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1'-Acetoxychavicol Acetate Increases Proteasome Activity by Activating cAMP-PKA Signaling

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List of abbreviations:

ACA, 1'-acetoxychavicol acetate; UPS, ubiquitin-proteasome system; A β , amyloid β ; APP, amyloid precursor protein; PKA, cAMP-protein kinase A; SAMP8, senescence-accelerated mouse prone 8; SAMR1, senescence-accelerated mouse resistant/1; MTT, 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; 3'-ACA: 1-acetoxy-3-(4-acetoxyphenyl)-2-propen; HPA: 1-hydroxy-3-(4-acetoxyphenyl)-2-propen, EGTA: ethylene glycol tetraacetic acid.

Abstract

Protein degradation systems are critical pathways for the maintenance of protein homeostasis. The age-dependent attenuation of the proteasome activity contributes to age-related neurodegenerative processes. The molecule 1'-acetoxychavicol acetate (ACA) is naturally obtained from the rhizomes and seeds of *Zingiberaceae* plants, such as *Languas galangal* and *Alpinia galangal*, and exhibits anti-carcinogenic effects. Recently, we have shown that ACA protected the age-related learning and memory impairments in senescence-accelerated mice and maintained cognitive performance. Therefore, we here examined the effects of ACA on the protein degradation systems and cell protection against neurotoxicity in differentiated PC12 cells. ACA increased proteasome activity in PC12 cells. Increased proteasome activity occurred during the initial stages of ACA treatment and lasted at least 9 h. The activity returned to control levels within 24 h. The increase in proteasome activity by ACA was suppressed by H-89, which is a cAMP-dependent protein kinase A inhibitor. ACA increased the adenylate cyclase activity and therefore the intracellular cAMP levels. Furthermore, ACA recovered the initial cell viability, which were reduced after the addition of the amyloid β -protein fragment to neuronally differentiated PC12 cells. The effects of ACA on amyloid toxicity were reduced after treatment with MG132, a proteasome inhibitor. These results demonstrated a neuroprotective effect of ACA via activation of cAMP/cAMP-dependent protein kinase A signaling in neuronally differentiated PC12 cells.

Keywords

1'-acetoxychavicol acetate, proteasome, AMP-dependent protein kinase A, adenylate cyclase, cAMP; amyloid β , PC12 cells

Introduction

In mammalian cells, misfolded, old and short-lived proteins are degraded by the ubiquitin-proteasome system (UPS) [1-3]. The UPS has a wide variety of functions ranging from cell-cycle control and transcription to development [4]. Aging is a key factor that reduces UPS function, including proteasome activity [5]. The aging-related reduction in proteasome activity contributes to the accumulation of misfolded proteins and neuropathologic processes, as in Alzheimer's disease and Parkinson disease [6].

Characteristic neuropathological features of the early phase of Alzheimer's disease, the most common neurodegenerative disorder, are the accumulation of senile plaques, the formation of neurofibrillary tangles, and selective neuronal death in the brain [7]. Amyloid β ($A\beta$) is the central component of the plaques. $A\beta$ peptides containing 40 or 42 amino acids are produced by the sequential cleavage of amyloid precursor protein (APP) by β -secretase and γ -secretase [8]. APP is ubiquitinated and degraded by the UPS. These findings suggest that UPS is important for APP processing and $A\beta$ production.

Recent studies have focused on manipulating the UPS via cAMP signaling in neurons. For example, in neuronally differentiated PC12 cells, cAMP analogs prevented apoptosis and the intense diffuse ubiquitin staining induced by proteasome inhibitors [9]. Moreover, proteasome inhibition in rat cerebral cortical neurons caused early accumulation of detergent-soluble ubiquitinated proteins, leading to caspase activation and tau pathology. These events were mitigated by the cAMP analog dibutyryl-cAMP, which was shown to stimulate proteasome activity [10]. Recently, Myeku et al. have reported that tau-driven 26S proteasome impairment and cognitive dysfunction can be prevented early in the disease by activating cAMP-protein kinase A (PKA) signaling [11]. These

results support the notion that activation of cAMP/PKA signaling in neurons could be a promising strategy to promote protein degradation via the UPS.

The molecule (1'S)-acetoxychavicol acetate (ACA) (Fig. 1) is naturally derived from the rhizomes and seeds of *Zingiberaceae* plants, such as *Languas galangal* and *Alpinia galangal*. Recently, we have shown that this compound ameliorates age-related spatial memory deterioration [12]. In senescence-accelerated mouse prone 8 (SAMP8) mice fed a control diet containing 0.02% ACA for 25 weeks, the learning ability in the Morris water maze test was significantly enhanced in comparison with that of mice fed the control diet alone. Furthermore, in the Y-maze test, SAMP8 mice showed decreased spontaneous alterations in comparison with senescence-accelerated mouse resistant/1 (SAMR1) mice, a homologous control, which was improved by ACA pretreatment. We also found that SAMR1 mice did not show histological abnormalities, whereas histological damage was observed in the CA1 region of the hippocampus in SAMP8-control mice; SAMP8-ACA mice were observed to be similar to SAMR1 mice [12]. However, the effects of ACA on proteasome activity remain unclear.

In this study, we investigated the effects of ACA on proteasome activity and intracellular cAMP levels in neuronally differentiated PC12 cells.

Results

We have reported that ACA affects on adipogenesis in 3T3-L1 cells at the concentration of 2.5 or 5 μ M [13]. Therefore, we examined the chymotrypsin-like proteasome activity in neuronally differentiated PC12 cells at the concentration of up to 5 μ M. As shown in Fig.2A, ACA increased the proteasome activity in a dose-dependent manner. The increase in the activity occurred within 1

h and remained for at least 9 h (Fig. **2B**). Furthermore, we examined the effect of those concentration of ACA on the cells viability after cultured for 24 h using neuronally differentiated PC12 cells. ACA had no neurotoxicity at the concentration of up to 5 μ M (Fig. **3**).

