Carnosic acid suppresses the production of amyloid－β 1-42 by inducing the metalloprotease gene TACE/ADAM17 in SH-SY5Y human neuroblastoma cells.

（カルノシン酸はSH-SY5Yヒト神経芽腫細胞においてメタロプロテアーゼ遺伝子TACE/ADAM17の誘導によりアミロイドβ（1-42）の産生を抑制する）

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ABSTRACT

A hallmark of Alzheimer's disease (AD) is the aggressive appearance of plaques of amyloid beta (Aβ) peptides, which result from the sequential cleavage of amyloid precursor protein (APP) by the β- and γ-secretases. Aβ production is evaded by alternate cleavage of APP by the α- and γ-secretases. Carnosic acid (CA) has been proven to activate the transcription factor Nrf2, a main regulator of the antioxidant response. We investigated the effects of CA on the production of Aβ 1-42 peptide (Aβ42) and on the expressions of the related genes in SH-SY5Y human neuroblastoma cells. The treatment of cells with CA suppressed Aβ42 secretion (61% suppression at 30 μM). CA treatment enhanced the mRNA expressions of an α-secretase *TACE (tumor necrosis factor-α-converting enzyme*, also called *a disintegrin and metalloproteinase-17, ADAM17*) significantly and another α-secretase *ADAM10* marginally; however, the β-secretase *BACE1 (β-site APP-cleaving enzyme-1)* was not increased by CA. Knockdown of *TACE* by siRNA reduced soluble-APPα secretion enhanced by CA and partially recovered the CA-suppressed Aβ42 secretion. These results suggest that CA reduces Aβ42 production by activating TACE without promoting BACE1 in human neuroblastoma cells. The use of CA may have a potential in the prevention of Aβ-mediated diseases, particularly AD.

Key words: Carnosic acid; Aβ42; TACE; ADAM17; Alzheimer's disease; SH-SY5Y

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; BACE1, β-site APP-cleaving enzyme-1; BCA, bicinchoninic acid; BSA, bovine serum albumin; CA, carnosic acid; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate; CHX, cycloheximide, DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; ECE 1, endothelin-converting enzyme-1; ELISA, enzyme-linked immumosorbent assay; FBS, fetal bovine serum; HRP, horseradish peroxidase; IDE, insulin-degrading enzyme; MAPK, mitogen-activated protein kinase; MT1-MMP, membrane type-1 matrix metalloprotease; NEP, neprilysin; NF-kappaB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGF, nerve growth factor; Nrf2, nuclear factor-erythroid 2-related factor 2; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; PS1, presenilin-1; PVDF, polyvinylidene fluoride; RT-PCR, reverse transcription-polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; sAPPα, soluble APPα; sAPPβ, soluble APPβ; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TACE, tumor necrosis factor-α-converting enzyme

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia affecting up to one-third of individuals who reached the age of 80 (Tanzi and Bertram, 2005). The important pathological features of AD are known to be the aggregation and accumulation of plaques of amyloid beta (Aβ) peptides and to be the phosphorylation and tangles of the neurofibrillary protein tau (Hardy and Selkoe, 2002). Aβ is produced by the sequential cleavage of amyloid precursor protein (APP) by β-secretase together with γ-secretase (its main part is presenilin-1; PS1) (Gandy, 2005; Mattson, 2004). β-Secretase includes the aspartic protease BACE1 (β-site APP-cleaving enzyme-1), which cleaves APP at the N-terminus of the Aβ domain (β-site), and thereby catalyzes the first and important step in Aβ generation (Vassar et al., 1999). An N-terminal soluble ectodomain fragment (~100-kDa, termed sAPPβ) of APP produced by β-secretase seems to have a proapoptotic function (Nikolaev et al., 2009). The production of Aβ can be prevented if APP is cleaved first, by α-secretase, at the α-site within the Aβ domain. In addition, α-secretase generates an N-terminal soluble form termed sAPPα, which has been reported to have neurotrophic and neuroprotective properties (Furukawa et al., 1996; Meziane et al., 1998; Stein et al., 2004). Importantly, promoting α-site APP-cleavage may provide a therapeutic approach for AD because it

can reduce Aβ production (Fahrenholz, 2007). Some different membrane-tethered proteases of the ADAM (a disintegrin and metallopoteinase) family, especially ADAM10 and ADAM17 (also called TACE, tumor necrosis factor-α-converting enzyme), have been implicated in APP cleavage by α-secretase (Kojro and Fahrenholz, 2005).

