Yokukansan, a Traditional Japanese Medicine, Enhances the l-DOPA-Induced Rotational Response in 6-Hydroxydopamine-Lesioned Rats: Possible Inhibition of COMT

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The aim of the present study was to investigate the effects of the traditional Japanese medicine yokukansan (YKS) on the function of dopamine (DA) in the rat nigrostriatal system. Unilateral 6-hydroxydopamine lesions were produced in the rat nigrostriatal system. Despite a marked loss in the striatal immunoactivity of tyrosine hydroxylase on the lesion side, striatal serotonin (5-HT) immunoactivity was not affected. Treatment using l-3,4-dihydroxyphenylalanine (l-DOPA) in conjunction with benserazide for 15 d induced abnormal involuntary movements (AIMs) such as locomotive (rotational response), axial, forelimb, and orolingual movements in the lesioned rats. The l-DOPA-treated locomotive and axial, but not forelimb and orolingual, AIMs were significantly increased and prolonged by the pre-administration of YKS. We next investigated the effects of YKS on the production of DA from l-DOPA in 5-HT synthetic RIN 14B cells. RIN 14B cells produced DA and its metabolite, 3-methoxytyramine (3-MT), following l-DOPA treatment. YKS significantly augmented DA production and inhibited its metabolism to 3-MT in a manner similar to the catechol-O-methyltransferase (COMT) inhibitor entacapone. YKS and some alkaloids (corynoxeine: CX, geissoschizine methyl ether: GM) in Uncaria hook, a constituent herb of YKS, also inhibited COMT activity, indicating that the augmenting effect of YKS on l-DOPA-induced DA production in 5-HT synthetic cells was due to the inhibition of COMT by CX and GM. Our results suggest that YKS facilitates the DA supplemental effect of l-DOPA, and that COMT inhibition by CX and GM contributes, at least in part, to the effects of YKS.

Key words yokukansan; dopamine; abnormal involuntary movement; catechol-O-methyltransferase; l-3,4-dihydroxyphenylalanine (l-DOPA); 6-hydroxydopamine

Yokukansan (YKS) is a traditional Japanese medicine comprising seven dried medicinal herbs. It has been approved by the Japanese Ministry of Health, Labour and Welfare as a treatment for neurosis, insomnia, and irritability in children. Recently, YKS was reported to reduce the behavioral and psychological symptoms of dementia (BPSD), including agitation, aggressiveness, and hallucinations, not only in patients with Alzheimer’s disease (AD) but also in those with dementia with Lewy bodies (DLB), Parkinson’s disease (PD), or PD with dementia (PDD), which are characterized by progressive dopamine (DA) neuronal loss. These clinical findings suggested that YKS acts on the DA nerve system, but it does not show the extrapyramidal side effects of the typical or atypical antipsychotic drugs used in the treatment of BPSD, which have a DA D2 receptor antagonistic effect.

Extrapyramidal symptoms such as motor dysfunction and catalepsy, which are induced particularly by a DA deficit or DA D2 receptor blockade, were also absent in various animal models treated with YKS. An in vitro competitive binding assay showed that YKS did not bind to various DA receptor subtypes. However, YKS ameliorated anxiety and decrease in extracellular DA concentrations in the prefrontal cortex (PFC) of aged rats. The findings of these animal studies also suggested that YKS modulated dopaminergic activities in the brain; however, the detailed effects of YKS on the dopaminergic system remain unclear.

Rats with unilateral 6-hydroxydopamine (6-OHDA)-lesioned or dopaminergic cell bodies of the midbrain raphe nuclei are considered, at least in part, to promote the conversion of exogenous l-DOPA to DA in the striatum of the treatment for neurosis, insomnia, or irritability in children. Recently, YKS was reported to reduce the behavioral and psychological symptoms of dementia (BPSD), including agitation, aggressiveness, and hallucinations, not only in patients with Alzheimer’s disease (AD) but also in those with dementia with Lewy bodies (DLB), Parkinson’s disease (PD), or PD with dementia (PDD), which are characterized by progressive dopamine (DA) neuronal loss. These clinical findings suggested that YKS modulated dopaminergic activities in the brain; however, the detailed effects of YKS on the dopaminergic system remain unclear.