PKA has been associated with phosphorylation of the 19S multi-subunit [14-16]. Therefore, we investigated the association of this kinase with the increase in proteasome activity by the treatment with ACA. H-89, a PKA inhibitor, has no effect on proteasome activity compared to control. However, H-89 suppressed significantly proteasome activity which was increased by ACA. The proteasome activity of neuronally differentiated PC12 cells treated with ACA and H-89 had no statistical difference compared to the activity of neuronally differentiated PC12 cells treated with H-89 (Fig. **4A**).

cAMP binds to the PKA regulatory subunit and induces the dissociation of the tetrameric PKA holoenzyme, resulting in the activation of PKA catalytic subunits [17]. To investigate the activation of PKA, we measured the effect of ACA on intracellular cAMP levels in neuronally differentiated PC12. As shown in Fig. **4B**, ACA increased intracellular cAMP levels.

The synthesis of cAMP is catalyzed by adenylate cyclase. Therefore, we wanted to ascertain the effect of ACA on adenylate cyclase activity. As shown in Fig. **4C**, ACA increased the adenylate cyclase activity.

The impairment of proteasome activity may be involved in the progression of neurodegenerative diseases [18]. Therefore, we examined whether the upregulation of proteasome activity through ACA affects the levels of neurotoxicity in well-established human Alzheimer's disease *in vitro* models. As shown in Fig. **5A**, A β reduced the viability of neuronally differentiated PC12 cells. However, the treatment of ACA recovered the cell viability in a dose-dependent manner.

To examine whether the recovery effect of ACA on cell viability is involved in the upregulation of proteasome activity, MG132, a well-established proteasome inhibitor, inhibited the recovery effect of ACA on cell viability in A β -treated neuronally differentiated PC12 cells. On the other hand, the cell viability of neuronally differentiated PC12 cells treated with ACA, MG132 and A β had no statistical difference compared to the cell viability of neuronally differentiated PC12 cells treated with MG132 and A β (Fig. **5B**).

It is considered that ACA undergoes transformation to form 3'-ACA, which is then hydrolyzed to HPA and acetic acid (Fig. **6**). It is conceivable that ACA is received such a structural change *in vivo*. Therefore, we examined the effect of ACA, 3'-ACA and HPA on the proteasome activity in neuronally differentiated PC12 cells. As shown Fig. **7**, ACA and its biotransformers, 3'-ACA and HPA, enhanced the proteasome activity to a similar level as observed with ACA.

Discussion

The present study showed that ACA induced protease activity by activation of cAMP/PKA signaling in neuronally differentiated PC12 cells. We also showed that ACA increased adenylate cyclase activity and, therefore, intracellular cAMP levels.

The increase in proteasome activity was observed during the early phase. Therefore, one possible mechanism involves the post-translational modification of the proteasome subunit. Phosphorylation is a well-studied post-translational modification. Under physiological conditions, the proteasome typically consists of two multi-subunit complexes, a 20S proteasome and a pair of 19S regulatory particles. Both 20S and 19S multi-subunits undergo phosphorylation for their subsequent activation [19, 20]. Cyclic AMP-dependent protein PKA has been associated with phosphorylation of the 19S multi-subunit [14, 15]. In this study, we showed that H-89, a PKA inhibitor, suppressed the ACA-induced protease activity. These results suggested that ACA increased proteasome activity via the PKA pathway.

There are several reports that proteasome function is regulated by PKA. Metcalfe et al. showed that dibutyryl-cAMP stimulated proteasomes and mitigated proteasome inhibition induced by prostaglandin J2 in cultured cortical neurons. They proposed that targeting cAMP/PKA to boost proteasome activity in a sustainable manner could offer an effective approach to avoid early accumulation of detergent-soluble ubiquitinated proteins and later caspase activation and tau cleavage, possibly preventing/delaying Alzheimer's disease neurodegeneration [10]. Myeku et al. also reported that tau-driven 26S proteasome impairment and cognitive dysfunction can be prevented early in disease by activating cAMP-PKA signaling [21]. Furthermore, increasing PKA activity is also important in Huntington's disease prevention. Lin et al. showed that decreased PKA activity was involved in proteasome impairment in mouse Huntington's disease models [22]. Though proteasomes are activated by PKA-induced phosphorylation, PKA regulatory subunits are also proteasome substrates. Lin et al. also showed that a phosphomimetic proteasome subunit mutant increased proteasome activity and reduced Huntington aggregates.

Hansen *et al.* showed that forskolin, an adenylate cyclase activator, induced neurite outgrowth of neuronally differentiated PC12 cells and is mediated by activation of the PKA signaling pathway

[23]. Furthermore, Sanchez *et al.* reported that dibutyryl-cAMP promotes neurite outgrowth in human SH-SY5Y cells and neuronally differentiated PC12 cells [24]. In addition, Lambeng *et al.* showed that intracellular cAMP protected against oxidative stress when used alone and in association with nerve growth factor and endothelial growth factor in PC12 cells [25].

