Growth Hormone-Releaser Diet Attenuates Cognitive Dysfunction in Klotho Mutant Mice via Insulin-Like Growth Factor-1 Receptor Activation in a Genetic Aging Model

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Background: It has been recognized that a defect in *klotho* gene expression accelerates the degeneration of multiple age-sensitive traits. Accumulating evidence indicates that aging is associated with declines in cognitive function and the activity of growth hormone (GH)/insulin-like growth factor-1 (IGF-1).

Methods: In this study, we examined whether a GH-releaser diet could be effective in protecting against cognitive impairment in *klotho* mutant mice.

Results: The GH-releaser diet significantly induced the expression of IGF-1 and IGF-1 receptors in the hippocampus of *klotho* mutant mice. *Klotho* mutant mice showed significant memory impairments as compared with wild-type mice. In addition, the *klotho* mutation significantly decreased the expression of cell survival/antiapoptotic factors, including phospho-Akt (p-Akt)/phospho-glycogen synthase kinase3β (p-GSK3β), phospho-extracellular signal-related kinase (p-ERK), and Bcl-2, but significantly increased those of cell death/proapoptotic factors, such as phospho-c-jun N-terminal kinase (p-JNK), Bax, and cleaved caspase-3 in the hippocampus. Treatment with GH-releaser diet significantly attenuated both decreases in the expression of cell survival/antiapoptotic factors and increases in the expression of cell death/proapoptotic factors in the hippocampus of *klotho* mutant mice. In addition, *klotho* mutation-induced oxidative stress was significantly attenuated by the GH-releaser diet. Consequently, a GH-releaser diet significantly improved memory function in the *klotho* mutant mice. GH-releaser diet-mediated actions were significantly reversed by JB-1, an IGF-1 receptor antagonist.

Conclusion: The results suggest that a GH-releaser diet attenuates oxidative stress, proapoptotic changes and consequent dysfunction in *klotho* mutant mice by promoting IGF-1 expression and IGF-1 receptor activation.

Keywords: *klotho* gene; Cognitive dysfunction; GH-releaser diet; Receptor, IGF type 1

INTRODUCTION

The *klotho* gene was named after the Greek goddess who purportedly spun the thread of life; this gene has been shown to encode a type I membrane protein with an extracellular domain [1-3]. The *klotho* gene is mainly expressed in tissues that function in the regulation of calcium and phosphate homeostasis, including the distal convoluted tubules in the kidney, as well as parathyroid hormone-secreting cells and the epithelium of the choroid plexus of the brain [3,4]. The brain, pituitary gland, skeletal muscle, and many other peripheral organs also show detectable levels of *klotho* gene expression [3]. *Klotho* proteins have been predicted to be localized on the cell surface of *klotho*-expressing cells because the *klotho* gene encodes a type 1 membrane protein. However, a large amount of *klotho* protein is detectable in the cytoplasm; its intracellular distribution mostly overlaps with the endoplasmic reticulum and Golgi apparatus [4]. The extracellular domain of *klotho*, as a coreceptor for fibroblast growth factor, plays an important role in maintaining phosphate and calcium homeostasis [5]. The secreted form of *klotho*, which is produced by shedding of the extracellular domain of *klotho*, is secreted into the blood and cerebrospinal fluid and shows various physiological effects, including the modulation of growth factor signaling [3,6].

Klotho mutant mice, which are defective in *klotho* gene expression, develop multiple age-related syndromes even at the age of 4 to 5 weeks, including growth retardation, hypogonadotropic hypogonadism, rapid thymic involution, skin atrophy, sarcopenia, vascular calcification, osteopenia, pulmonary emphysema, cognition impairment, hearing disturbance, and motor neuron degeneration; they typically die prematurely around 2 months of age [6]. In contrast, the introduction of a normal *klotho* gene into these mutant mice improves their phenotypes [4], and overexpression of this gene in normal wild-type mice significantly extends their lifespans [7]. Thus, the *klotho* gene may function as an age suppressor gene that extends the lifespan when overexpressed and accelerates aging when disrupted [8].

Although *klotho* mutant mice are considered to be a novel animal model for accelerated human aging, these mice do not reveal some phenotypes usually seen in aged humans, such as brain atrophy with the deposition of amyloid or senile plaques [4,9,10]. In previous studies, however, we reported cognitive impairment in *klotho* mutant mice [9,11]. We showed that the antideath gene/protein, Bcl-2 or Bcl-xL, was downregulated while the prodeath molecule, Bax, was upregulated in the hippocampus of these mutant mice [9]. Our recent study has suggested that inactivation of the janus kinase 2/signal transducer and activator of transcription 3 signaling axis and M1 muscarinic cholinergic receptor downregulation play a critical role in cognitive impairment in *klotho* mutant mice [11]. Immunocytochemically, Li et al. [12] indicated that synaptic structures and synaptophysin were reduced in number and expression, respectively, in the CA3 region of these animals. These results have suggested that the *klotho* gene may be essential for maintaining cognitive function in aging organisms.

The activities of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) decline with aging [13], which could contribute to age-related cognitive performance decay [14]. Although GH might exert a positive effect in preventing the development of Alzheimer disease (AD), exogenous GH treatment in healthy elderly participants or AD patients is not an attractive tool due to its potentially harmful side effects, injection requirements and high cost [15]. Thus, methods for increasing endogenous GH secretion and subsequent IGF-1 synthesis by oral intake might be a better alternative. As the oral administration of amino acids (i.e., arginine, glutamine, glycine, and lysine) has been found to increase the release of endogenous GH [16], supplementation with these amino acids might be a beneficial pharmacological intervention. We previously demonstrated that a "GH-releaser diet" significantly attenuated β-amyloid (Aβ) (1-42)-induced memory impairment [17] via stimulation of the hippocampal IGF-1 receptor [18].

Although previous reports suggest that *klotho* may have an inhibitory effect on insulin/IGF-1 activity, the mechanism by which *klotho* modulates the activity of insulin/IGF-1 receptors in cognitive function remains unknown. Therefore, we examined whether the GH-releaser diet could effectively protect against cognitive impairment in *klotho* mutant mice. In this study, the GH-releaser diet significantly attenuated memory deficits, and these effects were significantly counteracted by treatment with GH or IGF-1 receptor antagonists.

