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Partial agonistic effect of yokukansan on human recombinant serotonin 1A receptors expressed in the membranes of Chinese hamster ovary cells

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ABSTRACT

Ethnopharmacological relevance: Yokukansan (YKS) is a traditional Japanese medicine consisted of seven medicinal herbs and has been used for treatment of neurosis, insomnia, and behavioral and psychological symptoms of dementia (BPSD) in Japan.
Aim of the study: The aim of the present study is to clarify the intrinsic activity of YKS on serotonin (5-HT)1A and 5-HT2A receptors and also to determine the constituent herbs which are responsible for the effect of YKS.
Materials and methods: The dry powdered extracts of YKS, seven constituent herbs, and YKS-analogues which were produced by eliminating one of the constituent herbs from YKS in the manufacturing process, were used for the evaluation. Competitive binding assays for 5-HT receptors and [^{35}S]GTPγS binding assays for the evaluation of agonistic/antagonistic activity were performed using Chinese hamster ovary cell membranes stably expressing human recombinant 5-HT1A or 5-HT2A receptors.
Results: YKS (6.25–400 μg/ml) concentration-dependently inhibited the binding of [^{3}H]8-OH-DPAT to 5-HT1A receptors. The IC_{50} value was estimated to be 61.2 μg/ml. In contrast, YKS failed to inhibit the binding of [^{3}H]ketanserin to 5-HT2A receptors. Only Uncaria hook (3.13–50 μg/ml), of the seven constituent herbal extracts, inhibited the [^{3}H]8-OH-DPAT binding to 5-HT1A receptors in a concentration-dependent manner, and the IC_{50} value was estimated to be 7.42 μg/ml. The extracts of YKS or Uncaria hook increased [^{35}S]GTPγS binding to 5-HT1A receptors to approximately 50% of that of a full agonist, 5-HT. Both the competitive binding and [^{35}S]GTPγS binding of YKS to 5-HT1A receptors were remarkably attenuated by eliminating Uncaria hook from YKS, but it was almost unchanged when one of the other constituent herbs was eliminated from YKS.
Conclusion: These results suggest that YKS has a partial agonistic effect on 5-HT1A receptors, which is mainly attributed to Uncaria hook.

1. Introduction

In patients with Alzheimer's disease (AD), not only core symptoms, such as cognitive impairment, but also behavioral and psychological symptoms of dementia (BPSD), such as aggression, anxiety, and hallucinations, often emerge. BPSD is a serious problem for caregivers, and because its severity and the care burden show a positive correlation, therapy for BPSD is considered to be as important as therapy for the core symptoms (Nagaratnam et al., 1998; Tanji et al., 2005). To date, antipsychotic medicines have been used for treatment of BPSD. However, the drugs induce extrapyramidal symptoms and other adverse events, and in consequence, they decrease the quality of life and increase the difficulty of maintaining activities of daily living. In addition, the U.S. Food and Drug Administration warned that mortality was increased in elderly patients with dementia who used atypical antipsychotic medicines (www.fda.gov/cder/drug/advisory/antipsychotics.htm., 2005). Thus, new remedies without adverse effects have been sought.

Yokukansan (YKS), a traditional Japanese medicine referred to as a "*kampo* medicine" in Japan, has been approved by the Ministry of Health, Labour, and Welfare of Japan as a remedy for neurosis, insomnia, and night crying in children. Recently, YKS has been reported to ameliorate such BPSD as hallucinations, agitation, and aggressiveness in patients with AD, dementia with Lewy bodies (DLB), and other forms of senile dementia (Iwasaki

Abbreviations: AD, Alzheimer's disease; BPSD, behavioral and psychological symptoms of dementia; CHO, Chinese hamster ovary; GTPγS, guanosine-5′-[γ-thio]triphosphate; PCA, *p*-chloroamphetamine; 5-HT, 5-hydroxytriptamine (serotonin); YKS, yokukansan.

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et al., 2005a,b; Mizukami et al., 2009; Shinno et al., 2007, 2008). The usual adult dose for treatment of YKS is 7.5 g/day orally in 3 divided doses before or between meals. For example, Iwasaki et al. (2005a) demonstrated that treatment of YKS (2.5 g × 3 times/day) for 4 weeks improved BPSD and activity of daily living in a randomized, observer-blind, controlled trial using 52 patients with mild-to-severe dementia according to DSM-IV criteria. Mizukami et al. (2009) demonstrated the effectiveness and safety of YKS (2.5 g × 3 times/day for 4 weeks) for treatment of BPSD in a randomized cross-over study using 106 patients diagnosed as having AD or DLB. However, the mechanism underlying the effectiveness of YKS is still unclear.

The pathologic hallmarks of AD are neuronal loss and a decrease in the number of synapses, in addition to senile plaques (aggregations of extraneuronal amyloid β protein) and neurofibrillary tangles (accumulations of intraneuronal phosphorylated tau protein). In addition to the neuronal loss, cholinergic deficits in the cerebral cortex, hippocampus, medial septum, and nucleus of Meynert (Araujo et al., 1998; Coyle et al., 1985; Whitehouse et al., 1982), noradrenergic deficits in the locus coeruleus (Bondareff et al., 1982; Mann et al., 1984), and serotonergic deficits in raphe nuclei (Aletrino et al., 1992; Halliday et al., 1992; Yamamoto and Hirano, 1985) have been demonstrated. The serotonergic system, which projects from the raphe nuclei to the forebrain area, is suggested to be important in the pathophysiology of behavioral and psychological symptoms (aggression, anxiety, and depression) that are related to cognitive dysfunction (Kepe et al., 2006; Lai et al., 2003; Lanctôt et al., 2001).

