RESEARCH PAPER

Acta Neurobiol Exp 2021, 81: 393–404
DOI: 10.55782/ane-2021-042

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Identification of traditional Japanese Kampo medicines and crude drugs that upregulate brain-derived neurotrophic factor in human peripheral cells

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The neurotrophic hypothesis of depression, which suggests that decreased hippocampal brain-derived neurotrophic factor (BDNF) levels cause depression, has become increasingly popular. BDNF, a member of the neurotrophin family, promotes neuronal differentiation and survival. BDNF is synthesized in various peripheral tissues, as well as in the brain. Considering that peripheral BDNF can be transported into the brain across the blood-brain barrier, substances with the ability to upregulate BDNF activity in peripheral tissues may be useful in the management of depression. Previously, we demonstrated that the human kidney adenocarcinoma cell line ACHN produces BDNF; hence, this cell line was employed for screening upregulators of peripheral BDNF. Here, we aimed to identify Kampo (traditional Japanese) medicines and their crude drug components that upregulate BDNF levels using ACHN cells. Chotosan, Hochuekkito, Kososan, and Ninjinyoeito, Kampo medicines used in treating psychiatric disorders, increased BDNF levels in the culture media of ACHN cells. Furthermore, Chinpi (*Citrus unshiu* peel), a crude drug contained in these four Kampo medicines, as well as Onji (*Polygala tenuifolia* root), and Saiko (*Bupleurum falcatum* root) elevated BDNF levels in ACHN cells. Chinpi, showing strong BDNF elevating effect, increased *BDNF* mRNA expression. Inhibitors of protein kinase B, mitogen-activated protein kinase kinase, and cAMP-dependent protein kinase, involved in the transcription of *BDNF*, attenuated Chinpi-induced BDNF elevation. Our results suggest that Chinpi and Kampo medicines containing Chinpi can promote the production of BDNF in peripheral tissues, potentially alleviating depression symptoms.

Key words: depression, neurotrophic hypothesis, brain-derived neurotrophic factor, ACHN cells, Kampo medicine, crude drug

INTRODUCTION

Depression is one of the most common life-threatening psychiatric disorders worldwide. The core symptoms of depression are depressed mood, loss of interest, anhedonia, sleep disturbances, lack of energy, sadness, and suicidal tendencies (Cui, 2015). Currently, selective serotonin reuptake inhibitors and serotonin-noradrenaline reuptake inhibitors are the first-line drugs for the treatment of depression. These medications are based on the monoamine hypothesis of depression, which states that decreased monoamine levels in the brain cause the disease. However, these drugs are not effective in approximately 30% of patients with depression (Papakostas et al., 2008), and they cause adverse effects, such as sleep disorders, sexual dysfunctions, and digestive symptoms (Antai-Otong, 2004; Baldwin et al., 2006). Therefore, a novel treatment strategy for depression is required.

Received 5 February 2021, accepted 14 October 2021

Duman et al. (1997), suggested the neurotrophic hypothesis of depression, which states that brain-derived neurotrophic factor (BDNF) deficiency in the hippocampus is the underlying cause of depression. BDNF, a member of the neurotrophin family, is involved in the survival, growth, plasticity, and differentiation of neurons (Chao et al., 2006; Park and Poo, 2013). Supporting the relationship between depression and BDNF, administration of BDNF in the hippocampus elicits an antidepressant-like effect in rats (Shirayama et al., 2002). Furthermore, it has been reported that the mRNA and protein levels of BDNF in the hippocampus are decreased in various rodent model of depression (Li et al., 2007; 2015; Lee et al., 2013; Thakare et al., 2017).

BDNF is synthesized not only in the brain but also in various other organs, such as the kidney, liver, heart, and testes (Lommatzsch et al., 1999; Endlich et al., 2018). Since BDNF is transported from the peripheral circulation into the brain across the blood-brain barrier (BBB) (Poduslo and Curran, 1996; Pan et al., 1998), peripheral BDNF upregulators may have the potential to augment brain BDNF levels and elicit an antidepressant effect without penetrating the central nervous system (CNS). Previously, we demonstrated that the human kidney adenocarcinoma cell line ACHN secretes BDNF, suggesting that this cell line is useful for identifying peripheral BDNF upregulators. We further showed that methanol extracts of foxtail millet increased the production of BDNF in ACHN cell line (Nakajima et al., 2020).

Kampo (traditional Japanese) medicines such as Chotosan, Hangekobokuto, Kamikihito, Kamisyoyosan, and Ninjinyoeito, have been used clinically to treat psychiatric disorders such as depression and neurosis. Furthermore, Kampo medicines, including Chotosan, Hochuekkito, Kososan, and Sansoninto, reportedly elicit antidepressant-like effects in rodents and elevate BDNF levels in the hippocampus of mice (Zhao et al., 2011; Ito et al., 2017; Lim et al., 2018; Sawamoto et al., 2018). Accordingly, specific crude drugs contained in Kampo medicines may have the potential to promote BDNF production.

In this study, we investigated the BDNF upregulation effect of Kampo medicines, such as Chotosan, Hangekobokuto, Hochuekkito, Kamikihito, Kamisyoyosan, Kososan, Ninjinyoeito, and Sansoninto using ACHN cells. Subsequently, we evaluated the BDNF upregulation effect of crude drugs contained in Kampo medicines that increased BDNF in ACHN cells. Additionally, we investigated the effects of crude drugs, identified as upregulating the abundance of BDNF, on *BDNF* mRNA expression in ACHN cells.

