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ORIGINAL ARTICLE

Protective effect of *S*-allyl-L-cysteine against endoplasmic reticulum stress-induced neuronal death is mediated by inhibition of calpain

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Abstract Endoplasmic reticulum (ER) stress, implicated in various neurodegenerative processes, increases the level of intracellular Ca^{2+} and leads to activation of calpain, a Ca^{2+}-dependent cysteine protease. We have shown previously that *S*-allyl-L-cysteine (SAC) in aged garlic extracts significantly protects cultured rat hippocampal neurons (HPNs) against ER stress-induced neurotoxicity. The neuroprotective effect of SAC was compared with those of the related antioxidant compounds, L-cysteine (CYS) and *N*-acetylcysteine (NAC), on calpain activity in HPNs and also in vitro. SAC, but not CYS or NAC, reversibly restored the survival of HPNs and increased the degradation of α-spectrin, a substrate for calpain, induced by tunicamycin, a typical ER stress inducer. Activities of μ- and m-calpains in vitro were also concentration dependently suppressed by SAC, but not by CYS or NAC. At submaximal concentration, although ALLN (5 pM), which blocks the active site of calpain, and calpastatin (100 pM), an endogenous calpain-inhibitor protein, additively inhibited μ-calpain activity in vitro in combination with SAC, the effect of PD150606 (25 μM), which prevents interaction of Ca^{2+} with the Ca^{2+}-binding site of calpain, was unaffected by SAC. In contrast, SAC (1 mM) significantly reversed the effect of PD150606 at a concentration that elicited supramaximal inhibition (100 μM), but did not affect ALLN (1 nM)- and calpastatin (100 nM)-induced inhibition of μ-calpain activity. These results suggest that the protective effects of SAC against ER stress-induced neuronal cell death are not attributable to antioxidant activity, but to suppression of calpain through interaction with its Ca^{2+}-binding site.

Keywords *S*-allyl-L-cysteine · Calpain · Endoplasmic reticulum stress · Hippocampal neuron

Introduction

S-allyl-L-cysteine (SAC), the most abundant organosulfur molecule in aged garlic extract, has long been used as a common dietary supplement and traditional medicine. SAC is a derivative of the amino acid cysteine in which an allyl group has been added to the sulfur atom (Fig. 1). SAC has been reported to have multiple biological effects such as antioxidant (Ray et al. 2011), anticancer (Thomson and Ali 2003), and antihepatotoxic activities (Kodai et al. 2007). In the CNS, SAC shows neurotrophic activity, as demonstrated in cultured rat hippocampal neurons (HPNs) (Moriguchi et al. 1997), and improves learning deficits in senescence-accelerated mice (Nishiyama et al. 2001).

The endoplasmic reticulum (ER) plays crucial roles in various cellular processes including protein folding, protein trafficking, and intracellular Ca^{2+} regulation. ER can be divided into two types: Smooth ER and rough ER. The rough ER is studded with ribosomes on its outer surface and plays a key role in protein synthesis. On the other hand, the smooth ER is rich in enzymes that synthesize lipids and membrane phospholipids and participates in steroid synthesis. Rough

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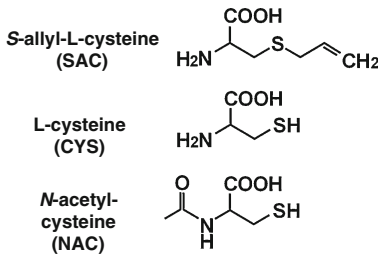

Fig. 1 Chemical structure of cysteine derivatives used in the present study

ER and smooth ER are interconnected, not physically discrete, and the relative proportion of each quickly changes. Disturbances of rough and smooth ER function induce cellular damage, resulting in apoptotic cell death. Various physiological and pathological conditions such as glucose deprivation, Ca^{2+} depletion and exposure to free radicals lead to the accumulation of misfolded proteins in the ER, a condition known as ER stress (Kaufman 1999). Recently, ER stress and ER stress-induced cell death have been shown to be involved in various neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (Lindholm et al. 2006). We and other groups have shown that several proteins are involved in ER stress-induced apoptosis, including a transcription factor, C/EBP homologous protein (CHOP), caspase-12, and c-Jun N-terminal kinase (JNK) (Gorman et al. 2012). Among them, caspase-12 was identified as the first ER-associated member of the caspase family and shown to be proteolytically activated under ER stress in rodents (Nakagawa et al. 2000). Moreover, caspase-12-deficient embryonic fibroblast cells are resistant to pharmacological inducers of ER stress such as tunicamycin (TM) (an inhibitor of glycosylation in the ER), thapsigargin (TG) (an inhibitor of ER-specific calcium ATPase), or befeldin A (BFA) (an inhibitor of ER-Golgi transport) (Nakagawa et al. 2000). We have shown previously that SAC protects against amyloid β-peptide (Aβ)- and TM-induced cell death in differentiated PC12 (Ito et al. 2003) cells and HPNs (Kosuge et al. 2006). The increases in cleaved caspase-12 induced by $A\beta_{25-35}$ were also reversed by simultaneously applied SAC (Ishige et al. 2007). Moreover, SAC provides a significant neuroprotective effect against caspase-12-dependent ER stress-induced neuronal death and its potentiation by Aβ through regulation of calpains, a family of Ca^{2+}-dependent neutral cysteine proteases, or Ca^{2+}–calpain interaction in organotypic hippocampal slice cultures (OHCs) (Imai et al. 2007). However, the underlying mechanisms responsible for the neuroprotective effect of SAC against ER stress-dependent apoptosis are not fully understood.

