Nerve Growth Factor-Inducing Activity of *Hericium erinaceus* in 1321N1 Human Astrocytoma Cells

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Neurotrophic factors are essential to maintain and organize neurons functionally; thereby neurotrophic factor-like substances or their inducers are expected to be applied to the treatment of neurodegenerative diseases such as Alzheimer's disease. In the present study, we firstly examined the effects of ethanol extracts of four edible mushrooms, *Hericium erinaceus* (Yamabushitake), *Pleurotus eryngii* (Eringi), *Grifola frondosa* (Maitake), and *Agaricus blazei* (Himematsutake), on nerve growth factor (NGF) gene expression in 1321N1 human astrocytoma cells. Among the four mushroom extracts, only *H. erinaceus* extract promoted NGF mRNA expression in a concentration-dependent manner. In addition, secretion of NGF protein from 1321N1 cells was enhanced by *H. erinaceus* extracts, and the conditioned medium of 1321N1 cells incubated with *H. erinaceus* extract enhanced the neurite outgrowth of PC12 cells. However, hericenones C, D and E, constituents of *H. erinaceus*, failed to promote NGF gene expression in 1321N1 cells. The enhancement of NGF gene expression by *H. erinaceus* extracts was inhibited by the c-jun N-terminal kinase (JNK) inhibitor SP600125. In addition, *H. erinaceus* extracts induced phosphorylation of JNK and its downstream substrate c-Jun, and increased c-fos expression, suggesting that *H. erinaceus* promotes NGF gene expression *via* JNK signaling. Furthermore we examined the efficacy of *H. erinaceus in vivo*. ddY mice given feed containing 5% *H. erinaceus* dry powder for 7 d showed an increase in the level of NGF mRNA expression in the hippocampus. In conclusion, *H. erinaceus* contains active compounds that stimulate NGF synthesis *via* activation of the JNK pathway; these compounds are not hericenones.

Key words nerve growth factor; *Hericium erinaceus*; astrocytoma; hericenone

Senile dementia is a serious social problem. In particular, Alzheimer's disease, for which there is currently no effective therapy, is the most common senile dementia. Alzheimer's disease patients have notable abnormalities in cholinergic neurons in the basal forebrain.[1)] Neurotrophic factors have potent biological activities, such as preventing neuronal death and promoting neurite outgrowth, and are essential to maintain and organize neurons functionally.[2)] Glial cells support neurons by releasing neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3, and glial-derived neurotrophic factor (GDNF). In particular, it is assumed that functional deficiency of NGF is related to Alzheimer's disease and plays a part in the etiology of the disease process.[3)]

It is known that NGF levels are decreased in the basal forebrains of Alzheimer's disease patients, and in the frontal cortices of undemented patients with senile plaques.[4,5)] Furthermore, intracerebroventricular administration of NGF eliminates degeneration and resultant cognitive deficits in rats after brain injury,[6)] and it enhances the retention of passive avoidance learning in developing mice.[7)] In aged rats, intracerebral infusion of NGF partly reverses cholinergic cell body atrophy and improves the retention of spatial memory.[8)] In addition, intranasal administration of NGF ameliorates neurodegeneration and reduces the numbers of amyloid plaques in transgenic anti-NGF mice (AD11 mice), in which have a progressive neurodegenerative phenotype resembling Alzheimer's disease.[9)] Therefore, NGF is expected to be applied to the treatment of Alzheimer's disease.[10)]

However, neurotrophic factors are proteins, and so are unable to cross the blood–brain barrier; they are also easily metabolized by peptidases. Therefore, their application as a medicine for the treatment of neurodegenerative disorders is assumed to be difficult. Alternatively, research has been carried out on low-molecular weight compounds that promote NGF biosynthesis, such as catecholamines,[11,12)] benzoquinones,[13)] fellutamides,[14)] idebenone,[15)] kansuinin, ingenol triacetate, jolkinolide B,[16)] dictyophorines,[17)] scabronines,[18)] hericenones,[18—21)] erinacins,[22—24)] and cyrneines.[25)]

Hericium erinaceus is a mushroom that grows on old or dead broadleaf trees. *H. erinaceus* is taken as a food in Japan and China without harmful effects. Hericenones C—H[2,19—21)] and erinacines A—I[22—24)] were isolated from the fruit body and mycelium of *H. erinaceus*, respectively, all of which promote NGF synthesis in rodent cultured astrocytes. These results suggest the usefulness of *H. erinaceus* for the treatment and prevention of dementia. However, the detailed mechanism by which *H. erinaceus* induces NGF synthesis remains unknown.

