Antidepressive-like effect of a Kampo (traditional Japanese) medicine, kososan (Xiang Su San) in a stress-induced depression-like mouse model: Proteomic analysis of hypothalamus

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ABSTRACT

Aim: A Kampo medicine, kososan (KS), has been used clinically for the treatment of certain depression-like symptoms. Our previous studies using the stress-induced depression-like model mice, showed that oral KS treatment leads to an antidepressive-like effect via the normalization of dysfunction of hypothalamic-pituitary-adrenal axis. In the present study, proteomic analysis was used to identify brain hypothalamus proteins that are affected by KS treatment.

Methods: KS was orally administered to stress-induced depression-like model mice at 1.0 g/kg/day for 9 days, and the hypothalamus was then analyzed using agarose 2-D gel electrophoresis, followed by mass spectrometry-based protein identification, and then western blot analysis and immunohistochemical analysis.

Results: The expression of eight proteins was increased or decreased in the hypothalamus of the model mice, but recovered on oral KS treatment. Among them, metabotropic glutamate receptor 2 (mGluR2) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase 1 (CNPase1) were identified as depression-related proteins. Western blot of the hypothalamus confirmed these results and showed that the expression of CNPase2 was opposite to that of CNPase1. On immunohistochemistry, mGluR2 expression in the hypothalamus paraventricular nucleus was downregulated and CNPase2 expression was upregulated in the cerebral fornix in the model mouse, but KS returned them to normal.

Conclusion: mGluR2 and CNPase in the hypothalamus are associated with the antidepressive-like activity of KS.

KEY WORDS: 2′,3′-cyclic nucleotide 3′-phosphodiesterase, antidepressant, kososan, metabotropic glutamate receptor 2, proteomic analysis, Xiang Su San

INTRODUCTION

A Kampo (traditional Japanese) medicine, kososan (Xiang Su San in Chinese), is composed of five medicinal herbs (cyperi rhizoma, perillae herba, aurantii nobilis pericarpium, glycyrrhizae radix, and zingiberis rhizoma) and has been used clinically for treatment of the depression-like symptoms associated with the initial stage of the common cold, anorexia, food-related allergic urticaria, irritable bowel syndrome, chronic fatigue syndrome, insomnia, and autonomic imbalance. It has also been suggested that kososan can clinically alleviate the depression induced by interferon (IFN)-α therapy for hepatitis C [1]. Our previous studies using two animal models for depression, stress- and IFN-α-induced depression-like model mice, demonstrated that oral kososan treatment leads to an antidepressive-like effect via the normalization of the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which is strongly associated with the pathogenesis of depression [2,3].

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We have also reported that kososan ameliorated the stress-induced decreases in the number of neural progenitor cells in the hippocampal dentate gyrus and orexin-A-positive cells in the lateral hypothalamic area [4,5]. This is supporting evidence that kososan may be beneficial for the treatment of depression. The mode of action of the antidepressive-like activity of kososan in a stress-induced depression-like mouse model, however, remains to be fully elucidated. In the present study, we used proteomic analysis to identify brain hypothalamus protein(s) that are affected by kososan treatment in a mouse model using agarose 2-D gel electrophoresis followed by mass spectrometry (MS)-based protein identification.

METHODS

Animals

We used 7-week-old male ddY mice (Japan SLC, Hamamatsu, Japan) that weighed 35–40 g at the beginning of the experiment. The mice were housed under conditions of constant temperature (23 ± 2°C) and humidity (55 ± 10%), with food and water available ad libitum (unless otherwise specified) and with a 12/12 h light–dark cycle (08:00–20:00 hours) during the stress procedure. Animal experiments were approved by the Institutional Animal Care and Use Committee for Kitasato University and performed in accordance with Guide for the Care and Use of Laboratory Animals in the Kitasato University and the National Research Council Guide for the Care and Use of Laboratory Animals in Japan.

Drugs

The component herbs of kososan were as follows: cyperi rhizoma (rhizome of *Cyperus rotundus* L.), 4.0 g (lot no. AE7951, Tsumura, Tokyo, Japan); perillae herba (leaf of *Perilla frutescens* Britton var. *acuta* Kudo), 2.0 g (lot no. B04401, Tsumura); aurantii nobilis pericarpium (pericarp of *Citrus unshiu* Markovich), 3.0 g (lot no. AD7971, Tsumura); glycyrrhizae radix (root of *Glycyrrhiza uralensis* Fisher), 2.0 g (lot no. 8661621, Uchida Wakan-yaku, Tokyo, Japan), and zingiberis rhizoma (rhizome of *Zingiber officinale* Roscoe), 0.5 g (lot no. AK8761, Tsumura). Specimens were deposited at Laboratory of Biochemical Pharmacology for Phytomedicines, Kitasato Institute for Life Sciences, Kitasato University, Japan. Kososan extract was prepared as previously described (yield, 28%) [2–5]. Toledomin (milnacipran hydrochloride) tablet was purchased from Asahi Kasei Pharma (Tokyo, Japan).

