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Inhibition of Glycogen Synthase Kinase-3β by Falcarindiol Isolated from Japanese Parsley (*Oenanthe javanica*)

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ABSTRACT: A new biological activity of falcarindiol isolated from Japanese parsley (*Oenanthe javanica*) using the mutant yeast YNS17 strain (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) was discovered as an inhibitor of glycogen synthase kinase-3β (GSK-3β). Falcarindiol inhibited GSK-3β in an ATP noncompetitive manner with a K_i value of 86.9 μM using a human enzyme and luminescent kinase assay platform. Falcarindiol also both suppressed gene expression of glucose-6-phosphatase (G6Pase) in rat hepatoma H4IIE cells and protected mouse neuroblastoma HT22 cells from glutamate-induced oxidative cell death at 10 μM. During an oral glucose tolerance test (OGTT), the blood glucose level was significantly decreased in the rats treated with oral administration of *O. javanica* extract containing falcarindiol (15 mg/kg). These findings indicate that Japanese parsley could be a useful food ingredient against type-2 diabetes and Alzheimer's disease.

KEYWORDS: *Japanese parsley, Oenanthe javanica, falcarindiol, Saccharomyces cerevisiae, GSK-3β, glucose-6-phosphatase, glutamate-induced oxidative cell death, oral glucose tolerance test*

INTRODUCTION

Japanese parsley, *O. javanica*, is an Apiaceae vegetable and a perennial herb with distinctive aroma. It has been reported that *O. javanica* has antiviral, antihepatitis, and antidiabetic effects.[1−3] Falcarindiol (heptadeca-1,9(*Z*)-diene-4,6-diyne-3,8-diol), one of the polyacetylene compounds, has been isolated from Apiaceae and is identified in the aerial parts of the Apiaceae used for food such as carrots, celery, and Japanese parsley.[4−6] Falcarindiol has shown biological characteristics such as anti-inflammatory effects, antibacterial activity, cytotoxic activity against several tumor cell lines, and induction activity of phase 2 drug-metabolizing enzymes and antioxidant enzymes.[6−9]

GSK-3β is a ubiquitous serine/threonine kinase that inactivates glycogen synthase through its phosphorylation and is involved in the molecular pathogenesis of several human diseases such as type-2 diabetes and Alzheimer's disease.[10−12] GSK-3β plays a central role in the regulation of insulin action and glucose metabolism. GSK-3β protein levels and total activities are elevated in type-2 diabetic muscle independent of obesity and inversely correlated with both glycogen synthase activity.[11] GSK-3β activity is altered in Alzheimer's disease brain, and it has been postulated that GSK-3β mediates β-amyloid-induced neurotoxicity and presenilin-1 mutation pathogenic effects.[12] Recent studies have widely reported that type-2 diabetes increases the risk of developing Alzheimer's disease.[13−15] Type-2 diabetes is associated with brain tau-protein hyperphosphorylation and increased activity of GSK-3β, a key kinase of tauprotein.[13] Thus deregulation of GSK-3β activity has been implicated in the pathogenesis of human diseases such as type-2 diabetes and Alzheimer's disease.

It has been reported that several small-molecule inhibitors of GSK-3β showed anti-type-2 diabetes, anti-Alzheimer's disease, anticancer, and anti-inflammation effects.[16] Thus, novel synthetic GSK-3β inhibitors have been developed as medicine. The food functional compound curcumin was reported as an inhibitor of GSK-3β.[17] However, there is no other in vivo study of GSK-3β inhibitors from food ingredients.

To find a new GSK-3β inhibitor from food ingredients, methanol extracts of vegetables were examined using a unique phenotypic screening system that evaluated the growth restored activity caused by inhibition of Ca^{2+}-signal transduction in the mutant yeast, YNS17 strain (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ).[18−20] The Ca^{2+}-signaling pathways for yeast growth regulation are composed of several signaling molecules such as the Ca^{2+} channel, calcineurin, Mck1 GSK-3β, Mpk1 mitogen activated protein kinase.[18,19] Recently we reported that 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC), isolated from Japanese pungent spice Wasabi (*Wasabia japonica*) using this screening system, inhibited human GSK-3β.[21]

In this study, we identified an active compound as falcarindiol from some Apiaceae vegetables, especially Japanese parsley (*O. javanica*, Japanese name Seri) using the mutant yeast. One of the molecular targets for falcarindiol was GSK-3β, and it exhibited physiological activities against different cell models of type-2 diabetes and Alzheimer's disease which are

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serious diseases in Japan. Falcarindiol was also effective against diabetic rats.