Calpains change the structure and function of substrate molecules and play a pivotal role in a wide variety of biological processes including cell division, differentiation, and migration (Goll et al. 2003). Calpains are sensitive to increases in the level of intracellular Ca^{2+} for activation. The best-characterized calpains are two ubiquitously expressed isozymes, μ-calpain and m-calpain. While μ-calpain is activated in the presence of micromolar concentrations of Ca^{2+}, activation of m-calpain requires millimolar concentrations (Goll et al. 2003). Interestingly, it has been shown that activated calpain activates caspase-12 proteolytically (Nakagawa and Yuan 2000). Furthermore, embryonic fibroblasts from calpain-deficient mice show attenuated activation of caspase-12 and are resistant to ER stress-induced apoptosis (Tan et al. 2006). Therefore, calpain seems to play a critical role in activation of caspase-12 during ER stress-induced apoptosis.

In the present study, we sought to determine the effect of SAC on calpains that play crucial roles in caspase-12-dependent neuronal death in HPNs and, using an in vitro assay system, found that SAC directly modulated the activation of calpains in a concentration-dependent manner.

Materials and methods

Materials

The chemicals that were used in the study were purchased from a variety of suppliers. [3-(4, 5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), TM, L-cysteine (CYS), *N*-acetylcysteine (NAC), and *N*-acetyl-Leu–Leu-Nle-CHO (ALLN) were obtained from Sigma-Aldrich (St Louis, MO, USA); TrypLE Express™ and Neurobasal medium, FBS, and B27 supplement from Invitorogen (Madison, WI, USA); calpastatin, 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD150606), purified μ-calpain, and purified m-calpain from Calbiochem (San Diego, CA, USA).

Preparation of cultured rat hippocampal neurons (HPNs)

All efforts were made to minimize the number of animals used and their suffering. All experiments with animals complied with the Guidelines for Animal Experiments at Nihon University. HNPs were prepared as described previously (Kosuge et al. 2003). Briefly, hippocampi were isolated from the brains of embryonic day 18 Wistar rats and treated with TrypLE Express™ and 0.01 % deoxyribonuclease at 37 °C for 15 min. The cells were suspended in Neurobasal medium containing B27 supplement and plated at a density of 5.0×10^5 cells/cm^2 on poly-L-lysine-coated 6- or 24-well culture plates (ASAHI GLASS CO. LTD, Tokyo, Japan). They were then cultured at 37 °C in humidified 5 % CO_2/95 % air for 7–8 days.

MTT reduction assay

The MTT reduction assay, which has been widely used for measuring cell viability, was executed as described previously (Kosuge et al. 2003). Briefly, the cells were incubated with MTT (0.25 mg/mL) for 4 h at 37 °C, and the reaction was stopped by adding a solution of 50 % dimethylformamide and 20 % SDS, pH 4.8. The next day, the amount of MTT formazan product was determined by measuring its absorbance with a microplate reader at a wavelength of 570 and 655 nm.

Western blotting

Western blots were performed as reported previously (Miyagishi et al. 2012). HNPs were treated with TM in the absence or presence of cysteine derivative. After this treatment, these cells were lysed in lysis buffer (25 mM Hepes–NaOH, pH 7.4, 10 mM EGTA, 10 mM EDTA, 50 mM NaCl, 1 % TritonX-100, 0.5 % SDS, 1 % sodium deoxycholate, 20 mM 2-mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)) for 1 h on ice. Protein extracts were loaded on SDS–polyacrylamide gel electrophoresis (5 % polyacrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in blocking buffer (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 5 % skim milk) for 1 h at room temperature and incubated with anti-α-spectrin monoclonal antibody (1:2,000; Millipore, Billerica, MA, USA) overnight at 4 °C. After washing, the membranes were incubated for 1 h at room temperature with a secondary antibody for visualization of specific band intensities with an enhanced chemiluminescence detection system. Optical density on the blots was measured with Scion imaging software (Scion, Frederick, MD, USA). Quantitative results were expressed as the ratio of the band intensity of the protein of interest to the band intensity of β-actin.

In vitro calpain activity assay

Enzymatic activities of μ- and m-calpains were determined using a Calpain-Glo^{TM} protease assay kit (G8501, Promega, Madison, WI, USA) with a luminogenic succinyl calpain substrate following the manufacturer's protocol. Briefly, 50 μL of Calpain-Glo^{TM} Reagent was added to 50 μL of sample containing cysteine derivative and/or a variety of calpain inhibitors with 1 μM Ca^{2+} (for μ-calpain) or 1 mM Ca^{2+} (for m-calpain) in each well of a white 96-well plate. Samples were incubated at room temperature for 10 min and analyzed using a plate-reading luminometer (FlexStation3, Molecular Devices, CA, USA). The measured value minus the background without drug was normalized to calpain activity from vehicle-treated samples (control; 100 %) and expressed as a percentage of the control. Curve fitting was performed using the sigmoidal dose–response (variable slope) with the aid of GraphPad Prism (version 4.0; GraphPad software, San Diego, CA, USA). Inhibitor concentrations required to produce 50 % inhibition of enzyme activity (IC_{50}) were calculated using percentage inhibitions of enzyme activity.