The UPS degrades numerous proteins, particularly short-lived nuclear and cytosolic proteins [26]. UPS dysfunction has been related to aging and a broad array of chronic neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases, in which the accumulation of ubiquitin-conjugated misfolded protein aggregates causes proteotoxicity [27]. Furthermore, reduced proteasomal chymotrypsin-like activity affects longevity and promotes the development of metabolic abnormalities [28]. In cultured human fibroblasts, proteasome inhibitors shorten the replicative life span and induce a senescent-like phenotype [29]. However, elevated proteasome activity extends the replicative lifespan in *Saccharomyces cerevisiae* through a mechanism distinct from the known longevity pathways [30]. These studies suggest that maintaining and increasing proteasome activity play important roles in optimizing a healthy life span. In the present study, we showed that ACA protects neuronally differentiated PC12 cells against A β (25-35) toxicity via proteasome activation. The protective effect of ACA was reduced by the addition of MG132, a proteasome inhibitor.

Many studies have suggested that an impairment of glucose metabolism may contribute to the cognitive deficits that are observed in Alzheimer's disease [31, 32]. We found that ACA also increased the plasma concentrations of β -hydroxybutyric acid, a major ketone body in blood, and palmitic acid levels in SAMP8 mice from serum metabolite profiles, [12]. Therefore, our results suggest that ketone bodies contribute to the maintenance of the cognitive performance of SAMP8 mice. On the other hand, we have reported that ACA causes a significant decrease in the activity of glycerol 2-phosphate dehydrogenase, a key enzyme in triglyceride synthesis, in 3T3L1 adipocytes

and inhibits cellular lipid accumulation following the activation of AMPK [13]. Fatty acids released from adipocytes may be transferred to the liver and oxidized to ketone bodies by oxidation. Then, the produced ketone bodies are transferred to the brain. In hepatocytes, activated AMPK increases fatty acid oxidation by inactivating acetyl-CoA carboxylase and upregulating the genes that are involved in fatty acid oxidation. Thus, activated AMPK has various metabolic effects.

Furthermore, there are some reports that cAMP pools affect the activation of AMPK. In the present study, we demonstrated that ACA caused a neuroprotective effect via the activation of cAMP/cAMP-dependent protein kinase A signaling in neuronally differentiated PC12 cells.

Taken together, these data suggest that ACA increases the cellular cAMP and/or the activation of AMPK and protects neuronal cells in the hippocampus by induction of proteasome activity and increases in fuels to maintain cognitive performance.

Recently Sagawa et al have showed an interesting report that TM-233, an analog of ACA, induced cell death in myeloma cells by inhibiting both JAK/STAT and proteasome activities [33]. We also reported that ACA inhibited cell proliferation in Ehrlich ascites tumor cells [34, 35] and in Hela cells and murine adenocarcinoma [36]. On the other hand, ACA induced cytoprotective enzymes, such as phase II enzymes, via the Nrf2 signaling pathway in normal intestinal epithelial cell line [37]. The present study also demonstrated a neuroprotective effect of ACA via enhancing proteasome activity in neuronally differentiated PC12 cells. These results suggest that ACA has different effects between tumor cells and normal cells. Further research is needed on the involvement of proteasome in different actions of ACA.

In conclusions, our data demonstrate that ACA upregulates proteasome activity via the cAMP-PKA pathway in neuronally differentiated PC12 cells. ACA may be considered a potential drug candidate for Alzheimer's disease and other diseases caused by the deregulation of the proteasome.

Materials and Methods

Materials

Dr. H. Azuma (Graduate School of Engineering of Osaka City University) provided the racemic-ACA (purity: 99.5%), 3'-ACA (purity: >99%) and HPA (purity: >99%). The chemical structure of ACA, 3'-ACA and HPA were shown in Fig. 6. ACA, 3'-ACA and HPA were dissolved in dimethyl sulfoxide (DMSO). A β (25-35) was purchased from Sigma.

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and MG132 (purity: 98.3%) were purchased from Wako Pure Chemical Industries, Ltd. Horse serum and fetal bovine serum were purchased from Nichirei Bioscience Inc. H-89 (purity: >99%) was purchased from Nacalai Tesque. All other chemicals used in this study were special grade commercial products.

Cell culture

Rat pheochromocytoma PC12 cells were provided by the National Institutes of Biomedical Innovation, Health and Nutrition in Japan. PC12 cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C for 3-4 days in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal bovine serum. Subsequently, the cells were washed and re-plated at a concentration of 2.5×10^5 cells/mL in fresh medium. To initiate differentiation, the PC12 cells were treated with nerve growth factor at a concentration of 50 ng/mL for 2 days. The ACA in DMSO was diluted in culture medium immediately before use (final DMSO concentration of <0.5%). In all experiments, control cultures were composed only of medium, DMSO and neuronally differentiated PC12 cells.

Preparation of A β fragment

A β (25-35) was dissolved in sterile distilled water at a concentration of 2 mM and incubated at 37°C for 4 days to form the aged A β fragment. The aged A β solution was stored at -20°C.

Cell viability

Cell viability was measured using an MTT assay. Neuronally differentiated PC12 cells were seeded in 96-well culture plates at a density of 2.5×10^4 cells per well. After differentiation, the medium was replaced, and the neuronally differentiated PC12 cells were treated with ACA and/or MG132, a well-established protease inhibitor, for 1 h. After incubation, 10 μ L of 5 mg/mL MTT was added to the medium, and the neuronally differentiated PC12 cells were incubated for an additional 4 h. The medium was eliminated, and 200 μ L of DMSO was added to each well. The plate was shaken for 10 min to extract the reduced MTT. The reduced MTT was measured in a micro plate reader at 535 nm. The cell viability was expressed as a percentage against the control group (assuming the control group to be 100%).

Chymotrypsin-like proteasome activity assay

The proteasome activity was measured using a Proteasome-Glo Chymotrypsin-like Cell-Based Assay kit (Promega). Neuronally differentiated PC12 cells were seeded in 96-well culture plates at a density of 2.5×10^4 cells per well. After differentiation, the medium was replaced, and the neuronally differentiated PC12 cells were treated with ACA or its analogs for 1 to 24 h. After incubation, cells were assayed according to the manufactures protocol.