MATERIALS AND METHODS

Plant Materials. *O. javanica*, *Cryptotaenia canadensis* subsp. *japonica*, *Angelica keiskei*, *Daucus carota* subsp. *sativus*, *Apium graveolens* var. *dulce*, and *Petroselinum crispum* were purchased from a local market in Morioka, Japan. *O. javanica* was used for the isolation of biological compounds.

Chemicals and Reagents. Difco yeast-peptone-dextrose (YPD) broth and YPD agar were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). FK506 was kindly given by Fujisawa Pharmaceutical Co., Ltd. (the present Astellas Pharma Inc., Tokyo, Japan). GSK-3β inhibitor-I (TDZD-8, 2-(3-iodobenzylthio)-5-(pyridin-4-yl)-1,3,4-oxadiazole) and GSK-3β inhibitor-II (2-thio(3-iodobenzyl)-5-(1-pyridyl)-[1,3,4-]-oxiadiazole) were purchased from Calbiochem (Darmstadt, Germany). Paper disks (8 mm) were purchased from Toyo Roshi (Tokyo, Japan). Human recombinant GSK-3β and substrate peptide (phospho-glycogen synthase peptide-2 (PGSP-2), YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE), were purchased from Millipore Co. (Billerica, WI, USA). Kinase-Glo plus luminescent kinase assay platform was purchased from Promega Co. (Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM), N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP), and insulin (bovine) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum albumin was purchased from EquitechBio (Kerrville, TX, USA). Horse serum (GIBCO) was purchased from Life Technologies Corp., (Carlsbad, CA, USA). Newborn calf serum was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan). Bovine serum albumin, olive oil, and dexamethasone were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). CDP-star reagent was obtained from Tropix (Bedford, MA, USA). Other chemicals were of the best grade commercially available.

Activity of the Methanol Extract of Apiaceae Vegetables against the Mutant Yeast. All aerial parts of *O. javanica*, *C. canadensis* subsp. *japonica*, *A. keiskei*, *A. graveolens* var. *dulce*, and *P. crispum* and underground parts of *D. carota* subsp. *sativus* were dried at room temperature and extracted with methanol. The methanol extract (10 mg/mL) was used for the assay and analysis by HPLC.

Isolation of Falcarindiol. Methanol extracts from *O. javanica* showed the most potent growth zone around an inhibitory zone on the plates, and we selected it for isolation of the biological compound. Fresh *O. javanica* (834.6 g) was dried at room temperature (55.1 g), and the active component was extracted with methanol (15.7 g). The extract was diluted with an excess of water and extracted twice with ethyl acetate. After evaporating ethyl acetate, the organic layer (1.23 g) was dissolved in methanol and was chromatographed by preparative TLC (20 cm × 20 cm × 2 mm, chloroform:methanol = 13:1, 114.6 mg). Finally, falcarindiol was purified by reverse-phase HPLC using a CAPCELL PAK C18 column (250 mm × 20 mm i.d.; Shiseido Co., Ltd., Tokyo, Japan), eluting with 75% methanol at 10 mL/min, monitoring by UV (220 nm) (25.4 mg).

Identification of Falcarindiol. Identification of the active constituents was performed by MS (JMS-700, JEOL Ltd., Tokyo, Japan), NMR (EX-400, JEOL Ltd.), UV (UV mini 1240, Shimazu Co., Kyoto, Japan), and optical rotation (DIP1000, Nihon Bunko Co., Tokyo, Japan). Data obtained are as follows: HR-FAB-MS m/z $(M + H - H_2O)^+$, calcd for $C_{17}H_{22}O$ 243.1749, found 243.1743; ^{13}C NMR δ_C ($CDCl_3$, 100 MHz, reference: solvent 77.0 ppm), 117.3, (C-1, t), 135.7 (C-2, d), 63.5 (C-3, d), 78.2 (C-4, s), 70.3 (C-5, s), 68.7 (C-6, s), 79.9 (C-7, s), 58.6 (C-8, d), 127.6 (C-9, d), 134.7 (C-10, d), 27.7 (C-11, t), 29.2 (C-12, t), 29.1 (C-13, t), 29.1 (C-14, t), 31.8 (C-15, t), 22.6 (C-16, t), 14.1 (C-17, q); the UV spectrum (nm) (ε) in methanol, 233 (1179), 245 (1083), 258 (639), 284 (127). All physicochemical properties such as molecular formula, chemical shift for carbon, and UV spectrum were identical with reported data for falcarindiol.[5]