In the present study, we examined the NGF-inducing activity of ethanol extracts of *H. erinaceus* in 1321N1 human astrocytoma cells. The results obtained indicate that *H. erinaceus* has NGF-inducing activity, but that its active compounds are not hericenones. Furthermore, ICR mice given feed containing 5% *H. erinaceus* dry powder for 7 d showed an increase in the level of NGF mRNA expression in the hippocampus.

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MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). FCS was from Biological Industries (Kibbutz Beit Haemek, Israel). HS was from ICN Biochemicals, Inc. (Costa Mesa, CA, U.S.A.). Tri Pure Isolation Reagent was from Roche Diagnostics (Indianapolis, U.S.A.). Oligo(dT)primer and NGF ELISA Kit $E_{max}^{®}$ Immunoassay System were from Promega Co., Ltd. (Madison, WI, U.S.A.). Rever Tra Ace was from Toyobo Co., Ltd. (Tokyo, Japan). Syber Premix Ex Taq was from Takara Bio Inc. (Shiga, Japan). U0126 was from Sigma Aldrich Japan (Tokyo, Japan). SP600125 was from BIOMOL (Plymouth Meeting, PA, U.S.A.). A23187, SB203580 and GF109203X were from Calbiochem (San Diego, CA, U.S.A.). H89 was from Seikagaku Corporation (Tokyo, Japan). Anti-NGF was from Boehringer Mannheim (Mannheim, Germany). Anti-phospho-ERK (Thr202/Tyr204) antibody, Anti-ERK antibody, anti-phosho-JNK (Thr183/Tyr185) antibody, anti-JNK antibody, anti-phospho-c-Jun (Ser63) antibody, and anti-c-Jun antibody were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Real-time PCR was carried out using an Opticon real-time PCR system (Bio-Rad Laboratories Inc., Japan). Dextrin was from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). ddY mice were purchased from Japan SLC Inc. (Shizuoka, Japan). *Hericium erinaceus*, *Pleurotus eryngii*, *Grifola frondosa*, and *Agaricus blazei* were cultured by Hokuto Corporation in its facilities (Nagano, Japan).

Cell Culture 1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml). PC-12 cells were grown in DMEM supplemented with 10% FCS, 5% HS, penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were cultivated in an incubator containing 5% CO_2 at 37 °C.

Preparation of Mushroom Extracts Fresh fruiting bodies of *H. erinaceus*, *P. eryngii*, *G. frondosa*, and *A. blazei* were lyophilized and powdered. The dry powder (5 g) of mushrooms was extracted with 150 ml of ethanol for 2 h at room temperature, and *H. erinaceus* ethanol extract (499 mg), *P. eryngii* ethanol extract (386 mg), *G. frondosa* ethanol extract (328 mg) and *A. blazei* ethanol extract (426 mg) were obtained. Similarly, *H. erinaceus* was extracted with H_2O and ethyl acetate, and *H. erinaceus* H_2O extract (1734 mg), and *H. erinaceus* ethyl acetate extract (257 mg) were obtained. The extracts were stored at −30 °C before use.