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In the present study, we evaluated the effects of YKS on AIMs induced by repeated L-DOPA treatment in unilat- eral 6-OHDA-lesioned rats with the aim of characterizing the effects of YKS on motor function in the nigrostriatal DA system. We then investigated the effects of YKS on L-DOPA- induced DA production in 5-HT neurons using 5-HT synthetic RIN 14B cells.25 The inhibitory effects of YKS and its cons- tituents on catechol-O-methyltransferase (COMT) activity were also determined in an attempt to identify the responsible mechanisms and active components.

MATERIALS AND METHODS

Animals Male Wistar rats (Charles River, Yokohama, Japan) weighing 120–130 g at the beginning of the experiment were used. They were housed in groups of three to four rats under a 12-h light/dark cycle with free access to food and water. The experimental protocols used in the study were approved by the Ethical Committees of Animal Experimentation at the University of Miyazaki.

YKS and Test Substances YKS is composed of seven dried medicinal herbs: Atractylodes Lancea Rhizome (ALR: 4.0 g, rhizome of Atractylodes lancea De Candolle), Poria Sclerotium (PS: 4.0 g, sclerotium of Wolfiporia cocos Ryvarden et Gilbertson), Cnidium Rhizome (CR: 3.0 g, rhizome of Cnidium officinale Makino), Uncaria Hook (UH: 3.0 g, hook of Uncaria rhynchophylla Miquel), Japanese Angelica Root (JAR: 3.0 g, root of Angelica acutiloba Kitagawa), Bupleurum Root (BR: 2.0 g, root of Bupleurum falcatum Linne), and Glyceryrhriza (GR: 1.5 g, root and stolon of Glyceryrhriza uralensis Fisher). YKS and its seven constituent medicinal herbs used in the present study were supplied as a dry powdered extract by Tsumura & Co. (Tokyo, Japan). Thus, each plant material served. Scores from 0 to 4 indicated the severity of each AIM monitored period during which the given behavior was ob- served. Each of the four subtypes on the basis of the proportion of the

AIMs were evaluated as described previously13,31) with modifications. The rats in each group were observed for 1 min every 10 min for 180 min after the injection of L-DOPA. AIMs were classified into four subtypes: locomotive movement (ro- tational response, i.e., increased locomotion to the contralat- eral side of the lesion), axial movement (dytsonic posturing or choreiform twisting of the neck and upper body to the contra- lateral side of the lesion), forelimb movement (abnormal and purposeless movements of the forelimbs and digits to the con- tralateral side of the lesion), and orolingual movement (empty jaw movements and tongue protrusion to the contralateral side of the lesion). A score from 0 to 4 was assigned to each rat for each of the four subtypes on the basis of the proportion of the monitoring period during which the given behavior was ob- served. Scores from 0 to 4 indicated the severity of each AIM subtype (0: absent, 1: occasional, i.e., present < 50% of the time; 2: frequent, i.e., present > 50% of the time; 3: continu- ous, but interrupted by strong sensory stimuli and 4: continu- ous, not interrupted by strong sensory stimuli).

Immunohistochemistry in Brain Sections Rats were...
deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) after the AIMS test and intracardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. The brain was removed and postfixed for 1 h in the same fixative and cryoprotected in 10% sucrose for 1 h and 30% sucrose in PB overnight. Coronal serial sections (thickness: 50 µm) including the striatum, substantia nigra, or dorsal raphe nuclei were prepared for immunohistochemical staining. \(^{25}\) Sections for tyrosine hydroxylase (TH) or 5-HT immunohistochemical staining were incubated in 10% normal horse or goat serum for 1 h at room temperature, and incubated at 4°C overnight with a primary antibody to TH (diluted 1:20 000; mouse monoclonal; Sigma-Aldrich) or 5-HT (1:3000; rabbit polyclonal; ImmunoStar, Hudson, WI, U.S.A.) dissolved in PB containing 0.1% Triton X-100, 0.1% sodium azide, and 2% bovine serum albumin. All sections were obtained by the Vector ABC immunohistochemical method (Vectorstain Elite kit; Vector, Burlingame, CA, U.S.A.) with diaminobenzidine as the chromogen. These sections were mounted on gelatin-coated glass slides and analyzed with light microscopy.