Assay of intracellular cAMP level

cAMP levels were measured by using a Cyclic AMP Direct EIA Kit (Arbor). Neuronally differentiated PC12 cells were incubated with ACA. After incubation with ACA for 30 min, the neuronally differentiated PC12 cells were collected and washed with PBS. Next, the cells were lysed in sample diluent for 10 min at room temperature. The lysed cells were sonicated on ice in a

DU-250 Bioruptor (Cosmo Bio) with a maximum output power of 250 W. After centrifugation at $13,000 \times g$ for 10 min at 4°C , the supernatants were assayed. Endogenous phosphodiesterases were inactivated in the sample diluent. A clear microtiter plate coated with an antibody to capture sheep IgG was provided and a neutralizing plate primer solution was added into the wells. Then, a cAMP-peroxidase conjugate was added to the wells. The binding reaction was initiated by the addition of a sheep antibody to cAMP into each well. After a 2 h incubation, the plate was washed, and the substrate was added. After a short incubation, the reaction was stopped, and the intensity of the generated color was detected via a microplate reader (wavelength = 450 nm). Total protein content was measured using the Bradford method, with bovine serum albumin as the standard [38].

Assay of adenylate cyclase activity

Adenylate cyclase activity was determined by measuring the production of cAMP using HPLC [39]. Neuronally differentiated PC12 cells were incubated with ACA. After incubation with ACA for 30 min, the cells were collected and washed with phosphate buffered saline. Then, the cells were lysed in sample buffer (40 mM Tris/HCl, pH 7.5, 3.3 mM MgSO_4 , 10 mM theophylline, 1 mM CaCl_2 , and 1 mM ethylene glycol tetraacetic acid (EGTA)). The cells were sonicated on ice in a DU-250 Bioruptor with a maximum output power of 250 W. After centrifugation at $13,000 \times g$ for 10 min at 4°C , the supernatants were assayed. The adenylate cyclase activity assay buffer contained 40 mM Tris/HCl, pH 7.5, 3.3 mM MgSO_4 , 10 mM theophylline, 1 mM CaCl_2 , 1 mM EGTA, 20 μM GTP, and 6 mM ATP. The reaction was started by the addition of 0.3 ml of sample solution to 0.3 mL of adenylate cyclase activity assay buffer. The reaction mixture was incubated at 30°C for 45 min and terminated by the addition of 4% (w/v) trichloroacetic acid. cAMP was separated on a silica 5C8 4E column (4.6×250 mm, particle size 5 μm , Shodex) using solvents A (0.1 M KH_2PO_4 , pH 3.0) and B (methanol). The flow rate was 2 mL/min. The elution gradient was 100% solvent A for 5 min, followed by a gradient to 50% methanol by 5.1 min. Fifty percent of the methanol was continued until 9 min, and then the gradient was returned to 100% solvent A by 9.1 min. The column was

filled with solvent A before the next sample was injected. The produced cAMP level was measured at 260 nm with Jasco UV-1570 (JASCO).

Statistical analysis

The data are presented as the means \pm SD. Statistical analyses were performed using Statcel3 the usefull add-in forms on excel statistical software (OMS publishing Inc.). Significant difference in assay values was evaluated using ANOVA followed by the Tukey test. A value of $p < 0.05$ indicated a statistically significant difference.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Legends of Figures

Fig. 1 The structure of ACA

Fig. 2 Effect of ACA on proteasome activity

(A) Neuronally differentiated PC12 cells were incubated with 2.5 or 5 μ M ACA for 1 h. (B)

Neuronally differentiated PC12 cells were incubated with 2.5 μ M ACA for 1 - 24 h. (A, B) The

proteasome activity was measured using a Proteasome-Glo Chymotrypsin-like Cell-Based Assay kit (Promega). The results are presented as the means \pm S.D. (N=4). * p <0.05, ** p <0.01 compared with the control.

Fig. 3 Effect of ACA on cell viability of neuronally differentiated PC12

Neuronally differentiated PC12 cells were incubated with 2.5 or 5 μ M ACA for 24 h. Cell viability was assayed using the MTT assay. The results are presented as the means \pm S.D. (N=4).

Fig. 4 Effect of inhibitor of kinase on proteasome activity increased by ACA treatment

(A) Effect of ACA and H-89, a PKA inhibitor, on proteasome activity. **(B)** Effect of ACA on intracellular cAMP levels in neuronally differentiated PC12. **(C)** Effect of ACA on AC activity in neuronally differentiated PC12. **(A)** Neuronally differentiated PC12 cells were incubated with 10 μ M H-89 for 30 min prior to treatment with 2.5 μ M ACA for 1 h. The proteasome activity was measured using a Proteasome-Glo Chymotrypsin-like Cell-Based Assay kit (Promega). **(B)** Neuronally differentiated PC12 cells were incubated with 1.25, 2.5, or 5 μ M ACA for 30 min. Intracellular cAMP levels were measured using a Cyclic AMP Direct EIA Kit (Arbor). **(C)** Neuronally differentiated PC12 cells were incubated with 2.5 μ M ACA for 30 min. The adenylate cyclase activity was measured using an HPLC system. The results are presented as the means \pm S.D. (N=4). * p <0.05, ** p <0.01 compared with the control. † p <0.05 compared with ACA.

Fig. 5 Effect of ACA on cell protection against neurotoxicity

(A) The protective effect of ACA against A β (25-35)-induced toxicity. **(B)** The effect of MG132 on the cell viability of neuronally differentiated PC12 cells recovered from A β -induced toxicity through ACA treatment. Neuronally differentiated PC12 cells were incubated with 2.5 μ M ACA and/or 100 nM MG132 for 1 h, followed by treatment with 20 μ M A β (25-35) for 20 h. Cell

viability was assayed using the MTT assay. The results are presented as the means \pm S.D. (N=4).