Statistical analysis

Values are expressed as mean ± SEM. Statistical significance was assessed by one-way analysis of variance followed by Tukey's multiple range tests. Statistical significance was defined as a probability value of <5 %.

Results

Effects of cysteine derivatives on TM-induced neuronal cell death in HPNs

Exposure of the HPNs to TM (10 μg/mL) for 24 h resulted in a significant decrease (53 %) of neuronal survival in the MTT reduction assay (Fig. 2a). Consistent with previous results (Kosuge et al. 2003), the TM-induced decrease in MTT reduction activity was rescued by simultaneous application of SAC in a concentration-dependent manner, and the maximal effect of SAC was observed at 1 μM, with a recovery of up to approximately 85 %.

To elucidate the possible mechanism of the neuroprotection of SAC against TM-induced neuronal cell death in HPNs, these effects were compared with those of two cysteine derivatives, CYS and NAC. HPNs were treated with 10 μg/mL TM in the absence or presence of 1 mM SAC, CYS or NAC, because the latter two were reported to exert a neuroprotective effect against oxidative stress-induced neuronal cell death at a concentration of 1 mM (Arakawa et al. 2007; Lu et al. 2011). As shown in Fig. 2b, the TM-induced attenuation of neuronal survival was reversed by the simultaneously applied SAC (1 mM) with a recovery of 76 %, whereas the same concentrations of CYS and NAC had no effect on the attenuated MTT levels.

Effects of cysteine derivatives on TM-induced α-spectrin degradation in HPNs

Next, we investigated the effects of SAC and two cysteine derivatives on calpain activities in TM-treated HPNs. Calpain activity was monitored by quantitative analysis of full-length α-spectrin (240 kDa), a neuron-specific cytoskeletal protein, which undergoes proteolysis by activated

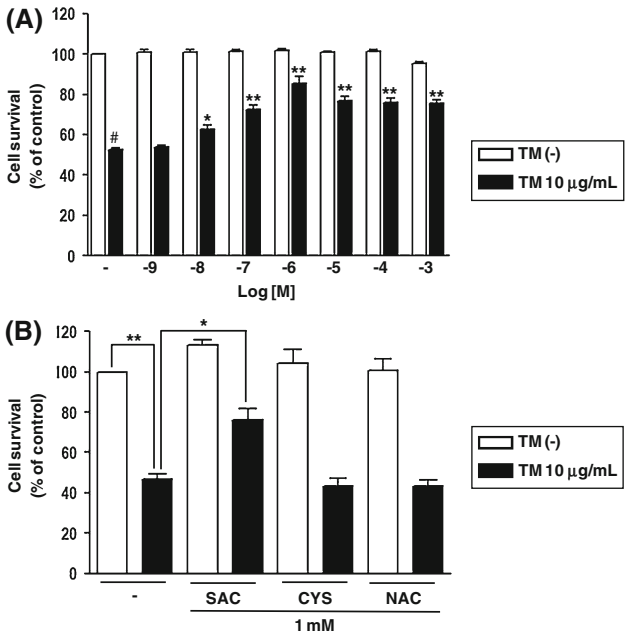

Fig. 2 Effects of cysteine derivatives on TM-induced neuronal cell death in HPNs. **a** Various concentrations of SAC were added simultaneously with 10 μg/mL TM for 24 h. The viability of HPNs was assessed by the MTT reduction assay. Values are expressed as the relative percentages of surviving cells. Each value represents the mean ± SEM for six different experiments. $^{\#}P < 0.001$ as compared to vehicle; $^{*}P < 0.01$, $^{**}P < 0.001$ as compared to 10 μg/mL TM alone. **b** SAC (1 mM), NAC (1 mM), and CYS (1 mM) were added simultaneously with 10 μg/mL TM for 24 h. The viability of HPNs was assessed by the MTT reduction assay. Values are expressed as the relative percentages of surviving cells. Each value represents the mean ± SEM for four different experiments. $^{*}P < 0.01$, $^{**}P < 0.001$

