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Glucagon-like peptide-1 protects the murine hippocampus against stressors *via* Akt and ERK1/2 signaling

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ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disease characterized by cognitive dysfunction and neuronal cell death in the hippocampus and cerebral cortex. Glucagon-like peptide-1 (GLP-1) is an insulinotropic peptides. GLP-1-associated medicines are widely used as treatments for type 2 diabetes. In addition, they have been shown to ameliorate pathology in AD mouse models. Here, we investigated the effects of GLP-1 on different stressors in murine hippocampal HT22 cells. GLP-1 (7–36) prevented H_2O_2-, L-glutamate-, tunicamycin-, thapsigargin-, and amyloid β_{1-42}-induced neuronal cell death in a concentration-dependent manner. GLP-1 (7–36) treatment for 1 h significantly increased phosphorylated Akt and extracellular signal-regulated kinase 1 and 2 (ERK1/2) when compared with vehicle-treatment. These results suggest that GLP-1 (7–36) is protective against these stressors *via* activation of survival signaling molecules, such as Akt and ERK1/2 in HT22 cells. In conclusion, GLP-1 and activators of the GLP-1 receptor might be useful targets for the treatment of AD.

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease that is characterized by cognitive dysfunction and neuronal cell death, particularly in the hippocampus and cerebral cortex [1,2].

Amyloid β (Aβ) and tau protein play key roles in the progression of AD. Aβ aggregates and forms oligomers that induces neuronal cell death in the hippocampus and cerebral cortex [3]. Tau is polymerized and forms intracellular neurofibrillary tangles, which induce cell death [4]. It had been reported that Aβ causes neuronal cell death *via* the inducting of mitochondrial dysfunction [5], unfolded protein response [6–9], synaptic dysfunction [10], and activation of glia cells [11]. These findings suggest that oxidative stress and endoplasmic reticulum (ER) stress play a critical role in AD pathology.

Glucagon-like peptide-1 (GLP-1) is an insulinotropic peptide secreted from L-cells in the small gut [12]. When food is consumed, GLP-1 acts on pancrestic β-cells, inducing a release of insulin that results in lower blood glucose (commonly referred to as the incretin effect) [13]. GLP-1 binds to the GLP-1 receptor (GLP-1R), which is in the subfamily of type 2 G-protein coupled receptors, inducing an elevation of cyclic adenosine monophosphate (cAMP) and causing the incretin effect [14]. GLP-1 is quickly degraded by dipeptidyl peptidase-IV (DPP-IV); therefore, its half-life is approximately 2 min in rodents [15]. GLP-1 analogues which are resistant to cleavage by DPP-IV or DPP-IV inhibitors, are widely used for clinical treatment of type 2 diabetes [16,17].

Recently, it had been reported that GLP-1R is expressed in neurons in the central nervous system [18]. Moreover, low levels of GLP-1 are produced in the brain, such as in the nucleus of the solitary tract, postrema, and caudal brain stem [19–21]. These reports suggest that GLP-1 and GLP-1R dominant signals may play critical roles in the brain. Interestingly, GLP-1R knockout mice exhibit cognitive impairments, suggesting that GLP-1R is needed for memory formation [22]. Furthermore, GLP-1R agonists improve cognitive dysfunction and decrease Aβ deposition in AD animal models [23,24]. GLP-1 and GLP-1R agonists are neuroprotective against oxidative and Aβ stress in SH-5YSY and PC12 cell lines

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; AMP, adenosine monophosphate; ATF-4, activating transcription factor-4; D-MEM, Dulbecco's modified Eagle's Medium; DMSO, dimethyl sulfoxide; DPP-IV, dipeptidyl peptidase IV; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1 and 2; FBS, fetal bovine serum; GLP-1, glucagon-like peptide; GLP-1R, GLP-1 receptor; HRP, horseradish peroxidase; MAPK, mitogen activator protein kinase; PBS, phosphate buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; TBS-T, Tris-buffered saline with 0.05% Tween 20.

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[23,25]; however, further investigations are needed to elucidate the role of GLP-1 in neuronal cell death in the hippocampus.