RT-PCR 1321N1 cells were seeded into 12-well plates and allowed to grow to confluence. Twenty-four hours before the incubation with mushroom extracts, the medium was replaced with serum-free DMEM. The mushroom extract was dissolved in DMSO at 50 mg/ml and then further diluted with serum-free DMEM to the appropriate concentration. 1321N1 cells were incubated with the mushroom extracts for 3 h. Total RNA of 1321N1 cells was extracted using Tri Pure Isolation Reagent according to manufacturer's protocol. First-strand cDNA primed by Oligo(dT) primer was prepared from total RNA (1 μg) using Rever Tra Ace, and was diluted with water by 5 times to use as a template for the real-time PCR analysis. The primers for amplification and the sizes of respective PCR products were as follows: NGF (sense: 5′-CCAAGGGAGCAGTTTCTATCCTGG-3′, and antisense: 5′-GGCAGTTGTCAAGGGAATGCTGAAGTT-3′, for 189 bp), β-actin (sense: 5′-AGGGAAATCGTGCGTGACAT-3′, antisense: 5′-TCCTGCTTGCTGATCCACAT-3′, for 467 bp), c-fos (sense: 5′-GTTCTCGGGTTTCAACGCGGACTACGAGGC-3′, antisense: 5′-GGCACTAGAGACGGACAGATCTGCGCAAAAGTCC-3′, for 922 bp). Real-time PCR was carried out in a 20-μl solution volume containing SYBR Premix (10 μl), RT template (3 μl), water (6 μl) and primers (1 μl). The amplification programs were as follows: NGF, 94 °C for 5 s, 61 °C for 20 s, and 72 °C for 15 s, for 35 cycles; β-actin, 94 °C for 10 s, 56 °C for 20 s, and 72 °C for 30 s, for 22 cycles; and c-fos, 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s, for 39 cycles. The levels of NGF and c-fos mRNA were normalized to that of the corresponding β-actin mRNA.

Assay for Neurite Outgrowth in PC12 Cells 1321N1 cells were seeded into 24-well plates and allowed to grow to confluence. Twenty-four hours before the incubation with *H. erinaceus* extracts or hericenone D, the medium was replaced with serum-free DMEM. After the incubation with *H. erinaceus* extracts or hericenone D for 48 h, the medium was collected, and centrifuged to remove cells.

PC-12 cells were seeded in a 24-well plate at a density of 7×10^4 cells/well and cultivated for 24 h. After aspiration of the medium, DMEM containing 10% FCS and 5% HS (100 μl), and 1321N1 cell culture medium prepared as described above (400 μl) were added and incubated for 48 h. On the other hand, PC-12 cells were stimulated with *H. erinaceus* extract or NGF directly for 48 h. PC-12 cells were observed under a phase-contrast microscope and neurite outgrowth was regarded as a sign of differentiation. About one hundred PC-12 cells in each well were evaluated. Cells bearing neurites longer than one cell diameter were regarded as differentiated cells. Data are expressed as means±S.E.M. of the values for three wells.

Enzyme Immunoassay of NGF 1321N1 cells were seeded into 24 well plates and allowed to grow to confluence. Twenty-four hours before the incubation with mushroom extracts, the medium was replaced with serum-free DMEM. The mushroom extract was dissolved in DMSO at a concentration of 50 mg/ml, and then diluted with serum-free DMEM to the appropriate concentration. 1321N1 cells were incubated with mushroom extracts for 24 h, and then the culture medium was collected. The NGF content in the medium was measured by ELISA ($E_{max}^{®}$ Immunoassay System, Promega) according to the procedures of the manufacturer, except using anti-NGF secondary antibody.

Immunoblot Analysis After drug incubation, the medium was removed and cells were lysed in lysis buffer (75 mM Tris–HCl, 2% SDS, 10% glycerol, 3% 2-mercapto-ethanol, and 0.003% bromophenol blue) for 15 min at room temperature and scraped. Lysates were subjected to SDS-PAGE on a 10% polyacrylamide gel. Separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, U.S.A.) using a semidry blot apparatus. The membrane was blocked 3% skim milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (TBST) for 40 min at room temperature. The membrane was washed with TBST and incubated with primary antibody overnight at 4 °C. Then, the membrane was washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 2

h at room temperature. The immunoreactive proteins were detected using an ECL Western blotting detection system.

Isolation and Analysis of Hericenones C, D, and E Fresh fruiting bodies of *H. erinaceus* (6.0 kg) were extracted with ethanol. The extract was concentrated and fractionated by solvent partition between chloroform and water. The chloroform fraction (25.0 g) was subjected to silica gel column chromatography using toluene–acetone as the eluent. The toluene–acetone (9 : 1) eluate was subjected to silica gel column chromatography using hexane–ethyl acetate. The hexane–ethyl acetate (10 : 1) eluate was further purified by ODS column chromatography using methanol to give hericenone C (210.9 mg), D (35.4 mg), and E (48.0 mg). Their spectral data (^{1}H-NMR, ^{13}C-NMR, IR, and HR-FAB-MS) completely agreed with the data reported.[20]

Hericenone C, D, and E in the extract of *H. erinaceus* were analyzed using HPLC (Shimadzu LC-10 series, SPD-6AV UV-VIS detector Shimadzu set at 260 nm, with a μbondapak C18 3.9×300 mm, 10 μm column (Waters)). The solvent used for separation was acetonitrile and the flow rate was 1.0 ml/min.