Quantitative HPLC Analysis of Falcarindiol. Quantitative analysis of falcarindiol was carried out on a SPD-6AV detector (Shimazu Co.) consisting of a HPLC pump (L-6000, Hitachi Co. Tokyo, Japan) and a Chromatopak (C-R6A, Shimazu Co.). Separations were performed on a Senshu pak PEGASIL ODS (150 mm × 4.6 mm i.d., 5.0 μm, Senshu Kagaku Co., Ltd. Tokyo, Japan). The column temperature was maintained at room temperature, and the mobile phases consisted of methanol−water (75:25 (v/v)). Flow rate with an injection volume of 10 μL was 1.0 mL/min. A standard solution of falcarindiol in methanol for standard curve was prepared by serial dilution of 1.0 mg/mL falcarindiol stock solution. The calibration curve consisted of five different concentrations (0.0625, 0.125, 0.25, 0.5, 1.0 mg/mL) and was performed in duplicate. An aliquot (10 μL) of each standard solution was subjected to HPLC analysis. The linearity for falcarindiol detection was established by plotting the peak area (y) versus concentration (x) of each analyte. The calibration curve showed good linear regression ($R^2 > 0.9816$) within the test ranges. A solution of methanol extract of each Apiaceae (1 mg/mL in methanol) was injected into HPLC for analysis (10 μL).[22]

Screening of Ca^{2+}-Signal Transduction Inhibitors. The strain used in this study was YNS17 strain (MATa *zds1::TRP1 erg3::HIS3 pdr1::hisG URA3::hisG pdr3::hisG*) that is a derivative of strain W303-1A.[20] The screening was performed on YPD agar plates containing 0.3 M $CaCl_2$ using YNS17 strain according to the procedure described previously.[23] Briefly, samples were dissolved in methanol, and 40 μL aliquots of the solution were applied to 8 mm paper disks. The paper disks containing each compound were placed on the surface of a YPD agar plate containing YNS17 strain and 0.3 M $CaCl_2$. After 3 days of incubation at 28 °C, the inhibitory activity of the Ca^{2+}-signal transduction was determined by the diameter of the growth zone and/or inhibition zone of the cells. The immunosuppressive drug FK506 (0.02 μg/disk) was used as a positive control.

GSK-3β Assay. GSK-3β assays were performed using the Kinase-Glo plus luminescent kinase assay platform in a white 96-well plate.[21] Briefly, PGSP-2 (20 μM) was mixed with recombinant human GSK-3β (0.022 unit) in a total volume of 50 μL of assay buffer (8 mM MOPS (pH 7.0), 1 mM EDTA, 5 μM ATP, 10 mM $MgCl_2$) in the presence or absence of inhibitors (2 μL of DMSO). After 3 h incubation at 30 °C, the enzymatic reaction was stopped by the addition of 50 μL of Kinase-Glo reagent. Glow-type luminescence was measured after 10 min using a Powerscan HT spectrophotometer (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). GSK-3β inhibitor-I inhibited GSK-3β at IC_{50} = 95.6 μM in our assay. ATP-competitive analysis was performed varying both ATP levels (5, 10, and 20 μM) and falcarindiol concentrations (0, 10, 20, and 50 μM) with a constant concentration of PGSP-2 (20 μM). PGSP-2-competitive analysis was performed varying both PGSP-2 (2.5, 5, and 10 μM) and falcarindiol concentrations (0, 10, 20, and 50 μM) with a constant concentration of ATP (5 μM).