In this study, we investigated the protective effects of GLP-1 against hippocampal neuronal cell death induced by AD-associated stressors, such as oxidative stress, ER stress, and Aβ neurotoxicity.

2. Material and methods

2.1. Materials

GLP-1 (7–36) (Bachem AG, Bubendorf, Switzerland) was dissolved in phosphate buffered saline (PBS; pH 7.4). $A\beta_{1-42}$ peptide (Abcam, Cambridge, UK) was dissolved in dimethyl sulfoxide (DMSO), followed by dilution in PBS according to the manufacturer's instructions. Oligomers/fibrils of $A\beta_{1-42}$ were created by incubating a 2 mM stock solution at 37 °C for 2 h.

2.2. Cell cultures

Mouse hippocampal HT22 cells were a generous gift from Yoko Hirata Ph.D. (Gifu University, Gifu, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (D-MEM; Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg mL^{-1} streptomycin (Meiji Seika) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were passaged by trypsinization every 2 days, and maintained in a 10 cm dish (BD Biosciences, Franklin Lakes, NJ, USA).

2.3. H_2O_2- or L-glutamate-induced cell death assay

HT22 cells were seeded at 3×10^3 cells per well into 96-well plates (BD Biosciences), and incubated for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Following this, the medium was replaced with fresh medium containing 1% FBS and 200 μM H_2O_2 or 3 mM L-glutamate, with or without 0.01–1 μM GLP-1 or trolox (Wako, Osaka, Japan) and incubated for 24 h at 37 °C.

Nuclear staining assays were carried out after 24-h incubation. Cell death was assessed by combination staining with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI; Molecular Probes). Images were collected by using an inverted epifluorescence microscope (IX70; Olympus. Co., Tokyo, Japan). The number of cells per condition was counted by a single observer who was blind to the treatment groups (Y.Y., M.I., or S.T.) with the aid of image-processing software (Image-J, version 1.33f; National Institutes of Health, Bethesda, MD, USA).

2.4. Thapsigargin- or tunicamycin-induced cell death assay

HT22 cells were seeded at 3×10^3 cells per well into 96-well plates and incubated for 24 h at 37 °C in the same conditions as described in Section 2.3. Following this, the medium was replaced with fresh medium containing 1% FBS and 0.01–1 μM GLP-1. Cells were incubated for 1 h, then, 50 nM thapsigargin or 50 mg·mL^{-1} tunicamycin was added and incubated for 24 h at 37 °C. Nuclear staining assays were carried out after 20 or 24 h of thapsigargin or tunicamycin-incubation, respectively.

2.5. Aβ-induced cell death assay

HT22 cells were seeded at 1×10^3 cells per well into 96-well plates and incubated for 24 h at 37 °C in the same conditions as described in Section 2.3. Following this, the medium was replaced with fresh medium containing 1% FBS and 2 μM Aβ, with or without 0.01–1 μM GLP-1, and incubated for 48 h at 37 °C. Nuclear staining assays were carried out after 48 h-incubation.

2.6. Western blot analysis

HT22 cell were seeded to 24 well plates at 15,000 cells per wells and incubated for 24 h. Cells were treated with GLP-1 (7–36) (1 μM) or PBS for 1 h, washed with PBS, and harvested. Western blot analysis was carried out as previously described [26]. The primary antibodies used were as follows: rabbit anti-phospho-protein kiniase B (p-Akt; S473; 1:1000; Cell Signaling), rabbit anti-Akt (1:1000; Cell Signaling), rabbit anti-phospho-extracellular signal-regulated kinase 1 and 2 (p-ERK1/2; 1:1000; Cell Signaling), rabbit anti-ERK1/2; 1:1000; Cell Signaling), and mouse anti-β-Actin (1:2000; Sigma–Aldrich). The secondary antibodies used were follows: goat anti-rabbit horseradish peroxidase (HRP)-conjugated and goat anti-mouse HRP-conjugated (1:1000; Pierce biotechnology, Inc. MA, USA).