**Cell Culture Study**

RIN 14B cells (No. 09-2059; DS Pharma Biomedical Co., Ltd., Osaka, Japan) were cultured and maintained at 37°C in 5% CO₂ and 95% air with 95% relative humidity in RPMI 1640 medium (Life Technologies Co., Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS: ICN Biomedical Inc., Santa Ana, CA, U.S.A.) and antibiotics (50 µg/mL penicillin and 50 µg/mL streptomycin). In the DA production assay, cells were seeded and cultured in 10% sucrose for 1 h in the same fixative and cryoprotected in 10% sucrose for 1 h in 30% sucrose in PB overnight. Coronal serial sections (Waters, Tokyo, Japan). The homogenate was collected in a plastic tube and kept at 4°C for 30 min. After centrifugation at 10 000 × g for 30 min, the supernatant was centrifuged at 100 000 × g for 10 min, the supernatant was subjected to a 96-well plate solution (HBSS, Sigma-Aldrich), 100 µL of test solution (L-DOPA, L-DOPA+test substance) or vehicle (HBSS) was added. These treated cells were incubated for 0, 0.01, 1, 2, 3, 5, or 8 h at 37°C. The reaction was stopped by the addition of 100 µL of 0.2 M perchloric acid containing 2 mM ethylenediaminetetraacetic acid (EDTA)-2Na and 8 mM sodium metabisulfite. Cells were homogenized in a well with a sonicator (Ultrasonic Homogenizer, SMT Co., Ltd., Tokyo, Japan). The homogenate was collected in a plastic tube and kept at 4°C for 30 min. After centrifugation at 10 000 × g and 4°C for 10 min, the supernatant was filtered with a 0.45-µm filter (Ultra free-MC-HV, Millipore, Billerica, MA, U.S.A.). The filtrate was used to determine DA and its metabolites by high-performance liquid chromatography (HPLC-ECD). \(^{25}\) Enactone (COMT inhibitor), NSD-1015 (AADC inhibitor), and pargyline (COMT inhibitor), which were purchased from Sigma-Aldrich, were used to identify candidate enzymes for the production and metabolism of DA.

**HPLC-ECD Assay for the Determination of DA and Its Metabolites**

The chromatographic conditions used for the determination of DA and its metabolites were as follows: column, Eicom pak SC-5ODS (a hydrophobic reverse-phase C18 HPLC column, 3.0Φ×150 mm long, 5-µm particle size, Eicom, Tokyo, Japan); mobile phase, 0.1 M citrate/acetate buffer (pH 3.2) containing 15% methanol, 13.4 µM EDTA-2Na, and 786 µM sodium I-octanesulfonate (Dojindo, Kumamoto, Japan); flow rate, 0.5 mL/min; column temperature, 25°C; applied potential to an Ag/AgCl reference electrode, +750 mV; and working electrode, graphite electrode. Authentic DA, 3-MT, and DOPAC, purchased from Sigma-Aldrich, were used as standards for identification and quantification. The chromatographic data were processed with Empower software (Waters, Tokyo, Japan).

**Immunocytochemistry for Cultured Cells**

RIN 14B cells (40000 cells/well in 300 µL) were seeded on glass slides with 8-well chambers (Thermo Fisher Scientific Inc., Rochester, NY, U.S.A.) and cultured at 37°C for 3 d. After the cells in each well were washed with HBSS, 100 µL of HBSS or L-DOPA solution dissolved in HBSS was added, followed by incubation at 37°C for 1 h. After the HBSS or L-DOPA solution was removed, the remaining cells in the well were fixed with 300 µL of 0.1 M sodium cacodylate buffer (pH 7.4) containing 5% glutaraldehyde and 1% sodium metabisulfite at 4°C for 1 h. The fixed cells were reduced with 0.01 M phosphate buffer saline (PBS) containing 0.01% Tween 20 and 1% sodium metabisulfite at room temperature for 30 min and were then used for immunocytochemistry. Immunohistochemical staining was performed according to a previously reported procedure. \(^{20}\) The primary antibody used was a mixture of anti-5-HT rabbit serum (AB125; Chemicon, Temecula, CA, USA; 1:400) and an anti-DA mouse monoclonal antibody (MAB5300; Chemicon; 1:1000) diluted with 0.01 M PBS containing 0.01% Tween 20. The second antibody used was a mixture of fluorescein-conjugated donkey anti-rabbit IgG (AP182F; Chemicon; 1:200) and rhodamine-conjugated donkey anti-mouse IgG (AP192R; Chemicon; 1:200) diluted with the same 0.01% Tween 20-PBS. The stained cells were observed and analyzed using a deconvolution fluorescence microscope system (BioRevo, BZ-9000, Keyence, Osaka, Japan); 5-HT-, DA-, and double (5-HT/DA)-labeled structures displayed green (fluorescein), red (rhodamine), and yellow (fluorescein/rhodamine) fluorescence, respectively.

