Modulation of Radioligand Binding to the GABA<sub>A</sub>-benzodiazepine Receptor Complex by a New Component from Cyperus Rotundus

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Four sesquiterpenes, β-selinene, isocurcumenol, nootkatone and aristolone and one triterpene, oleanolic acid were isolated from the ethylacetate fraction of the rhizomes of Cyperus rotundus and tested for their ability to modulate γ-aminobutyric acid (GABA<sub>A</sub>)-benzodiazepine receptor function by radioligand binding assays using rat cerebrocortical membranes. Among these compounds, only isocurcumenol, one of the newly identified constituents of this plant, was found to inhibit [3H]Ro15-1788 binding and enhance [3H]flunitrazepam binding in the presence of GABA. These results suggest that isocurcumenol may serve as a benzodiazepine receptor agonist and allosterically modulate GABAergic neurotransmission via enhancement of endogenous receptor ligand binding.

Key words Cyperus rotundus; isocurcumenol; neuromodulation; γ-aminobutyric acid (GABA<sub>A</sub>)-benzodiazepine receptor; radioligand binding

The benzodiazepine receptor (BZR) is an important component of the γ-aminobutyric acid (GABA<sub>A</sub>) receptor complex, the activation of which enhances the actions of GABA<sub>A</sub>, an inhibitory neurotransmitter, on the Cl<sup>-</sup> conductance of the neuronal membrane. Three functionally distinct classes of compounds are known to interact with the BZR to modulate GABA interactions with its receptor and its associated Cl<sup>-</sup> channel function. These are agonists (e.g. diazepam), inverse agonists (e.g. methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) and antagonists (e.g. flumazenil). Since the first description of the BZR, many investigators have attempted to identify the 'endogenous' BZR ligand. 1,4-Benzodiazepines, l-β-carboline-3-carboxylate, isoin and the diazepam binding peptide inhibitor have been proposed<sup>(1)</sup> to be natural BZR ligands, that is to say neurotransmitters or hormones synthesized in situ, released in response to specific physiological signals which then interact with the BZR. Up to the present time, flavonoids,<sup>(2,3)</sup> biflavonoids,<sup>(2,3)</sup> terpenes,<sup>(4,5)</sup> and quinolines<sup>(5)</sup> isolated from medicinal plants have been introduced as BZR agonists.

As part of our ongoing search for sedative agents from natural sources,<sup>(6–8)</sup> we evaluated the activity of the rhizomes of Cyperus rotundus L. (Cyperaceae), which have been used as an analgesic and as a sedative drug in Korean folk medicine.<sup>(9)</sup> Constituents of this plant have mainly been examined for terpenoids: besides a few saponins<sup>(10–12)</sup> and alkaloids,<sup>(13)</sup> more than 20 sesquiterpenes<sup>(14–21)</sup> have been isolated. However, phytochemical research on this plant has not been thoroughly performed. Moreover, the effects of its constituents on GABAergic neurotransmission have not been reported yet.

Herein, we describe the isolation of some components, including new sesquiterpenes, from the active fraction of Coxyri Rhizoma and the agonistic property of a newly identified constituent from this plant on the rat cerebrocortical BZR.

MATERIALS AND METHODS

Materials and Animals The dried rhizomes of C. rotundus were purchased from an oriental drugstore in Youngchon, Korea, and identified by Prof. Byung-Soo Kang, College of Oriental Medicine, Dongguk University, Kyongju, Korea. A voucher specimen of this plant material is deposited at the Herbarium (No. SKK-C018) at Sungkyunkwan University, Seoul, Korea. [3H]Flunitrazepam and [3H]Ro15-1788 were obtained from Dupont-NEN (Boston, MA, U.S.A.). Ro15-1788 was donated by Hoffmann-Roche, Switzerland. Scintillation cocktail (Aquadish-2) was purchased from Packard Instruments B.V. Chemical Corporations (Groningen, Netherlands). Bichinchoic acid protein assay kit was provided by Pierce Chemicals (Rockford, IL, U.S.A.). Male Sprague-Dawley rats weighing 250—350 g were used for the receptor preparation. Animals were kept in a regulated environment (21±1 °C) with a 12 h light-dark cycle. For analytical instruments: see reference.<sup>(22)</sup>

Extraction and Isolation The dried and pulverized rhi- zomes of the plant (600 g) were extracted with n-hexane (2×21) at 40°C to yield a hexane fraction (4.5 g). The residue was extracted with methanol and successively with ethylacetate to furnish the corresponding methanol fraction (94.5 g) and ethylacetate fraction (10.2 g). The active ethylacetate fraction gave at least five components by TLC (CH<sub>3</sub>C<sub>2</sub>H<sub>2</sub>) and CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 10:1:0.1) using phosphomolybdic acid spray reagent (Aldrich Co.). This fraction was chromatographed on a silica gel column (230—400 mesh, Merck; 3×50 cm) with the above solvents, affording four sesquiterpenes and one triterpene. Structural determinations of these compounds were based on comparison of GC/MS (HP5973 MSD system) and NMR (Varian XL-200, 200 MHz) spectra with published data, as follows (Rf value in CH<sub>2</sub>Cl<sub>2</sub>): β-selinene (0.95),<sup>(11)</sup> isocurcumenol (0.77),<sup>(23)</sup> nootkatone (0.56),<sup>(24)</sup> aristolone (0.37),<sup>(25)</sup> and oleanolic acid (0.56, in CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 10:1:0.1).<sup>(18)</sup> Among these compounds, isocurcumenol, nootkatone and aristolone were isolated for further studies.