**p<0.01 compared with the control. ††p<0.01 compared with the single A β or MnCl₂ treatment.

Fig. 6 The transformation of ACA

3'-ACA: 1-acetoxy-3-(4-acetoxyphenyl)-2-propen; HPA: 1-hydroxy-3-(4-acetoxyphenyl)-2-propen.

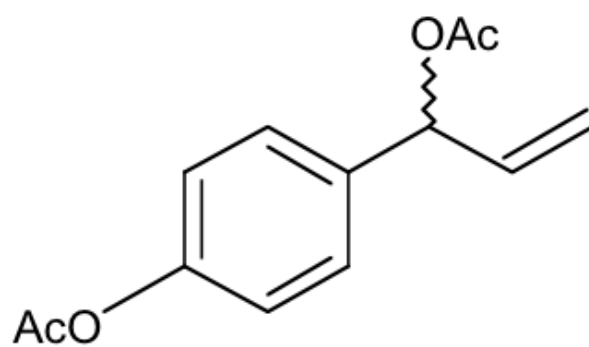
Fig. 7 Effect of ACA, 3'-ACA and HPA on proteasome activity

Neuronally differentiated PC12 cells were incubated with 2.5 μ M ACA, 3'-ACA or HPA for 1 h.

The proteasome activity was measured using a Proteasome-Glo Chymotrypsin-like Cell-Based

Assay kit (Promega). The results are presented as the means \pm S.D. (N=4). **p<0.01 compared with the control.