**Catechol-O-methyltransferase (COMT) Inhibition Assay**

An *in vitro* COMT assay was performed according to a previously reported procedure. \(^{23}\) Briefly, 25 µL of the test substance or vehicle as the control, an equal volume of 20 µg/mL COMT (51–27aa, COM0905, ATGen Co., Gyeonggi, South Korea), and 200 µM 6,7-dihydroxydopamine (as amlotenin: Sigma-Aldrich) as a substrate were mixed in a flat-bottomed 96-well black plate (MicroFluor™, Dynatec Laboratories Inc., Rochester, NY, U.S.A.) on ice. The mixture was preincubated at 37°C for 5 min, after which 25 µL of 100 µM S-(5′-adenosyl)-L-methionine (AdoMet, Sigma-Aldrich) was added. One hundred microliters of the reaction mixture was further incubated at 37°C for 60 min. Fluorescence intensity was determined at excitation and emission wavelengths of 355 nm and 460 nm, respectively, using the Infinite M200 plate reader (Tecan, Grödig, Austria). U-0521 (MP Bio Japan K.K., Tokyo, Japan) was used as a specific COMT inhibitor.

The test substance and ascleutin used in this assay were dissolved in dimethyl sulfoxide (DMSO) and diluted with 100 mM PB containing 5 mM MgCl₂ and 20 mM L-cysteine (pH 7.4), for a final DMSO concentration of 0.5% in 100 µL of the reaction mixture. COMT and AdoMet were dissolved in the same buffer.

**Statistical Analysis**

Data are presented as the mean±standard error of the mean (S.E.M.). Data from the behavioral experiment were analyzed by two-way ANOVA with repeated measures (group×time) followed by Bonferroni’s
post hoc tests. The significance of the differences between groups in the cell culture experiments was assessed by one-way ANOVA followed by Bonferroni’s post hoc tests. The significance level in each statistical analysis was accepted at p<0.05.

RESULTS

Effects of YKS on AIMs Induced by Repeated l-DOPA Treatment During the chronic drug treatment period, l-DOPA induced the gradual development of four subtypes of AIMs: locomotive (rotational), axial, forelimb, and orolingual movements, which was consistent with our previous findings.13) The rotational behavior induced by l-DOPA was tight, covering a small diameter, and the animals displayed a nose-to-tail posture as a result of twisting of the spine.

The pre-administration of a single dose of YKS [1 g/kg, per os (p.o.)] significantly increased and prolonged the l-DOPA-induced locomotive [group: F(1, 12)=7.3, p<0.05; time: F(1, 12)=499.4, p<0.01; group×time: F(1, 17)=2.7, p<0.01] and axial [group: F(1, 12)=13.6, p<0.01; time: F(1, 12)=1170.6, p<0.01; group×time: F(1, 17)=2.3, p<0.01] AIMs on day 15. YKS-induced enhancement of locomotive movement first appeared 30 min after the injection of l-DOPA, preceding that of axial movement. In contrast, no significant alteration by YKS was observed in the l-DOPA-induced forelimb [group: F(1, 12)=3.8, p=0.075; time: F(1, 12)=662.0, p<0.01; group×time: F(1, 17)=1.4, p=0.147] or orolingual [group: F(1, 12)=14.6, p=0.334; time: F(1, 12)=1122.0, p<0.01; group×time: F(1, 17)=0.414, p=0.981] AIMs (Fig. 1). Thus, YKS significantly enhanced locomotive and axial movements but not forelimb and orolingual movements induced by repeated l-DOPA treatment.

Immunohistochemistry in 6-OHDA-Lesioned Rats Figure 2 shows typical TH and 5-HT immunoreactivities in the striatum, and their nucleus origin regions (substantia nigra and ventral tegmental area for TH and dorsal raphe nuclei for 5-HT) in 6-OHDA-lesioned rats. TH immunoreactivities in the left striatum and ipsilateral substantia nigra and ventral tegmental area completely disappeared following the injection of 6-OHDA into the left MFB, relative to those in the non-treated right controls. In contrast, no significant differences were observed in 5-HT immunoreactivities between both sides of the striatum and dorsal raphe nuclei.