Three-dimensional high-performance liquid chromatography

Three-dimensional high-performance liquid chromatography (HPLC) of kososan extract was performed as previously described [2,3]. 3-D HPLC profile of an aqueous kososan extract is shown in Figure 1. UV absorption clearly showed the presence of the following major constituents in kososan extract: liquiritin, liquiritigenin and glycyrrhizin (originating from glycyrrhizae radix); rosmarinic acid and caffeic acid (perillae herba); hesperidin, narirutin and nobiletin (auratii nobilis pericarpium).

Stress-induced depression-like model mouse

The stress-induced depression-like model mouse [2] was prepared using a combination of the modified forced swimming (FS) [6,7] and the chronic mild stress [8,9] approaches (Fig. 2). On day 11, the total duration of immobility of the mice during the 5 min forced swimming test (FST) was measured.

Drug treatment

The kososan extract or milnacipran (pulverized Toledomin tablet with a mortar) was dissolved or suspended in distilled water, respectively. Previous studies showed that oral kososan or milnacipran leads to an antidepressive-like effect in stress-induced depression-like model mice when given at a dose of 1.0 g/kg or 60 mg/kg, respectively [2]. Therefore, kososan (1.0 g/kg) or milnacipran (60 mg/kg, used here as a positive control) was administered orally once daily for 11 days (Fig. 2). LY341495 (2-[(1*S*,2*S*)-2-carboxycyclopropyl]-3-(9*H*-xanthen-9-yl)-D-alanine; Tocris Bioscience, Ellisville, MO, USA), which is a selective metabotropic glutamate receptor 2/3 (mGluR2/3) antagonist, was dissolved in 1/15 mol/L phosphate buffer (pH 8.0). LY341495 (0.03 mg/kg) was injected i.p. at 30 min before FST on day 11 (Fig. 2).

Agarose 2-D electrophoresis

Sample preparation

Mice were anesthetized with inhalation of isofluran (Mylan Seiyaku, Tokyo, Japan) and perfused transcardially with cold phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Brains were collected and the hypothalamus excised and stored at −80°C. Frozen tissues were fractionated using ReadyPrepProtein Extraction Kit (Membrane II; Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Briefly, hypothalamus (approx. 20 mg) was disrupted using a Teflon glass homogenizer in 0.2 mL Lysis Buffer (Bio-Rad Laboratories). The homogenate was centrifuged at 3000 × *g* at 4°C for 10 min, and the supernatant was added into 60 mL of Membrane Protein Concentrating Reagent (Bio-Rad Laboratories) and stirred gently for 60 min on ice. After stirring, the mixture was centrifuged at 100 000 × *g* at 4°C for 60 min. The pellet was washed with Lysis Buffer and sonicated in the mixture of 1 mL 2-D Rehydration/Sample Buffer 1 (Bio-Rad Laboratories; 7 mol/L urea, 2 mol/L thiourea, 1% w/v ASB-14, 40 mmol/L Tris base, 0.001% Bromophenol Blue (Bio-Rad Laboratories)) and 10 μL of ReadyPrep TBP Reducing Reagent (Bio-Rad Laboratories; 200 mmol/L tributylphosphine in 1-methyl-2-pyrrolidinone) with Bioruptor UCD-200T (Cosmo Bio, Tokyo, Japan). The supernatant was dialyzed against water and lyophilized. The lyophilizate was applied to ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories) and vortexed in the mixture of 2-D

Rehydration/Sample Buffer 1 and ReadyPrep TBP Reducing Reagent. The suspensions were centrifuged at 16 000 × *g* at 20°C for 20 min, and the supernatant (membrane or hydrophilic protein fraction, respectively) underwent agarose 2-D electrophoresis.

Agarose 2-D electrophoresis

Two-dimensional electrophoresis was performed according to the procedure given by Oh-Ishi *et al.* [10–13].

Identification of proteins on 2-D electrophoresis

Protein separated on agarose 2-D electrophoresis was identified using in-gel tryptic digestion of the protein followed by MS as described previously [13].

Western blot

Expression of protein was determined on immunoblotting as previously described [13,14], with minor modification. In brief, the hypothalamus of brain was fractionated using ReadyPrep-Protein Extraction Kit (Membrane II) as described in the previous section. The membrane protein fraction was dissolved in 100 μL of sodium dodecylsulfate (SDS) sample buffer, heated at 94°C for 4 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of lysate protein were run on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare). Nitrocellulose blots were blocked with 1% bovine serum albumin (BSA; Blot Qualified, Promega, Madison, WI, USA) in Tris-buffered saline (TBS), and then incubated with rabbit anti-mouse mGluR2/3 (1:500, Sigma-Aldrich) or monoclonal anti-mouse

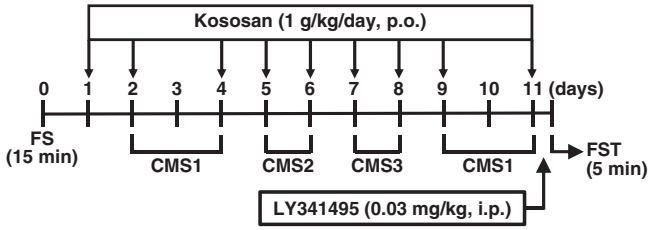

Figure 2 | Schedule for preparation of stress-induced depression-like model mouse and drug treatment. CMS, chronic mild stress; FS, forced swimming; FST, forced swimming test.