NGF mRNA Expression in Mouse Brain Tissue Male ddY mice aged 5 weeks and weighing 33—35 g at the beginning of the experiments, were used. Mice were divided into a control group and a *H. erinaceus* group, so that the average of their body weights was equalized. While the control group was given normal diet (MF, Oriental Yeast, Tokyo, Japan) containing 5% dextrin, the *H. erinaceus* group was given MF containing 5% freeze-dried powder of *H. erinaceus*. Animals were given feed and water freely and maintained under controlled conditions at a temperature of 24±1 °C, relative humidity of 45±5%, and 12-h light cycle (09:00—21:00). Mice were fed an experimental diet for 1 or 7 d. At the end of the experiment, mice were killed by cervical dislocation, and the cerebral cortex and hippocampus were dissected out. The tissues were rapidly homogenized in Tri Pure Isolation Reagent (Takara, Japan) and extracted according to the manufacturer's protocol. Then, NGF mRNA expression was examined by RT-PCR.

Statistical Methods Data are expressed as means±S.E.M. Significant differences ($p<0.05$) were determined by one-way ANOVA, followed by a Tukey's test.

RESULTS

Firstly, we investigated the NGF-inducing activity of four edible mushrooms, *H. erinaceus*, *P. eryngii*, *G. frondosa*, and *A. brazei*, in 1321N1 human astrocytoma cells. The NGF mRNA level in 1321N1 cells was significantly increased by the calcium ionophore A23187 at a concentration of 1 μM (positive control), and by the ethanol extract of *H. erinaceus* at a concentration of 100 μg/ml. However, no significant increase in NGF mRNA levels was induced by the ethanol extracts of the other mushrooms (Fig. 1). In addition, the NGF protein level in the culture medium was also increased by the ethanol extract of *H. erinaceus*, while the ethanol extracts of other three mushrooms failed to induce such an increase (Fig. 2). Moreover, we investigated the effects of H_2O and ethyl acetate extracts of *H. erinaceus* on NGF mRNA expression compared with the effects of the ethanol extract. The ethanol and ethyl acetate extracts promoted NGF mRNA expression in a concentration-dependent manner with similar potency. However, the H_2O extract did not increase NGF mRNA expression (Fig. 3).

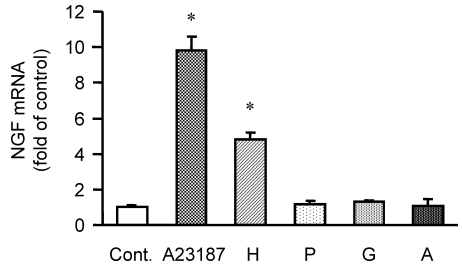

Fig. 1. Effects of Mushroom Extracts on NGF mRNA Expression in 1321N1 Cells

1321N1 cells were stimulated with ethanol extract (100 μg/ml) of *H. erinaceus* (H), *P. eryngii* (P), *G. frondosa* (G), *A. blazei* (A), and A23187 (1 μM, as a positive control) for 3 h at 37 °C, and then NGF gene expression was examined by RT-PCR. Values represent the means±S.E.M. ($n=3$). *$p<0.05$ *vs.* control (Cont.).

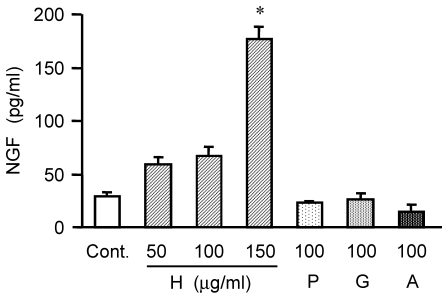

Fig. 2. Effects of Mushroom Extracts on the Secretion of NGF from 1321N1 Cells

1321N1 cells were stimulated with ethanol extract (50, 100, 150 μg/ml) of *H. erinaceus* (H), ethanol extract (100 μg/ml) of *P. eryngii* (P), *G. frondosa* (G), *A. blazei* (A), and A23187 (1 μM, as a positive control) for 24 h at 37 °C. The amount of NGF protein in the culture media of 1321N1 cells was measured by ELISA. Values represent the means±S.E.M. ($n=3$). *$p<0.05$ *vs.* control (Cont.).