Carnosic acid (CA), a low-molecular electrophilic compound in rosemary (*Rosmarinus officinalis* L.), promotes the activation of the transcription factor Nrf2 (nuclear factor-erythroid 2 related factor 2), a master regulator of the antioxidant response (Satoh et al., 2008a,b; Takahashi et al., 2009). CA induces the Nrf2-dependent expression of *nerve growth factor (NGF)* in T98G glioblastoma cells and normal human astrocytes (Mimura et al., 2011). Moreover, CA and edaravone, a free radical scavenger, synergistically enhance NGF expression in both Nrf2- and phosphoprotein-dependent manners in human astrocytes (Yoshida et al., 2011). Importantly, CA has been reported to prevent neurotoxin-induced neuronal cell death (Park et al., 2008) and to promote neurite outgrowth of neuronal cells (Kosaka et al., 2010) by inducing various signaling pathways including Nrf2 (Johnson et al., 2008).

We hypothesized that CA possesses a molecular-biological potential for inducing neuroprotective reactions to manage Aβ production or alter the course of its accumulation process. In particular, the balance of α-cleavage and β-cleavage of APP is critical to determine the production of Aβ. Aβ 1-42 peptide (Aβ42) is more

hydrophobic and oligomerizes more rapidly than Aβ40 (Bitan el al., 2002), implying that Aβ42 is an important therapeutic target (Kirkitadze and Kowalska, 2005). The purpose of this study was to investigate the effect of CA treatment on the processing Aβ42 in SH-SY5Y human neuroblastoma cells. We also examined the involvement of α-, β- and γ-secretases in the CA-modulated Aβ42 production, and tried to assess their possible mechanisms.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) containing GlutaMAX™-I, Advanced DMEM/F12, fetal bovine serum (FBS), Zy max™ horseradish peroxidase (HRP)-conjugated anti-goat IgG and Lipofectamine™ RNAiMAX transfection reagent were from Invitrogen™/LifeTechnologies. CA was kindly donated by Nagase Co., Ltd. SB203580, an inhibitor of p38 mitogen-activated protein kinase (MAPK), was from Biomol. U0126, an inhibitor of MAPK/extracellular signal-regulated kinase kinase 1/2 (MEK1/2), SP600125, an inhibitor of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), BAY11-7082, a pIkappaB-alpha inhibitor, SN50, an inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB), and a protein kinase B (Akt) inhibitor were from Calbiochem/Merck. LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor was from Cell Signaling. The NucleoSpin® RNA II total RNA isolation kit was from Macherey-Nagel GmbH & Co. KG. siRNAs for *TACE*, *ADAM10*, *Nrf2* and *IDE* were from Qiagen. iScript™ cDNA synthesis kit and SsoFast™ EvaGreen® Supermix solution for quantitative PCR were from Bio-Rad Laboratories, Inc. Oligonucleotide primers for reverse transcription-polymerase chain reaction (RT-PCR) and real-time

quantitative PCR were custom synthesized by Fasmac/Greiner Japan. The enzyme-linked immunosorbent assay (ELISA) kits for Ab42 (Human/Rat bAmyloid (42) ELISA Kit *Wako*, High-Sensitive) and for Ab40 (Human/Rat bAmyloid (40) ELISA Kit *Wako* II) and cycloheximide (CHX) were from Wako Pure Chemical Industries, Ltd. The Human sAPPα (highly sensitive) Assay Kit and the Human sAPPb-w (highly sensitive, wild type) Assay Kit were from Immuno-Biological Laboratories Co., Ltd. (IBL). The bicinchoninic acid (BCA™) protein assay kit was from Pierce®/Thermo Scientific. Polyvinylidene fluoride (PVDF) membranes and a Luminata™ Crescendo western HRP substrate were from Millipore Corporation. Anti-TACE (C2C3) and anti-APP (C2C3, C-terminal) antibodies were from GeneTex Inc. Anti-rabbit IgG-HRP and anti-mouse IgG-HRP were from Santa Cruz Biotechnology. An anti-actin antibody, bovine serum albumin (BSA) and CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate) were from Sigma-Aldrich.