Immunocytochemistry of RIN 14B Cells Figure 3 shows the ability of RIN 14B cells to synthesize DA using immunocytochemical stains with anti-5-HT and anti-DA antibodies. 5-HT immunoreactive cells were observed in the untreated control group, whereas DA immunoreactive cells were absent. However, DA-positive cells were detected following the addition of l-DOPA (1 nmol/mL) to the culture medium. A photograph merging 5-HT and DA-positive cells showed that 5-HT and DA were synthesized by the same cells.

DA Synthesis after Addition of l-DOPA to RIN 14B Cells Changes in DA concentrations followed by the addition of l-DOPA to RIN 14B cells are shown in Fig. 4. DA levels rapidly increased with addition of l-DOPA (1 nmol/mL) to the medium. This elevated level plateaued after 1–3 h and then gradually decreased. Based on these results, the following experiments aimed at characterizing the effects of test substances on l-DOPA-induced increases in DA synthesis were performed 3 h after the incubation.

Effects of Entacapone, NSD-1015, and Pargyline on l-DOPA-Induced DA and 3-MT Levels in RIN 14B Cells Figure 5A shows the effects of entacapone (COMT inhibitor) on DA and 3-MT (a DA metabolite catalyzed by COMT) levels in the RIN 14B cells. Neither DA nor 3-MT was detected in the l-DOPA-untreated cells (data not shown). However, DA and 3-MT levels markedly increased with the addition of l-DOPA (1 nmol/mL). This increase in DA levels was significantly augmented by the concomitant addition of entacapone (0.1 and 0.3 nmol/mL) [F(2, 6)=809.7, p<0.001], whereas the
L-DOPA-induced increase in 3-MT levels was completely blocked by the concomitant addition of entacapone.

Figure 5B shows the effects of NSD-1015 (AADC inhibitor) on the DA and 3-MT levels in the RIN 14B cells. NSD-1015 (3 and 10 nmol/mL) significantly inhibited L-DOPA-induced increases in DA and 3-MT levels in a concentration-dependent manner [DA: F(2, 6)=1499, p<0.001; 3-MT: F(2, 6)=246, p<0.001].

Figure 5C shows the effects of pargyline (MAO inhibitor) on DA and 3-MT levels. Pargyline (1.0 nmol/mL) significantly augmented L-DOPA-induced increases in DA and 3-MT levels [DA: F(2, 6)=74.0, p<0.001; 3-MT: F(2, 6)=127.5, p<0.001]. In this experiment, DOPAC, which is a DA metabolite catalyzed by MAO, was not detected in the L-DOPA-untreated cells, similar to DA and 3-MT, but was detected (11.0±0.6 nM) following the addition of L-DOPA (1 nmol/mL). The L-DOPA-induced increase in DOPAC levels was blocked by the concomitant addition of the MAO inhibitor pargyline (0.1 and 1.0 nmol/mL) in a concentration-dependent manner (3.8±0.2 nM at 0.1 nmol/mL of pargyline and non-detection at 1.0 nmol/mL of pargyline).

Effects of YKS on L-DOPA-Induced Increases in DA and 3-MT Levels in RIN 14B Cells

Figure 6 shows the effects of YKS on L-DOPA-induced increases in DA and 3-MT levels. Neither DA nor 3-MT was detected following the addition of YKS (500 µg/mL) to the medium of the
l-DOPA-untreated cells (data not shown). Marked increases in DA and 3-MT concentrations were observed with the addition of l-DOPA (1 nmol/mL). This increase in DA levels was significantly augmented by the concomitant addition of YKS (50–500 µg/mL) in a concentration-dependent manner [YKS 150 µg/mL: F(3, 8)=25.3, p<0.01; YKS 500 µg/mL: F(3, 8)=25.3, p<0.001], whereas the l-DOPA-induced increase in 3-MT levels was inhibited by the concomitant addition of YKS in a concentration-dependent manner [F(3, 8)=58.9, p<0.001].