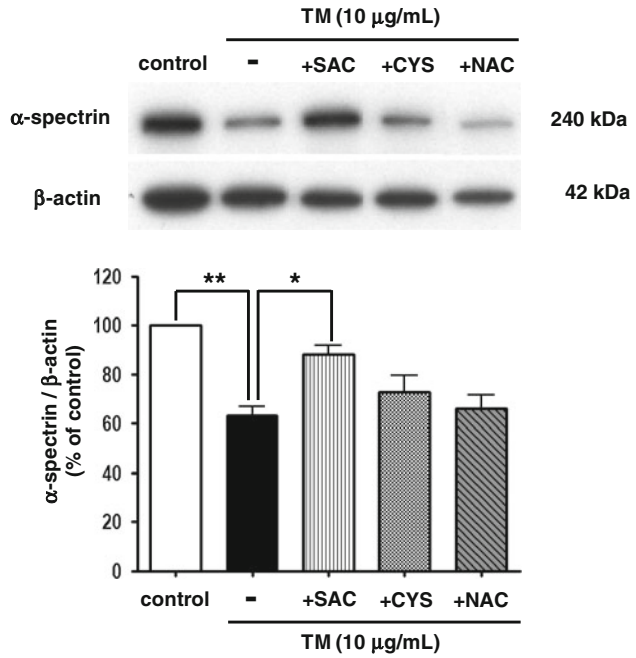

Fig. 3 Effects of cysteine derivatives on TM-induced α-spectrin degradation in HPNs. **a** The expression levels of full-length α-spectrin (240 kDa) were investigated by Western blotting after exposure to TM (10 μg/mL) in the absence or presence of SAC (1 mM), NAC (1 mM), and CYS (1 mM) for 24 h. Anti-β-actin antibody was used as an internal control. **b** Amounts of full-length α-spectrin were assessed by densitometric analysis, and the relative level of each band was calculated as described in "Materials and methods". Each value represents the mean ± SEM for four different experiments. $^{*}P < 0.05$, $^{**}P < 0.001$

calpain. Exposure of the HPNs to TM for 24 h resulted in a 63 % decrease of full-length α-spectrin levels (Fig. 3), and the decrease in the expression was significantly reversed by simultaneous application of 1 mM SAC, with a recovery of 90 %, whereas neither NAC nor CYS rescued the reduced level of expression (Fig. 3). In the absence of TM, none of these derivatives affected the levels of full-length α-spectrin (data not shown).

Effects of calpeptin and cysteine derivatives on μ-calpain and m-calpain activity

Calpeptin is a typical synthesized inhibitor of calpain (Carragher 2006). Using a synthetic substrate for calpain (Suc-LLVY-Glo), we characterized calpeptin inhibition of recombinant μ-calpain and m-calpain activity in an in vitro assay system. As shown in Fig. 4a, μ-calpain activity was suppressed by calpeptin in a concentration-dependent manner ($IC_{50} = 0.064$ nM). Similarly to μ-calpain, calpeptin also concentration dependently decreased the activity of m-calpain ($IC_{50} = 0.422$ μM) (Fig. 4b). This value is comparable to the IC_{50} of m-calpain measured under similar conditions (Tsujinaka et al. 1988). The activities of μ- and m-calpains were completely blocked by calpeptin at 10 nM and 10 μM, respectively (Fig. 4a, b).

Next, the effects of SAC and two cysteine derivatives on calpain activity were examined using the same in vitro calpain assay system. Addition of SAC suppressed both μ- and m-calpain activity in a concentration-dependent manner (Fig. 4c, d), and the inhibitory potency of SAC for μ-calpain was higher than that for m-calpain. Statistically significant attenuation of the activity was observed at concentrations of 10 nM and higher for μ-calpain and 1 mM and higher for m-calpain. Unlike calpeptin, SAC did not completely suppress μ- and m-calpains at relatively higher concentrations, the maximal inhibition being 45 and 63 % of control, respectively (Fig. 4c, d). Unlike SAC, CYS and NAC had no effect on the activity of μ-calpain and m-calpain in this system (Fig. 4c, d).

Combined effects of SAC and ALLN on μ-calpain activity

To elucidate the possible interaction of calpain and SAC, we examined the inhibitory activity of calpain inhibitor in