METHODS

Animals

All animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Humane Care and Use of Laboratory Animals (NIH Publication No. 85 23, 1985; www.dels.nas.edu/ila). The present study was performed in accordance with the Institute for Laboratory Research guidelines for the care and use of laboratory animals. Mice were maintained under a 12-hour light/12-hour dark cycle and fed *ad*

libitum. Since *klotho* mutant mice are infertile, wild-type and *klotho* mutant mice were generated by crossing heterozygous *klotho* mutant mice [4,9,11]. Prior to weaning, tail specimens were collected from each animal, and DNA was extracted to genotype wild-type- and *klotho* mutant mice by polymerase chain reaction (PCR) using primer pairs specific for each genotype; 5′-TTGTGGAGATTGGAAGTGGACGAAAGAG-3′ (forward), 5′-CGCCCCGACCGGAGCTGAGAGTA-3′ (*klotho* mutant reverse), 5′-CTGGACCCCCTGAAGCTG-GAGTTAC-3′ (wild-type reverse). The products were amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using the following PCR parameters: an initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes, followed by electrophoresis on 1% agarose gels with ethidium bromide and photography under ultraviolet (UV) light. Six-week-old wild-type or *klotho* mutant mice were used in the present study.

Guide cannula implantation and intracerebroventricular infusion

Forty-day-old mice were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A stainless steel guide cannula (AG-4, Eicom, Kyoto, Japan) was implanted into the right lateral ventricle (stereotaxic coordinates: 0.5 mm posterior to the bregma, 1 mm right to the midline, and 2 mm ventral to the dura) [11,18]. Microinfusion into the lateral ventricle was performed through a microinfusion cannula (AMI-4, Eicom) at a rate of 1 µL/min using a microinjection pump (CMA/100, CMA, Solna, Sweden). The microinfusion cannula was kept in place for 1 minute after infusion to avoid backflow. Guide cannula implantation did not affect the behavior of the subjects. Subsequent to guide cannula implantation, each animal was housed in a single cage in order to safely maintain the integrity of the implantation during the experimental period.

Drug treatment

Drug treatment started after 24 hours of recovery from the guide cannula implantation. Mice received a GH-releaser diet [17,18], which is composed of 5% GH-releaser (a mixture of l-arginine/l glutamine/l-lysine/glycine at a ratio of 37:30:18.5:14.5) and 95% vehicle diet (45% sucrose, 20% casein, 15% cornstarch, 7% corn oil, 3% cellulose, 3% mineral mixture, 1.5% vitamin mixture, 0.3% dl-methionine, and 0.2% choline bitartrate), or a vehicle diet during the entire experi-
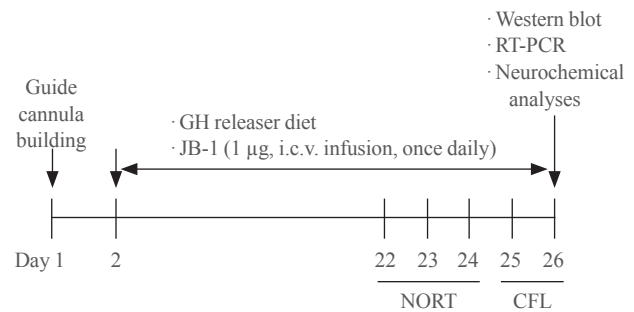

Fig. 1. Experimental schedule for evaluating the effect of a growth hormone (GH)-releaser diet on the cognitive dysfunction in *klotho* mutant mice. Mice received a GH-releaser diet or a vehicle diet during the entire experimental period. JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, was infused (1 µg/5 µL/5 min, once daily for 25 days) into the lateral ventricle. Twenty days after the GH releaser diet began, a novel object recognition test (NORT) and a conditioned fear learning test (CFL) were performed. Mice were sacrificed for Western blot, reverse transcription-polymerase chain reaction (RT-PCR), or neurochemical analyses at 15 minutes after the CFL.

mental period (25 days). JB-1 (IGF-1 receptor antagonist, Bachem Bioscience, Inc., King of Prussia, PA, USA) was dissolved in artificial cerebrospinal fluid (aCSF; 150 mM NaCl, 1.8 mM CaCl$_2$, 1.2 mM MgSO$_4$, 2 mM K$_2$HPO$_4$, 10 mM glucose, pH 7.4) and infused (1 µg/5 µL/5 min, once daily for 25 days) into the lateral ventricle. The dose of JB-1 used in this study was selected based on previous studies [18]. Twenty days after beginning the GH-releaser diet, novel object recognition test and conditioned fear learning test were performed. Mice were sacrificed for Western blot, reverse transcription-PCR (RT-PCR) or neurochemical analyses at 15 minutes after the test trial of the conditioned fear learning test (Fig. 1).

Novel object recognition test

The novel object recognition test was performed as described previously with a slight modification [9,11]. During the training session, two different objects were floor-fixed in a symmetric position from the center of the open field box (40×40×35 high cm), 20 cm from each and 10 cm from the nearest wall, and each mouse was allowed to explore the objects for 10 minutes. Twenty-four hours after the training session, mice were subjected to the retention session. One of the familiar objects used during training was replaced by a novel object, and then each mouse was allowed to explore the objects for 10 minutes. Time spent exploring each object was recorded and analyzed with a video tracking system (EthoVision, Noldus, the Netherlands). Novel object recognition was expressed as an "exploration of a

novel object (%)," the percentage of time spent exploring the novel object over the total time spent exploring both objects.

Conditioned fear learning test

Mice were placed in a neutral cage (30×30×35 high in cm), and the freezing response was measured for 1 minute in the absence of sound (preconditioning phase). In the conditioning phase, each mouse was placed in a training cage (25×30×11 high in cm) equipped with a metal floor, and the mice were allowed to explore the cage freely for 2 minutes, and then a 15-second tone (85 dB) was delivered (conditioned stimulus). During the last 5 seconds of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus though a shock generator. This procedure was repeated four times at 15-second intervals. Twenty-four hours after the conditioning, context-dependent tests were carried out. Mice were placed in the training cage, and the freezing response was measured for 2 minutes in the absence of the conditioned stimulus [9,11].