Cell Culture. Rat hepatoma H4IIE cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin (50 units/mL)−streptomycin (50 μg/mL) at 37 °C under 5% CO_2 in a humidified atmosphere.[24] Mouse hippocampal HT22 cells (kindly provided by David Schubert in the Salk Institute, La Jolla, CA, USA) derived from the HT4 cell line were grown in DMEM supplemented with 5% of heat-inactivated horse serum and 5% of precolostrum newborn calf serum at 37 °C under 5% CO_2 in a humidified atmosphere.[25]

Glucose-6-phosphatase Gene Expression. H4IIE cells were seeded onto a 60 mm dish at a density of 8×10^5 cells/dish in 3 mL of DMEM with 10% heat-inactivated FBS at 37 °C. After the cells were grown to confluence at 37 °C, the cells were washed three times with phosphate buffered saline and then incubated in DMEM containing 0.1% bovine serum albumin for 24 h. Then, the cells were treated with or without dexamethasone (0.5 μM)/Bt_2cAMP (100 μM) and in the absence or presence of various concentrations of test reagent for 24 h.[24]

Northern Blotting. Specific cDNA probes for the detection of G6Pase mRNA transcripts by Northern blotting were synthesized as described previously.[24] Total RNA was prepared by the AGPC

method.[26] Twelve milligrams of total RNA from H4IIE cells was separated on a 1.2% agarose−formaldehyde gel and transferred to a positively charged nylon membrane (GE Healthcare Bioscience, Tokyo, Japan). After UV cross-linking, membranes were hybridized with digoxigenin-labeled cDNA probes generated by rTaq DNA polymerase reaction for 12 to 16 h at 50 °C in hybridization solution (5 × SSC, 50% formamide, 50 mM sodium phosphate buffer (pH 7.0), 7% sodium dodecyl sulfate (SDS), 2% blocking reagent, and 0.1% *N*-lauroysarcosine). Membranes were washed twice 2 × SSC−0.1% SDS for 15 min at 68 °C. Specific hybridization was detected with an anti-digoxigenin antibody conjugated with the alkaline phosphatase, and blots were developed with the CDP-star reagent. To estimate the relative intensity of each band, X-ray films were scanned and analyzed using NIH Image.

Glutamate-Induced Cell Cytotoxicity. HT22 cells were dissociated and seeded into 24-well plates at a density of 8×10^4 cells/well in 500 μL of medium. Five hours after seeding, the cells were exposed to 5 mM glutamate with or without several different concentrations of GSK-3β inhibitors and falcarindiol. Twenty-four hours after the treatment, the viability of HT22 cells was determined by using the method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction (MTT assay).[25] To the cell culture medium in each well was added MTT. After 2 h of incubation at 37 °C, cells were solubilized with a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). The absorbance at 570 nm was measured on the following day with a microplate reader.

Animals. Male spontaneously diabetic GK (Goto-Kakizaki) rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed in individual stainless cages in a temperature and humidity controlled room with a 12 h light/dark cycle. The animals were allowed free access to water and the CRF-1 diet (Oriental Yeast Co., Ltd. Tokyo, Japan) during one week for acclimatization.

Oral Glucose Tolerance Test (OGTT) of Acute Feeding Test. Fresh *O. javanica* (1000.0 g) was dried at room temperature (83.19 g) and extracted with methanol. The extract (17.70 g) was suspended in 10% methanol and then extracted twice with 1 volume of ethyl acetate. After evaporation of ethyl acetate (1.66 g) the organic layer was diluted in methanol. The concentration of falcarindiol in the ethyl acetate extract was determined by HPLC. After evaporation of methanol, the ethyl acetate extract was diluted to 28 mg/mL (falcarindiol: 1.5 mg/mL) in olive oil. The GK rats were divided into two groups, each containing six rats: namely, control groups and groups fed with *O. javanica* extracts (OJ). After 20 h fasting, the control groups received a single dose of glucose (2 g/kg p.o.) and 2 mL of olive oil, whereas a single dose of the glucose (2 g/kg p.o.) and the solution of *O. javanica* extracts (in olive oil, 280 mg/kg (falcarindiol: 15 mg/kg)) was administered p.o. to the OJ groups. Blood glucose was measured at 0, 30, 60, 90, 120, 150, 180, 210, and 240 min via the tail vein with a blood glucose test meter (ACCU-CHECK Active, Roche Diagnostics Co., Tokyo, Japan). The areas under the curve (AUC) of changes in the blood glucose were determined from time 0 to 240 min after glucose administration. This study was approved by the Japan Food Research Laboratories (Tokyo, Japan).

Statistical Analysis. Data are means ± SD or ± SEM. Differences in the mean values among groups were assessed using the Tukey−Kramer multiple comparisons test (Instat Ver. 3.0). The level of significance was set at $P < 0.05$ for all statistical tests.