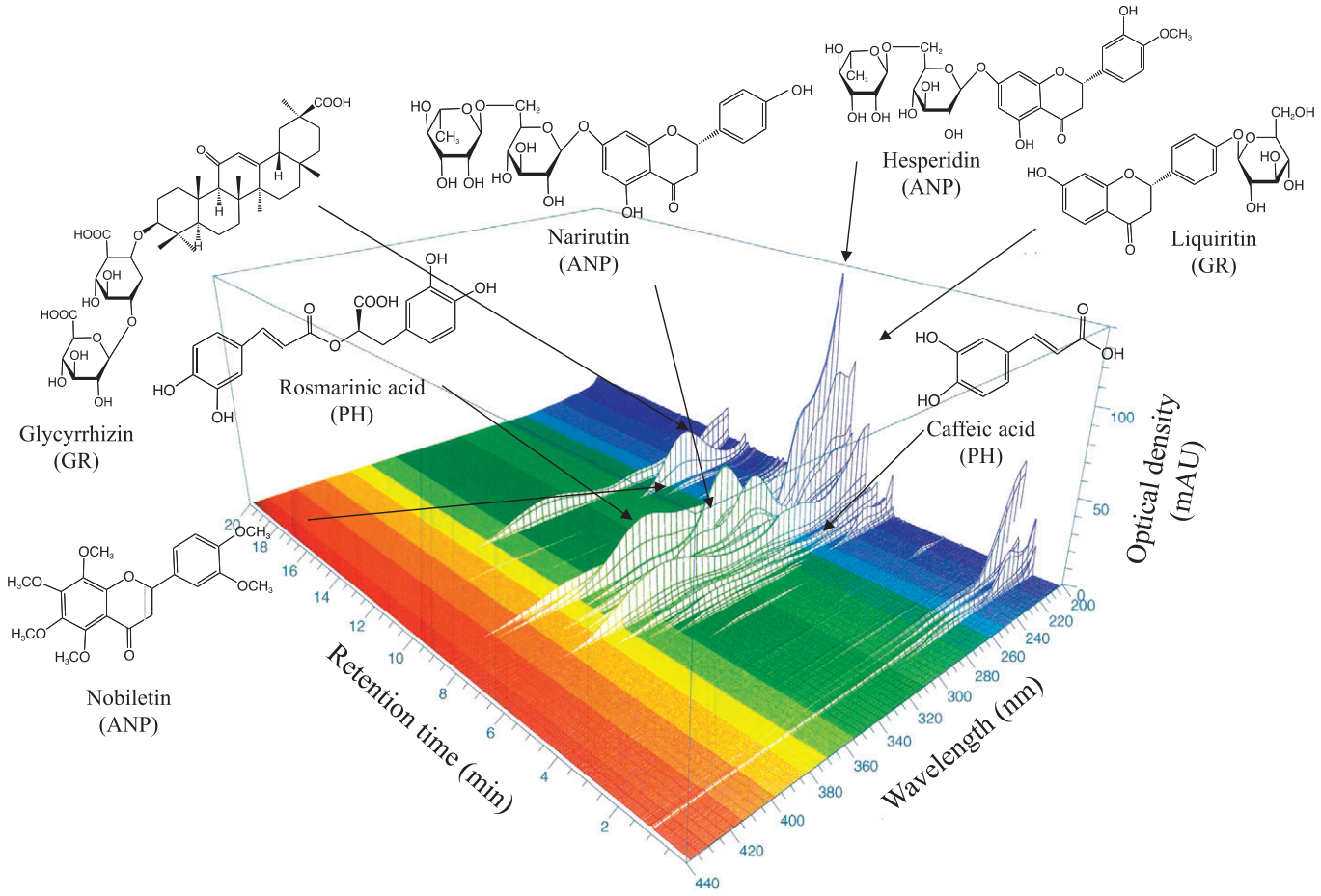

Figure 1 | Three-dimensional high-performance liquid chromatography profile of kososan extract.

2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; 1:500, Sigma-Aldrich) in TBS containing 1% BSA. After washing with TBS containing 0.1% Triton X-100 (TBST), the blots were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:2000. Immunoreactive mGluR2/3 or CNPase protein was detected using the enhanced chemiluminescent protocol (Perkin Elmer Life Sciences, Waltham, MA, USA).

Brain fixation and tissue storage

Mice were anesthetized with isofluran and perfused transcardially with cold PBS containing protease inhibitor cocktail (Nacalai Tesque) and subsequently with cold 4% paraformaldehyde phosphate buffer solution. Brains were collected and post-fixed in 4% paraformaldehyde phosphate buffer solution at 4°C overnight. Serial coronal sections (50 mm in thickness) were obtained throughout the hypothalamus using a Linear Slicer PRO7 (Dosaka EM, Kyoto, Japan) and were stored in PBS containing 0.1% NaN_3 at 4°C until needed for subsequent experiments.

