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Decreased levels of RGS4 in the paraventricular nucleus facilitate GABAergic inhibition during the acute stress response

Soonwoong Jung, Hyeonwi Son, Dong Hoon Lee, Gu Seob Roh, Sang Soo Kang, Gyeong Jae Cho, Wan Sung Choi, Hyun Joon Kim*

Department of Anatomy and Convergence Medical Science, Bio Anti-aging Medical Research Center, Institute of Health Sciences, Gyeongsang National University Medical School, Jinju, 52727, Republic of Korea

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ABSTRACT

A healthy acute stress response requires both rapid increase and rapid clearance of blood corticosteroids. We previously showed that regulators of G-protein signaling 4 (RGS4), which decreases in the paraventricular nucleus (PVN) during acute stress, forms a complex with the $GABA_B$ receptor. In the present study, we show that this decrease in RGS4 levels in the PVN during an acute stress response facilitates the return of blood corticosteroids to basal levels. Moreover, the effect of RGS4 decrease is attenuated by a $GABA_B$ receptor antagonist. These results suggest that RGS4 in the PVN regulates blood corticosteroid-related $GABA_B$ receptor signaling during the acute stress response.

1. Introduction

The classic endocrine acute stress response begins with hypothalamus-pituitary-adrenal (HPA) axis activation, which results in the release of corticosteroids [cortisol in humans and corticosterone (CORT) in mice] via the modulation of corticotrophin-releasing hormone (CRH) in the paraventricular nucleus (PVN). The termination of the acute stress response is marked by a return to basal levels of these increased corticosteroids [1]. Although various stressful stimuli can initiate CRH release and the subsequent release of corticosteroids from the adrenal gland, the termination of the stress response is regulated mainly by GABAergic signaling, which inhibits CRH release from the PVN [2–4]. GABAergic neurotransmission is highly plastic, undergoing dynamic alterations in response to changes in the environment, such as following both acute and chronic stress [5].

GABAergic signaling can be modulated by metabotropic as well as ionotropic receptors [6,7]. The $GABA_B$ receptor ($GABA_BR$), which is the primary metabotropic receptor for GABA and is abundant in the PVN, is a G-protein coupled receptor (GPCR). GPCR-mediated signaling can be modulated by regulators of G-protein signaling (RGS) proteins via the GTPase-activating property of the RGS domain [8–10]. During the acute stress response, numerous GPCRs modulate several neural activities, including CRH release, cognition processes, fear memory consolidation, and inhibition of CRH release [2,11,12].

RGS4 is a member of the B/R4 group of RGS proteins, which have both an alpha-helix and RGS domain [13,14]. RGS4 is abundant in the central nervous system (CNS), especially in stress response-related regions [15]. We have previously identified RGS4 as an acute stress responsive RGS protein, the expression of which is decreased in the PVN of mouse hypothalamus in response to 2 h of acute immobilization stress (AIS) [16,17]. We have also found the direct complex of RGS4 and $GABA_B$ subunit in the PVN. Moreover, the RGS4 bound to $GABA_BR$ were decreased in the PVN 2 h after AIS [17].

Based on these findings, we hypothesized that a decrease in RGS4 may enhance the GABAergic signaling that is necessary for the termination of the acute stress response. To prove this hypothesis, we performed a local knockdown of RGS4 in the PVN via in vivo electroporation of RGS4 short-hairpin RNA (shRNA) and assessed changes in blood CORT levels during the acute stress response.

* Corresponding author. Department of Anatomy and Convergence Medical Science, Bio Anti-aging Medical Research Center, Institute of Health Sciences, Gyeongsang National University Medical School, 816 Beongil 15, Jinju-daero, Jinju, Gyeongnam, 52727, Republic of Korea. Tel.: 82 55 751 8734.
E-mail address: kimhj@gnu.ac.kr (H.J. Kim).

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2. Materials and methods

2.1. Animals

Nine-week-old male C57BL/6 mice (SPF grade, KOATEC, Korea) were used in all studies. Male mice were housed in a temperature-controlled (22 °C) environment under a 12-h light/dark cycle (lights on at 6 AM) with free access to laboratory chow and water. Animals were habituated for 1 week before the experiments, and treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and standard guidelines for laboratory animal care at the animal facility of the Gyeongsang National University (GLA-120120-M0001).

2.2. Preparation of RGS4-shRNA

Plasmids for RGS4-shRNA and scrambled shRNA purchased from OriGene (OriGene Technologies, Inc., Rockville, MD, USA) were constructed with cytomegalovirus (CMV) promoter (#TG510988, OriGene Technologies, Inc.) and turbo green fluorescent protein (tGFP) on the C-terminus. These plasmids have been validated for transient transfection and gene expression with GFP. The RGS4-shRNA expression plasmid has a 29-nucleotide gene-specific sequence inserted immediately downstream of a U6 promoter in the plus orientation, a seven-nucleotide loop, and a 29-nucleotide sequence in the reverse complement, followed by a TTTTTT termination sequence. The shRNA sequence used was 5′-ggcttcctgcctgaggagtgcaaaggaca-3′. Plasmids were collected from *Escherichia coli* culture by midi-prep and in vitro transfection was confirmed via microscope and Western blot analysis (data not shown).