More recently, we demonstrated that YKS abolished *p*-chloroamphetamine (PCA: serotonergic neurotoxin)-induced aggressive and impaired social behaviors as well as serotonin (5-HT) 1A receptor agonist buspirone or 5-HT2A receptor antagonist ketanserin (Terawaki et al., 2007). Therefore, we hypothesized that the ameliorative effects of YKS may be due to agonism or antagonism of 5-HT1A or 5-HT2A receptors like buspirone or ketanserin.

In the present study, to examine the hypothesis, we evaluated the binding and intrinsic activity (agonistic/antagonistic activity) of the extracts of YKS on 5-HT1A or 5-HT2A receptors in vitro by competitive binding and [^{35}S]GTPγS binding assays, using the membranes of CHO (CHO-h5-HT1A) or CHO-K1 (CHO-K1-h5-HT2A) cells which stably expressing human recombinant 5-HT1A or 5-HT2A receptors. Furthermore, to determine the constituent herbs which are responsible for the effect of YKS, the extracts of seven constituent medicinal herbs, and seven kinds of YKS-analogues, which were produced by eliminating one of the constituent herbs from YKS in the manufacturing process, were evaluated.

2. Materials and methods

2.1. Drugs and reagents

Yokukansan (YKS) is composed of seven dried medicinal herbs: *Atractylodes lancea* rhizome (4.0 g, rhizome of *Atractylodes lancea* De Candolle), Poria sclerotium (4.0 g, sclerotium of *Poria cocos* Wolf), Cnidium rhizoma (3.0 g, rhizome of *Cnidium officinale* Makino), Japanese Angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa), Bupleurum root (2.0 g, root of *Bulpleurum falcatum* Linné), glycyrrhiza (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher), and Uncaria hook (3.0 g, thorn of *Uncaria rhynchophylla* Miquel). Regarding ingredients contained in YKS extract, 25 compounds have been identified by three-dimensional high-performance liquid chromatographic analysis (Mizukami et al., 2009). The dry powdered extracts of YKS with all seven constituent medicinal herbs, and seven kinds of YKS-analogues, which were produced by eliminating one of the constituent herbs in the manufacturing process, were supplied by Tsumura & Co. (Tokyo, Japan), and were used in the present studies. In brief, each plant material was authenticated by identification of external morphology and marker compounds of plants specimens, according to the methods of Japanese Pharmacopoeia and our company's standard. The seven material herbs were extracted with purified water at 95 °C for 1 h, and the extraction solution was separated from the insoluble waste and concentrated by removing water under reduced pressure. Spray drying was used to produce a dried extract powder. The yield of the extract was about 15.9%. The quality was standardization based on the Good Manufacturing Practice defined by the Ministry of Health and Welfare of Japan.

The membranes of Chinese hamster ovary (CHO) cells or CHO-K1 cells which stably expressing human recombinant 5-HT1A or 5-HT2A receptors, for binding assays were purchased from PerkinElmer (Waltham, MA, USA). Radioligands [^{3}H]8-OH-DPAT (NET929, 170.2 Ci/mmol) and [^{3}H]ketanserin (NET791, 67.0 Ci/mmol) were also purchased from PerkinElmer, and [^{35}S]GTPγS (SJ-1308, 1033 Ci/mmol) was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Serotonin, ketanserin, mianserin, dithiothreitol (DTT), guanosine-5′-diphosphate (GDP), guanosine-5′-[γ-thio]triphosphate (GTPγS), metergoline, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl cyclohexanecarboxamide tri-hydrochloride (WAY-100635) were purchased from Sigma (St. Louis, MO, USA). Other reagents used for the binding analysis were purchased from commercial sources.

2.2. Binding effects of YKS or constituent herbs on 5-HT1A and 5-HT2A receptors

Extracts of YKS, its seven constituent herbs, and YKS-analogues were used as test substances. Various concentrations of each test substance were prepared by dissolving it in 50% DMSO.

Competitive binding assays for 5-HT receptors were carried out with slight modifications of the method described by May et al. (2003). In brief, the membranes of CHO (CHO-h5-HT1A) or CHO-K1 (CHO-K1-h5-HT2A) cells which stably express human recombinant 5-HT1A or 5-HT2A receptors were used for the competitive binding assays.

In the 5-HT1A receptor binding assay, 5.25 μl of the test substance solution or vehicle (50% DMSO) was incubated in duplicate with 500 μl of the CHO-h5-HT1A membrane solution (60–92 μg protein/ml) and 20 μl of 40 nM [^{3}H]8-OH-DPAT in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1% ascorbic acid, 0.5 mM EDTA, and 10 mM $MgSO_4$ for 60 min at 25 °C. Non-specific binding was determined by adding 10 μM metergoline.

In the 5-HT2A receptor binding assay, 5.25 μl of the test substance solution or vehicle (50% DMSO) was incubated with 500 μl of the CHO-K1-h5-HT2A membrane solution (60–92 μg protein/ml) and 20 μl of 13 nM [^{3}H]ketanserin in the same 50 mM Tris–HCl buffer for 60 min at 25 °C. Non-specific binding was determined by adding 1 μM mianserin.

After incubation, 5-HT1A or 5-HT2A receptor–ligand complexes were isolated by rapid filtration through a Whatman GF/B filter using a cell harvester (Brandel MLR-48, Skatron Micro-96, PerkinElmer). The filters were rinsed four times with 3 ml of ice-cold 50 mM Tris–HCl buffer and dried. Radioactivity (cpm) trapped on the dried filter was measured using a liquid scintillation counter (PerkinElmer).

The specific binding was defined by subtracting non-specific binding from total binding, and expressed as the percentage inhibition using the following formula: Inhibition