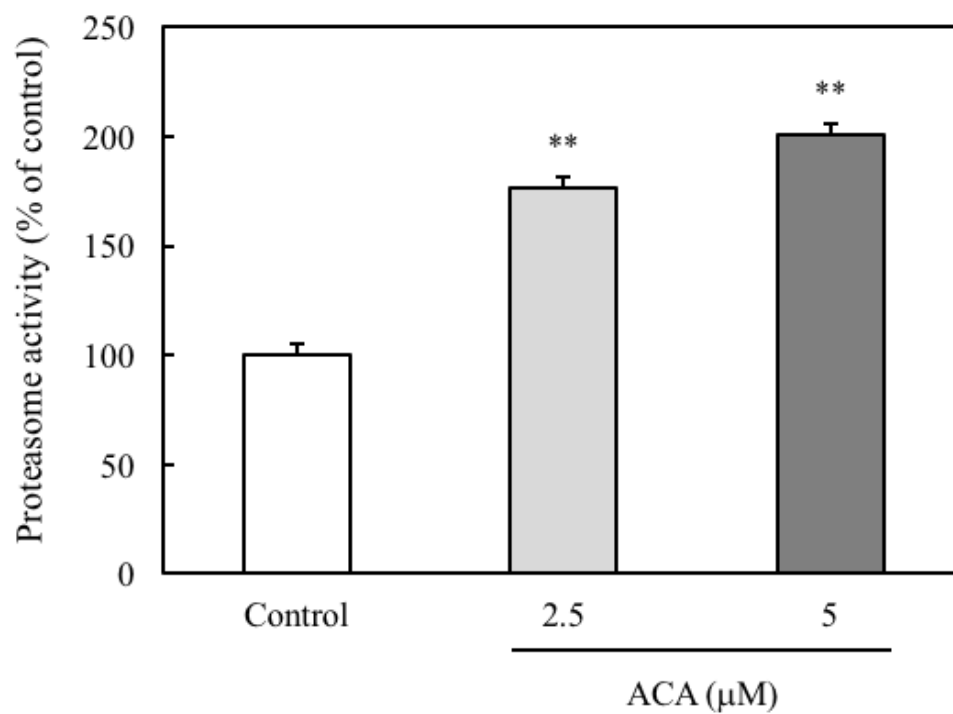
Fig. 1



AcO: CH₃COO-

Fig. 2

A



B

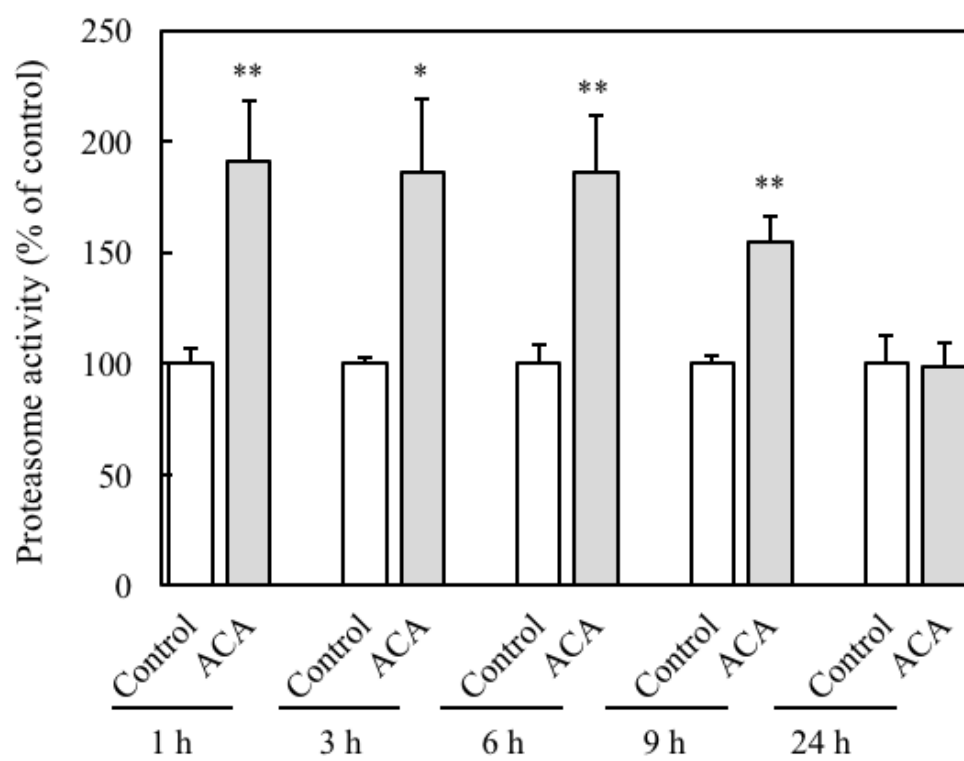


Fig. 3