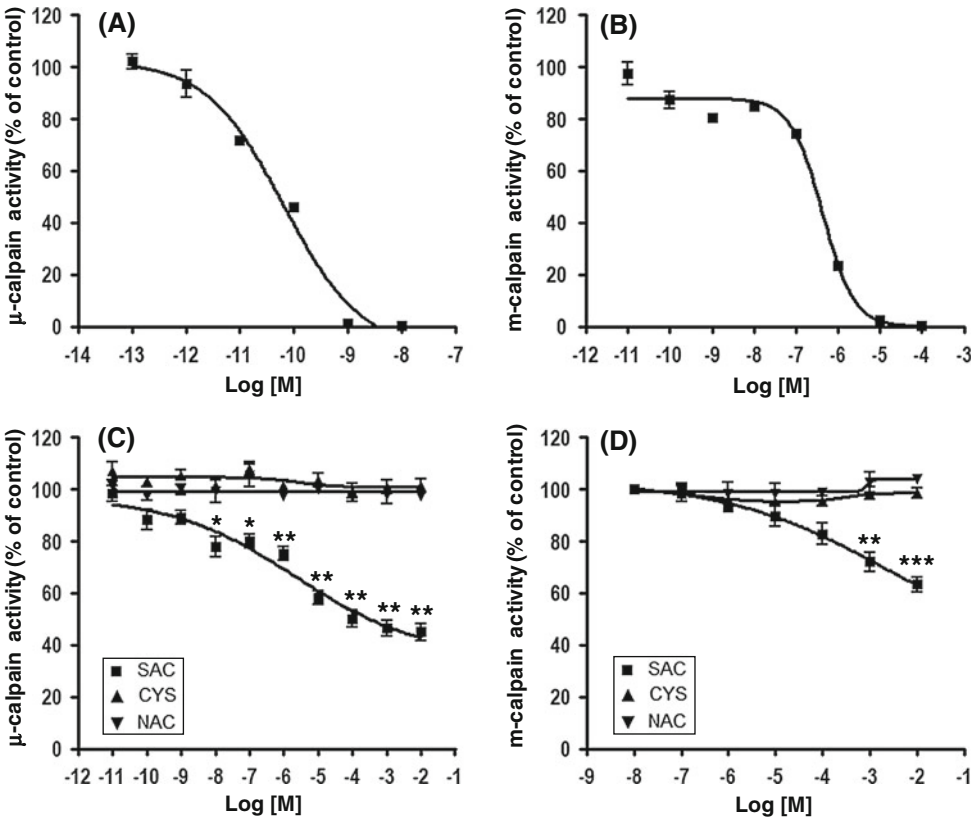

Fig. 4 Effects of calpeptin and cysteine derivatives on μ-calpain and m-calpain activity. Inhibition curves of μ-calpain (**a**) and m-calpain (**b**) with varying concentrations of calpeptin were created by curve fitting as described in "Materials and methods". Similarly, inhibition curves of μ-calpain (**c**) and m-calpain (**d**) with varying concentrations of SAC, CYS, and NAC were also created. Each value represents mean ± SEM for three (**a**, **b**) and four (**c**, **d**) different experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared to control (vehicle)

the absence and presence of SAC in this system. Treatment with ALLN, which acts on the active center of calpain (Carragher 2006), alone decreased the activity of μ-calpain in a concentration-dependent manner ($IC_{50} = 0.01$ nM) and completely blocked the activity at 10 nM (Fig. 5a). At submaximal concentration (5 pM), the inhibitory effect of ALLN on μ-calpain activity was comparable to that of 1 μM SAC, and the inhibitory effect of ALLN was potentiated by simultaneous application of 1 μM and 1 mM SAC (57 and 42 % of control, respectively) (Fig. 5b). In contrast, 1 mM SAC did not affect ALLN-induced inhibition at the supramaximal inhibitory concentration (1 nM) (Fig. 5c).

Combined effects of SAC and PD150606 on μ-calpain activity

As in the case of ALLN, the inhibitory activity of PD150606, which acts on the Ca^{2+}-binding site of calpain (Wang et al. 1996), was examined in the presence and absence of SAC. Treatment with PD150606 alone inhibited the decrease of μ-calpain activity in a concentration-dependent manner ($IC_{50} = 20.85$ μM) (Fig. 6a). Unlike ALLN, the inhibitory effect of 25 μM PD150606 on μ-calpain activity remained unaltered upon simultaneous application of 1 μM and 1 mM SAC (Fig. 6b); however, 1 mM SAC partially but significantly rescued the inhibition of PD150606 at the supramaximal inhibitory concentration (100 μM) (Fig. 6c).

Combined effects of SAC and calpastatin on μ-calpain activity

To determine whether SAC can modulate the effect of calpastatin, an endogenous calpain inhibitor, the effect of calpastatin on μ-calpain activity in the presence and absence of SAC was examined using this system. Treatment with calpastatin alone decreased the activity of μ-calpain in a concentration-dependent manner (Fig. 7a). Unlike ALLN and PD150606, calpastatin did not completely inhibit the activity, even at very high concentrations (maximal inhibition level = 85 %). At submaximal concentration (100 pM), the inhibitory effect of calpastatin on μ-calpain activity was potentiated by simultaneous application of 1 μM and 1 mM SAC (57 and 42 % of control, respectively) (Fig. 7b). As in the case of ALLN, 1 mM SAC did not affect calpastatin-induced inhibition at the supramaximal inhibitory concentration (100 nM) (Fig. 7c).

Discussion

S-Allyl-L-cysteine has already been shown to be absorbed in the gastrointestinal tract after oral administration and has