RESULTS

Restoring Activity of the Methanol Extract of Apiaceae Vegetables. All examined Apiaceae extracts except *A. graveolens* var. *dulce* (Serori) and *P. crispum* (Paseri) showed restored activity of the mutant yeast (growth zone: *O. javanica* (Seri); 24.7 mm, *C. canadensis* subsp. *japonica* (Mitsuba); 18.2 mm, *A. keiskei* (Ashitaba); 16.3 mm, *D. carota* subsp. *sativus* (Ninjin); 14.1 mm at 400 μg/disk). *O. javanica* (Seri) showed the most potent activity. The activity was parallel with that of peak area of HPLC analyses (quantity of falcarindiol) (Table 1).

Table 1. Falcarindiol Content of Apiaceae Vegetables

Apiaceae vegetables (Japanese name)	falcarindiol content (mg/g) (relative proportion)
Oenanthe javanica (Seri)	34.4 (100)
Cryptotaenia canadensis subsp. *japonica* (Mitsuba)	22.8 (66)
Angelica keiskei (Ashitaba)	3.8 (11)
Daucus carota subsp. *sativus* (Ninjin)	1.4 (4)
Apium graveolens var. *dulce* (Serori)	0.5 (1)
Petroselinum crispum (Paseri)	ND[a]

[a]Not detected.

Restoration of Compromised Growth of a Ca^{2+}-Sensitive YNS17 Strain by Falcarindiol. Figure 1 shows a typical phenotypic effect of falcarindiol on the compromised cell growth of a Ca^{2+}-sensitive yeast strain. Falcarindiol showed restored growth activity on YNS17 strain, detected as a growth zone around an inhibitory zone in a dose-dependent manner. GSK-3β specific inhibitor GSK-3β inhibitor-I showed a faint growth zone around the growth inhibitory zone of YNS17 strain on the plate.[23] Since the restored growth phenotypes of falcarindiol and GSK-3β inhibitor-I against YNS17 strain were similar, it is likely that one of the new molecular targets of falcarindiol is GSK-3β.

Figure 1. Restored growth activity of falcarindiol against YNS17 strain of *S. cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ). The paper disks containing falcarindiol were placed on the surface of a YPD agar plate containing YNS17 strain (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) and 0.3 M $CaCl_2$. The plate was incubated at 28 °C for 3 days. 1: 40 μg/disk. 2: 20 μg/disk. 3: 10 μg/disk. 4: 5 μg/disk. 5: 2.5 μg/disk. 6: 1.25 μg/disk. 7: FK506 (0.02 μg/disk).

Determination of the Inhibition Pattern on GSK-3β. To identify the new molecular target of falcarindiol, the inhibition activity of falcarindiol on GSK-3β was directly examined in the luminescent kinase assay method using recombinant human GSK-3β as an enzyme and phosphopeptide as a substrate in vitro. Falcarindiol inhibited GSK-3β with an IC_{50} value of 83.0 μM. Falcarindiol showed an ATP noncompetitive binding mode to GSK-3β by Dixon plot (K_i = 86.9 μM, Figure 2A) and showed a PGSP-2 competitive binding mode to GSK-3β with by Dixon plot (K_i = 46.5 μM, Figure 2B). These data showed that falcarindiol is a new ATP noncompetitive GSK-3β inhibitor and might be expected to be a useful functional compound as anti-type-2 diabetes, anti-Alzheimer's disease, and anti-inflammation agent.

Falcarindiol Suppresses Glucose-6-phosphatase Gene Expression. To assess the GSK-3β inhibition activity of falcarindiol and type-2 diabetes therapeutic potential, we analyzed the gene expression of G6Pase in H4IIE rat hepatoma cells. G6Pase is a rate-controlling enzyme of gluconeogenesis.[27]