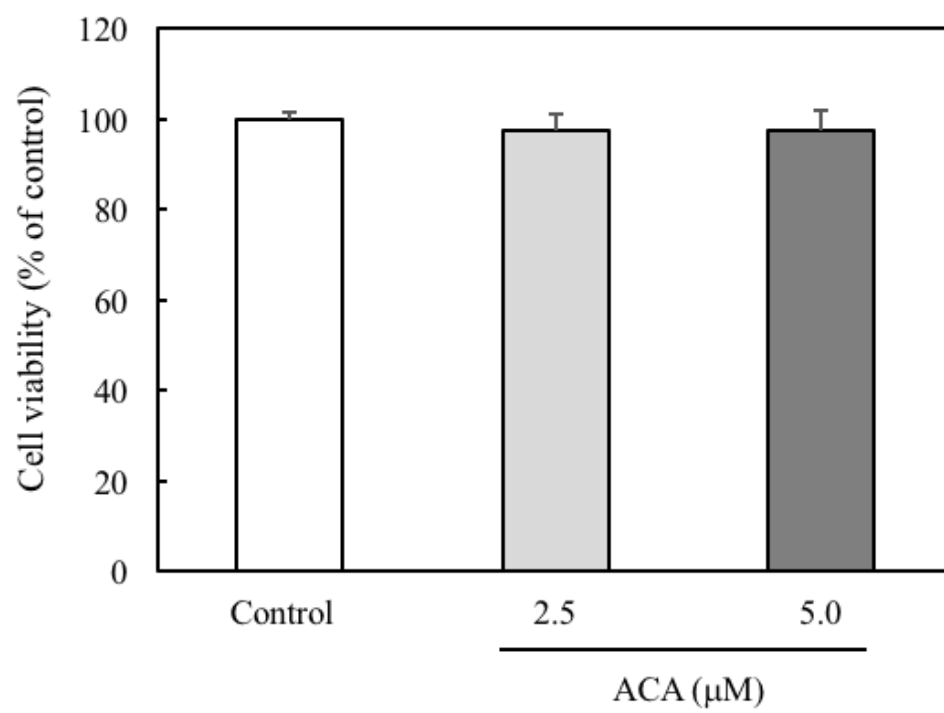


Fig. 4

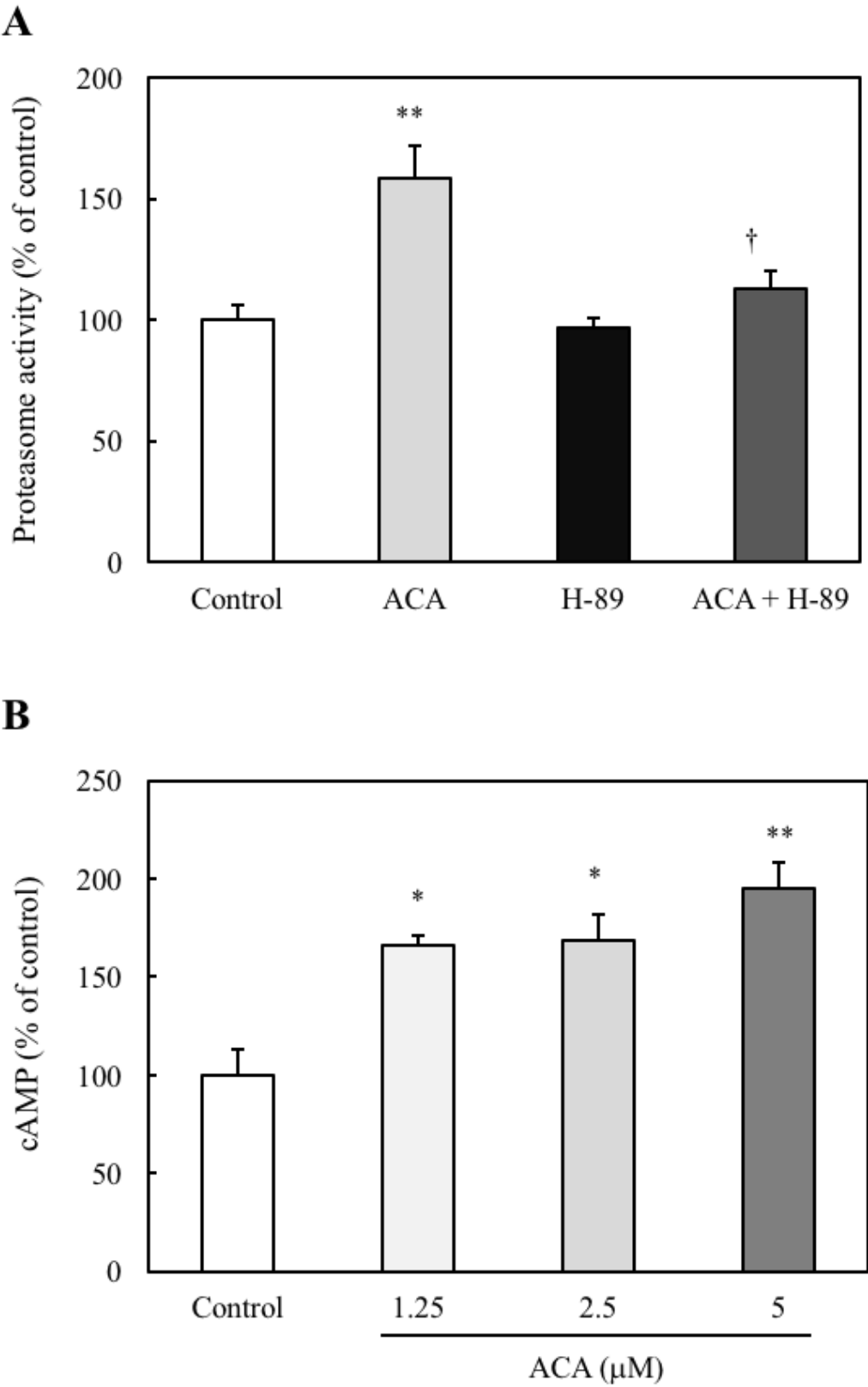


Fig. 4

C

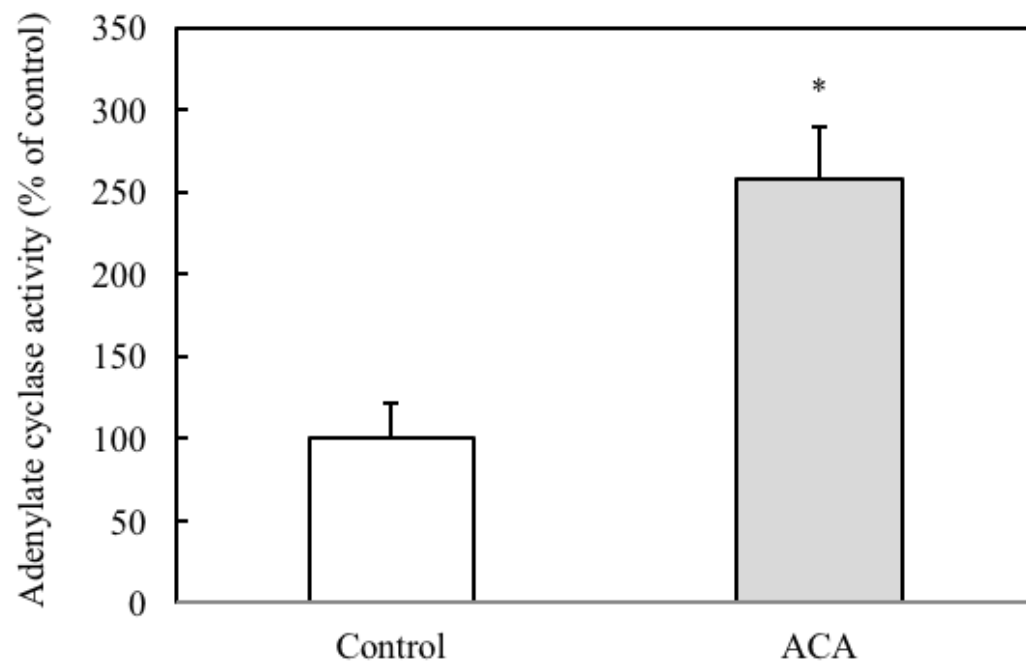


Fig. 5