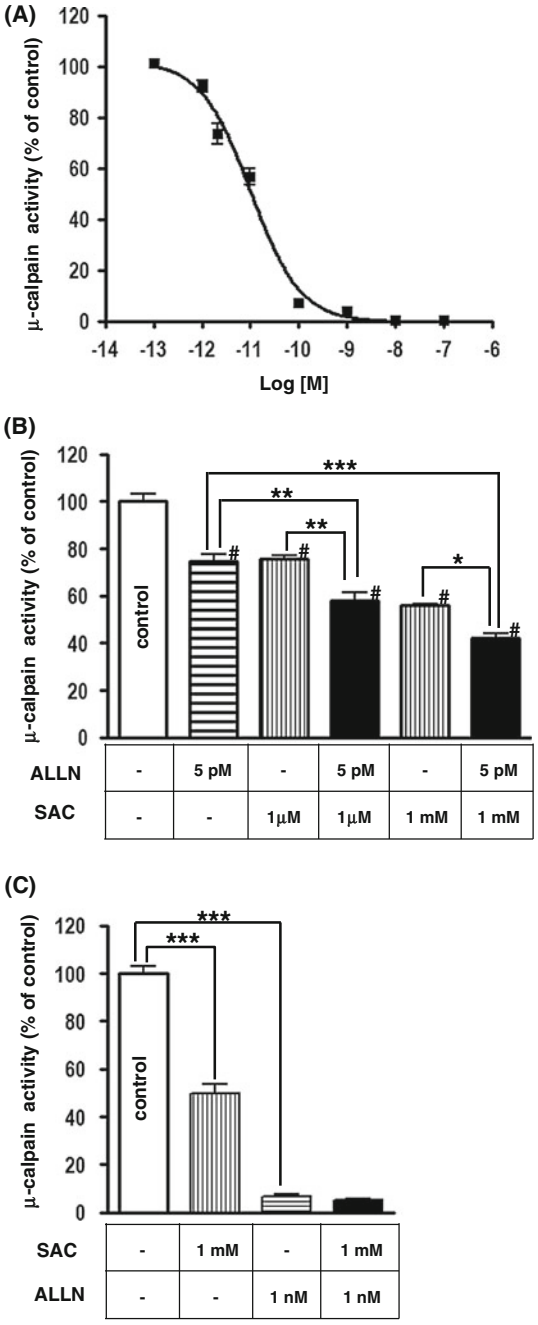

Fig. 5 Effects of SAC on inhibition of μ-calpain by ALLN. **a** Inhibition curve for μ-calpain with varying concentrations of ALLN was created by curve fitting as described in "Materials and methods". **b** ALLN (5 pM) with or without SAC (1 μM or 1 mM) incubated with 100 nM μ-calpain. **c** ALLN (1 nM) with or without SAC (1 mM) incubated with 100 nM μ-calpain. The calpain activity was examined using an in vitro assay system as described in "Materials and methods". Each value represents the mean ± SEM for three (**a**) or four (**b**, **c**) different experiments. $^{\#}P < 0.001$ as compared to control (vehicle), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

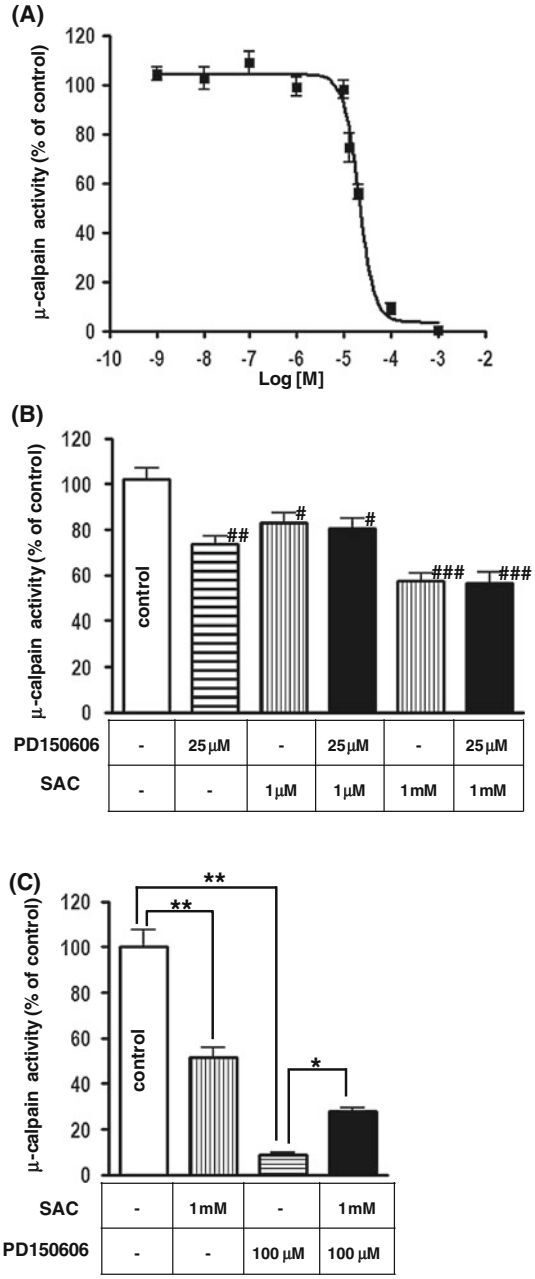

Fig. 6 Effects of SAC on inhibition of μ-calpain by PD150606. **a** Inhibition curve of μ-calpain with varying concentrations of PD150606 was created by curve fitting as described in "Materials and methods". **b** PD150606 (25 μM) with or without SAC (1 μM or 1 mM) incubated with 100 nM μ-calpain. **c** PD150606 (100 μM) with or without SAC (1 mM) incubated with 100 nM μ-calpain. The calpain activity was examined using an in vitro assay system as described in "Materials and methods". Each value represents the mean ± SEM for four different experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared to control (vehicle), $^{*}P < 0.05$, $^{**}P < 0.001$

been using for the treatment of patients with hypertension with no obvious signs of toxicity (Ried et al. 2010). Thus, SAC may be a useful therapeutic agent with few harmful effects. We have demonstrated previously that SAC exerts significant neuroprotective activity against ER stress-induced neurotoxicity in HPNs (Kosuge et al. 2003, 2006) and OHCs (Imai et al. 2007). A growing body of evidence