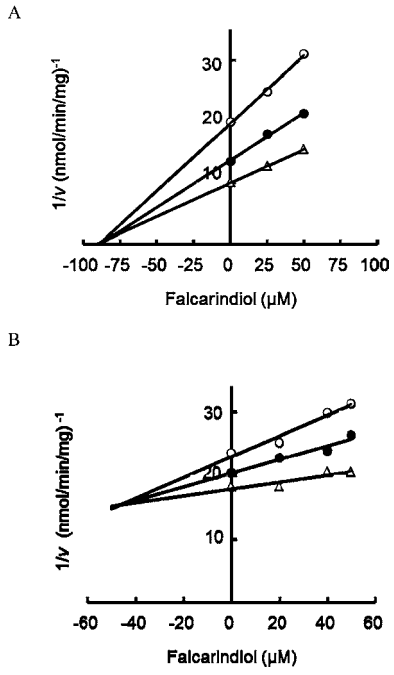

Figure 2. Dixon plot of falcarindiol against GSK-3β. A: Double-reciprocal plot of kinetic analyses was performed using three ATP concentrations (5 (○), 10 (●), 20 (△) μM) and three falcarindiol concentrations (0, 25, 50 μM). The GSK-3β reaction was performed as described in the text with a constant concentration of PGSP-2 (20 μM). B: Double-reciprocal plots of kinetic analyses were performed using three PGSP-2 concentrations (2.5 (○), 5 (●), 10 (△) μM) and four falcarindiol concentrations (0, 20, 40, 50 μM) with a constant concentration of ATP (5 μM). The GSK-3β reaction was performed as described in the text.

In rat hepatoma cells, GSK-3β inhibitors (lithium, SB216763, and SB415286) reduced the dexamethasone/Bt_2cAMP-induced gene expression of G6Pase.[28] Figure 3 shows that treatment of H4IIE cells with falcarindiol blocks dexamethasone/Bt_2cAMP-induced gene expression of G6Pase in a dose dependent manner. These results showed that falcarindiol can inhibit GSK-3β and may therefore have therapeutic potential for lowering blood glucose levels.

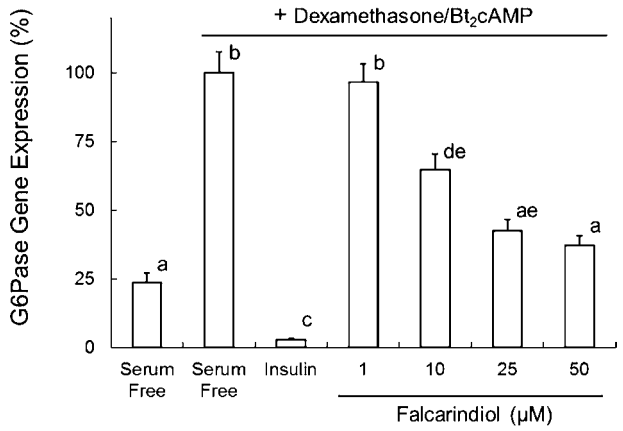

Figure 3. Repression of G6Pase expression by falcarindiol. H4IIE cells were incubated with or without dexamethasone (0.5 μM)/Bt_2cAMP (100 μM) and falcarindiol (1, 10, 25, 50 μM). Expression of G6Pase was measured, normalized to the level of GADPH mRNA, and expressed as means ± SEM from three separate experiments performed in duplicate. Means with different letters are significantly different (*P < 0.05).

Falcarindiol Inhibited Glutamate Induces Cell Death. To assess the GSK-3β inhibition activity of falcarindiol and Alzheimer's disease therapeutic potential, we analyzed the inhibition activity of falcarindiol against the glutamate induced cell death. HT22 cells were induced to oxidative stress and neurotoxicity by exposure to high concentrations of glutamate.[25] GSK-3β inhibitor (LiCl) indeed led to an increased tolerance of HT22 to glutamate.[29] HT22 cells were treated with 5 mM glutamate in the presence or absence of falcarindiol and incubated for 24 h. Cell survival was quantified by conducting the MTT assay. In control HT22 cells, glutamate induced global cell death after 24 h due to oxidative stress. In contrast, the cells treated with GSK-3β inhibitor-I and GSK-3β inhibitor-II (10 μM) were resistant to the oxidative stress (Figure 4A).