2.7. Statistical analyses

All data were expressed as the means ± standard error of the mean (S.E.M.). Statistical comparisons were made using a two-tailed Student's *t*-test or one-way ANOVA followed by Dunnett's test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. GLP-1 (7–36) protected HT22 cells against oxidative stress-induced cell death

Firstly, we investigated the effects of GLP-1 (7–36) on oxidative stress-induced hippocampal cell death using HT22 cells. We assessed cell death by nuclear staining with Hoechst 33342, to distinguish apoptotic and normal cells, and PI, to distinguish necrotic and apoptotic cells (Fig. 1A). H_2O_2 is toxic because it increases intracellular reactive oxygen species (ROS); therefore it is a recognized model for oxidative stress-induced toxicity in neuronal cells [27]. Incubation with H_2O_2 (200 μM) for 24 h increased the number of PI-positive cells. This effect was ameliorated when cells were pre-treated with GLP-1 (7–36) or trolox, which is a vitamin E mimic and well-known anti-oxidant (Fig. 1B). H_2O_2 (200 μM) significantly increased the percentage of dead cells and this effect was significantly ameliorated with 0.01–1 μM GLP-1 (7–36) pre-treatment in a concentration-dependent manner (Fig. 1C).

Next, we investigated the effect of GLP-1 (7–36) on glutamate-induced oxidative stress. HT22 cells do not possess ionotropic glutamate receptors; therefore, they are not susceptible to glutamate-induced excitotoxicity [28]. Consequently, high concentrations of glutamate induce cell death in HT22 cells *via* oxidative neurotoxicity [29]. L-glutamate increased cell death, and this effect was ameliorated with 0.01–1 μM GLP-1 (7–36) treatment (Fig. 1D). These results suggested that GLP-1 (7–36) was protective against oxidative stress-induced cell death.

3.2. GLP-1 (7–36) protected HT22 cells from ER stress-induced cell death

To investigate whether GLP-1 (7–36) is protective against ER stress-induced HT22 cells death, we used two ER stress inducers, tunicamycin (a glycosylation inhibitor) and thapsigargin (an ER