2.3. Electroporation of shRNA into PVN

Mice were anesthetized by xylazine/tiletamine/zolazepam (0.5/1 μl/g, i.p.) and fixed to a stereotaxic device. We opened the cranium and drilled a hole at the coordinates of the PVN: −0.8 mm from the bregma, ±0.2 mm lateral, and −4.7 mm in depth. Electrodes were located at the exact target depth and retracted by 0.3 mm to infuse DNA-containing buffer, which was injected at a flow rate of 0.25 μl/min via a microinjector (KD Scientific, Holliston, MA USA). Following buffer injection, electrodes were re-inserted at the target coordinates for 30 s. We calculated the appropriate voltage for electroporation by measuring actual resistance and via the following formula: $V = R \times a$, where R is the measured resistance and a is a constant value (a = 10 here). Electroporation was performed 10 times (pulse cycle: 2 ms on, 98 ms off) via an in vivo electroporator (CUY21Vivo − SQ, BEX, Tokyo, Japan). Targeting and expression of shRNA were confirmed by microscopic observation of coronal brain sections (Fig. 1).

2.4. Determination of blood corticosterone levels

Blood corticosterone level measurements were performed as previously described [17]. Briefly, mouse blood was collected in vacutainers containing K^3EDTA and centrifuged for plasma isolation at 1000 × g for 15 min at 4 °C. Samples were stored at −80 °C until the assay was performed. Quantification of plasma corticosterone levels was carried out using a corticosterone EIA kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's protocol.

2.5. Western blot analysis

Western blot analysis was performed as previously described [17]. Briefly, protein samples (20 μg each) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 1% bovine serum albumin and 5% skim milk and incubated with primary antibodies (anti-RGS4, 1:500, SC-6204, Santa Cruz Biotechnology, Dallas, TX, USA). Bound antibodies were detected with an enhanced chemiluminescence detection kit (Amersham Biosciences, Munich, Germany) according to the manufacturer's protocol. Each band density was read by SigmaGel software (Sigma–Aldrich, St. Louis, MO, USA) to quantify the results. Each density was normalized using the corresponding α-tubulin density as an internal control.

2.6. Statistical analysis

Western blot results were assessed by Student's unpaired t-tests or one-way analysis of variance (ANOVA) in GraphPad (GraphPad Software, La Jolla, CA, USA) and SigmaPlot (Systat Software, San Jose, CA, USA). Bonferroni post hoc tests were used for multiple comparisons. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1. RGS4 shRNA reduces RGS4 expression in the PVN and alters blood corticosterone levels during the acute stress response

Plasmids for RGS4 shRNA used in the present study contained both shRNA and a tGFP coding sequence with a CMV promoter. Therefore, the expression of shRNA in the PVN region was confirmed by tGFP expression 6 days after surgery (Fig. 1A and B). We confirmed the local knockdown of RGS4 expression in the hypothalamic region by Western blot analysis ($F_{(2,15)} = 32.45$, $p < 0.01$); the scrambled shRNA plasmid did not affect RGS4 expression levels (Fig. 1C). Mice transfected with RGS4 shRNA or scrambled shRNA and mock control were exposed to 2 h of immobilization stress (IM), and blood CORT levels were assayed by enzyme immunoassay. The local knockdown of RGS4 in the PVN did not influence CORT levels immediately after 2 h of IM. However, CORT levels were significantly reduced by RGS4 shRNA 2 h after the termination of IM (Fig. 2, $F_{(2,15)} = 730.2$, $p < 0.01$). This result suggests that the reduction of RGS4 may be involved in the termination but not the initiation or main event of the acute stress response.

3.2. $GABA_B$ receptor antagonist reverses the effect of RGS4 shRNA on CORT levels 2 h after IM

We recently showed that RGS4 and $GABA_BR$ form a direct complex [17]. Therefore, we hypothesized that a reduction in RGS4 may enhance GABAergic signaling, which would subsequently return CORT to basal levels over an appropriate time interval. To prove this hypothesis, we administered the $GABA_BR$ antagonist CGP 54626 30 min prior to IM. This $GABA_BR$ antagonist reversed the effect of shRNA in a dose-dependent manner (Fig. 3, $F_{(5,59)} = 38.17$, $p < 0.01$), which suggests that the reduction in CORT levels due to RGS4 shRNA may be mediated by enhanced $GABA_BR$ signaling. We further hypothesized that a $GABA_BR$-specific agonist would exert a similar effect on changes in CORT levels, if the return of CORT to basal levels is regulated by RGS4 action via $GABA_BR$. Thus, we injected the $GABA_BR$-specific agonist baclofen (2.5 mg/kg) 30 min prior to IM (Fig. 4). We found that baclofen decreased CORT levels both during the IM and 2 h after IM ($t_8 = 10.35$, $p < 0.01$), which indicates that both $GABA_BR$ and RGS4 in the PVN are associated with the termination of the acute stress response.