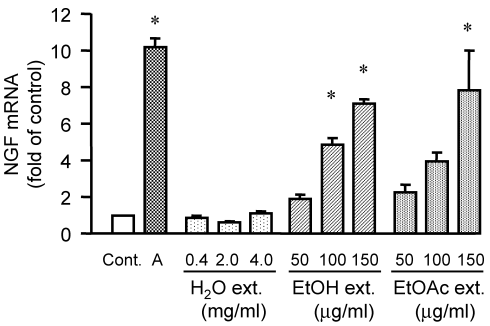

Fig. 3. Effects of *H. erinaceus* Extract on NGF mRNA Expression in 1321N1 Cells

1321N1 cells were stimulated with H_2O extract (0.4, 2.0, 4.0 mg/ml), ethanol extract (50, 100, 150 μg/ml), and ethyl acetate extract (50, 100, 150 μg/ml) for 3 h at 37 °C, and then NGF gene expression was examined by RT-PCR. Values represent the means±S.E.M. ($n=3$). *$p<0.05$ *vs.* control (Cont.).

Next, we examined the NGF mRNA-inducing activity of hericenone C, D and E, components of *H. erinaceus* reported to be stimulators of NGF biosynthesis in mouse astroglial cells at 33 μg/ml.[20] However, hericenones C, D and E did not increase NGF mRNA expression at 10—100 μg/ml in 1321N1 cells (Fig. 4). Furthermore, we failed to demonstrate that hericenones C, D, and E stimulated NGF mRNA expression in primary cultured rat astroglial cells (data not shown). Moreover, we analyzed the levels of hericenones C, D, and E

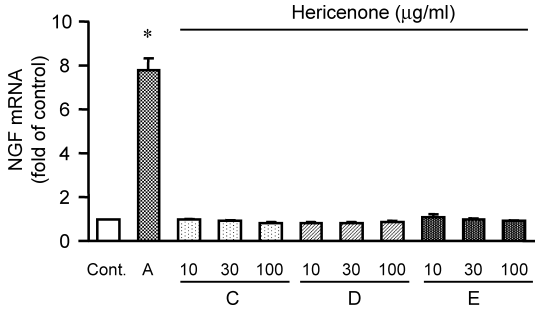

Fig. 4. Effects of Hericenones C, D, and E on NGF mRNA Expression in 1321N1 Cells

1321N1 cells were stimulated with hericenones C, D, and E (10, 30, 100 μg/ml) for 3 h at 37 °C, then NGF gene expression was examined by RT-PCR. Values represent the means±S.E.M. (n=3). * p<0.05 *vs.* control (Cont.).

in the ethanol extract of *H. erinaceus* by HPLC, and their concentrations in 100 μg/ml ethanol extract of *H. erinaceus* were 20 ng/ml for hericenone C, 4 ng/ml for hericenone D, and 2 ng/ml for hericenone E.

To investigate the physiological effects of *H. erinaceus* on neurite outgrowth *via* NGF, PC-12 cells were cultivated for 2 d in the conditioned medium of 1321N1 cells that had been incubated with the ethanol extract of *H. erinaceus* for 24 h. Considering that NGF significantly promoted neurite outgrowth in PC-12 cells at concentrations of 10 and 100 ng/ml, but not at 0.1 and 1 ng/ml, NGF level in the conditioned medium of 1321N1 cells with *H. erinaceus* extract (Fig. 2) was too low to promote neurite outgrowth in PC-12 cells. Furthermore, the ethanol extract of *H. erinaceus* did not promote neurite outgrowth in PC12 cells directly. However, neurite outgrowth was significantly promoted by the conditioned medium of 1321N1 cells incubated with *H. erinaceus* ethanol extract at concentrations of 125 and 250 μg/ml for 24 h. On the other hand, hericenone D did not show the effect such as ethanol extract of *H. erinaceus* (Fig. 5).