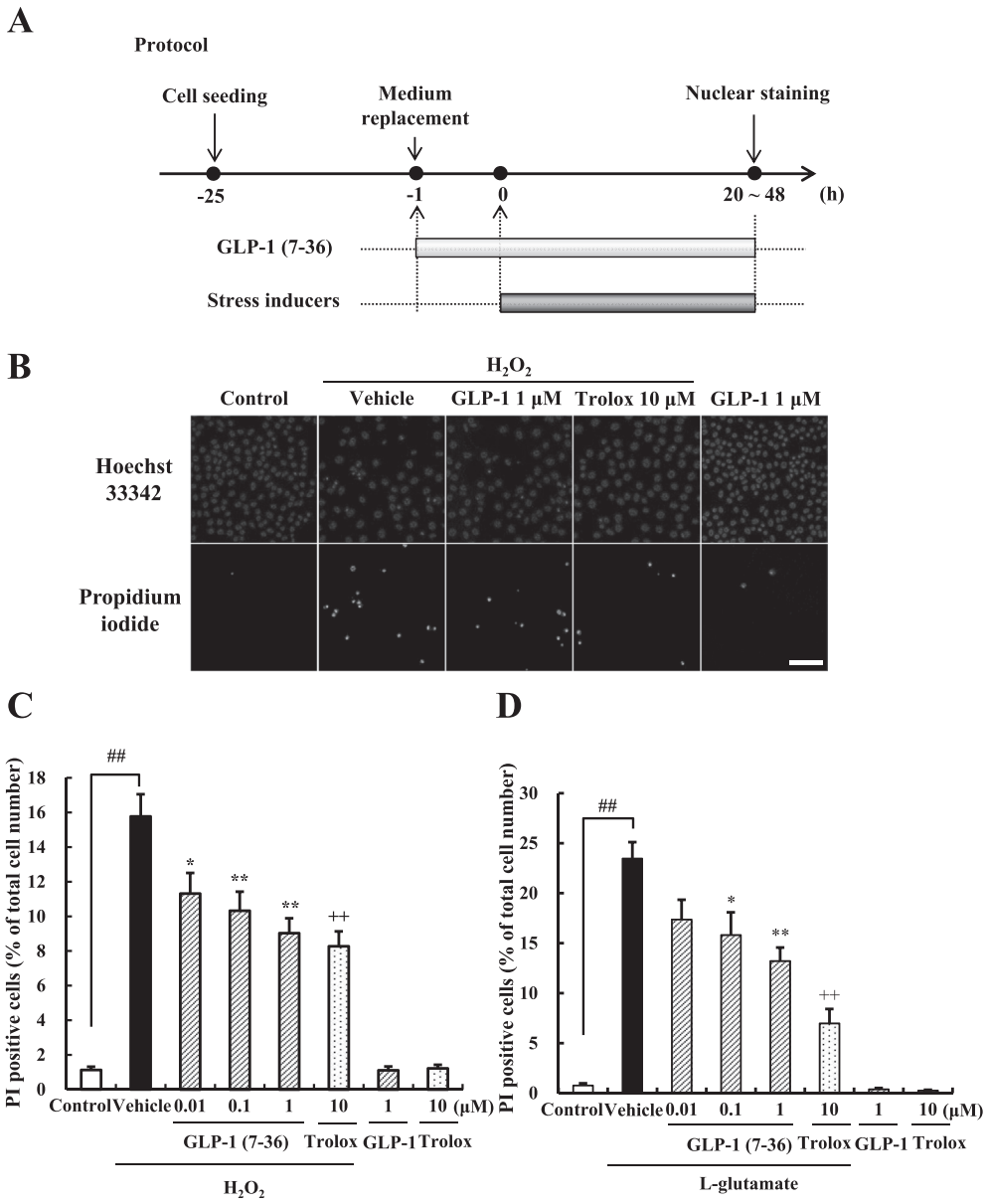

Fig. 1. GLP-1 (7–36) protects HT22 cells against oxidative stress-induced cell death. (A) A representative protocol of the cell death assay is shown. GLP-1 (7–36) was added 24 h after cell seeding. Stress inducers were added to the cells 1-h later. Cells were incubated in following conditions: H_2O_2 for 24 h, L-glutamate for 24 h, thapsigargin for 20 h, tunicamycin for 24 h, and Aβ for 48 h. After incubation, cell death was evaluated by nuclear staining. (B) Representative fluorescence micrographs of Hoechst 33342 (blue) and propidium iodide (PI: red) in HT22 cells. Cells were treated with vehicle, with or without GLP-1 (7–36), followed by 200 μM H_2O_2 or vehicle for 24 h. Scale bar represents 100 μm. (C) The number of Hoechst 33342 or propidium iodide (PI) positive cells was measured and the cell death was expressed as a percentage of PI-positive cells divided by Hoechst 33342-positive cells. (D) GLP-1 (7–36) protected cells against oxidative stress induced by 3 mM L-Glutamate for 24 h $^{\#\#}P < 0.01$ versus control (Student's *t*-test).*P < 0.05; **P < 0.01 versus vehicle (Dunnett's test). $^{++}P < 0.01$ versus vehicle (Student's *t*-test). Each column represents mean ± S.E.M. n = 9–12.

calcium inhibitor) [30,31]. GLP-1 (7–36) was protective against tunicamycin- and thapsigargin-induced cell death (Fig. 2A and B). These results suggested that GLP-1 was also protective against ER stress.

3.3. GLP-1 (7–36) protected HT22 cells against Aβ-induced cell death

Aβ, which plays a key role in AD pathology, induces neuronal cell apoptosis in part *via* oxidative stress, ER stress, and the other sressors [5–9,32]. Treatment with 0.1–1 μM GLP-1 (7–36) significantly prevented $A\beta_{1-42}$-induced cell death (Fig. 3). These results suggested that treatment with GLP-1 (7–36) protected HT22 cells against AD-associated stress-induced cell death.