Western blot analysis

Tissues were homogenized in a lysis buffer, containing 200 mM Tris HCl (pH 6.8), 1% SDS, 5 mM ethyleneglycol tetraacetic acid, 5 mM ethylenediaminetetraacetic acid, 10% glycerol, 1×phosphatase inhibitor cocktail I, 1×protease inhibitor cocktail. The supernatant fraction was subsequently centrifuged at 30,000 g for 30 minutes. Proteins (20 to 50 μg/lane) were separated by 10% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Following transfer, membranes were preincubated with 5% nonfat milk and incubated overnight at 4°C with anti-Akt (1:5,000, Cell Signaling Technology, Inc., Danvers, MA, USA), antiphospho-Akt (anti-p-Akt) at Thr308 (1:1,000, Cell Signaling Technology, Inc.), antiglycogen synthase kinase3β (anti-GSK3β; 1:3,000, BD Biosciences, San Jose, CA, USA), antiphospho-GSK3β at Ser9 (anti-p-GSK3β; 1:750, Cell Signaling Technology, Inc.), anticalcium/calmodulin-dependent kinase II (anti-CaMKII; 1:1,000, Millipore, Bedford, MA, USA), anti-phospho-CaMKII (anti-p-CaMKII; 1:1,000, Millipore), antiextracellular signal-related kinase (anti-ERK; 1:5,000, Cell Signaling Technology, Inc.), anti-phospho-ERK (anti-p-ERK; 1:1,000, Cell Signaling Technology, Inc.), anti-c-jun N-terminal kinase (anti-JNK; 1:2,000, Cell Signaling Technology, Inc.), anti- phosphor-JNK (anti-p-JNK; 1:1,000, Cell Signaling Technology, Inc.), anti-p38 (1:1,000, Cell signaling Technology, Inc.), antiphospho-p38 (anti-p-p38; 1:500, Cell Signaling Technology,

Inc.), anti-cAMP responsive element binding protein (anti-CREB; 1:1,000, Cell Signaling Technology, Inc.), anti-phospho-CREB (anti-p-CREB; 1:500, Cell Signaling Technology, Inc.), Bcl-2 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bcl-xL (1:1,000, Cell Signaling Technology, Inc.), Bax (1:500, Santa Cruz Biotechnology, Inc.), or cleaved caspase-3 (1:1,000, Cell Signaling Technology, Inc.). After incubation with primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antirabbit immunoglobulin G (IgG; 1:1,000, GE Healthcare, Piscataway, NJ, USA), antimouse IgG (1:1,000, Sigma, St. Louis, MO, USA) or antigoat IgG (1:1,000, Sigma) for 2 hours. Subsequent visualization was performed using the ECL plus enhanced chemiluminescence system (GE Healthcare). Relative band intensities were quantified using PhotoCapt MW version 10.01 (Vilber Lourmat, Marne la Vallée, France), and each intensity was then normalized to the β-actin intensity.

RT-PCR

Expression of IGF-1 and IGF-1 receptors was assessed using semi-quantitative RT-PCR to analyze mRNA levels. Total RNA was isolated from hippocampal tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription reactions were carried out using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) and random oligonucleotide primers for 1 hour at 37°C. PCR amplification was performed using 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes and extension at 72°C for 1 minute. The following primer sequences were used: IGF-1 specific primers [18], forward: 5′-GGA CCA GAG ACC CTT TGC GGG G -3′ and reverse: 5′-GGC TGC TTT TGT AGG CTT CAG TGG-3′; IGF 1 receptor specific primers [19], forward: 5′-GAC ATC CGC AAC GAC TAT CAG -3′ and reverse: 5′-GTA GTT ATT GGA CAC CGC ATC-3′; glyceraldehyde-3-phosphate dehydrogenase specific primers [13], forward: 5′ ACC-ACA-GTC-CAT-GCC-ATC-AC-3′ and reverse: 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under UV light. The quantitative analysis of mRNA was performed using PhotoCapt MW version 10.01.

Determination of malondialdehyde

Hippocampal tissues were homogenized in phosphate buffered saline (PBS) to measure malondialdehyde (MDA), protein carbonyl and reactive oxygen species. The amount of lipid peroxi-

dation was determined by measuring the accumulation of thiobarbituric acid-reactive substances in hippocampal homogenates and is expressed as MDA content. In brief, 0.1 mL of the homogenate (or standard solutions prepared daily from 1,1,3,3-tetra-methoxypropane) and 0.75 mL of the working solution (thiobarbituric acid 0.37% and perchloric acid 6.4%, 2:1 v/v) were mixed and heated to 95°C for 1 hour. After cooling (10 minutes in an ice water bath), the flocculent precipitate was removed by centrifugation at 3,200 ×g for 10 minutes. The supernatant was neutralized and filtered prior to injection on an octadecylsilyl 5 μm column. The mobile phase consisted of 50 mM PBS (pH 6.0):methanol (58:42, v/v). Isocratic separation was performed with 1.0 mL/min flow rate and detection at 532 nm using a UV/VIS HPLC-Detector (Model 486, Waters Associates, Milford, MA, USA) [20].

Determination of protein carbonyl

The extent of protein oxidation was assessed by measuring the content of protein carbonyl groups, which was determined spectrophotometrically with the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure [20] as described by Oliver et al. [19]. The results are expressed as nmoL of DNPH incorporated/mg protein based on the extinction coefficient for aliphatic hydrazones of 21 mM^{-1} cm^{-1}. Protein was measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Statistical analyses

Data were analyzed using the SPSS version 19.0 (IBM Co., Armonk, NY, USA). Three-way analysis of variance was performed for the effect of the *klotho* gene mutation, the GH-releaser diet and JB-1. *Post hoc* multiple pair-wise comparisons with Bonferroni's correction were then followed. *P* values of <0.05 were considered statistically significant.

RESULTS

Effect of GH-releaser diet on the mRNA expression of IGF-1 and IGF-1 receptor in the hippocampus of *klotho* mutant mice

As shown in Fig. 2, *klotho* mutant mice treated with aCSF and vehicle diet showed significantly reduced hippocampal IGF-1 and IGF-1 receptor mRNA expression, compared to wild-type mice (*P*<0.01). These decrements in the levels of IGF-1 and IGF-1 receptor mRNA were significantly blocked by the GH-releaser diet (IGF-1, *P*<0.01; IGF-1 receptor, *P*<0.05). In contrast, the GH-releaser diet did not affect hippocampal IGF-
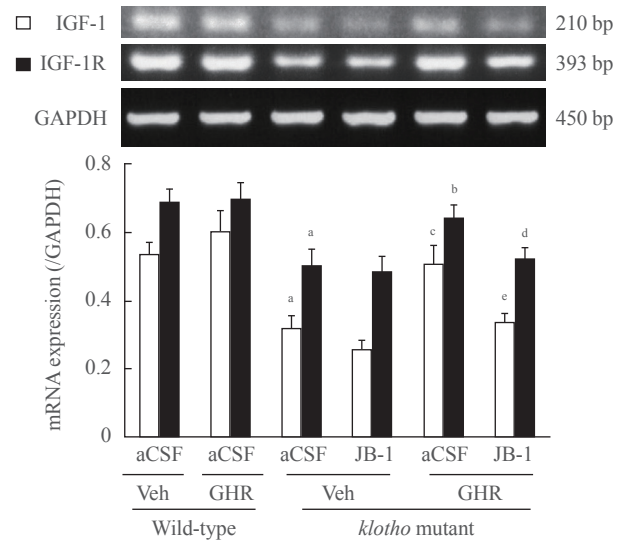