METHODS

Reagents

Kampo medicines were obtained from Tsumura & Co. (Tokyo, Japan). All crude drugs were purchased from Tochimoto Tenkaido Co., Ltd (Osaka, Japan) or Daido Pharmaceutical Co., Ltd (Toyama, Japan) (Table 1). The Human Free BDNF Quantikine ELISA Kit was purchased from R&D Systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM), RNAlater, and TRIzol were purchased from Life Technologies (Carlsbad, CA, USA), and ReverTra Ace was purchased from Toyobo (Osaka, Japan). The SYBR Fast qPCR Mix was purchased from Takara Bio, Inc. (Shiga, Japan). Primers for amplifying human *BDNF* and 18S ribosomal RNA were obtained from GeneNet (Fukuoka, Japan) or Greiner Bio-One (Kanagawa, Japan). All other reagents, including 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of methanol extracts from Kampo medicines or crude drugs

The methanol extracts were prepared according to the methods described in our previous study (Nakajima et al., 2020). Briefly, the Kampo medicines were crushed using a mortar and pestle, whereas the crude drugs were crushed using a Tube Mill 100 control (IKA, Staufen, Germany). The crushed samples (5 g) were gently agitated in methanol for 12 h at room temperature. After centrifugation at 15,000 × *g* for 5 min, the supernatant was collected and dried by spraying gaseous nitrogen. The residue was dissolved in DMSO to obtain a final concentration of 40 or 80 mg/mL.

Cell culture

The ACHN cells were cultured in DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

The MTT assay was performed as described in our previous studies (Nakajima et al., 2018; 2020). Briefly, the cells were seeded in 96-well plates at 1×10^4 cells/well and cultured for 24 h. The test methanol extracts (0–100 μg/mL) were added to the culture medium. Af-

Table 1. List of crude drugs used in this study.

Japanese name	English name	Scientific name	Medicinal content
Bakumondo	Ophiopogon Root	*Ophiopogon japonicas*	root
Bofu	Saposhnikovia Root and Rhizome	*Saposhnikovia divaricata*	root
Bukuryo	Poria Sclerotium	*Wolfiporia cocos*	sclerotium
Byakujutsu	Atractylodes Rhizome	*Atractylodes japonica, A. macrocephala*	rhizome
Chinpi	Citrus Unshiu Peel	*Citrus unshiu, C. reticulata*	pericarp
Chotoko	Uncaria Hook	*Uncaria rhynchophylla, U. sinensis, U. macrophylla*	hook
Gomishi	Schisandra Fruit	*Schisandra chinensis*	fruit
Hange	Pinellia Tuber	*Pinellia ternate*	tuber
Jio	Rehmannia Root	*Rehmannia glutinosa* var. *purpurea, R. glutinosa*	root
Kanzo	Glycyrrhiza	*Glycyrrhiza uralensis, G. glabra*	root and stolon
Keihi	Cinnamon Bark	*Cinnamomum cassia*	bark
Kikuka	Chrysanthemum Flower	*Chrysanthemum morifolium, C. indicum*	capitulum
Kobushi	Cyperus Rhizome	*Cyperus rotundus*	rizhome
Ninjin	Ginseng	*Panax ginseng*	root
Ogi	Astragalus Root	*Astragalus membranaceus, A. mongholicus*	root
Onji	Polygala Root	*Polygala tenuifolia*	root or root bark
Saiko	Bupleurum Root	*Bupleurum falcatum*	root
Sekko	Gypsum	–	–
Shakuyaku	Peony Root	*Paeonia lactiflora*	root
Shokyo	Ginger	*Zingiber officinale*	rizhome
Shoma	Cimicifuga Rhizome	*Cimicifuga foetiga, C. simplex, C. dafurica*	rizhome
Sojutsu	Atractylodes Lancea Rhizome	*Atractylodes lancea, A. chinensis*	rizhome
Soyo	Perilla Herb	*Perilla frutescens* var. *crispa*	leaf and tip of branch
Taiso	Jujube	*Zizyphus jujube* var. *inermis*	fruit
Toki	Japanese Angelica Root	*Angelica acutiloba, A. acutiloba* var. *sugiyamae*	root

Gomishi was purchased from Daido Pharmaceutical Co., Ltd (Toyama, Japan). Other crude drugs were purchased from Tochimoto Tenkaido Co., Ltd (Osaka, Japan).

ter 24 h of cultivation, MTT (200 μg/mL) was added to each well, and the cells were cultured for an additional 4 h. After removing the culture medium, the formazan crystals produced in cells were dissolved in DMSO. The optical densities were measured at 570 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Values are expressed as the ratio of the optical density of treated cells to that of the control cells.

Measurements of BDNF concentrations in the culture medium

The ACHN cells were seeded into 96-well plates at 4 × 10^4 cells/well in DMEM and cultured for 24 h. Fresh DMEM with or without various concentrations of test extracts was added to each well and the cells were cultured for an additional 24 h. The culture medium was collected as a specimen. Thereafter, the BDNF concen-

trations in the culture media were measured using the Human Free BDNF Quantikine ELISA Kit according to the manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from ACHN cells with the TRIzol reagent and reverse-transcribed into complementary DNA (cDNA) with ReverTra Ace. Reaction mixture, containing 10 µL 2 × SYBR Fast qPCR Mix, 0.4 pmol sense and antisense primers, and 2 µL diluted cDNA, was loaded into a 96-well plate. PCR amplification was conducted under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. The PCR was performed using the CFX Connect Real-Time System (Bio-Rad, Tokyo, Japan). The following sense and antisense primers were used: human BDNF forward 5′-TTTGGTTGCATGAAGGCTGC-3′, and reverse 5′-GCCGAACTTTCTGGTCCTCA-3′; 18S ribosomal RNA forward 5′-GTAACCCGTTGAACCCCATT-3′, and reverse 5′-CCATCCAATCGGTAGTAGCG-3′. Transcript levels were estimated from the respective standard curves and normalized to 18S ribosomal RNA expression (internal control).