3.4. GLP-1 (7–36) increased Akt and ERK1/2 phosphorylation in HT22 cells

It has been reported that GLP-1 binding to GLP-1R activates intracellular signaling proteins such as Akt [33,34] and ERK1/2 [35,36]. Activation of Akt and ERK1/2 plays an important role in neuronal survival [37–40]. We investigated whether Akt and ERK1/2 phosphorylation was affected in HT22 cells 1- h after GLP-1 (7–36) treatment (Fig. 4A). GLP-1 (7–36) significantly increased p-Akt in HT22 cells when compared with the PBS-treated group (Fig. 4B and D). In addition, p-ERK1/2 was significantly increased in cells treated with GLP-1 (7–36) (Fig. 4C and E). These results suggested that treatment with GLP-1 (7–36) increased Akt and ERK1/2 phosphorylation in HT22 cells.

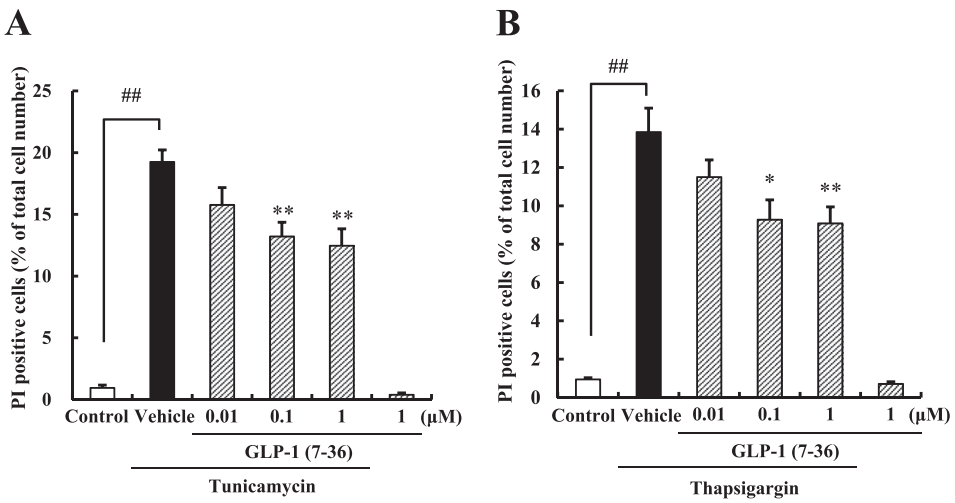

Fig. 2. GLP-1 (7–36) protected HT22 cells against ER stress-induced cell death. (A, B) Results of the cell death assay after treatment with ER stressors. The number of Hoechst 33342- or PI-positive cells was measured and cell death was expressed as a percentage of PI-positive cells divided by Hoechst 33342-positive cells. (A) GLP-1 (7–36) protected cells against ER stress induced by tunicamycin at 50 ng·mL^{-1} for 24 h (n = 9–12). (B) GLP-1 (7–36) protected cells against ER stress induced by thapsigargin at 50 nM for 20 h (n = 6). $^{\#\#}P < 0.01$ versus control (Student's *t*-test). $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle (Dunnett's test). Each column represents mean ± S.E.M.

4. Discussion

In the present study, we evaluated the effects of GLP-1 (7–36) on different stressors of HT22 cells and revealed that GLP-1 (7–36) was protective against oxidative stress-, ER stress-, and Aβ-induced cell death. It has been previously reported that GLP-1 and GLP-1R agonists protect against apoptosis [23,34,41–43]; however to the best of our knowledge, this is the first report to show that GLP-1 is protective in hippocampal neuronal cells.

Since the hippocampus is significantly affected in AD, the protection of hippocampal neurons is a promising therapeutic strategy. GLP-1R is expressed in the brain; GLP-1R KO mice show impairments in synaptic plasticity and memory formation, which is partly dependent on hippocampal function [22]. Other studies have shown that GLP-1R agonists improve cognitive impairment in animal models of AD [44,45]. Furthermore, treatment with a DPP-IV inhibitor increases the amount of GLP-1 in the brain and improves cognitive impairment in a high fat diet- and streptozotocin-induced AD rat model [46,47]. These reports suggest that GLP-1 may be involved in the pathology of AD.