Immunohistochemistry

All staining was conducted using 48-well plates for free-floating immunohistochemistry. For mGluR2 immunohistochemistry, free-floating sections were incubated in 3% H_2O_2/80% methanol for 40 min at room temperature (RT). After washing in PBS, sections were blocked for 2 h with 1% BSA (Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.3% Triton X-100 (PBST) and incubated for 18 h at 4°C with mouse anti-mGluR2 monoclonal [mG2Na-s] antibody (1:1000, Abcam, San Carlos, CA, USA). Sections were then rinsed in PBST, incubated for 2 h at RT with biotinylated goat anti-mouse IgG (1:200; Vector Laboratories), rinsed in PBS and incubated for 2 h at RT with ABC solution (ABC *Elite* Kit, Vector Laboratories). mGluR2-positive cells were visualized by incubating sections with diaminobenzidine (DAB) solution (2/3 mg/mL in TBS containing 0.1% H_2O_2, Sigma-Aldrich). Sections were mounted on silane-coated slides and dried, and were then dehydrated, and coverslipped using Permount (Fisher Scientific International, Fair Lawn, NJ, USA). For CNPase2 immunohistochemistry, free-floating sections were incubated in 3% H_2O_2/80% methanol for 20 min at −20°C, followed by a rinse in PBST. Sections were then incubated in 10 mmol/L sodium citrate buffer (pH 6.0) for 30 min at 95°C (to enhance antigenicity) and rinsed in PBST. After blocking for 3 h with 1% BSA in PBST, sections were incubated overnight at 4°C with rabbit anti-CNPase2 specific antiserum (1:500; prepared by Medical & Biological Laboratories, Nagoya, Japan). After rinsing in PBST, sections were incubated for 2 h at RT with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories), which was followed by incubation with ABC solution for 3 h at RT. CNPase2-positive cells were visualized by incubating sections with DAB solution. Sections were then mounted and coverslipped as described. All immunohistochemical procedures included negative controls that lacked primary antibodies. No staining was detected in the controls. Positive cells were detected and processed using a Keyence BZ-8000 microscope (Keyence, Osaka, Japan).

Statistical analysis

Results are presented as mean ± SEM. The significance of differences between more than three experimental groups was analyzed with one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison using KaleidaGraph Ver.4.0 (Synergy Software, HULINKS, Tokyo, Japan). The significance of differences between two experimental groups was analyzed on independent *t*-test. Differences were considered significant at $P < 0.05$, and $P < 0.1$ was considered as tending toward significance.

RESULTS

2-D electrophoresis of hypothalamus

Because the present study confirmed that kososan extract reduced the immobility time on FST significantly at a dose of 1.0 g/kg/day for 11 days, as reported previously [2], proteomic analysis was performed using the hypothalamus from the stress-unchallenged or stress-challenged mice given water or kososan extract. Of the protein spots of membrane protein fractions of mice hypothalamus compared on 2-D electrophoresis, the expression of five was altered in stress-challenge compared with non-stressed mouse, and recovered by kososan treatment (Fig. 3a). These proteins were identified as aconitase 2 (spots 1,2), CNPase1 (spot 3), mGluR2 precursor (spot 4) and voltage-dependent anion channel 2 (VDAC2; spot 5) on in-gel tryptic digestion of the proteins followed by HPLC separation and MS (Table 1). These results were reproduced in three independent animal experiments. When the protein spots of hypothalamus hydrophilic protein fractions from stress-unchallenged mice treated with water vehicle, or stress-challenged treated with water vehicle, or stress-challenged treated with kososan were compared on 2-D electrophoresis, the expression of two protein spots was upregulated in stress-challenged mice compared with non-stressed mice, and lowered on kososan treatment (Fig. 3b). These proteins were identified as malate dehydrogenase (spot 6) and ubiquinol cytochrome c reductase core protein 2 (spot 7; Table 1).

Immunoblotting of mGluR2/3 and CNPase in hypothalamus

To verify the 2-D electrophoresis findings, we conducted immunoblotting of the proteins that have been reported to be associated with depression among the six altered proteins. On western blotting of membrane protein fraction of hypothalamus using antibody targeted at mGluR2/3, kososan upregulated the reduced dimer (207 kDa) and monomer (100 kDa) of mGluR2/3 in stress-challenged mice (Fig. 4a). Western blotting of membrane protein fraction of hypothalamus using