Statistical analyses

Values are expressed as the mean ± standard deviation (SD). The differences between groups were analyzed with the two-sample *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Differences were considered significant at $P<0.05$.

RESULTS

Effects of Kampo medicine methanol extracts on BDNF levels in ACHN cells

We examined the viability of ACHN cells treated using different concentrations (0, 25, 50, or 100 µg/mL) of Kampo medicine methanol extracts by the MTT assay. Although the Hangekobokuto extract significantly increased ACHN cell viability at a concentration of 100 µg/mL, other methanol extracts did not affect the viability of ACHN cells up to 100 µg/mL (Table 2). Therefore, the test concentrations of Hangekobokuto extracts were 25 and 50 µg/mL for observing its effect on BDNF levels in ACHN cells and those of the other Kampo extracts were 50 and 100 µg/mL. Methanol extracts of Chotosan, Hochuekkito, Kososan, and Ninjinyoeito significantly increased the BDNF levels in ACHN cells from 1.22 to 1.45-fold at 50 µg/mL and from 1.42 to 1.55-fold at 100 µg/mL respectively, but the other extracts had no effect (Fig. 1).

Effects of crude drug methanol extracts on BDNF levels in ACHN cells

Table 3 shows the constituent crude drugs of Kampo medicines used in this study. We investigated the effects of the methanol extracts obtained from the crude drug constituents of Kampo medicines that had induced upregulation of BDNF in ACHN cells. The viability of ACHN cells was examined by MTT assay (Table 4). Sekko, a crude drug of Chotosan, was not evaluated for its effect on BDNF production as no res-

Table 2. Effects of Kampo medicine methanol extracts on the cell viability of ACHN cells.

Kampo	Cell viability of ACHN cells (%)			
	0 (µg/mL)	25 (µg/mL)	50 (µg/mL)	100 (µg/mL)
Chotosan	100.0 ± 4.4	101.9 ± 4.2	97.5 ± 2.4	95.0 ± 4.8
Hangekobokuto	100.0 ± 4.2	102.5 ± 4.9	108.1 ± 5.4	120.9 ± 5.8*
Hochuekkito	100.0 ± 4.1	97.9 ± 4.0	95.9 ± 4.2	95.3 ± 3.4
Kamikihito	100.0 ± 7.4	97.2 ± 5.3	93.3 ± 3.4	94.7 ± 3.6
Kamisyoyosan	100.0 ± 4.0	100.9 ± 3.7	99.2 ± 3.7	97.1 ± 2.9
Kososan	100.0 ± 9.3	97.6 ± 7.8	101.9 ± 5.4	105.9 ± 6.0
Ninjinyoeito	100.0 ± 1.5	104.1 ± 6.3	103.5 ± 5.1	105.6 ± 4.2
Sansoninto	100.0 ± 5.7	99.7 ± 5.6	95.3 ± 3.6	108.3 ± 10.8

MTT assay was performed to determine the concentration for examining the BDNF up-regulating effect. ACHN cells were treated each Kampo medicine at 0–100 µg/mL for 24 h. Data are shown relative to the viability of control cells (100%). Data are expressed as the mean ± SD (n=6). Tukey's test; *$P<0.05$ *vs.* control (0 µg/mL).