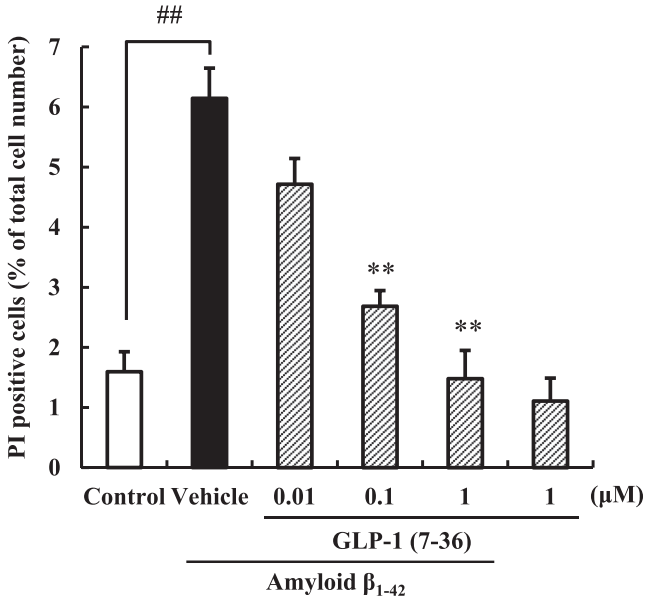

Fig. 3. GLP-1 (7–36) protected HT22 cells against Aβ-induced cell death. The number of Hoechst 33342- or PI-positive cells was counted and cell death was expressed as a percentage of PI-positive cells divided by Hoechst 33342-positive cells. GLP-1 (7–36) protected cells against cell death induced by Aβ at 2 μM for 48 h (n = 6). $^{\#\#}P < 0.01$ versus control (Student's *t*-test). $^{**}P < 0.01$ versus vehicle (Dunnett's test). Each column represents mean ± S.E.M.

We investigated the effects of GLP-1 (7–36) against oxidative- and ER stress-induced cell death using HT22 cells. These two stressors are involved in various diseases, such as AD, stroke, and Parkinson's disease [41,43,48–50] and induce cell apoptosis. For example, Aβ, which is toxic to neuronal cells, produces ROS [32]; therefore, the oxidative stress could contribute to cell apoptosis. ER stress, which is caused by unfolded protein reactions, is also induced in AD pathology and causes cell apoptosis [8]. Several signaling pathways, such as the caspase cascade, are activated by these stressors; therefore, inhibition of these signals is a useful therapeutic targets for AD [51]. Molecules activated in survival signaling cascades, such as Akt and ERK1/2, may also play important roles [37–40]. During phosphatidylinositol 3 kinase (PI3K)/Akt signaling, the serine/threonine kinase Akt (protein kinase B) binds to phosphatidylinositol phosphate 3, which results in phosphorylation at Ser473 and downstream signaling leading to cellular functions, such as metabolism, growth, proliferation, neuronal survival, transcription, and apoptosis [38]. ERK1/2 is activated in the primary mitogen activator protein kinase (MAPK) signaling cascade and mediates proliferation, growth, and survival in the nervous system [39]. Moreover, Akt and ERK1/2 signaling reduce neuronal cell death *via* inhibition of caspase-9 activation [40,52].

Finally, we showed that GLP-1 increased Akt and ERK1/2 phosphorylation, which is consistent with a previous report using a different neuronal cell line [42]. These results suggest that GLP-1 activates cell survival signaling pathways to inhibit the apoptosis induced by various stressors.

In conclusion, GLP-1 is neuroprotective against AD-associated-stressors *via* the activation of proteins associated with cellular survival, such as Akt and ERK1/2 in HT22 cells. These results suggest that GLP-1, and activators of GLP-1R, are useful targets for the treatment of AD.

Conflict of interest

None.