Fig. 2. Effect of a growth hormone (GH)-releaser diet on the mRNA expression of insulin-like growth factor-1 (IGF-1) and IGF-1 receptor in the hippocampus of *klotho* mutant mice. Each value is the mean±SEM of six animals. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]*P*<0.01 vs. wild-type mice treated with aCSF and vehicle diet; [b]*P*<0.05; [c]*P*<0.01 vs. *klotho* mutant mice treated with aCSF and vehicle diet; [d]*P*<0.05; [e]*P*<0.01 vs. *klotho* mutant mice treated with aCSF and GH releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

1 and IGF-1 receptor mRNA expression in wild-type mice. JB-1, an IGF-1 receptor antagonist, significantly blocked GH-releaser diet-mediated increases in IGF-1 and IGF-1 receptor mRNA expression in *klotho* mutant mice (IGF-1, *P*<0.01; IGF-1 receptor, *P*<0.05).

***Klotho* mutation significantly decreased the expressions of cell survival/antiapoptotic factors but increased those of cell death/proapoptotic factors in the hippocampus**

Total Akt was not changed in *klotho* mutant mice treated with aCSF and a vehicle diet, while the p-Akt/Akt ratio was significantly decreased compared to wild-type mice (*P*<0.01) (Fig. 3A). The GH-releaser diet significantly attenuated (*P*<0.01) a decrease in hippocampal p-Akt levels in the *klotho* mutant mice. A decrease in the ratio of p-GSK3β/GSK3β in *klotho* mutant mice (*P*<0.01) (Fig. 3B) was also significantly increased after the GH-releaser diet (*P*<0.01). JB-1 significantly blocked these actions on the hippocampal ratio of p-Akt/Akt (*P*<0.01) and p-GSK3β/GSK3β (*P*<0.05) in *klotho* mutant mice (Fig. 3A, B). A GH-releaser diet did not affect the expression of CaMKII and p-CaMKII in wild-type or *klotho* mutant
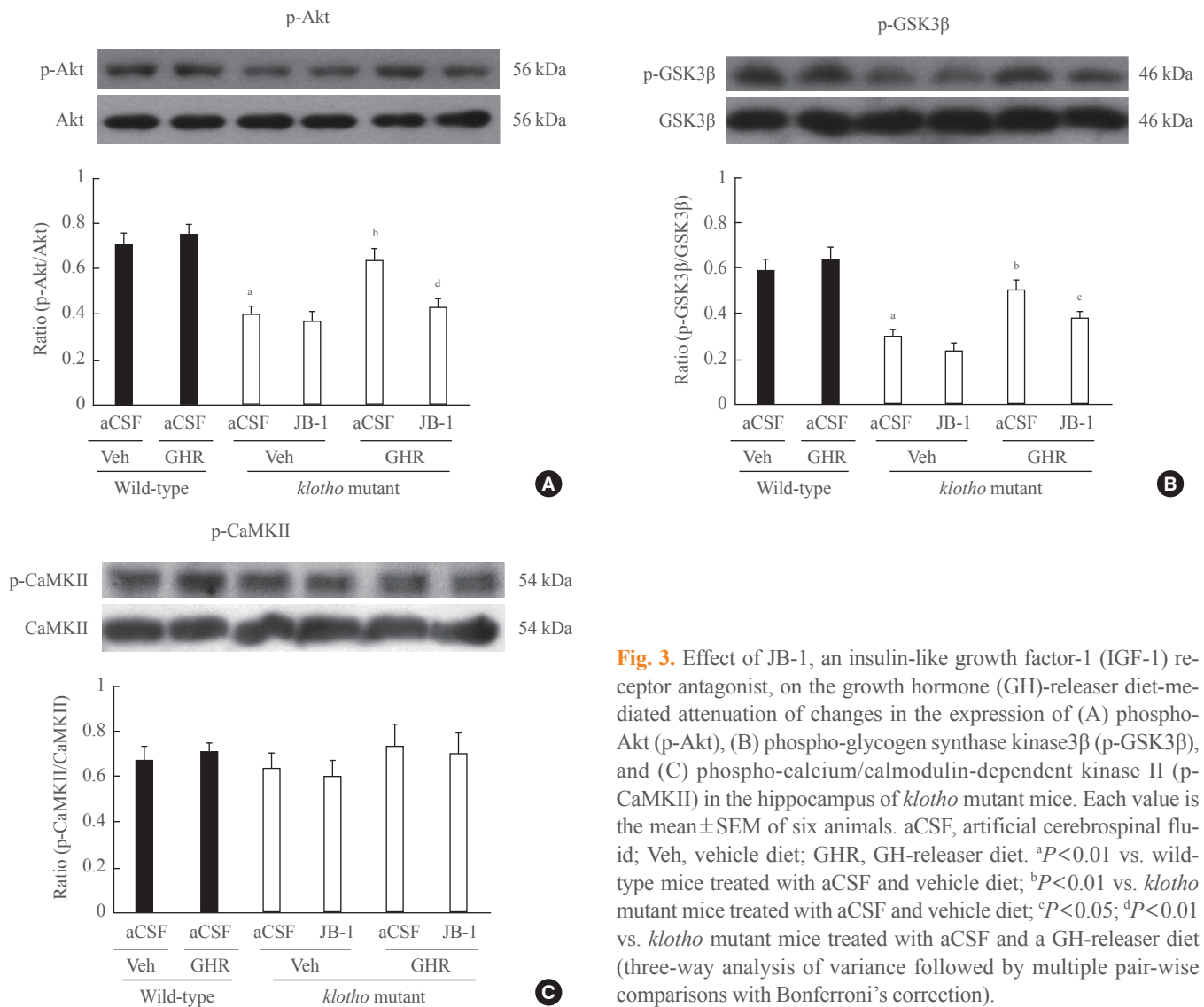
p-Akt

p-Akt   56 kDa
Akt   56 kDa

p-GSK3β

p-GSK3β   46 kDa
GSK3β   46 kDa

A

B

p-CaMKII

p-CaMKII   54 kDa
CaMKII   54 kDa

C

Fig. 3. Effect of JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, on the growth hormone (GH)-releaser diet-mediated attenuation of changes in the expression of (A) phospho-Akt (p-Akt), (B) phospho-glycogen synthase kinase3β (p-GSK3β), and (C) phospho-calcium/calmodulin-dependent kinase II (p-CaMKII) in the hippocampus of *klotho* mutant mice. Each value is the mean±SEM of six animals. aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]$P<0.01$ vs. wild-type mice treated with aCSF and vehicle diet; [b]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and vehicle diet; [c]$P<0.05$; [d]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and a GH-releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

mice (Fig. 3C).