To clarify the mechanism underlying NGF induction by *H. erinaceus*, 1321N1 cells were pretreated with various kinase inhibitors, and then stimulated with ethanol extract of *H. erinaceus*. The promotion of NGF mRNA expression by the ethanol extract of *H. erinaceus* was significantly inhibited by the c-Jun N-terminal kinase (JNK) inhibitor SP600125, but not by the MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, the PKA inhibitor H89, or the PKC inhibitor GF109203X (Fig. 6). In fact, the ethanol extract of *H. erinaceus* induced phosphorylation of JNK and its downstream substrate c-Jun in a time-dependent manner (Fig. 7). Furthermore, the ethanol extract enhanced c-fos gene expression (Fig. 8).

To investigate the effect of *H. erinaceus in vivo*, we measured NGF mRNA expression in the cortex and hippocampus of mice administered *H. erinaceus*. The mice in the *H. erinaceus* group were fed a diet containing 5% dried powder of *H. erinaceus*, and the mice of control group were fed a diet containing 5% dextrin instead of *H. erinaceus* for 1 or 7 d. The level of NGF mRNA in the hippocampus of mice in the *H. erinaceus* group was significantly increased compared with that of mice in the control group at the 7th day. On the other hand, NGF mRNA expression in cortex was not increased by *H. erinaceus* during the test period (Fig. 9).

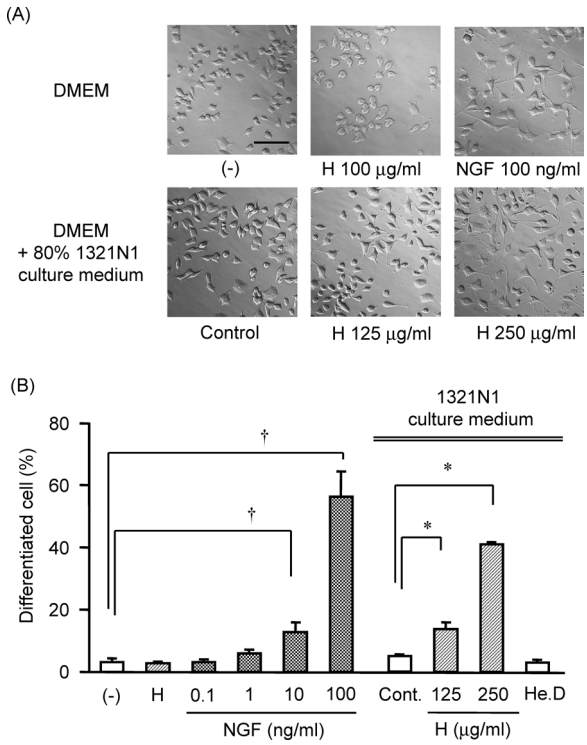

Fig. 5. Morphological Differentiation of PC12 Cells Induced by the Medium of 1321N1 Cells Conditioned with Ethanol Extract of *H. erinaceus*

(A) Morphological changes of PC12 cells. PC12 cells were directly stimulated with ethanol extract (100 μg/ml) of *H. erinaceus* (H) or NGF (100 ng/ml) for 2 d (upper figures). PC12 cells were stimulated for 2 d with 20% DMEM plus 80% 1321N1 culture medium conditioned with 125 or 250 μg/ml of ethanol extract of *H. erinaceus* (lower figures). Scale bar: 100 μm. (B) Evaluation of neurite outgrowth. PC12 cells were directly stimulated with ethanol extract (100 μg/ml) of *H. erinaceus* (H) or NGF (0.1—100 ng/ml) for 2 d (left). After 1321N1 cells were incubated for 2 d in DMEM containing 125 or 250 μg/ml of ethanol extract of *H. erinaceus* (H) or 30 μg/ml of hericenone D (He.D), PC12 cells were cultivated for an additional 2 d in 20% DMEM plus 80% 1321N1 culture medium (right). Values represent the means±S.E.M. (n=3). *, † p< 0.05.

