Potentiated Modulation of Pregnanolone on GABA_A Receptors in Behaviorally Stressed Borderline-Hypertensive Rats

Hyoung Chul CHOI,a Jong-Yeon KIM,b Jong-Bum LEE,c Yong-Hoon PARK,d Jae-Tae LEE,e Hun-Gu KANG,f Byung-jo KANG,g Kwang Yoon Lee,a and Jeoung-Hee HA*a

a Departments of Pharmacology, College of Medicine, Yeungnam University; b Psychiatry, College of Medicine, Yeungnam University; c Psychiatry, College of Medicine, Yeungnam University; d Pediatrics, College of Medicine, Yeungnam University; e Psychiatry, College of Medicine, Kyungbook National University; f Psychiatry, College of Medicine, Kyungbook National University; Taegu 700–412, Republic of Korea.

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The modulatory effects of behavioral stress on [3H]flunitrazepam, an agonist for the central-type benzodiazepine receptor complex, in borderline hypertensive rats (BHR) were examined. In repeatedly immobilized (for 2 weeks, for 2 h/d) BHR, enhancement of [3H]flunitrazepam binding to the receptor was observed to be potentiated. The percent enhancement of [3H]flunitrazepam binding in BHR was higher than that in normotensive control Wistar-Kyoto rats. Pregnanolone, a neuroactive steroid that has been reported to be a putative endogenous modulator in the stress response, concentration dependently enhanced [3H]flunitrazepam binding to the receptor. Enhancement of [3H]flunitrazepam binding was observed to be potentiated by the same immobilized stress, and the EC50 values of pregnanolone in BHR was significantly lower than those in controls and Emax values were higher. From the above results, it can be concluded that neural modulation to behavioral stress, especially in GABAergic neurotransmission, is exaggerated in BHR. We propose strain-specific differences of stress reactivity as an important pathogenetic factor in psychosomatic disorders including stress-induced hypertension. This is supported by reports showing exaggerated cardiovascular and sympathoadrenal responses to stress in BHR.

Key words borderline-hypertensive rat; behavioral stress; benzodiazepine receptor; neuroactive steroid

Borderline hypertensive rats (BHR) have been used extensively to study the interaction of environmental factors, genetics, and blood pressure. BHR are the first-generation progeny of a cross between spontaneously hypertensive rats (SHR) and control Wistar-Kyoto rats (WKR). The resting systolic blood pressure in BHR remains within the normotensive range throughout its lifetime. Interestingly, the genotype of BHR makes them particularly sensitive to the influence of environmental factors.1

The physiological significance of the roles of GABA_A in the mediation of stress-related responses is widely known. To abolish the aversive effects of behavioral stress, endogenous benzodiazepine receptor ligands have been proposed to modulate GABA transmission by activating the central-type benzodiazepine receptor (CBR) located in the GABA_A receptor complex. Neuroactive steroids, the other candidate for the endogenous modulator in the stress response, concentration dependently enhanced [3H]flunitrazepam binding to the receptor. Enhancement of [3H]flunitrazepam binding was observed to be potentiated by the same immobilized stress, and the EC50 values of pregnanolone in BHR was significantly lower than those in controls and Emax values were higher. From the above results, it can be concluded that neural modulation to behavioral stress, especially in GABAergic neurotransmission, is exaggerated in BHR. We propose strain-specific differences of stress reactivity as an important pathogenetic factor in psychosomatic disorders including stress-induced hypertension. This is supported by reports showing exaggerated cardiovascular and sympathoadrenal responses to stress in BHR.

MATERIALS AND METHODS

Animals Male WKR and BHR were used. To obtain BHR, breeding pairs of female SHR and male WKR were purchased from SLC (Japan) at 10 weeks of age. Litters were culled to 8 pups at birth and weaned from their mothers at 28 d. Animals were maintained on a 12-h light/dark cycle (lights on at 06:00), housed individually, and given ad libitum access to food and water. Rats were behaviorally stressed or killed in accordance with US guidelines (NIH publication # 85-23, revised in 1985).

Immobilization Stress Male rats, weighing 150—200 g, were subjected to immobilization stress as described by Nappi and Rivest.2 Briefly, the stress was produced by forcing the rats into immobilizer (Centrap cage, Fischer, Pittsburg, PA, U.S.A.) for 2 h (14:00—16:00) for 2 weeks. The animals were decapitated after termination of immobilization stress.