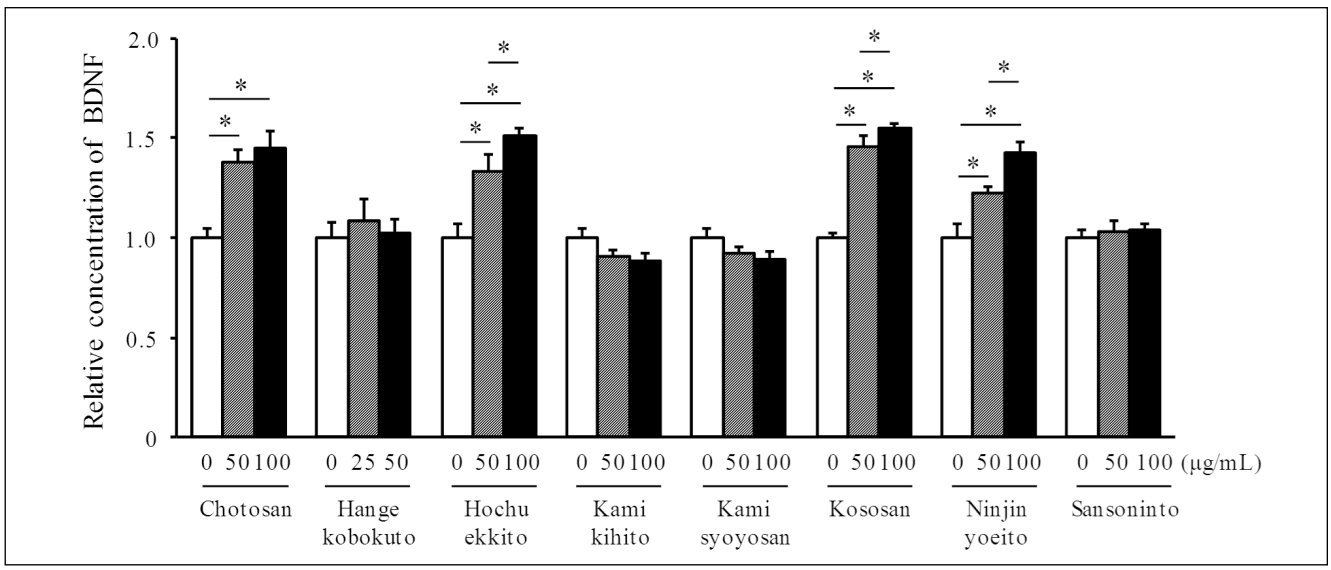

Fig. 1. Effects of Kampo medicine methanol extracts on BDNF concentrations in the culture medium of ACHN cells. Cells were cultured for 24 h with the methanol extracts of Chotosan, Hangekobokuto, Hochuekkito, Kamikihito, Kamisyoyosan, Kososan, Ninjinyoeito, and Sansoninto. BDNF levels were measured using ELISA. Each value represents BDNF levels of treated cells relative to that of the control (0 μg/mL). The data are expressed as the mean ± SD (n=6). Tukey's test; *P<0.05 *vs.* control (0 μg/mL).

idue was obtained following the methanol extraction procedure. Evaluation of the effects elicited by the crude drug methanol extracts on BDNF production was conducted using non-lethal concentrations of the crude drugs. The BDNF levels in the culture medium of ACHN cells treated with Chinpi (*Citrus unshiu* peel), Onji (*Polygala tenuifolia* root), or Saiko (*Bupleurum falcatum* root) methanol extracts were approximately 1.69, 1.12, and 1.24-fold higher than each control, respectively (Fig. 2). In contrast, Bofu, Byakujutsu, Chotoko, Gomishi, Kanzo, Keihi, Kikuka, Ninjin, Shakuyaku, Shoma, Sojutsu, and Soyo significantly decreased the BDNF levels than those of control (Fig. 2).

Thereafter, we evaluated whether Chinpi elevated BDNF in a dose-dependent manner. The BDNF concentrations in the culture medium of ACHN cells treated with 50, 100, and 150 μg/mL Chinpi methanol extracts was 1.53-, 1.67-, and 1.65-fold higher than that in the control, respectively (Fig. 3).

The mechanisms of underlying the effects of Chinpi and Keihi methanol extracts on BDNF levels in ACHN cells

Chinpi was found to strongly augment BDNF levels; hence, the effects of Chinpi extract on *BDNF* mRNA expression were evaluated using qRT-PCR. The *BDNF* mRNA expression level in ACHN cells was approximately 1.9-fold higher in the treated cells compared to the control (Fig. 4A).

BDNF gene transcription is regulated by the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and neuronal PAS domain protein 4 (NPAS4) signaling pathway (Finkbeiner et al., 1997; Lonze and Ginty, 2002; Pruunsild et al., 2011). CREB and NPAS4 are phosphorylated by the activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and cAMP-dependent protein kinase (PKA) signaling, as well as the calcium/calmodulin-dependent kinase (CaMK), and Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways (Gonzalez and Montminy, 1989; Lonze and Ginty, 2002; Speckmann et al., 2016; Blüthgen et al., 2017; Liu et al., 2017; Funahashi et al., 2019). To determine whether these kinases participate in the increase of BDNF by Chinpi, ACHN cells were cultured in the presence of Chinpi (100 μg/mL) plus Akt inhibitor MK-2206 (5 and 10 μmol/L), CaMK inhibitor KN-93 (1.25 and 2.5 μmol/L), MEK inhibitor U0126 (10 and 20 μmol/L), or PKA inhibitor H89 (10 and 20 μmol/L) for 24 h. The inhibitors were used at concentrations that did not affect the viability of ACHN cells in the presence of 100 μg/mL Chinpi (data not shown). MK-2206, U0126, and H89, but not KN-93, suppressed Chinpi-induced BDNF elevation (Fig. 4B-E).

Keihi substantially decreased BDNF levels in ACHN cells compared with the other test crude drug extracts. A significant decrease in the *BDNF* mRNA level was observed in cells treated with Keihi extract (Fig. 4F).

Table 3. Crude drugs contained in test Kampo medicines in this study.

Crude drug	Kampo Medicine							
	Choto san	Hange koboku to	Hochu ekkito	Kami kihito	Kami syoyo san	Koso san	Ninjin yoeito	Sanso ninto
Bakumondo	+	-	-	-	-	-	-	-
Bofu	+	-	-	-	-	-	-	-
Botanpi	-	-	-	-	+	-	-	-
Bukuryo	+	+	-	+	+	-	+	+
Byakujutsu	-	-	-	-	-	-	+	-
Chimo	-	-	-	-	-	-	-	+
Chinpi	+	-	+	-	-	+	+	-
Chotoko	+	-	-	-	-	-	-	-
Gomishi	-	-	-	-	-	-	+	-
Hakka	-	-	-	-	+	-	-	-
Hange	+	+	-	-	-	-	-	-
Jio	-	-	-	-	-	-	+	-
Kanzo	+	-	+	+	+	+	+	+
Keihi	-	-	-	-	-	-	+	-
Kikuka	+	-	-	-	-	-	-	-
Koboku	-	+	-	-	-	-	-	-
Kobushi	-	-	-	-	-	+	-	-
Mokko	-	-	-	+	-	-	-	-
Ninjin	+	-	+	+	-	-	+	-
Ogi	-	-	+	+	-	-	+	-
Onji	-	-	-	+	-	-	+	-
Ryuganniku	-	-	-	+	-	-	-	-
Saiko	-	-	+	+	+	-	-	-
Sanshishi	-	-	-	+	+	-	-	-
Sansonin	-	-	-	+	-	-	-	+
Sekko	+	-	-	-	-	-	-	-
Senkyu	-	-	-	-	-	-	-	+
Shakuyaku	-	-	-	-	+	-	+	-
Shokyo	+	+	+	+	+	+	-	-
Shoma	-	-	+	-	-	-	-	-
Sojutsu	-	-	+	+	+	-	-	-
Soyo	-	+	-	-	-	+	-	-
Taiso	-	-	+	+	-	-	-	-
Toki	-	-	+	+	+	-	+	-

(+); presence, (-); absence

Table 4. Effects of crude drug methanol extracts on the cell viability of ACHN cells.