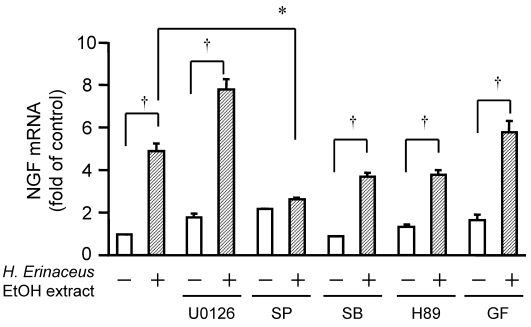

Fig. 6. Effects of U0126, SP600125, SB203580, H89, and GF109203X on *H. erinaceus*-Induced NGF mRNA Expression

1321N1 cells were preincubated with U0126 (10 μM), SP600125 (30 μM), SB203580 (3 μM), H89 (10 μM), and GF109203X (5 μM) for 20 m before the addition of *H. erinaceus* extract. After incubation with *H. erinaceus* ethanol extract (100 μg/ml) for 3 h, NGF mRNA expression was examined by RT-PCR. Values represent the means± S.E.M. (n=3). *, † p<0.05.

DISCUSSION

In the present study, we demonstrate that the ethanol extract of *H. erinaceus* promotes the synthesis of NGF in 1321N1 human astrocytoma cells. *H. erinaceus* alone had NGF-inducing activity among the four mushrooms examined. However, 100 μg/ml of the *H. erinaceus* ethanol extract

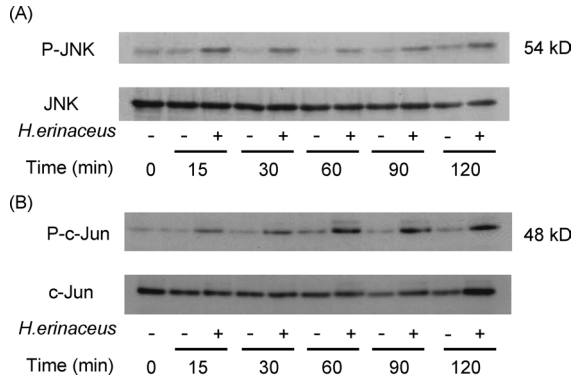

Fig. 7. *H. erinaceus*-Induced Phosporylation of JNK and c-Jun in 1321N1 Cells

1321N1 cells were incubated with ethanol extract of *H. erinaceus* (100 μg/ml) for the indicated times, and then JNK and c-Jun phosphorylation were analyzed by Western blotting. (A) Time-dependent increase in the level of phosphorylation of JNK induced by *H. erinaceus*. (B) Time-dependent increase in the level of phosphorylation of c-Jun induced by *H. erinaceus*.

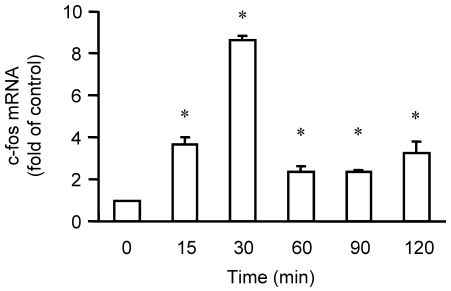

Fig. 8. Effects of *H. erinaceus* Ethanol Extracts on c-fos mRNA Expression in 1321N1 Cells

1321N1 cells were stimulated with ethanol extract of *H. erinaceus* (100 μg/ml) for 3 h at 37 °C, and then c-fos gene expression was examined by RT-PCR. Values represent the means±S.E.M. ($n=3$). $*p<0.05$ *vs.* 0 min.

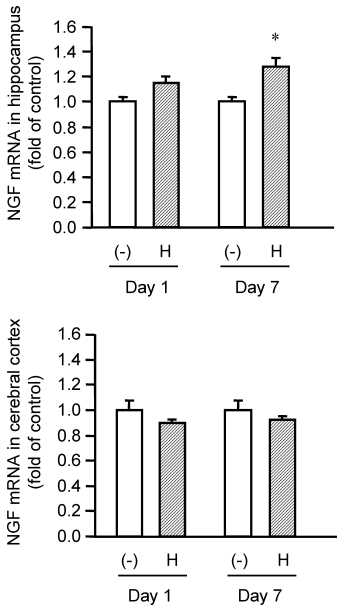

Fig. 9. NGF Gene Expression in the Brains of Mice Fed *H. erinaceus*

Mice were fed a diet containing 5% *H. erinaceus* or dextrin (control), and then NGF mRNA expression in their hippocampus and cortex was analyzed by RT-PCR. Values represent the means±S.E.M. (Day 1 and Day 7: $n=8$; Day 21: $n=3$). $*p<0.05$ *vs.* Control (−).

significantly increased NGF mRNA expression but not NGF protein synthesis. Therefore, it is possible that effective concentrations of *H. erinaceus* ethanol extract to induce NGF protein synthesis and secretion differs from that to induce NGF mRNA expression, because protein synthesis/secretion is regulated by several factors.