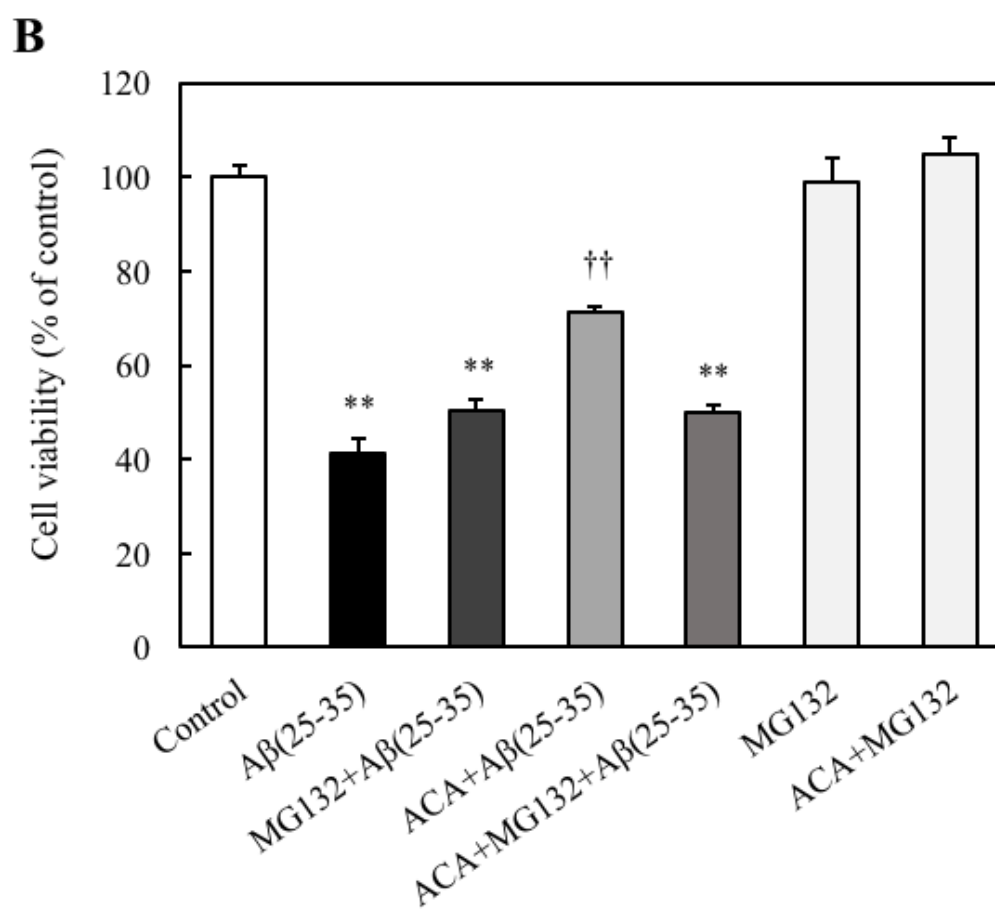
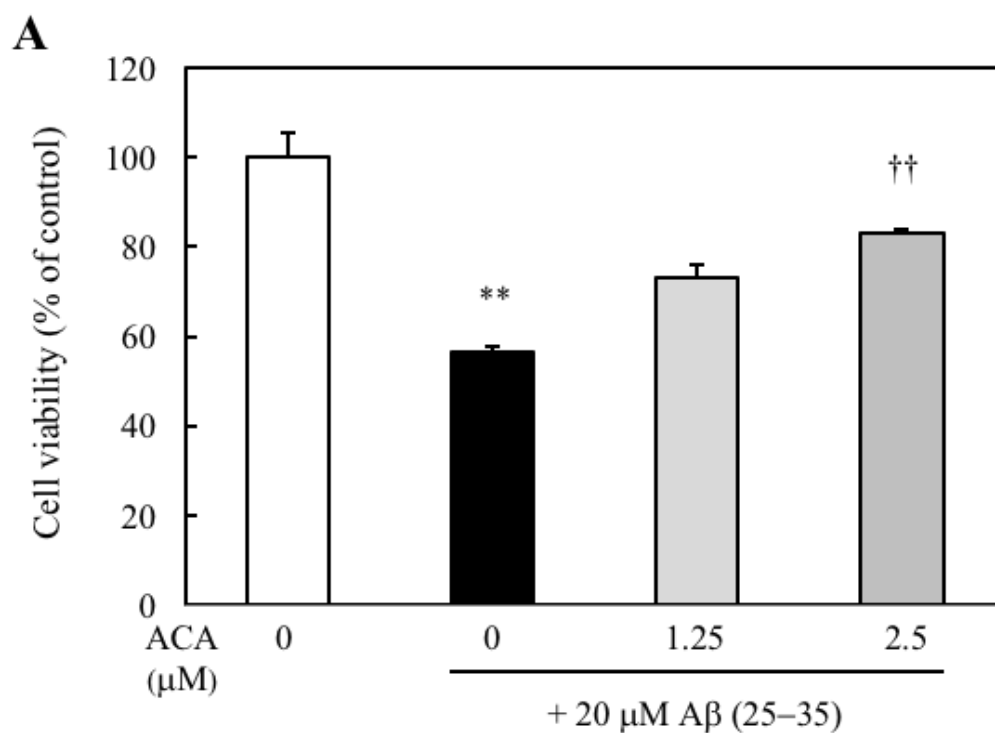


Fig. 6

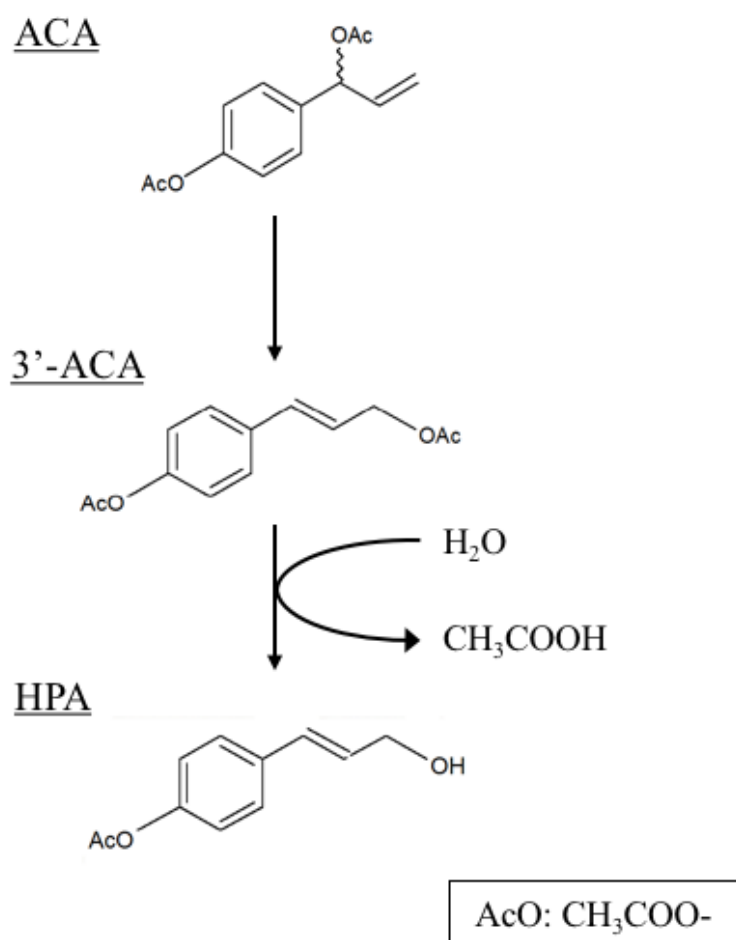


Fig. 7