Crude drug	Cell viability of ACHN cells (%)				
	0 (μg/mL)	12.5 (μg/mL)	25 (μg/mL)	50 (μg/mL)	100 (μg/mL)
Bakumondo	100.0 ± 3.8	–	97.9 ± 3.2	95.3 ± 3.1	97.9 ± 3.8
Bofu	100.0 ± 3.3	–	99.3 ± 1.9	96.9 ± 2.1	97.1 ± 2.5
Bukuryo	100.0 ± 6.5	–	94.5 ± 9.3	62.2 ± 5.4*	62.9 ± 4.0*
Byakujutsu	100.0 ± 4.6	–	94.9 ± 4.9	94.9 ± 3.6	100.2 ± 1.9
Chinpi	100.0 ± 3.1	–	101.0 ± 4.0	98.2 ± 5.5	96.8 ± 4.5
Chotoko	100.0 ± 5.7	–	107.0 ± 8.7	129.3 ± 5.4*	156.9 ± 4.7*
Gomishi	100.0 ± 4.7	–	102.7 ± 4.8	105.9± 5.6	105.3 ± 8.6
Hange	100.0 ± 6.8	–	98.2 ± 6.1	96.8 ± 7.4	90.1 ± 4.5
Jio	100.0 ± 4.8	–	100.6 ± 5.0	99.6 ± 7.1	98.1 ± 5.4
Kanzo	100.0 ± 4.3	99.6 ± 3.64	115.7 ± 2.6*	134.9 ± 4.2*	175.3 ± 5.8*
Keihi	100.0 ± 5.2	–	105.2 ± 4.1	82.5 ± 7.2*	25.6 ± 6.0*
Kikuka	100.0 ± 6.0	–	99.1 ± 10.9	104.0 ± 5.2	117.9 ± 5.9*
Kobushi	100.0 ± 3.5	–	107.6 ± 4.2	118.4 ± 8.0*	125.5 ± 6.2*
Ninjin	100.0 ± 8.9	–	99.5 ± 8.7	94.7 ± 3.8	97.2 ± 4.9
Ogi	100.0 ± 3.5	–	99.8 ± 5.7	105.2 ± 3.0	100.5 ± 6.0
Onji	100.0 ± 2.1	–	98.3 ± 2.5	101.9 ± 4.7	105.6 ± 3.3*
Saiko	100.0 ± 3.2	–	99.8 ± 5.0	98.0 ± 4.5	99.2 ± 5..4
Shakuyaku	100.0 ± 5.8	–	97.9 ± 6.0	102.1 ± 6.4	103.5 ± 3.4
Shokyo	100.0 ± 4.3	–	103.5 ± 3.4	107.0 ± 3.7	111.8 ± 6.1*
Shoma	100.0 ± 3.6	–	100.2 ± 4.1	99.4 ± 7.2	103.7 ± 7.5
Sojutsu	100.0 ± 4.5	–	100.1 ± 5.5	104.0 ± 5.2	105.4 ± 4.3
Soyo	100.0 ± 4.8	–	96.6 ± 8.4	117.7 ± 3.5*	127.8 ± 3.1*
Taiso	100.0 ± 1.4	–	95.8 ± 4.3	97.6 ± 2.8	95.5 ± 4.1
Toki	100.0 ± 3.7	–	99.8 ± 2.4	101.7 ± 5.4	101.6 ± 1.4

MTT assay was performed to determine the concentration for examining the BDNF up-regulating effect. ACHN cells were treated each crude drug at 0–100 μg/mL for 24 h. Data are shown relative to the viability of control cells (100%). Data are expressed as the mean ± SD (n=6). Tukey's test; *P<0.05 *vs.* control (0 μg/mL).