Inhibitory Effects of YKS, Constituent Herbs, and Components on COMT Activity

The inhibitory effects of YKS, its seven constituent herbs, and UH-derived alkaloid components on COMT activity were examined using human recombinant COMT. YKS (100–2000 µg/mL) inhibited COMT activity in a concentration-dependent manner, and marked inhibition (83.2%) was observed at the maximum concentration (2000 µg/mL) (Fig. 7). Among the seven constituent herbs (UH, PS, ALR, GR, CR, JAR, and BR: 100 µg/mL each), UH markedly inhibited COMT activity (84.5%), whereas the other six constituents did not inhibit its activity by more than 50% (Fig. 8). The inhibitory effects of the UH-derived seven alkaloid components (CX, GM, ICX, IRP, RP, HTE and HTI; 100 nmol/mL each) on COMT activity were then evaluated, and marked inhibition was observed with CX (72.4%), GM (65.1%), and ICX (51.1%) (Fig. 9). The inhibition of COMT activity by the other components was less than 40%. The inhibition of COMT activity by the specific inhibitor U-0521 (100 nmol/mL) used as the positive control in these three examinations verified that our COMT assay was appropriate for evaluating the inhibitory effects of test substances.

DISCUSSION

We have shown that l-DOPA-induced locomotive and axial AIMs were significantly increased and prolonged by the pre-administration of a single dose of YKS. Drug-induced locomotion, expressed as rotational behavior in rodents, was closely associated with the expression of limb, axial, and masticatory symptoms, at least with l-DOPA treatment. Assuming that these rotations were caused by axial dystonia and/or abnormal movements of the contralateral limb, the rotational behavior itself may be considered locomotive dyskinesia.34,35 The reason why YKS influenced only locomotive and axial movement, and why YKS reinforced only locomotive movement at early time of 30 min, could not be determined. Elucidation of these mechanisms will await further behavioral experiments. It is clear that YKS reinforced locomotive and axial movements caused by l-DOPA (DA supply) in unilateral striatal DA nerve destruction rat. We hypothesized that DA mediates these behavioral actions of YKS. Thus, the present result suggested that YKS enhanced the rotational response induced by repeated l-DOPA treatment.

The depletion of DA in the striatum ipsilateral to the
6-OHDA lesions was confirmed by an immunohistochemical examination for TH (Fig. 2) and was consistent with previous findings.26,28 We also confirmed the induction of AIMS by repeated l-DOPA treatment in 6-OHDA-lesioned rats in the present and previous studies.13 This induction may be due to the facilitation of neurotransmission of DA synthesized from exogenous l-DOPA in the unilateral DA-depleted striatum by 6-OHDA; in the striatum on the lesion side, DA was synthesized in 5-HT neurons instead of DA neurons because 5-HT neurons contain AADC, an enzyme for DA synthesis.19,20,36 This neural mechanism was supported by the presence of a nearly normal number of 5-HT immunoreactive nerve terminals in the DA-depleted striatum, as shown in Fig. 2.

Pre-administration of YKS significantly enhanced l-DOPA-induced locomotive and axial movements, whereas forelimb and orolingual movements were not altered. In general, the four subtypes of AIMS did not develop simultaneously; orolingual and forelimb dyskinesia occurred first, followed by axial dyskinesia and rotational behavior.37 The causal role of striatal l-DOPA in dyskinesia was previously demonstrated in a reverse microdialysis study; lower l-DOPA concentrations elicited stereotypic-like movements of the head, neck, and forelimb, while higher l-DOPA concentrations caused overtly abnormal movements with a broader body distribution and pronounced dystonic characteristic.38 Taken together, the results of the behavioral experiment suggest that YKS elevates striatal l-DOPA and DA concentrations when pre-administered before l-DOPA. A similar reinforcement was reported in
unilateral 6-OHDA-lesioned rats co-treated with L-DOPA and entacapone, a COMT inhibitor that has been clinically used as L-DOPA therapy for PD patients.39,40 These findings suggested that YKS had an inhibitory effect on COMT activity.

To test this hypothesis, we evaluated the effects of YKS on the synthesis and concentration of DA using cultured 5-HT synthetic cells, RIN-14B cells.23 DA was not detected under normal conditions or with YKS treatment alone. However, it was detected and/or increased with addition of L-DOPA to the cell culture medium (Figs. 3, 4), and the L-DOPA-induced increase in DA concentrations was enhanced by YKS (Fig. 6). These results confirmed that 5-HT neurons were able to synthesize DA if supplied L-DOPA, and also that L-DOPA as well as 5-HTP is the substrate for AADC contained in 5-HT neurons.19,20,36 L-DOPA-induced DA synthesis in serotonergic cells has also been demonstrated in another serotonergic cell line, RN46A-B14.41 DA-associated enzymes such as AADC, COMT, and MAO are considered to be possible factors increasing DA concentrations by YKS. We accordingly examined the effects of AADC, MAO, and COMT inhibitors on DA and its COMT-metabolite 3-MT levels, and the results were compared with those of YKS. Similar changes in the form of increased DA and decreased 3-MT were observed with the COMT inhibitor and YKS treatments (Figs. 5A, 6), suggesting that COMT activity is inhibited by active components contained in YKS.