Morphological differentiation of PC12 cells was promoted by the conditioned media of 1321N1 cells incubated with *H. erinaceus* ethanol extract, suggesting that *H. erinaceus* stimulates neuronal differentiation *via* an increment in the release of neurotrophic factors, including NGF, from glial cells. However, the NGF concentration elevated by *H. erinaceus* extract is assumed to be too low to promote neurite outgrowth in PC-12 cells. Therefore, other factors might be involved in neurite outgrowth. In fact, astrocytes secrete other neurotrophins, cytokines and growth factors, some of which influence morphological change or facilitate NGF-induced differentiation in PC12 cells.[2,26—28)]

It has been reported that phorbol esters enhance PKC-dependent NGF synthesis in primary mice astrocytes, and AP-1, one of the targets for PKC, was assumed to regulate NGF gene expression.[29)] In fact, there is an AP-1 consensus sequence (TRE: TPA-response element) downstream of the TATA box at the junction of the exon I/intron I region of the NGF gene.[30)] AP-1 consists of homo or hetero dimers of Jun/Jun or Fos/Jun, which bind to DNA at the AP-1 site TRE. In the present study, the enhancement of NGF gene expression by *H. erinaceus* was inhibited by the JNK inhibitor SP600125, and *H. erinaceus* caused phosphorylation of JNK. These results suggest that JNK is involved in the enhancement of NGF gene expression induced by *H. erinaceus*. JNK is the predominant kinase to phosphorylate c-Jun.[31)] Furthermore, *H. erinaceus* enhanced c-Jun phosphorylation and c-fos gene expression as well as JNK phosphorylation. The activation of AP-1 by *H. erinaceus* is assumed to participate in NGF gene expression downstream of JNK, but PKC is not involved in this signaling pathway, because of a lack of inhibitory action with the PKC inhibitor GF109203X.

It has been reported that the active components of *H. erinaceus* are the hericenones C—H, which stimulate NGF protein synthesis in mouse or rat astrocytes.[19—21)] However, hericenones C, D, and E did not exhibit NGF-promoting activity at all under the present experimental condition using 1321N1 human astrocytoma cells. In addition, the concentrations of hericenones in the ethanol extract were very low (the concentrations of hericenones C, D, and E in the 100 μg/ml ethanol extract of *H. erinaceus* were 20, 4, and 2 ng/ml, respectively) compared to their effective concentration (33 μg/ml) as shown in a previous report.[20)] These results, therefore, raise the possibility that *H. erinaceus* has unknown active compounds that promote NGF expression, other than hericenones, which are lipid-soluble (soluble in ethanol and/or ethyl acetate).

Furthermore, the oral administration of *H. erinaceus* increased NGF mRNA expression in the mouse hippocampus. This result suggests the possibility that the active compound could be absorbed into blood and delivered into the central nervous system through the blood–brain barrier. The hippocampus is postulated to encode working memory.[32)] The increase in the level of NGF mRNA in the hippocampus suggests the potential of *H. erinaceus* to act on the central nervous system *in vivo*. However, we could not elucidate why

H. erinaceus increased NGF mRNA expression in the hippocampus but not in the cortex. This difference might result from variations of expression level of signaling molecules related to JNK signaling pathway or kinetic difference of active components of *H. erinaceus* such as brain distribution and metabolism in these tissues.

On the other hand, the mycelia of *H. erinaceus* are known to contain erinacines, which also stimulate NGF synthesis.[22—24] It has been reported that oral administration of erinacine A significantly increases the level of NGF in the rat locus coeruleus and hippocampus, but not in the cerebral cortex.[33] However, it has not yet been reported that the fruit body of *H. erinaceus* contains erinacines. Thus, it is necessary to reevaluate whether fruit bodies contain erinacines, and to examine the existence of unknown derivatives with NGF-inducing activity in the fruit bodies of *H. erinaceus*.

In conclusion, *H. erinaceus* contains active compounds that stimulate NGF synthesis *via* activation of the JNK pathway; these compounds are not hericenones.

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