The ratio of p-ERK/ERK and p-CREB/CREB was significantly decreased in *klotho* mutant mice ($P<0.01$) (Fig. 4A, D). The GH releaser diet significantly increased the hippocampal ratio of p-ERK/ERK ($P<0.01$) and p-CREB/CREB ($P<0.05$) in *klotho* mutant mice, which was significantly reversed by JB-1 (p-ERK/ERK, $P<0.01$; p-CREB/CREB, $P<0.05$). On the other hand, the increase in the ratio of p-JNK/JNK in *klotho* mutant mice ($P<0.01$) was significantly decreased after the GH-releaser diet ($P<0.01$) (Fig. 4B). JB-1 significantly blocked these actions in *klotho* mutant mice ($P<0.05$). However, the GH-releaser diet did not affect the expression and phosphorylation of p38 (Fig. 4C).

The levels of Bax and cleaved caspase-3 were significantly

increased in the hippocampus of *klotho* mutant mice, whereas Bcl-2 and Bcl-xL were substantially decreased ($P<0.01$) (Fig. 5). Increases in the expression of Bax and cleaved caspase-3 were significantly reduced by the GH-releaser diet ($P<0.01$), which was reversed by JB-1 (Bax, $P<0.01$; cleaved caspase-3, $P<0.05$) (Fig. 5A, B). Decreases in the expression of Bcl-2 and Bcl-xL were significantly recovered in the presence of the GH-releaser diet in *klotho* mutant mice ($P<0.01$) (Fig. 5C, D). JB-1 significantly blocked these actions of the GH-releaser diet in *klotho* mutant mice (Bcl-2, $P<0.01$; Bcl-xL, $P<0.05$).

Effect of JB-1, an IGF-1 receptor antagonist, on the GH-releaser diet-mediated attenuation of lipid peroxidation

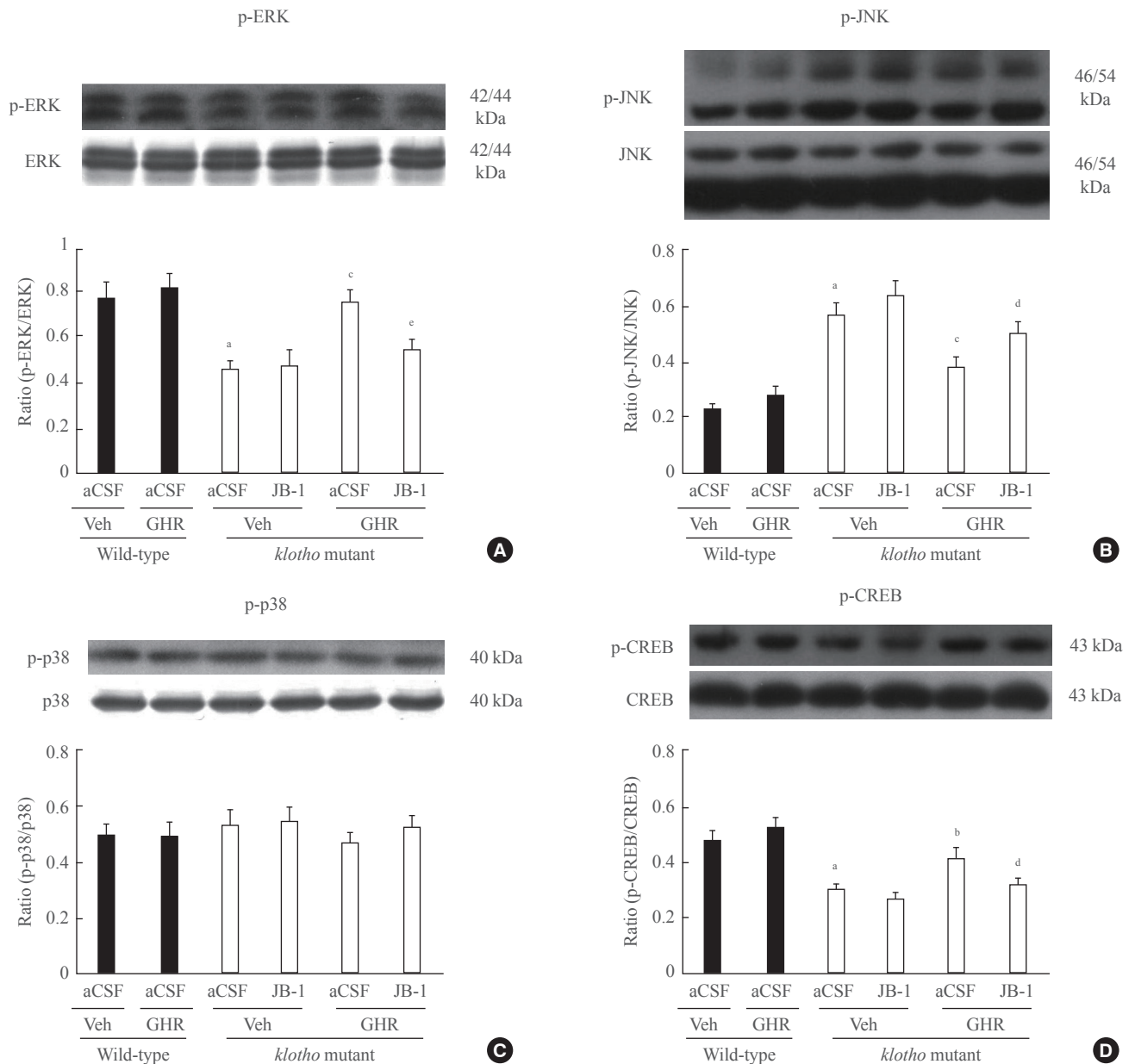To determine the effect of the GH-releaser diet on oxidative