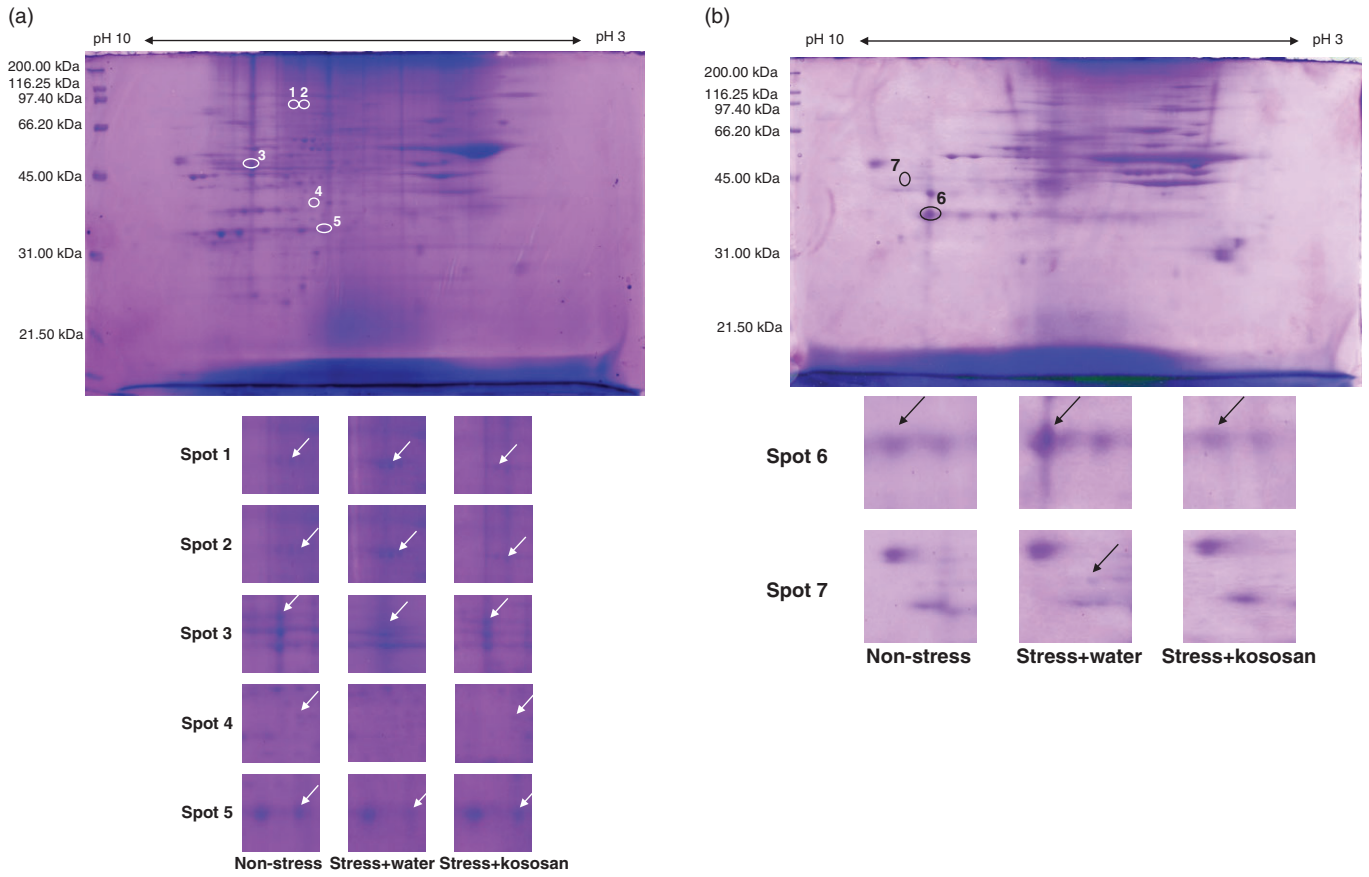

Figure 3 | Effect of kososan on protein expression in membrane protein fraction and hydrophilic protein fraction from hypothalamus of stress-induced depression-like model mice was measured on agarose 2-D electrophoresis. Kososan (1.0 g/kg/day) or water was given orally to stress-challenged or unchallenged mice once daily for 11 days. Hypothalamus was obtained after the FST and (a) membrane protein fraction and (b) hydrophilic protein fraction were prepared. Spots in agarose 2-D electrophoresis patterns of stress-unchallenged/water-treated mouse, stress-challenged/water-treated mouse and stress-challenged/kososan-treated mouse are shown.

antibody against CNPase showed that kososan upregulated the reduced CNPase 1 level (46 kDa) but kososan downregulated the increased CNPase 2 level (48 kDa, another isoform of CNPase) in stress-challenged mice (Fig. 4b). These results were reproduced in three independent animal experiments.

Immunohistochemistry of mGluR2 and CNPase2 in hypothalamus

To verify the immunoblotting findings, we conducted immunohistochemical analyses of mGluR2 and CNPase2 in the hypothalamus of mouse brain. Compared with stress-unchallenged mice (Fig. 5a), stress challenge decreased mGluR2 expression in the hypothalamic paraventricular nucleus (PVN; Fig. 5b), indicating that mGluR2 expression in the hypothalamic PVN of stress-induced depression-like model mice was downregulated by stress challenge. Kososan increased the expression of mGluR2 (Fig. 5c) as compared with the water-treated stress-challenged mice (Fig. 5b). Milnacipran also recovered the expression of mGluR2 (Fig. 5d). It is not possible to prepare CNPase1-specific antibody, because amino-acid sequence of CNPase1, an isoform of CNPase2, is absolutely identical to CNPase2 from the 21st amino acid to the C terminal (420th amino acid). Therefore antiserum was prepared against part of the N-terminal amino acid sequence (ftrkshtflpklffrk) of CNPase2, which bound to CNPase2 but not to CNPase1 (data not shown), and was used for immunohistochemistry. Stress challenge increased CNPase2 expression at the columns of fornix in hypothalamus (Fig. 6b) as compared with the stress-unchallenged mice (Fig. 6a). Kososan decreased the expression of CNPase2 (Fig. 6c) as compared with the water-treated mice (Fig. 6b). These results were reproduced in three independent animal experiments.