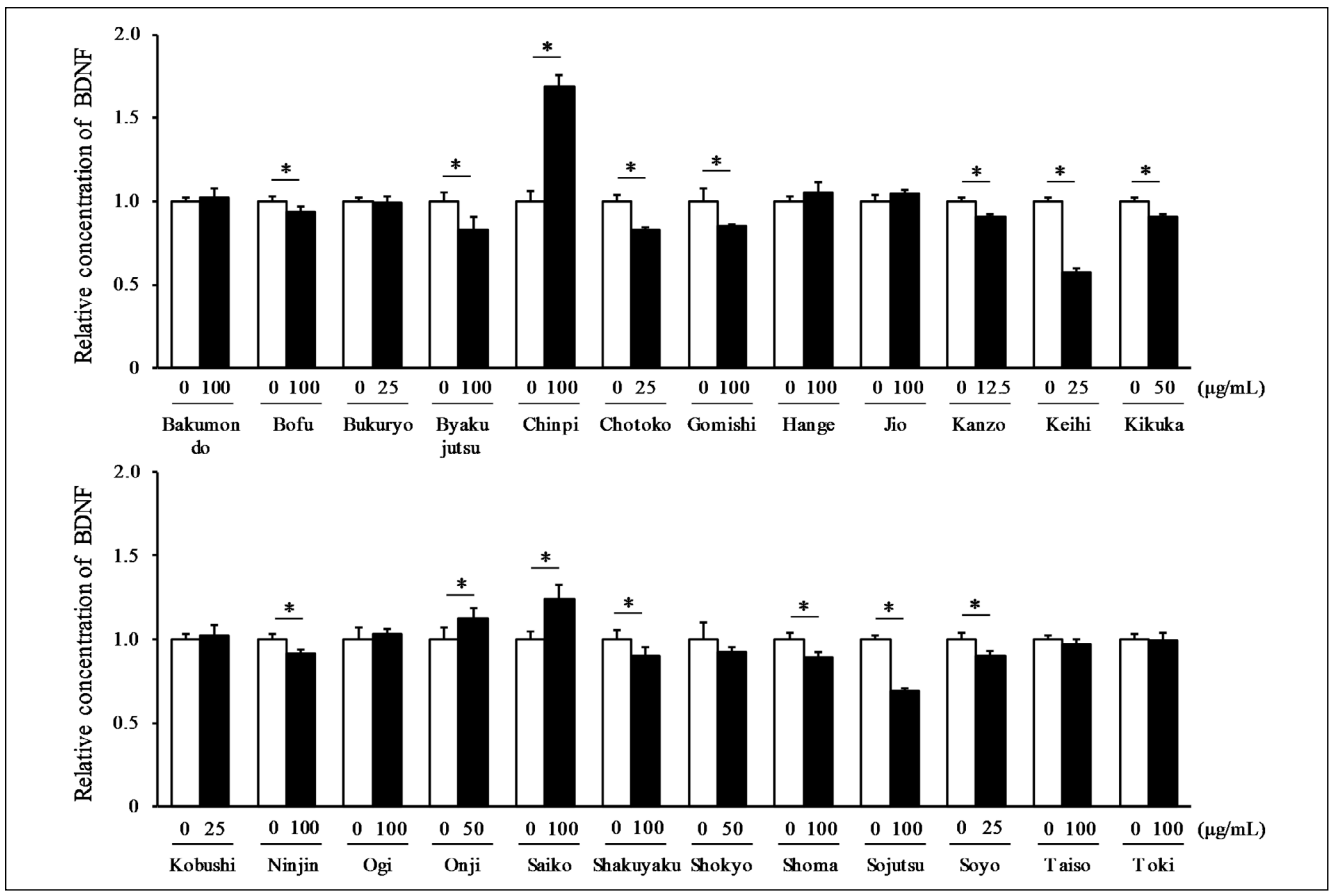

Fig. 2. Effects of crude drug methanol extracts on BDNF concentrations in culture medium of ACHN cells. Cells were cultured for 24 h with the methanol extract of each crude drug. BDNF levels were measured using ELISA. Each value represents BDNF levels of treated cells relative to that of the control (0 μg/mL). The data are expressed as the mean ± SD (n=6). Two-sample *t*-test; **P*<0.05 *vs.* control (0 μg/mL).

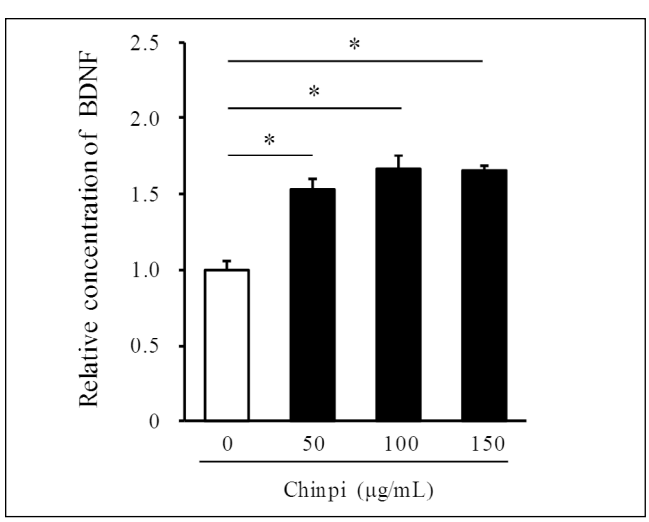

Fig. 3. Effects of Chinpi methanol extracts on BDNF concentrations in culture medium of ACHN cells. Cells were cultured for 24 h with the methanol extracts of Chinpi (50, 100, and 150 μg/mL). BDNF levels were measured using ELISA. Each value represents BDNF levels of treated cells relative to that of the control (0 μg/mL). The data are expressed as the mean ± SD (n=3). Tukey's test; **P*<0.05 *vs.* control (0 μg/mL).

DISCUSSION

In this study, we demonstrated that the methanol extracts of four Kampo medicines and those of Chinpi, Onji, and Saiko increased the production of BDNF in ACHN cells. To screen crude drugs with BDNF-upregulating activity efficiently, we identified Kampo medicines that elevated BDNF concentrations as the first step and found that Hochuekkito, Kososan, and Ninjinyoeito upregulated BDNF in ACHN cells in a concentration-dependent manner. Although Chotosan elevated BDNF levels, it did not show a concentration-dependent effect. Chotosan and Hochuekkito have reportedly upregulate BDNF levels in the brain of mice (Zhao et al., 2011; Lim et al., 2018). The BDNF can cross the BBB (Poduslo and Curran, 1996; Pan et al., 1998), and peripheral administration of BDNF was reported to elevate the BDNF protein level in murine hippocampus (Schmidt and Duman, 2010). Therefore, our findings suggested that peripheral BDNF upregulation caused by Chotosan and Hochuekkito may lead to increased BDNF levels