Fig. 4. Effect of JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, on the growth hormone (GH)-releaser diet-mediated attenuation of changes in the expression of (A) phospho-extracellular signal-related kinase (p-ERK), (B) phospho-c-jun N-terminal kinase (p-JNK), (C) phospho-p38 (p-p38), and (D) phospho-cAMP responsive element binding protein (p-CREB) in the hippocampus of *klotho* mutant mice. Each value is the mean±SEM of six animals. aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]$P<0.01$ vs. wild-type mice treated with aCSF and vehicle diet; [b]$P<0.05$; [c]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and vehicle diet; [d]$P<0.05$; [e]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and a GH-releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

stress, we examined lipid peroxidation (MDA) and protein oxidation (protein carbonyl) in the hippocampus of *klotho* mutant mice. The levels of MDA and protein carbonyl were significantly increased in *klotho* mutant mice (MDA, $P<0.05$; protein carbonyl, $P<0.01$). The GH-releaser diet significantly reduced the levels of MDA and protein carbonyl (MDA, $P<0.05$; protein carbonyl, $P<0.01$), and these effects were significantly reversed by JB-1 (protein carbonyl, $P<0.05$) (Fig. 6).
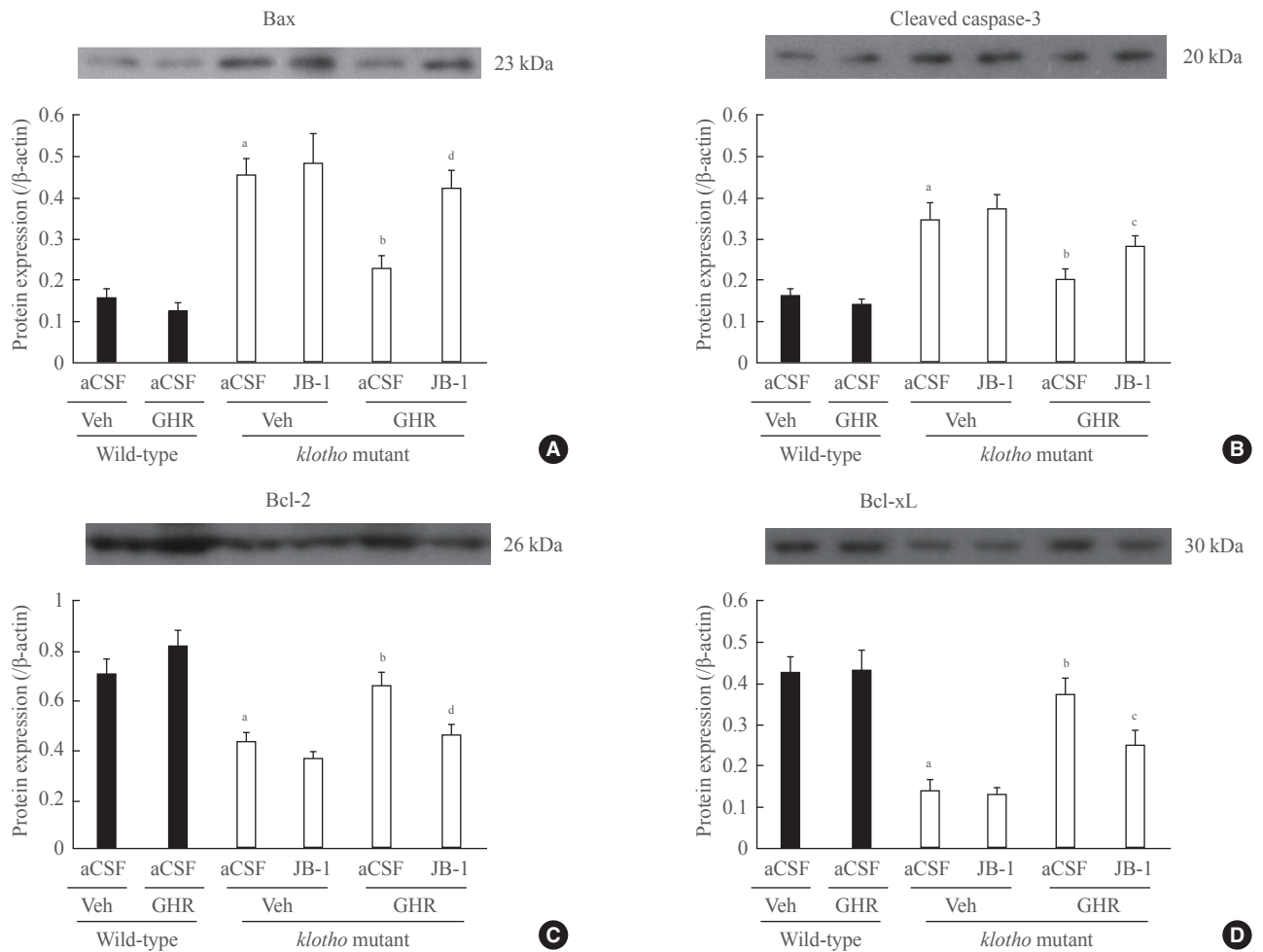
Fig. 5. Effect of JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, on the growth hormone (GH)-releaser diet-mediated attenuation of changes in the expression of (A) Bax, (B) cleaved caspase-3, (C) Bcl-2, and (D) Bcl-xL in the hippocampus of *klotho* mutant mice. Each value is the mean±SEM of six animals. aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]$P < 0.01$ vs. wild-type mice treated with aCSF and vehicle diet; [b]$P < 0.01$ vs. *klotho* mutant mice treated with aCSF and vehicle diet; [c]$P < 0.05$; [d]$P < 0.01$ vs. *klotho* mutant mice treated with aCSF and a GH-releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

Effect of JB-1 on the GH-releaser diet-mediated attenuation of cognitive dysfunction

In the training trial of the novel object recognition test, wild-type and *klotho* mutant mice did not show any exploratory preference between objects (data not shown). In the test trial, the exploratory preference for the novel object, as expressed in the recognition index, was significantly decreased in *klotho* mutant mice compared with wild-type mice ($P < 0.01$). The GH-releaser diet significantly increased the recognition index in *klotho* mutant mice ($P < 0.01$), and this cognitive enhancing effect of the GH-releaser was significantly inhibited by JB-1 ($P < 0.05$) (Fig. 7A).

During the preconditioning trial of the context-dependent

conditioned fear learning test, mice showed little freezing behavior, and no statistical difference was observed among groups. In the retention trials (24 hours after the conditioning trial), *klotho* mutant mice showed significantly less freezing response compared with wild-type mice ($P < 0.01$). The GH-releaser diet significantly enhanced the freezing response in *klotho* mutant mice ($P < 0.01$). This enhancement was significantly blocked by JB-1 ($P < 0.05$) (Fig. 7B). However, the GH-releaser diet affected none of these memory tests in the wild-type mice (Fig. 7).