Interestingly, Uchida et al.42 reported that YKS reduced methamphetamine-induced hyperlocomotion in normal mice, suggesting inhibitory effects of YKS on the dopaminergic system in the striatum. The discrepancy between their result and our enhancing effect of YKS on DA system may be due to the difference in animal models. Generally, the extracellular DA concentration in the synaptic cleft is controlled by several regulatory mechanisms, including release, reuptake, autoinhibition, and metabolism. Thus, inhibition of methamphetamine-induced hyperlocomotion by YKS suggested that YKS affected these regulatory mechanisms. In contrast, in our 6-OHDA-injected rats, because the nigrostriatal dopaminergic pathway of rats was severely depleted, DA neurotransmission in the striatum is thought to be mediated via 5-HT neurons that are not subject to normal regulation of DA concentration in the synaptic cleft. Given that administration of YKS to normal animals did not produce hyperactivity or catalepsy,43 YKS may act weakly on the DA system when DA neurons are normal. However, in cases when the dopaminergic system is degenerated, such as in 6-OHDA-injected rats and Parkinson’s disease, an increasing effect of YKS on the extracellular DA concentration by inhibition of COMT may appear, owing to dysregulation of the extracellular DA concentration at 5-HT nerve terminals.

An in vitro COMT inhibition assay showed that YKS inhibited COMT activity in a concentration-dependent manner (Fig. 7). YKS is composed of seven medical herbs. To date, ferulic acid, sinapic acid, 5-hydroxy-ferulic acid, and chlorogenic acid are known to be contained in CR, which is one of the constituents of YKS, and to have COMT-inhibitory effects.44 However, screening to clarify the active component showed that UH more potently inhibited COMT than it did the other six constituent herbs including CR (Fig. 8). We accordingly further investigated the inhibitory effects of seven UH alkaloids on COMT activity and found that CX, GM, and ICX significantly inhibited COMT activity (Fig. 9). We recently reported that CX and GM were both absorbed in the blood from the intestine and reached the brain across the blood–brain barrier in rats orally administered YKS, whereas ICX was not detected in the brain.45 We more recently reported that specific binding sites for GM were present in several brain regions including the frontal cortex, hippocampus, striatum, amygdala, central medial thalamic nucleus, and dorsal raphe nucleus of the rat brain, by receptor-autoradiography using [3H]GM.46 Taken together, these results suggest that CX and GM in UH are active components accounting for the COMT inhibition effect of YKS.

In the treatment of PD symptoms, COMT inhibitors (e.g., entacapone) are expected to mitigate side effects, such as wearing-off, caused by long-term administration of L-DOPA.47,48 YKS may have enhanced the rotational response induced by the repeated L-DOPA treatment by inhibiting COMT activity. Thus, the combination of L-DOPA and YKS may be useful for reducing the amount of L-DOPA administered to PD patients; however, further studies are needed. In addition, YKS improved BPSD such as hallucination, agitation, aggressiveness, and anxiety in patients with AD.1–3 Reduced dopaminergic activity in the striatum and PFC in 6-OHDA-lesioned rats caused discontinuous responses in the elevated plus-maze test,49 whereas decreased DA levels in the medial PFC increased anxiety induced by social defeat stress.50 Reduced PFC dopaminergic function or the blockade of DA receptors in the PFC impaired working memory.51–53 YKS ameliorated anxiety54 and impairments in working memory51 in aged rats by ameliorating age-related DA reductions in the PFC. The formation of DA by inhibition of COMT activity may be associated with these ameliorating effects of YKS.

In conclusion, our results including in vivo and in vitro experiments suggest that YKS has augmenting effects on L-DOPA treatment and that inhibition of COMT activity by components such as CX and GM may contribute, at least in part, to the effects of YKS.

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REFERENCES