Receptor-Binding Assay For the measurement of the modulatory effect of pregnanolone on [3H]flunitrazepam binding to the CBR, the cerebral cortex of rats (male, 250—350 g) was removed immediately after decapitation. Tissues were disrupted in 50 volumes of 50 mM Tris–citrate buffer (pH 7.4) and centrifuged at 20000 g for 20 min, and the pellets were resuspended in 50 volumes of 50 mM Tris–citrate buffer (pH 7.4) and recentrifuged. Final pellets were stored for assay at −70 °C after 3—5 washing procedures. The binding of [3H]flunitrazepam (85.0 Ci/mmol, NEN), an agonist of the CBR, to the membranes was assayed using a filtration technique. Each assay comprised triplicate samples containing 0.16 mg of protein suspended in 0.5 ml of 50 mM Tris–citrate buffer (pH 7.4) and incubated for 60 min at 4 °C using 1 nM of radiolabeled drugs. Nonspecific binding was determined in the presence of Ro15-1788 10 μM and represented about 10% of total binding. The assays were terminated by filtration through GF/B filters (Whatman) and three washes with 5 ml of ice-cold buffer using a harvesting apparatus (Brandel M-24R, Brandel Instruments, Gaithersburg, MD, U.S.A.). The radioactivity retained by the filters was measured in a liquid scintillation spectrometer (Wallac 1410, Turku, Finland) using 4 ml of scintillation solution (Packard

* To whom correspondence should be addressed. e-mail: jhha@medical.yeungnam.ac.kr © 2004 Pharmaceutical Society of Japan
instruments B.V. Chemical Corporations, Groningen, Netherlands) as a fluor. Protein was determined as previously reported using the bichinchonic acid method (Pierce Chemicals, Rockford, IL, U.S.A.).

**Chemicals** [3H]Flunitrazepam were purchased from DuPont-NEN (Boston, MA, U.S.A.). Ro15-1788 (donated by Hoffmann-La-Roche, Switzerland) was dissolved in absolute ethanol. Tris, citric acid, polyethylenimine, pregnanolone[5-β-pregnan-3-α-ol-20-one], and sucrose were purchased from Sigma (U.S.A.) and dissolved in distilled water. Scintillation cocktail (Aquasol-2) was purchased from Packard Instruments B.V. Chemical Corporations. Bichinchonic acid was obtained using the bichinchonic acid method (Pierce Chemicals, Evanston, IL, U.S.A.).

**RESULTS AND DISCUSSION**

As shown in Fig. 1, basal binding of [3H]flunitrazepam to the CBR in cerebral cortices differs in WKR and BHR before repeated immobilization stress for 2 weeks. In BHR, the basal binding (pmol/mg protein) of [3H]flunitrazepam before repeated immobilization stress, 73.8±0.11, significantly (p<0.05) lower than that of WKR, which was 87.9±0.2. In all groups, repeated immobilization stress significantly (p<0.05) enhanced [3H]flunitrazepam binding, compared with that in control rats. The percent enhancement of basal binding of [3H]flunitrazepam binding to the CBR in WKR and BHR was 3.1±0.1 and 11.0±0.2, respectively. The CBR, which is located on the GABA-Cl channel receptor complex, was observed to be upregulated after behavioral stress, therefore, increased positive allosteric modulation of GABA_A receptor by endogenous substances released by behavioral stress could reduce the averse effects in vivo. As shown in our experiment, [3H]flunitrazepam binding to the CBR was enhanced by repeated immobilization stress for 2 weeks, and the percent enhancement of [3H]flunitrazepam binding to the CBR in BHR was higher than in normotensive control WKR.

Neuroactive steroids are one of the endogenous substances that positively modulate the GABA_A-Cl channel receptor complex by increasing the binding affinity of endogenous benzodiazepine receptor agonist to the receptor, in association with behavioral stress. Their selective interaction with a benzodiazepine receptor agonist to the receptor, in association with behavioral stress. These effects are similar to those exhibited by other endogenous modulators of the GABA_A receptor complex such as endogenous benzodiazepine receptor ligands.

As shown in Fig. 2, pregnanolone, a neuroactive steroid, concentration dependently enhanced [3H]flunitrazepam binding to control and stressed rat cortices in experimental animals. Enhancement of [3H]flunitrazepam binding was observed to be potentiated by the same immobilized stress. In stressed BHR, the EC_{50} (μM) of pregnanolone enhancement of [3H]flunitrazepam binding, was 0.41±0.00, which was significantly (p<0.05) lower than that of control WKR, which was 0.82±0.02. The E_{max} (%) of pregnanolone enhancement was significantly (p<0.05) increased to 162.2±1.2 in stressed BHR compared with 151.2±1.2 in control BHR. EC_{50} values of pregnanolone enhancement in stressed WKR are shown in Table 1. However, no differences were observed between BHR and WKR in the EC_{50} and E_{max} values of unstressed animals.

Some studies indicated that BHR have exaggerated cardio-
vascular and sympathoadrenal responses to stress compared with normotensive WKR control.8—11) Consistent with these reports, in our study, BHR showed greater changes in mean arterial pressure after the immobilization, compared with WKR (data not shown). Recently, heightened neuronal activation of immediate early gene mRNA levels in BHR has been suggested, compared with control WKR.12) Consistent with these reports, in our experiments, BHR showed exaggerated reactivity to stress, particularly in the neuronal activation.

From the above results, it can be concluded that a neuronal modulation to behavioral stress, especially in the GABAergic neurotransmission, is exaggerated in BHR. We would like to propose strain-specific differences of stress reactivity as an important pathogenetic factor in psychosomatic disorders including stress-induced hypertension.

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