DISCUSSION

The present study demonstrated that the GH-releaser diet sig-
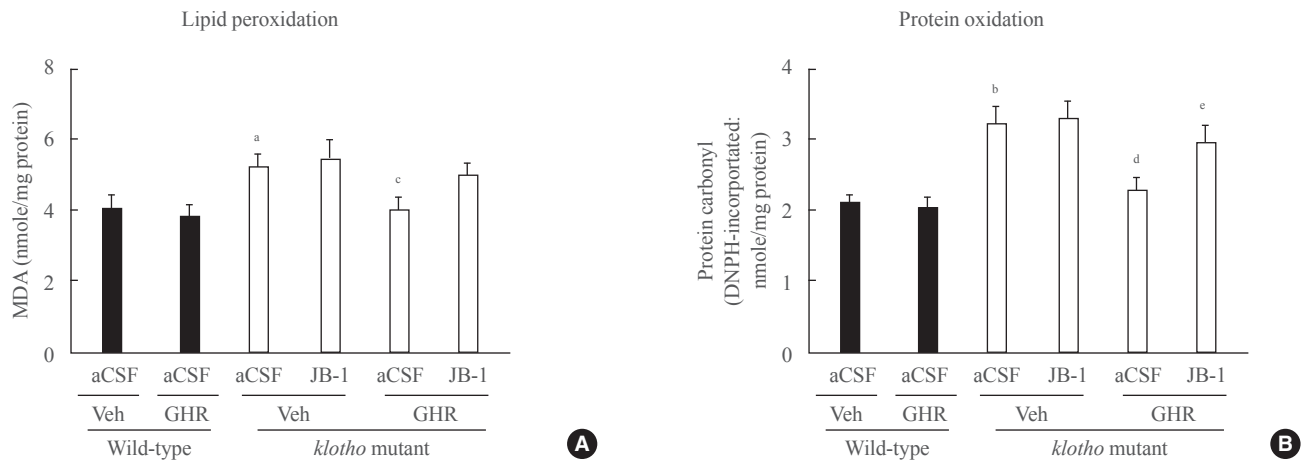
Lipid peroxidation

Protein oxidation

Fig. 6. Effect of JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, on the growth hormone (GH)-releaser diet-mediated attenuation of lipid peroxidation, as measured by (A) the malondialdehyde (MDA) level, and protein oxidation, as measured by (B) the protein carbonyl level in the hippocampus of *klotho* mutant mice. Each value is the mean±SEM of eight animals. DNPH, 2,4-dinitrophenylhydrazine; aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]$P<0.05$; [b]$P<0.01$ vs. wild-type mice treated with aCSF and vehicle diet; [c]$P<0.05$; [d]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and vehicle diet; [e]$P<0.05$ vs. *klotho* mutant mice treated with aCSF and a GH-releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

Novel object recognition test

Context-dependent conditioned fear learning test
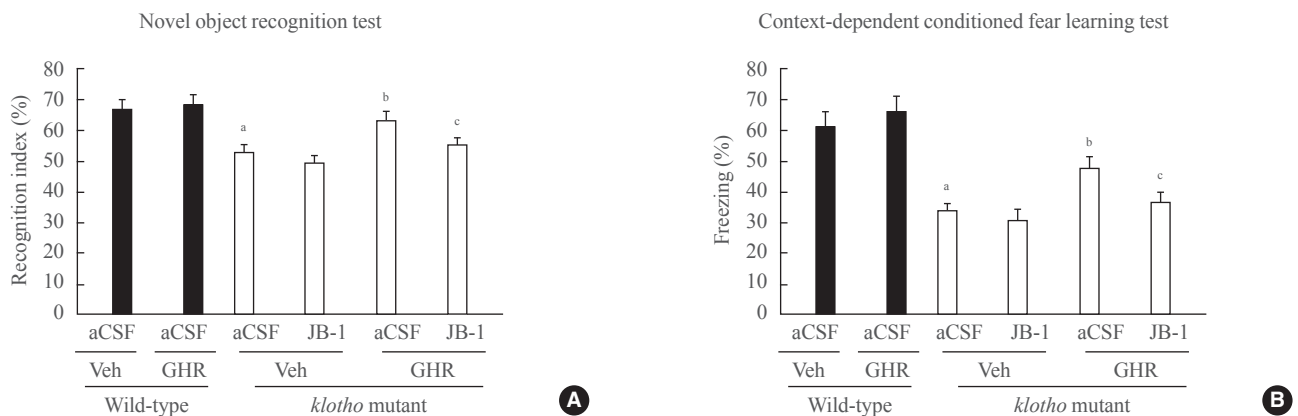
Fig. 7. Effect of JB-1, an insulin-like growth factor-1 (IGF-1) receptor antagonist, on the growth hormone (GH)-releaser diet-mediated attenuation of cognitive dysfunction, as evaluated by (A) a novel object recognition test and (B) a context-dependent conditioned fear learning test in *klotho* mutant mice. Each value is the mean±SEM of 10 animals. aCSF, artificial cerebrospinal fluid; Veh, vehicle diet; GHR, GH-releaser diet. [a]$P<0.01$ vs. wild-type mice treated with aCSF and vehicle diet; [b]$P<0.01$ vs. *klotho* mutant mice treated with aCSF and vehicle diet; [c]$P<0.05$ vs. *klotho* mutant mice treated with aCSF and a GH-releaser diet (three-way analysis of variance followed by multiple pair-wise comparisons with Bonferroni's correction).

nificantly attenuated memory deficits in *klotho* mutant mice. The GH-releaser diet was shown to affect hippocampal changes in IGF-1/IGF-1-receptor mRNA expression, phosphorylation of Akt/GSK3β and ERK/CREB and the levels of apoptosis-related factors. In addition, the GH-releaser diet significantly decreased oxidative stress, such as lipid peroxidation and protein oxidation. These effects of the GH-releaser diet were significantly counteracted by treatment with JB-1, an IGF-1 receptor antagonist, suggesting that GH-releaser diet-

mediated cognitive enhancement is stimulated via the IGF-1 receptor in *klotho* mutant mice.

The ability of *klotho* to inhibit insulin/IGF-1 receptors may contribute to its antiaging properties since partial inhibition of an insulin-like signaling pathway was reported as one of the evolutionarily conserved mechanisms for suppressing aging. *Klotho*-deficient mice are hypoglycemic, hypoinsulinemic and extremely sensitive to insulin [21], whereas *klotho*-overexpressing transgenic mice are resistant to insulin and IGF-1 [7].