Effect of LY341495 on kososan-induced reduction of immobility

Although oral kososan at a dose of 1.0 g/kg/day for 11 days significantly reduced the duration of immobility in the stressed mice (Fig. 7a, $P < 0.05$), i.p. injection of LY341495, mGluR2/3

Table 1 | Identified proteins in hypothalamus

Spot no.	Protein name	Data base accession no.	Experimental MW (kDa)	Theoretical MW (Da)	SEQ score	Coverage (%)[†]	Change of expression	
							Stress + water[‡]	Stress + kososan[§]
1	Aconitase 2	gi|18079339|ref|NP_542364.1|	95.0	85 445	56.0	7.0	↑	↓
2	Aconitase 2	gi|63101587|gb|AAH94462.1|	95.0	85 479	28.0	2.9	↑	↓
3	Cyclic nucleotide phosphodiesterase 1	gi|18257317|gb|AAH21904.1|	52.0	47 105	128.0	18.1	↓	↑
4	Metabotropic glutamate receptor 2 precursor	gi|82930987|ref|XP_914720.1|	40.5	95 865	24.4	3.9	↓	↑
5	Voltage-dependent anion channel 2	gi|6755965|ref|NP_035825.1|	35.4	31 714	74.2	14.4	↓	↑
6	Malate dehydrogenase, mitochondrial	gi|1200100|emb|CAA30274.1|	39.0	35 575	64.2	14.2	↑	↓
7	Ubiquinol cytochrome c reductase core protein 2	gi|13097348|gb|AAH03423.1|	45.0	48 206	60.2	13.3	↑	↓

[†] Protein coverage was defined as the percentage of the whole length of the protein sequence covered by matched peptides identified on LC-MS.
[‡] Comparison between non-stress and stress/water-administered groups.
[§] Comparison between stress/water-administered and stress/kososan-administered groups.

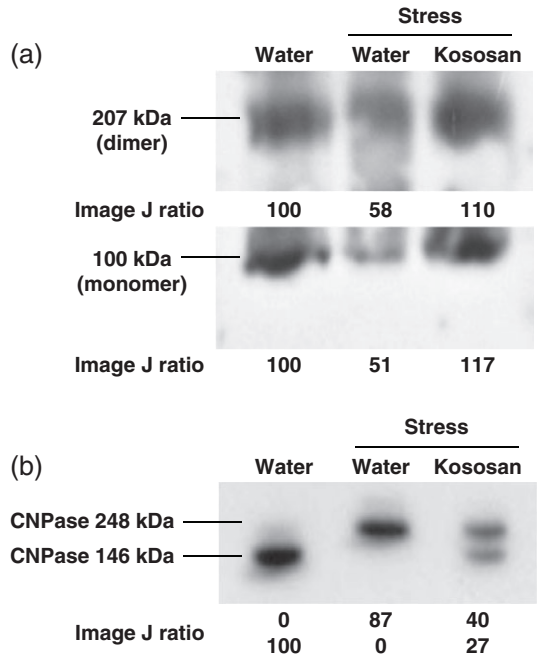

Figure 4 | Effects of kososan on protein expressions in membrane protein fraction from hypothalamus of stress-induced depression-like model mice were measured on western blot. Stress-challenged or unchallenged mouse was treated with kososan or water as described in legend of Figure 3. Hypothalamus was obtained after the FST and its membrane protein fraction was prepared. Western blot patterns of (a) mGluR2/3 and (b) CNPase.

antagonist, at the dose of 0.03 mg/kg showed a tendency to block the kososan-induced decrease in duration of immobility during FST (Fig. 7a, $P < 0.1$). LY341495, however, did not affect the duration of immobility in the stress-induced depression-like model mice (Fig. 7a) or the non-stress group (Fig. 7b).

DISCUSSION

We have reported that a Kampo medicine, kososan, which has been used for the treatment of depressive state in Japan, shows antidepressive-like activity in stress-induced depression-like model mice [2]. We also reported that kososan suppressed the hyperactivity of the HPA axis and ameliorated the glucocorticoid receptor protein expression in the hypothalamus PVN, which was downregulated in the depression-like model mice [2]. The mechanism of action of the antidepressive-like activity of kososan has not been fully elucidated. Therefore, we analyzed proteins in the hypothalamus, which are related to the antidepressive-like activity of kososan, using proteomic analysis. The expression of mGluR2 was downregulated in membrane protein fraction of hypothalamus of stress-induced depression-like model mice, and oral kososan recovered the expression of mGluR2 on agarose 2-D gel electrophoresis (Fig. 3a; Table 1). These results were confirmed on western blot using anti-mGluR2/3 antibody (Fig. 4a). Immunohistochemistry of brain section showed the expression of mGluR2 protein was reduced in the hypothalamus PVN of stress-induced depression-like model mice, and kososan increased the mGluR2