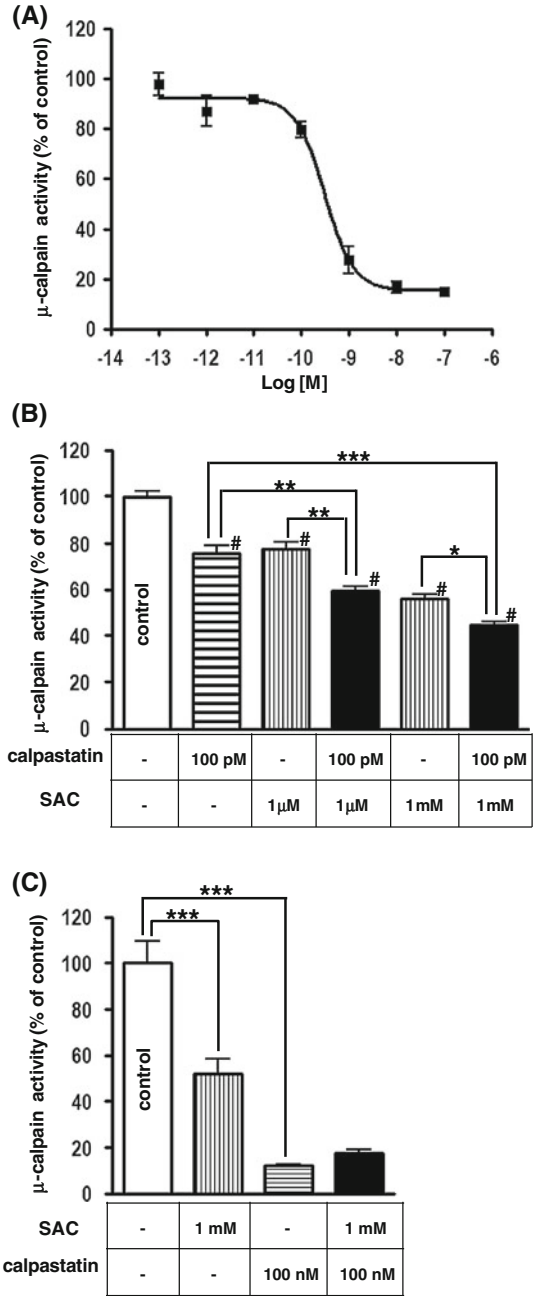

Fig. 7 Effects of SAC on inhibition of μ-calpain by calpastatin. **a** Inhibition curve of μ-calpain with varying concentrations of calpastatin was created by curve fitting as described in "Materials and methods". **b** Calpastatin (100 pM) with or without SAC (1 μM or 1 mM) incubated with 100 nM μ-calpain. **c** Calpastatin (100 nM) with or without SAC (1 mM) incubated with 100 nM μ-calpain. The calpain activity was examined using an in vitro assay system as described in "Materials and methods". Each value represents the mean ± SEM for three (**a**), four (**c**), and five (**b**) different experiments. $^{\#}P < 0.001$ as compared to control (vehicle), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

suggests that significant cross-talk occurs between the ER and mitochondria before neuronal cell death (Chen et al. 2012; Hedskog et al. 2013). Indeed, typical ER stressors, TG and BFA, have been shown to increase the levels of reactive oxygen species and induce cell death in HT22 murine hippocampal neuronal cells (Choi et al. 2010). On the basis of these findings, one possible explanation for the neuroprotective effect of SAC on Aβ- (Kosuge et al. 2003) and TM-induced hippocampal neuronal cell death (Kosuge et al. 2006) may be the antioxidant activity of SAC. Since two cysteine derivatives, CYS and NAC, have been shown to protect neurons from oxidative stress-induced damage induced by peroxynitrite (Lu et al. 2011) and 4-hydroxy-nonenal (Arakawa et al. 2007) at 1 mM, we sought to compare the effect of SAC with those of CYS and NAC at the same concentration (1 mM). Unlike SAC, CYS and NAC had no effect on TM-induced cell death in HPNs (Fig. 2b). These results suggest that the neuroprotective effect of SAC against TM-induced toxicity in HPNs may be attributable to suppression of ER stress-mediated signaling, and not to the oxidative stress-mediated cascade.

Recently, μ-calpain has been shown to be activated in the brains of AD patients (Liu et al. 2005) and mice transgenic for amyloid precursor protein (Vaisid et al. 2007). Furthermore, conjugated linoleic acid, a highly selective inhibitor of μ-calpain, inhibits Aβ-induced death in SH-SY5Y neuroblastoma cells (Lee et al. 2013). We have shown previously that pre-treatment of calpeptin suppressed TM-induced cell death in SK-N-SH neuroblastoma cells (Oda et al. 2008). Another calpain inhibitor, PD150606, has also been shown to attenuate TM-induced cell death in LLC-PK1 renal epithelial cells (Muruganandan and Cribb 2006). These results suggest that calpain inhibitors are protective against TM-induced cell death in different cell lines. We have shown that exposure of OHCs to TM significantly increased the activity of calpain (calpain-mediated proteolysis of α-spectrin) and the cleaved forms of caspase-12 (42 kDa) and caspase-3 (29 kDa), suggesting that the calpain-dependent caspase-12-related apoptotic pathway plays a pivotal role in TM-induced neuronal death in the hippocampus. Therefore, we investigated the potential role of calpain in TM-induced apoptosis in HPNs and measured calpain activity, as well as evaluated the effects of SAC and calpain inhibitors in vitro. In HPNs, we showed clearly that SAC suppressed the TM-induced degradation of full-length α-spectrin, a substrate for calpain, in these cells, whereas CYS and NAC had no effect on the decreased level of full-length α-spectrin (Fig. 3). Unlike calpeptin, SAC caused mild suppression of μ- and m-calpain activities in a concentration-dependent manner in the recombinant μ- and m-calpain assay in vitro (Fig. 4c, d), and significant decreases of μ-calpain and m-calpain activity were observed at concentrations of 10 nM and higher and 1 mM and higher, respectively. Taken together, these results suggest that μ-calpain is more sensitive to SAC than m-calpain, and that μ-calpain plays a