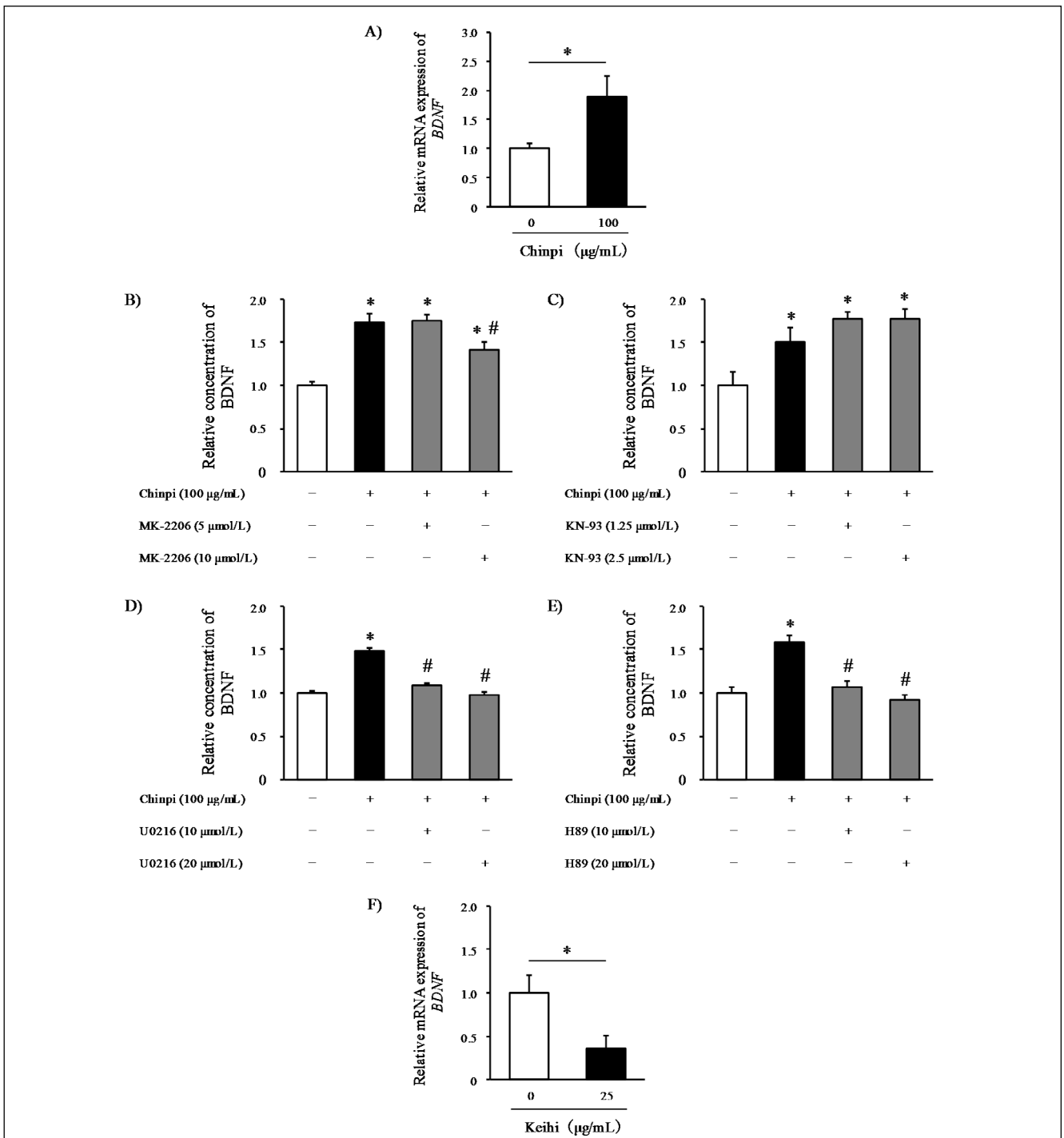

Fig. 4. The mechanisms of underlying the effects of Chinpi and Keihi methanol extracts on BDNF levels in ACHN cells. (A) The mRNA levels of *BDNF* in ACHN cells treated with 100 μg/mL Chinpi methanol extract. Each value represents *BDNF* mRNA levels of treated cells relative to that of the control (0 μg/mL). The data are expressed as the mean ± SD (n=5). Two-sample *t*-test; **P*<0.05 *vs.* control (0 μg/mL). (B–E) Effects of inhibition of Akt, CaMK, MEK, and PKA on Chinpi-induced BDNF upregulation in ACHN cells. ACHN cells were treated with Chinpi (100 μg/mL) and MK-2206 (Akt inhibitors), KN-93 (CaMK inhibitors), U0216 (MEK inhibitors), or H89 (PKA inhibitors) for 24 h. The levels of BDNF in ACHN cells treated with Chinpi and MK-2206 (B), KN-93 (C), U0216 (D) or H89 (E) were measured using ELISA. The data shown represent the mean ± SD (n= 5–6). **P*<0.05 *vs.* without Chinpi and inhibitor group (white bar). #*P*<0.05 *vs.* Chinpi only group (black bar). Akt: protein kinase B, CAMK: calcium/calmodulin-dependent kinase, MEK: mitogen-activated protein kinase kinase, PKA: cAMP-dependent protein kinase. (F) The mRNA levels of *BDNF* in ACHN cells treated with 25 μg/mL Keihi methanol extract. Each value represents *BDNF* mRNA levels of treated cells relative to that of the control (0 μg/mL). The data are expressed as the mean ± SD (n=5). Two-sample *t*-test; **P*<0.05 *vs.* control (0 μg/mL).

in the brain. Additionally, Kososan has been shown to prevent social-avoidance behavior in socially defeated mice (Ito et al., 2017), while Ninjinyoeito ameliorates depressive behavior in mice (Murata et al., 2018). Considering that these antidepressant-like effects are caused by hippocampal neurogenesis (Ito et al., 2017; Murata et al., 2018), and that BDNF initiates neurogenesis (Foltran and Diaz, 2016), peripheral BDNF upregulation induced by Kososan and Ninjinyoeito may participate in these antidepressant-like effects.