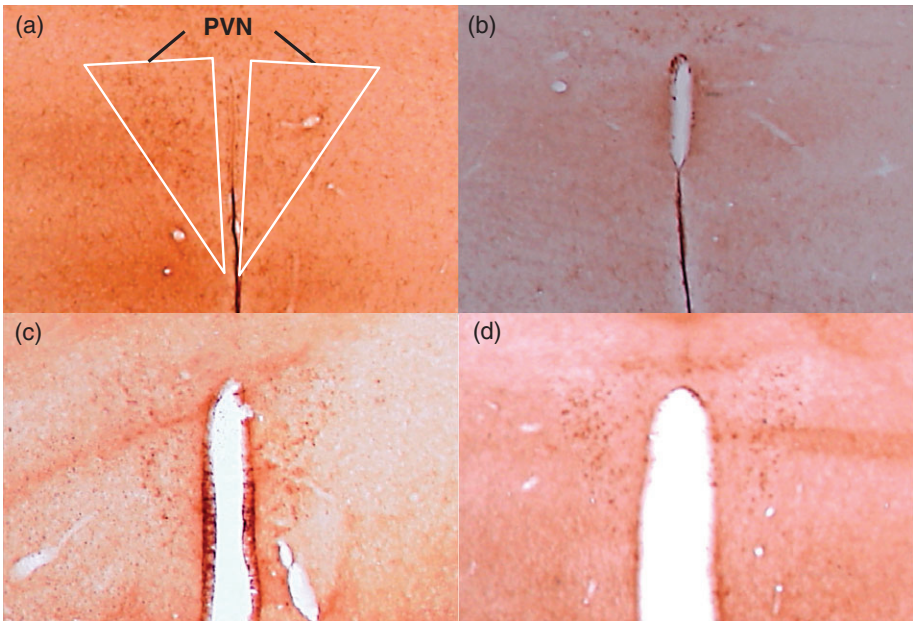

Figure 5 | Immunohistochemistry of the effect of kososan on mGluR2 protein expression in the hypothalamus of stress-induced depression-like model mice. Stress-challenged or unchallenged mice were treated with kososan, milnacipran (60 mg/kg/day) or water. Mice were perfused transcardially under deep anesthesia with PBS containing protease inhibitor followed by 4% paraformaldehyde after FST and the brains were removed. Analysis of mGluR2 protein was performed on serial 50 μm microslicer sections of the brains of mice (a) without or with stress treated with (b) water, (c) kososan or (d) milnacipran. mGluR2-positive cells (brown spots) were detected using Keyence BZ-8000 microscope. PVN, paraventricular nucleus.

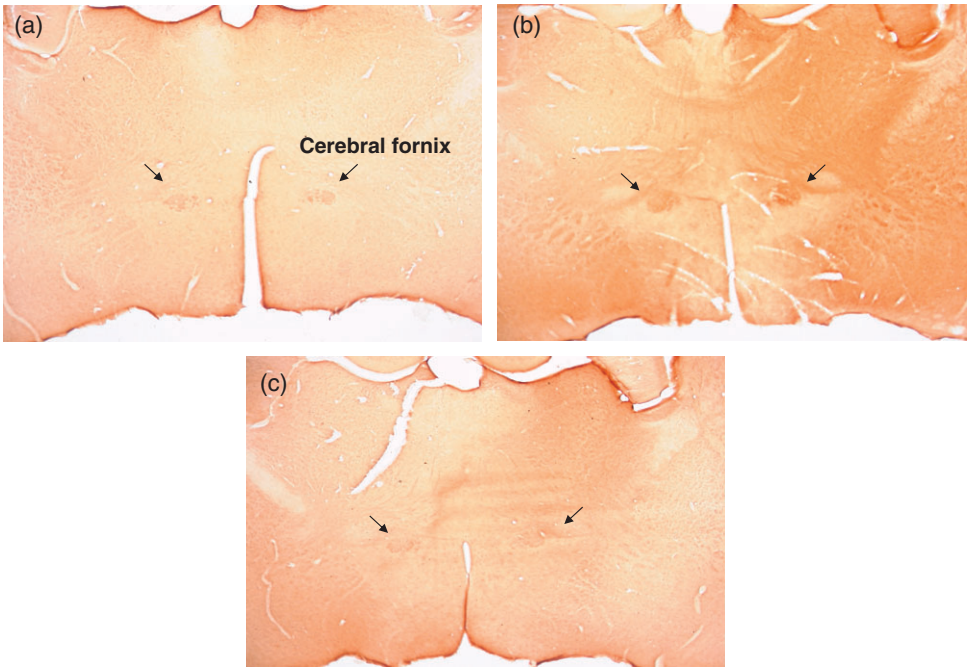

Figure 6 | Immunohistochemistry of the effect of kososan on CNPase2 protein expression in the hypothalamus of stress-induced depression-like model mice. Stress-challenged or unchallenged mice were treated with kososan, or water. Mice were perfused transcardially under deep anesthesia with PBS containing protease inhibitor followed by 4% paraformaldehyde after FST and the brains were removed. Analysis of CNPase2 protein was performed on serial 50 μm microslicer sections of the brains of mice (a) without or with stress treated with (b) water or (c) kososan. CNPase2-positive cells (brown spots) were detected using Keyence BZ-8000 microscope.