dominant role in TM-induced neuronal cell death. However, the specificities of the substrates for μ-calpain and m-calpain are very similar. Therefore, m-calpain might play a role, as least in part, in TM-induced neuronal cell death. In contrast, neither CYS nor NAC affected μ- and m-calpain activities in this system, suggesting that the sulfhydryl group did not play a role in the inhibition of calpains (Fig. 4c, d).

To characterize the interactive site(s) of μ-calpain for SAC, using an in vitro μ-calpain assay system we examined the possible interactions of SAC with three calpain inhibitors: ALLN, which interferes with the active center of calpain (Carragher 2006), PD150606, which interacts with the Ca^{2+}-binding site of calpain (Wang et al. 1996), and calpastatin, an endogenous and exclusive calpain inhibitor. When submaximal concentrations of calpain inhibitors were used, ALLN (5 pM) in combination with SAC (1 μM and 1 mM), and calpastatin (100 pM) in combination with SAC (1 μM and 1 mM) additively inhibited μ-calpain activity in vitro, whereas the inhibitory effect of PD150606 (25 μM) was unaffected (Figs. 5b, 6b, 7b). In contrast, at supramaximal concentration, SAC (1 mM) significantly reversed the inhibitory effect of PD150606 (100 μM), but did not affect ALLN (1 nM)- and calpastatin (100 nM)-induced inhibition of μ-calpain activity (Figs. 5c, 6c, 7c). These findings suggest that SAC interacts with the Ca^{2+}-binding site of μ-calpain, thus bringing about the conformational change that is necessary to activate the catalytic domain. However, the precise mechanism of interaction between SAC and the Ca^{2+}-binding site of μ-calpain still remains to be clarified.

It has been shown that depletion of calpastatin by multiple abnormally activated proteases accelerates calpain dysregulation in AD, thus leading to disruption of the cytoskeleton and neurodegeneration (Rao et al. 2008). Calpastatin may also participate in neurodegeneration where calpain plays an essential role in the ischemia-induced pathological cascade (Saido et al. 1997). Previous studies have shown that calpastatin inhibits calpain by occupying both sides of the active site cleft between domain I and domain II (Hanna et al. 2008) and the Ca^{2+}-binding site (Todd et al. 2003); however, the preferential site for calpastatin in calpain still remains speculative. Our data suggest that SAC may not interact with calpastatin at its binding site, since SAC markedly potentiates the inhibitory effects of calpastatin on μ-calpain at submaximal concentration (Fig. 7b), and the inhibitory effect of calpastatin at high concentration is unaltered by SAC (Fig. 7c). Taken together, the additive effects of SAC and calpastatin could be expected on the ER-dependent pathophysiological conditions.

It has been shown that the outcome of calpain inhibition depends on the potency of the inhibitors and the duration of inhibition. Inhibition of calpain by calpain inhibitor XI has been reported to protect photoreceptor cells against retinal degeneration in organotypic retinal explant cultures (Paquet-Durand et al. 2010). Although the protective effect of this compound was observed when the cells were exposed for short periods of time, a significant increase of TUNEL-positive cells was demonstrated after exposure for several days. Calpastatin reduced retinal degeneration-induced photoreceptor apoptotic cell death after both short and prolonged exposure (Paquet-Durand et al. 2010). In the present study, ALLN and PD150606 completely inhibited μ-calpain activity at higher concentrations in our in vitro assay system, whereas SAC and calpastatin partially inhibited the μ-calpain activity even at higher concentrations. Unlike potent synthetic calpain inhibitors, SAC exhibited a mild and weak inhibitory effect on μ-calpain activity, thus exhibiting protective effects against ER stress-dependent neuronal cell death in HPNs.

In conclusion, we have demonstrated for the first time that the protective effect of SAC against ER stress-induced neuronal cell death is at least partly attributable to direct inhibition of μ-calpain activity through binding of SAC to the Ca^{2+}-binding domain of this enzyme. These results directly support our previous study demonstrating that SAC provides a significant neuroprotective effect against ER stress-induced neuronal cell death and its potentiation by Aβ through regulation of calpain itself or Ca^{2+}–calpain interaction in OHCs. This novel effect of SAC on calpain may provide a therapeutic means of rescuing hippocampal neurons in patients with neurological and/or ischemic disorders associated with ER stress and/or calpain overactivation.

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Conflict of interest The authors declare that they have no conflict of interest.

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