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the first time from this plant.

Receptor Binding Assay For the preparation of receptors, cerebral cortices of male Sprague-Dawley rats were removed immediately after rapid decapitation according to the American Association for Accreditation of Laboratory Animal Caretaker Guidelines. 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 centrifuged. Final pellets were stored at −70°C after 3—5 times washing. The bindings of [3H]Ro15-1788 (78.0 Ci/mmol) and [3H]flunitrazepam (85.0 Ci/mmol) to the membranes were assayed using a filtration technique. Each assay comprised triplicate samples containing 0.16 mg protein suspended in 0.5 ml of 50 mM Tris–citrate buffer (pH 7.4) incubated for 60 min at 4°C in the presence of 1 nm radiolabeled ligands. Nonspecific binding was determined in the presence of 10 μM Ro15-1788 and represented about 10% of the total binding. The reactions were terminated by filtration through GF/B filters (Whatman) and three washes with 5 ml ice-cold buffer using a Harvesting apparatus (Brandel M-24R, Brandel Instruments, Gaithersberg, MD, U.S.A.). The radioactivity retained by the filters was measured in a liquid scintillation spectrometer (Wallac 1410, Turku, Finland) using 4 ml scintillation solution as a fluor. Protein was determined using a bichinchonic acid kit. In the competition assay for [3H]Ro15-1788 binding, increasing volumes of the extract or column fractions are added to the assay tubes. The amount of radioligand bound in the presence of the extracts is then compared to competition curves constructed using known concentrations of diazepam, a generic BZR agonist. Results were expressed in terms of mg diazepam equivalents/g tissue wet weight. Finally, ‘GABA shift’ assays were employed to determine the pharmacological characteristics of the constituent(s) interacting with BZR. Addition of GABA (final concentration of 0.1 mM) to the reaction mixture significantly (p<0.05) enhanced inhibition of [3H]flunitrazepam binding by the methanol fraction, indicating that the methanol fraction was agonistic to the receptor. In contrast, the hexane fraction showed much less diazepam equivalent value (32.8 ± 7.64) (Fig. 2). The hexane fraction inhibited [3H]flunitrazepam binding: in the presence of 10 μM GABA, the percent inhibition of [3H]flunitrazepam binding by the methanol fraction was 0.7 ± 0.42, and 19.5 ± 0.42, respectively. This positive GABA shift strongly suggests that component(s) in this fraction may have agonistic activity to BZR.

Results and Discussion

Activity-guided fractionation of the rhizomes of *Cyperus rotundus* and the isolation of five components (Fig. 1) from the active fraction were carried out and their abilities to modulate GABA, benzodiazepine receptor function through BZR were examined by receptor binding assays.

The methanol fraction inhibited the specific binding of [3H]Ro15-1788, a selective BZR antagonist, to rat cerebral cortical membranes, showing a diazepam equivalent (mg/g of weight) of 328.9 ± 7.64 (Fig. 2). The methanol fraction also inhibited the specific binding of [3H]flunitrazepam (see below), a selective BZR agonist. The addition of GABA (20 μM) to the reaction mixture significantly (p<0.05) enhanced inhibition of [3H]flunitrazepam binding by the methanol fraction. The percent inhibition of [3H]flunitrazepam binding by the methanol fraction in the absence and presence of GABA was 0.7 ± 0.42 and 19.5 ± 0.42, respectively. This positive GABA shift strongly suggests that component(s) in this fraction may have agonistic activity to BZR. In contrast, the hexane fraction showed much less diazepam equivalent value (39.2 ± 8.71) than the methanol fraction (Fig. 2). Based on these observations, the methanol fraction was further sub-fractionated with ethylacetate. The ethylacetate fraction exhibited about 5-fold higher diazepam equivalent value (1587.9 ± 28.20) than the methanol fraction (Fig. 2). The ethylacetate fraction enhanced [3H]flunitrazepam binding to the BZR. However, in the presence of GABA, the ethylacetate fraction inhibited [3H]flunitrazepam binding: in the presence of 20 μM GABA and 120 mM NaCl, percent inhibition of [3H]flunitrazepam binding (8.2 ± 1.18) was significantly (p<0.05) higher than that in the absence (−10.7 ± 1.43) (Fig. 3). These results suggested that the substance or substances acting as BZR agonist(s) may be contained in the ethylacetate fraction of *C. rotundus*.

Next, we isolated five components from the ethylacetate fraction and identified them as β-selinene, isocurcumenol, nootkatone, aristolone and oleanolic acid. Among them, isocurcumenol, nootkatone and aristolone were isolated for the first time from this plant. Except for isocurcumenol, the
been reported to be increased in the presence of GABA.\(^1\)

Endogenous BZR agonists found in mammalian brain has activity on the GAB\(_A\) receptor. Isocurcumenol significantly \(^2\) enhanced the binding of \[^{3}H\]flunitrazepam in the presence of GABA. \(^3\)

Therefore, the findings presented in this study suggest that isocurcumenol may allosterically modulate GABAergic neurotransmission via enhancement of interactions of GABA with its receptor in vivo.

Taken together, these observations may help to provide a pharmacological basis for the traditional applications of \(C.\ rotundus\) as a sedative.\(^9\)

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REFERENCES