements were analyzed using one-way analysis of variance (ANOVA) to determine statistical significance. Bonferroni’s test was used for post-hoc comparisons. A P value < 0.01 or 0.05 was considered statistically significant (13-15).

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