Secreted *klotho* had enough activity to inhibit insulin/IGF-1-induced autophosphorylation of the insulin/IGF-1 receptor [7,22] and conferred oxidative stress resistance via activation of FoxO transcription factors that are negatively regulated by insulin/IGF-1 signaling [23]. *Klotho*'s effects on insulin signaling or oxidative stress resistance may occur either via *klotho* expression in tissues involved in carbohydrate metabolism or as a hormone [24]. Therefore, it has become increasingly clear that partial or tissue-specific inhibition of the insulin-like signaling pathway is a mechanism for antiaging and life span extension. However, the mechanism by which secreted *klotho* inhibits activity of insulin/IGF-1 receptors in the brain remains to be determined.

Recently, Lorenzi and colleagues [25] demonstrated that the soluble *klotho* protein does not inhibit IGF-1 and/or insulin signaling, arguing against a direct role of *klotho* in insulin signaling. Conditioned media from cells expressing the *klotho* protein failed to inhibit IGF-1 signaling or insulin signaling in HEK cells, rat L6 or human HepG2 cells, although the *klotho* protein in conditioned media is bioactive [25]. Their results stand in contrast with the data published by Kurosu et al. [7], who showed potent inhibition of insulin and IGF-1 signaling by purified murine recombinant *klotho* protein in L6 cells. Those discrepancies may be due to the inactivation of *klotho* bioactivity during the purification procedure or to species restrictions. These findings that native, bioactive *klotho* protein fail to inhibit insulin signaling raises the possibility that the GH-releaser diet might attenuate cognitive impairment in *klotho* mutant mice via activation of IGF-1 signaling.

In the present study, the GH-releaser diet significantly attenuated memory deficits and reduced oxidative stress in *klotho* mutant mice. These results correlated with previously reported protective effects of a GH-releaser diet on Aβ (1-42)-induced learning impairment [17,18]. Given that the GH-releaser diet showed no significant changes in wild-type mice, we suggest that it can act more effectively to protect from various neuronal damages. To address the possibility that the neuroprotective effects reported in the present study were mediated by an IGF-1 pathway, we examined mRNA levels of IGF-1 and IGF-1 receptors in the hippocampus from *klotho* mutant mice. We detected significant increases in IGF-1 and IGF-1 receptors by the GH-releaser diet and further confirmed these effects of the GH-releaser diet using an IGF-1 receptor antagonist. The GH-releaser diet was also shown to decrease the levels of lipid peroxidation and protein oxidation, and treatment with IGF-1 receptor antagonist aggravated memory deficits in *klotho* mutant

mice. These results confirmed that the IGF-1 pathway might be involved in attenuating memory impairment in *klotho* mutant mice by reducing oxidative stress.

Furthermore, the present study indicated that the GH-releaser diet activated both the PI3K/Akt and the ERK pathways in the hippocampus of *klotho* mutant mice, which is in agreement with the study by Frago et al. [26]. Synthetic GH secretagogues (GHS) and the endogenous ligand ghrelin have been found to have cardioprotective properties in several *in vivo* studies [27,28], although the molecular mechanisms remain largely unknown. Two recent *in vitro* studies on cardiomyocytes and endothelial cells suggest that the antiapoptotic effects of GHS are mediated by activation of both Akt and ERK kinases [29] and by regulating the activity of caspase-3 and expression of Bax and Bcl-2 [30]. These findings are largely in agreement with the mechanisms proposed in the present study.

ERK pathways are known to exert multiple functions in mammalian cell proliferation, cell death, cell survival, and neuronal plasticity [31,32], and ERK or CREB activation is necessary for memory function [32]. Our findings are consistent with a previous report that found treatment with a selective ERK inhibitor resulted in significant deficits in the novel object recognition test in mice [33]. CREB and ERK1/2 phosphorylation can be suppressed by sublethal Aβ treatment in cortical neuronal cells [34], and ERK 1/2 and CREB phosphorylation increases after paired conditioning in the mouse hippocampus [35]. These results indicated that the GH-releaser diet significantly attenuated *klotho* knockout-induced cognitive impairment mainly via the ERK/CREB pathway.

Another finding in the present study was that GSK3β phosphorylation was increased following the GH releaser diet, which is the expected response after activation of the PI3K/Akt pathway [36]. Activation of the PI3K pathway and the downstream phosphorylation of Akt are known to mediate growth factor-induced neuronal survival *in vitro* [37]. pAkt promotes cell survival and can inhibit apoptosis by inactivating several proapoptotic targets, including Bad, GSK3β, and caspase 9. GSK3β has been shown to play a role in apoptosis and to be a key target of the PI3K/Akt survival signaling pathway [38]. Therefore, inhibition of GSK3β through phosphorylation could partly explain the neuroprotective effect of the GH-releaser diet. Activation of GSK3β has been linked to the triggering of caspase-dependent apoptosis [39], and the GH releaser-induced reduction of GSK3β activity offers additional support for the involvement of caspases in *klotho* mutant mice.

Interestingly, the GH-releaser diet did not affect hippocampal IGF-1 and IGF-1 receptor expression; consequently, all parameters were examined in the wild-type mice. Our previous data [17,18] showed that serum IGF-1 and hippocampal IGF-1/IGF-1 receptor expressions were maintained at a physiological level in the Aβ (42-1)-treated (negative control) mice in spite of increases in the serum and pituitary GH levels evoked by the GH-releaser diet, suggesting that the levels of GH and IGF-1 were under the normal homeostatic regulation. However, it has been reported that serum GH levels of *klotho* mutant mice are similar to those of wild-type mice [21] even though the serum IGF-1 levels of *klotho* mutant mice are significantly lower than those of wild-type mice, indicating that homeostatic feedback regulation of GH and IGF-1 is impaired in the *klotho* mutant mice. Similar to our results, Kashimada et al. [40] demonstrated that repeated GH treatment for 5 weeks normalizes the IGF-1 mRNA levels in the liver of *klotho* mutant mice. Thus, restoring the normal homeostatic regulation of GH and IGF-1 might be one possible mechanism for the cognitive enhancing effect of the GH-releaser diet.

In conclusion, we demonstrated, for the first time, that the GH-releaser diet markedly attenuated cognitive impairment in *klotho* mutant mice via IGF-1-receptor stimulation. We provided evidence that the protective effect of a GH-releaser diet is at least partly mediated through the PI3-kinase pathway, including activation of Akt and inhibition of GSK3β through phosphorylation. We also suggest that the GH-releaser effect is partly mediated by the ERK pathways, including downstream phosphorylation CREB. These GH-releaser diet-mediated actions were significantly reversed by an IGF-1 receptor antagonist, suggesting a role of the IGF-I receptor. However, further studies will be required to clarify the molecular mechanism by which the GH-releaser diet–mediated IGF-1-receptor stimulation prevents the cognitive impairment.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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