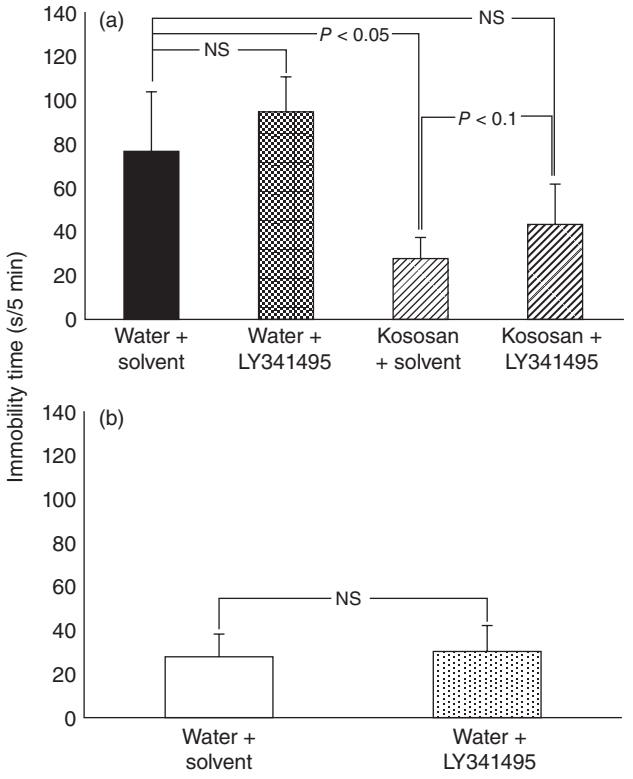

Figure 7 | Effect of mGluR2/3 antagonist, LY341495, against antidepressive-like effect of kososan on the stress-induced extension in the duration of immobility during the FST. Kososan (1.0 g/kg/day) was administered orally to stress-induced depression-like model mice once daily for 11 days. LY341495 (0.03 mg/kg) was injected i.p. in (a) stress-induced depression-like model mice or (b) non-stress mice at 30 min before FST on day 11. The duration of immobility was measured during a 5 min FST at 60 min after the final kososan treatment. Each column represents the mean ± SEM. ($n = 8–10$). Statistical analysis was conducted using Dunnett's test or *t*-test. NS, not significant.

protein expression (Fig. 5). To investigate whether mGluR2 expression was related to antidepressive-like activity of kososan, LY341495, an mGluR2/3 antagonist, was administered i.p., and LY341495 showed a tendency to prolong the immobility time compared with that of solvent treatment in kososan-treated mice (Fig. 7a). mGluR2 is a 7-transmembrane protein and regulates glutamatergic neurotransmission. mGluR2 and mGluR3 are coupled to Gi/Go proteins and are preferentially localized in the preterminal region of axons, where they negatively modulate neurotransmitter release [15]. Expression and function of mGluR2/3 has been reported to reduce in the hippocampus of spontaneously depressed Flinders Sensitive Line (FSL) rats, and pharmacological activation of mGluR2/3 shortens the time required for the therapeutic efficacy of conventional antidepressants in these rats [16–18]. It has also been reported that L-acetylcarnitine has antidepressive-like activity by the epigenetic regulation of mGluR2 in FSL rats and in mice exposed to chronic unpredictable stress, genetic and environmentally induced depression models, respectively, through enhancement of the transcription of *Grm2* encoding for mGluR2 [19]. These results suggest that upregulation of mGluR2 in hypothalamus PVN by kososan is associated with the antidepressive-like activity of kososan.

CNPase1 is one of several oligodendrocyte-specific markers, and was previously implicated in major depressive disorder [20,21] supporting the hypothesis that altered CNPase1 function may participate in the pathophysiology of psychiatric disorders. CNPase1 expression was downregulated and CNPase2, an isoform of CNPase1, was upregulated, and oral kososan upregulated the reduced CNPase1 expression and downregulated the increased CNPase2 expression in the hypothalamus of stress-induced depression-like model mice in the present study (Fig. 4b). CNPase1 and CNPase2 are encoded separately by different promoters of the same gene [22,23]. This suggests that kososan may have an antidepressive-like effect through normalization of CNPase gene transcription in the hypothalamus of depression-like model mice.

In the present study, aconitase 2, malate dehydrogenase and ubiquinol cytochrome c reductase core protein 2 were upregulated in hypothalamus of the stress-induced depression-like model mice and downregulated by kososan treatment (Fig. 3; Table 1). Similar results have been reported in hippocampal synaptoproteomics of learned helpless rats and tricyclic antidepressant nortriptyline treatment [24]. Aconitase 2 catalyzes the interconversion of citrate to isocitrate via cis-aconitate in the second step of the tricarboxylic acid cycle. Malate dehydrogenase catalyzes the reversible oxidation of malate to oxaloacetate, utilizing the NAD/NADH cofactor system in the citric acid cycle. Ubiquinol cytochrome c reductase core protein 2 is one of the subunits of mitochondrial complex III, a component of the mitochondrial respiratory chain [25]. The increased expression of the energy metabolism enzymes suggests an increase in the production of ATP as a response to stress in mice. Indeed, it has been shown that mitochondrial response to stress leads to increased expression and enzymatic activity of several subunits of the respiratory chain complexes [26]. Downregulation of aconitate 2 and malate dehydrogenase by kososan treatment suggests that kososan modulates these enzymes to correct stress-induced mitochondrial abnormalities in the depression-like model mice. VDAC is considered the main pathway for metabolite diffusion across the mitochondrial outer membrane and also thought to be involved in the mitochondrial apoptotic pathway [27]. In the present study, VDAC2 was downregulated in the hypothalamus of stress-induced depression-like model mice and upregulated by kososan treatment (Fig. 3a; Table 1). This may be due to the inhibition of apoptosis of neural cells though antidepressive-like activity of kososan. Elucidation of further mechanisms of action of the antidepressive-like effects of kososan using these proteins identified on proteomic analysis is now in progress.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest for this article.

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