In the second step of screening, we investigated the effects of 24 crude drugs contained in Kampo medicines that were found to induce the upregulation of BDNF in ACHN cells. The methanol extracts of Chinpi, Onji, and Saiko significantly increased the levels of BDNF. Interestingly, all Kampo medicines containing Chinpi elicited a BDNF-enhancing effect in ACHN cells, whereas those without Chinpi did not. These findings indicate that Chinpi may strongly contribute to BDNF upregulation. This also implies that Kampo medicines containing Chinpi may possess the potential to elevate the BDNF levels in peripheral tissues.

Among crude drugs, Keihi considerably decreased BDNF levels in ACHN cells, probably by downregulating *BDNF* mRNA. Ninjinyoeito containing Keihi significantly increased BDNF levels in this study. The components contained in Ninjinyoeito could interfere BDNF-decreasing effect by Keihi. However, it is unclear why the BDNF-elevating effect of Chinpi and Saiko contained in Ninjinyoeito was not completely offset by Keihi.

All Kampo medicines identified in this study contains Chinpi. Chinpi upregulated BDNF levels in ACHN cells at 50, 100 and 150 μg/mL. Its increasing effect was approximately from 1.5 to 1.7-fold higher than that of control. All Kampo medicines identified in this study upregulated BDNF levels in approximately 1.4-fold higher than that of control. The reason why the BDNF-increasing rate by Kampo medicines contained Chinpi was lower than the Chinpi alone is considered to be the presence of crude drugs that reduce BDNF in those Kampo medicines. Furthermore, the increases in BDNF levels induced by Kampo medicines were similar, which may be due to a similar amount of Chinpi contained in each Kampo medicine extract.

We also demonstrated that the methanol extracts of Chinpi increased *BDNF* mRNA expression in ACHN cells. Hence, transcriptional activation of *BDNF* gene would be involved in the increase of BDNF levels by Chinpi. The transcription of *BDNF* is regulated by the CREB and NPAS4 signaling pathway (Finkbeiner et al., 1997; Lonze and Ginty, 2002; Pruunsild et al., 2011). The phosphorylation of CREB and NPAS4 are activated *via* the Akt, CaMK, MEK/ERK, and PKA pathways (Gonzalez and Montminy, 1989; Lonze and Ginty, 2002; Speckmann et al., 2016; Blüthgen et al., 2017; Liu et al., 2017; Funahashi et al., 2019). Our investigation using inhibitor for each pathway suggest that the Akt, MEK, and PKA, but not CaMK, pathways may be involved in the BDNF-elevating effect of Chinpi. Chinpi contains abundant flavonoids such as hesperidin and nobiletin (Lu et al., 2006). Hesperidin upregulates the PKA (Wu et al., 2020). Meanwhile, nobiletin stimulates phosphorylation of Akt and MEK (Nagase et al., 2005; Li et al., 2018). On the basis of these reports and our results, it could be assumed that Chinpi flavonoids are involved in BDNF upregulation *via* the PKA, Akt, and MEK pathways. It is known that *BDNF* gene expression is regulated by at least nine promoters, such as *BDNF* promoters I, IV, and VI (Aid et al., 2007; Pruunsild et al., 2011), and CREB and NPAS4 activates *BDNF* promotor IV (Pruunsild et al., 2011). In this study, we revealed that PKA, Akt, and MEK pathways, which phosphorylates CREB and NPAS4, possibly involve in the BDNF upregulation by Chinpi. This results indicate that Chinpi may activate *BDNF* promotor IV.

Serum BDNF levels in healthy subjects have been reported as approximately 1.21-fold higher than those in patients with depression (Karege et al., 2005). Moreover, stress, a major factor in depression, reduces BDNF level in rodents (Duman and Monteggia, 2006; Kikusui et al., 2009). Even among healthy humans, the serum BDNF level is lower in individuals with occupational stress than in those who are not regularly exposed to stress (Mitoma et al., 2008). BDNF levels in the brain and blood show a positive correlation (Klein et al., 2011), and it has been reported that peripheral BDNF administration increases BDNF levels in the brain (Schmidt and Duman, 2010). Therefore, peripheral BDNF upregulators are expected to elicit prophylactic and antidepressant effect. Our results suggest that Chinpi and Kampo medicines including it may upregulate BDNF in the kidney and increase BDNF levels in the blood and brain. In the future, we would like to investigate the usefulness of primary kidney cells for the screening BDNF upregulators and *in vivo* studies are required to clarify whether these Kampo medicines and crude drugs upregulate BDNF and induce antidepressant-like effects.

CONCLUSION

We revealed that the methanol extracts of Chotosan, Hochuekkito, Kososan, and Ninjinyoeito and those of Chinpi, Onji, and Saiko upregulated BDNF levels in the culture medium of ACHN cells. The Akt, MEK, and PKA pathways may be involved in the BDNF-el-

evating effect of Chinpi. The findings of the present study may suggest that these Kampo medicines and crude drugs have the potential to elevate BDNF levels in peripheral tissues.

ACKNOWLEDGMENTS

This work was supported by the JSPS KAKENHI (grant number 19K20134).

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