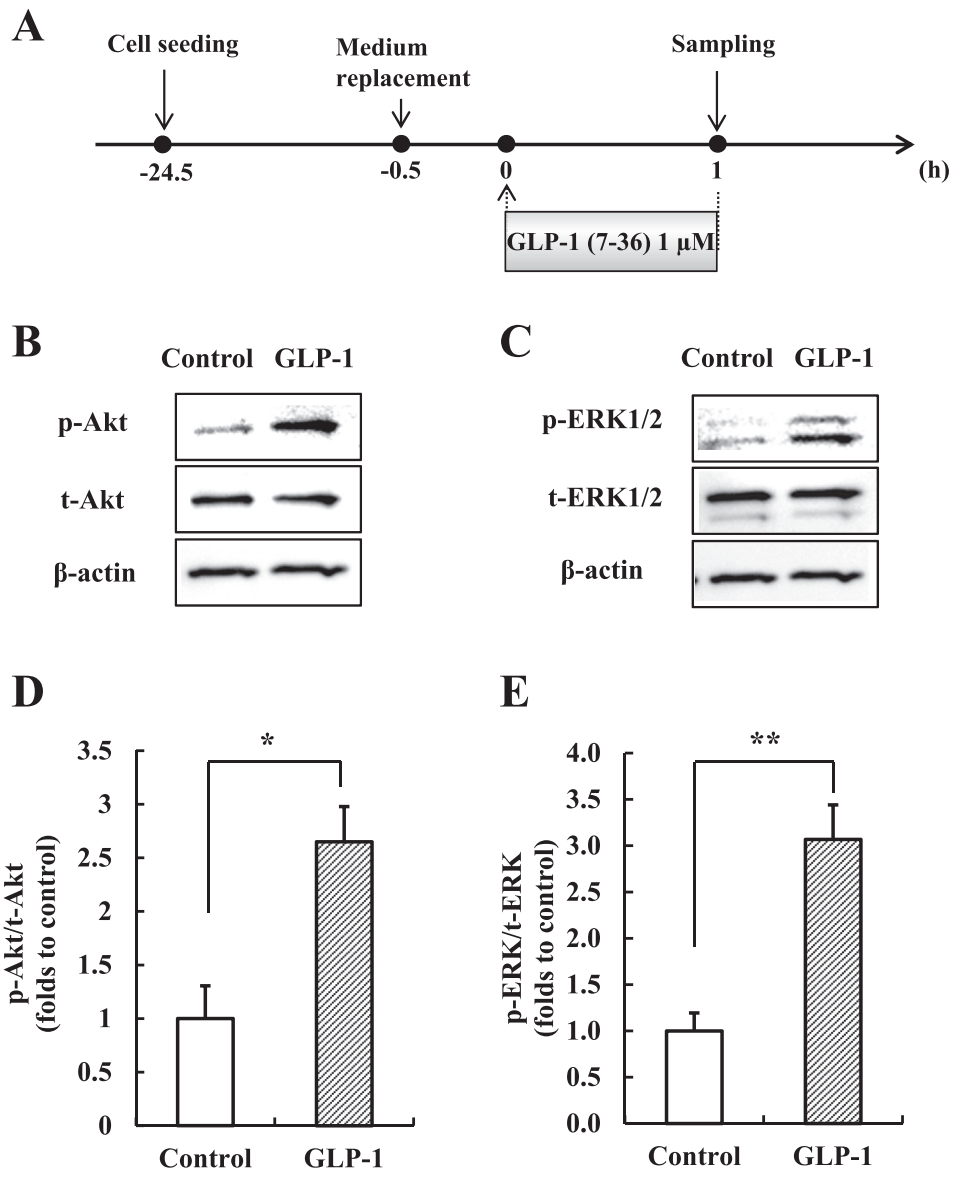

Fig. 4. GLP-1 (7–36) activated Akt and ERK1/2 signaling in HT22 cells. (A) A representative protocol of the western blot analysis. Sampling was performed 60 min after GLP-1 (7–36) treatment. (B, D) Representative images of immunoblotting showing p-Akt (S473), total Akt, p-ERK, total ERK, and β-Actin. P-Akt (S473) and p-ERK were increased in HT22 cells treated GLP-1 (7–36). (C, E) Quantitative analysis showed that GLP-1 (7–36) increased Akt (S473) and ERK phosphorylation. *$p < 0.05$; **$p < 0.01$ versus control (Student's *t*-test). Each column represents mean ± S.E.M. n = 4.

Transparency document

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