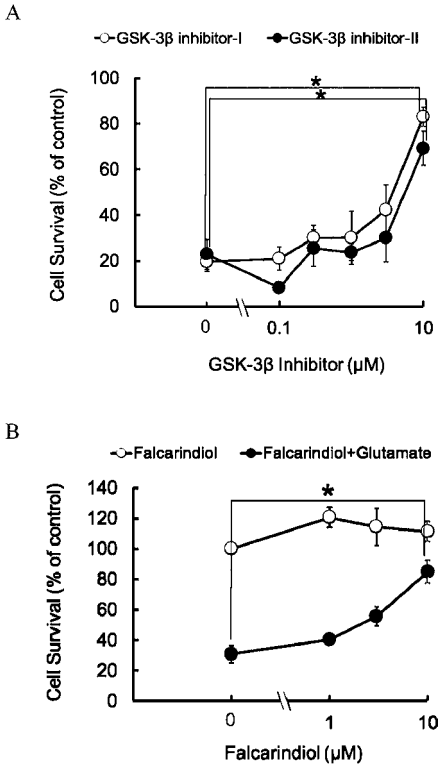

Figure 4. Neuroprotection by falcarindiol. Cell viability was the assessed by MTT assay. Significance of difference between no inhibitors and inhibitors at any concentration was determined by ANOVA (*P < 0.01). A: HT22 cells were exposed to various concentrations of GSK-3β inhibitor-I and GSK-3β inhibitor-II (0, 0.1, 0.3, 1.0, 3.0, 10 μM) for 30 min in the presence (5 mM) of glutamate. B: HT22 cells were exposed to various concentrations of falcarindiol (0, 1.0, 3.0, 10 μM) for 30 min in the presence (5 mM) or absence of glutamate.

Falcarindiol (10 μM) also protected HT22 cells against the oxidative glutamate toxicity in a dose dependent manner (Figure 4B). These results showed that falcarindiol might have a neuroprotective effect through the inhibition of GSK-3β in the cells.

OGTT Curve on Plasma Glucose Level of GK Rats in Acute Feeding Test. Since falcarindiol led to inhibition of G6Pase gene expression in H4IIE cells, the therapeutic potential for lowering blood glucose levels by falcarindiol or its ingredient vegetables was examined. We examined the effect

of *O. javanica* extract on OGTT and plasma glucose level of GK rats in an acute feeding test. GK rats fed with *O. javanica* ethyl acetate extracts dissolved in olive oil (280 mg/kg of body weight (falcarindiol: 15 mg/kg of body weight)). Figure 5A shows the acute effect of *O. javanica* extracts on blood glucose levels in GK rats. There were no significant differences in the initial blood glucose level (0 min) among the groups. OJ significantly decreased blood glucose level at 30, 60, 90, and 120 min compared to the control ($P < 0.05$) (Figure 5A). The total AUC was also significantly lower in the OJ group than in the control group ($P < 0.05$) (Figure 5B). These results suggest that *O. javanica* extract and/or falcarindiol has a glucose intolerance ameliorating effect in GK rats.

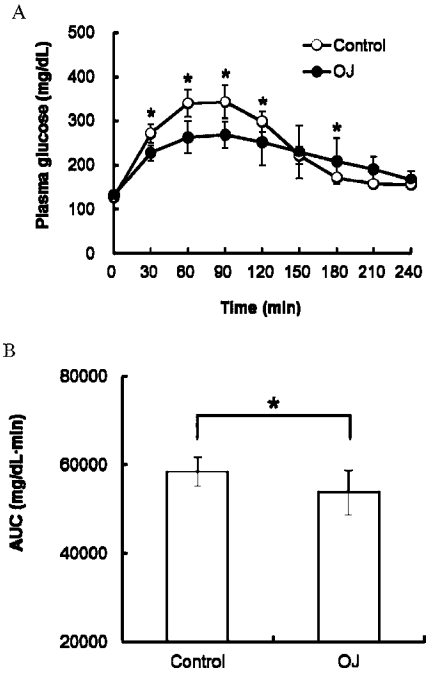

Figure 5. The effects of ethyl acetate extract of *O. javanica* on oral glucose tolerance tests in GK rats. A: The control groups were injected orally by single dose of glucose (2 g/kg of body weight) and 2 mL of olive oil. *O. javanica* exract groups (OJ) were injected orally by single dose of glucose (2 g/kg) and ethyl acetate extracts of *O. javanica* in olive oil (280 mg/kg (falcarindiol: 15 mg/kg)). Data are mean ± SD ($n = 6$). *$P < 0.05$ when compared with corresponding values of the control group. B: The area under the curve (AUC) of plasma glucose levels. Data are mean ± SD ($n = 6$, *$P < 0.05$).

DISCUSSION

This paper describes inhibition of GSK-3β by falcarindiol isolated from Japanese parsley, and it exhibited physiological activities against H4IIE cells, HT22 cells, and GK rats. Because GSK-3β is involved in so many pathways for cell function, selective and moderate inhibition may be the best way to reach the therapeutic goal and avoid or decrease potential side effects. Substrate competitive inhibitors are expected to be more specific and safer and to result in less toxic effects than ATP-competitive inhibitors. Actually, L803-mts, a substrate competitive inhibitor of GSK-3β ($IC_{50} = 40$ μM), improves glucose homeostasis in ob/ob mice with long-term treatment without toxic responses as judged by histopathology and blood chemistry analyses.[30] Although falcarindiol exhibits an IC_{50} in the high micromolar range, it is expected to be selective and show moderate inhibition to GSK-3β in vivo.

Falcarindiol showed ATP noncompetitive inhibition and substrate (PGSP-2) competitive inhibition by Dixon plot analysis. These results suggest that falcarindiol interacts not with the ATP-binding pocket but with the substrate binding site. ATP competitive inhibitors of GSK-3β usually interact with the P-loop of the ATP binding pocket on GSK-3β.[31] On the other hand, some substrate competitive inhibitors of GSK-3β interact with the primed phosphate binding pocket and/or Gln89-Asn95 loop of the substrate binding site on GSK-3β.[31] Because GSK-3β recognizes prephosphorylated substrates, the primed phosphate binding pocket and Gln89-Asn95 are key residues of the substrate binding site.[31] Falcarindiol may also interact with the primed phosphate binding pocket and Gln89-Asn95 loop on GSK-3β by hydrogen bond or covalent bond formation.

We isolated falcarindiol from *O. javanica* by activity (growth restoring activity against YNS17 strain)-guided fractionation. Although it has been reported that myristicin and falcarinol occur in *O. javanica*, we could not isolate them from *O. javanica* by activity-guided fractionation using YNS17 strain. Commercial myristicin did not show restoration of compromised growth of a Ca^{2+}-sensitive YNS17 strain even at 100 μg/disk, and the restoring activity of the methanol extracts of Apiaceae vegetables on YNS17 strain were identical with the quantity of falcarindiol.

In a typical phenotype of growth zone on the Ca^{2+}-signal transduction inhibitor screening plate, falcarindiol showed a clearer growth zone than GSK-3β inhibitor-I and 6-MSITC.[21,23] The differences of typical phenotype between falcarindiol, 6-MSITC, and GSK-3β inhibitor-I showed that this might be dependent on the different inhibition mechanisms to GSK-3β (ATP-noncompetitive inhibition, ATP-competitive inhibition, or allosteric inhibition). The relationship between the phenotype on the screening plates and GSK-3β inhibition mechanism needs further study.

Recent studies have reported that brain insulin resistance led to the overactivation of GSK-3β, which resulted in hyperphosphorylation of Tau.[14] Attendant increased oxidative stress leads to reactive oxygen species and ubiquitination, followed by misfolding of Tau.[14] Falcarindiol both suppressed gene expression of G6Pase in rat hepatoma cells and protected mouse neuroblastoma cells from glutamate-induced oxidative cell death dose dependently at 10 μM. These results indicate that falcarindiol might inhibit GSK-3β in different cell models and reduce the risk of Alzheimer's disease in brain insulin resistance.

Although the control value varies widely, a single injection of falcarindiol to GK rats using the same method and same dose (15 mg/kg) showed a glucose tolerance effect superimposable on that of the ethyl acetate extract of *O. javanica* (data not shown). This indicates that the main active compound in the extract of *O. javanica* is falcarindiol.

Falcarindiol is a new ATP-noncompetitive inhibitor of GSK-3β from food ingredient, and Apiacea vegetables containing falcarindiol were expected to prevent type-2 diabetes and Alzheimer's disease.

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■ ABBREVIATIONS

GSK-3β, glycogen synthase kinase-3β; G6Pase, glucose-6-phosphatase; PGSP-2, phospho-glycogen synthase peptide-2; YPD, yeast-peptone-dextrose; DMEM, Dulbecco's modified Eagle's medium; Bt_2cAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; AGPC, acid guanidinium phenol chloroform; GK, Goto-Kakizaki; OGTT, oral glucose tolerance test; AUC, area under the curve; ATCC, American Type Culture Collection; FBS, fetal bovine serum

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