2.2. Cell Culture

SH-SY5Y human neuroblastoma cells (ECACC No. 94030304) were from the European Collection of Cell Cultures and cultured in DMEM/F12 containing GlutaMAX™-I supplemented with 10% FBS. After cells had grown to 80% confluence, the medium was replaced with fresh Advanced DMEM/F12 containing 3% FBS for use in experiments. In

experiments with CHX, SB203580, U0126, SP600125, LY294002, Akt inhibitor, SN50 and BAY11-7082, cells were pretreated with the inhibitor for 1 h prior to the addition of CA. Except for SN50, all of the inhibitors and CA were dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO in culture medium was 0.1%, and control cells were treated with vehicle alone. SN50 was dissolved in phosphate-buffered saline (PBS, pH 7.4).

2.3. RNA interference (RNAi)

SH-SY5Y cells were transfected with non-silencing control siRNA or target gene siRNAs using a Lipofectamine™ RNAiMAX transfection reagent according to the manufacturer's protocol. The sequences of the non-silencing control siRNA and *Nrf2* siRNA (Hosoya et al., 2005) were as follows:

control siRNA-sense *(5'-UUCUCCGAACGUGUCACGUdTdT-3')*,

control siRNA-antisense *(5'-ACGUGACACGUUCGGAGAAdTdT-3')*,

Nrf2 siRNA-sense *(5'-GAGUAUGAGCUGGAAAAACdUdU-3')*,

and *Nrf2* siRNA-antisense *(5'-GUUUUUCCAGCUCAUACUCdUdU-3')*. The products named Hs_ADAM17_7 (Qiagen) for *TACE* siRNA, Hs_ADAM10_6 (Qiagen) for *ADAM10* siRNA and Hs_IDE_6 (Qiagen) for *IDE* siRNA were also used. After 24 h incubation, the cells were washed,

and fresh Advanced DMEM containing 3% FBS was added. The cells were then treated with CA. Conditioned medium was collected, and total RNA was extracted after incubation for up to 24 h.

2.4. Real-time quantitative PCR

Total RNA was extracted, and single-strand cDNA was synthesized using an iScript™ cDNA synthesis kit. A CFX96™ Real-Time PCR System (Bio-Rad) was used for quantitative analyses of *18S rRNA* and mRNA for *TACE, ADAM10* (Marshall et al., 2006), *BACE1* (Li et al., 2010), *PS1* (Nord et al., 2010), *APP* (Yasojima et al., 2001), *hemoxygenase-1 (HO-1)* (Mimura et al., 2011), *neprilysin (NEP)*, *insulin-degrading enzyme (IDE)*, *endothelin-converting enzyme-1 (ECE1)* (Wang et al., 2010) and *matrix metalloprotease-9 (MMP-9)* (Han et al., 2010). Single-stranded cDNA, prepared as above, was used as a template and amplified according to the manufacturer's specifications. The sequences of the sense (forward; -F) and antisense (reverse; -R) primers were as follows:

TACE-F (5'-TTGGTGGTAGCAGATCATCG-3'),

TACE-R (5'-CTGGGAGAGCCAACATAAGC-3'),

ADAM10-F (5'-TTTGATGATGGCGTACTTGG-3'),

ADAM10-R (5'-AGTTTGTCCCCAGATGTTGC-3'),

BACE1-F (5’-CCGGCGGGAGTGGTATTATG-3’),

BACE1-R (5’-GCAAACGAAGGTTGGTGGT-3’),

PS1-F (5’-GGTAAAGCCTCAGCAACAGC-3’),

PS1-R (5’-AAACAAGCCCAAAGGTGATG-3’),

APP-F (5’-ACGAAGAAGCCACAGAGAGA-3’),

APP-R (5’-TTCATTCTCATCCCCAGGTG-3’),

HO-1-F (5’-CCAGCAACAAAGTGCAAGATTC-3’),

HO-1-R (5’-TCACATGGCATAAAGCCCTACAG-3’),

NEP-F (5’-GCCTCAGCCGAACCTACAAG-3’),

NEP -R (5’-AATTTGCACAACGTCCTCAAGTT-3’),

IDE-F (5’-GCCGAAGCCTTGTCTCAACT-3’),

IDE -R (5’-CAAATAGGCCATGTTACAGTGCAA-3’),

ECE1-F (5’-GACGCCGATGAGAAGTTCATG-3’),

ECE1-R (5’-GCAAAACTTCCAGCGAGGAA-3’),

MMP-9-F (5’-TCCCTGGAGACCTGAGAACC-3’),

MMP-9-R (5’-GGCAAGTCTTCCGAGTAGTTT-3’),

18S rRNA-F (5’-ACTCAACACGGGAAACCTCA-3’),

and *18S rRNA-R (5’-AACCAGACAAATCGCTCCAC-3’)*.

The primers were used at 300 nM. The amplification was performed with a SsoFast™

EvaGreen® Supermix solution with the following reaction conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s, 55°C for 10 s and 60°C for 15 s; and an increase from 65-95°C, 0.1°C/s, for melting. Data were analyzed with CFX Manager™Version 2.1 software.

2.5. ELISA

Samples of cell-conditioned medium were mixed with 0.2% BSA and 0.075% CHAPS to minimize losses by adhesion. These were centrifuged briefly, and the supernatant was stored at -80°C until use. The levels of Ab42, Ab40, sAPPα or sAPPb in the media were determined using each ELISA kit mentioned above according to the manufacturer's instructions.

2.6. Immunoblot analysis

Cells cultured in a 35 mm-diameter dish were washed twice with ice-cold 20 mM PBS. The cells were then lysed in 50 μL of Laemmli's reducing sample buffer and incubated on ice for 30 min with occasional vortexing. Lysates were centrifuged at 10000 X *g* for 20 min at 4°C, and the extract supernatants were transferred to clean tubes. Aliquots (9 μL) of extracts were mixed with 1.8 μL of 6X loading buffer for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and boiled for 5 min. The

samples were subjected to electrophoresis on 7.5% or 10-20% SDS-polyacrylamide gels, and proteins were transferred onto PVDF m embranes. The membranes were blocked by incubating in 1X TBST buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 0.1% Tween-20) containing either 1% nonfat dry milk or 1% BSA for 1 h at room temperature. Then, the membranes were incubated with anti-TACE antibody (1:1000) or anti-APP antibody (1:1000), followed by HRP-conjugated anti-rabbit IgG (secondary antibody). The immunoreactive bands were visualized by using chemiluminescence detection reagents. The same blot was then reprobed using anti-actin antibody (1:2000).

2.7. Statistics

Values are expressed as means ± SD, and statistical significance was analyzed by Student's *t*-test. All probability (*P*) values were based on two-tailed tests, and $P < 0.05$ was considered significant.

3. Results

3.1. Influence of CA treatment on the secretions of Aβ42, Aβ40, sAPPα and sAPPb fragments

The concentration-dependent effects of CA on the levels of Aβ42, Aβ40, sAPPα and sAPPb fragments of full-length APP secreted from cultured SH-SY5Y human neuroblastoma cells are shown in Fig. 1. Untreated control cells constitutively released Aβ42 into cell-conditioned medium for 8 and 24 h. Either 8- or 24-h CA treatment significantly lowered Aβ42 production in a concentration-dependent manner. Compared with the control, Aβ42 production was reduced to 39% when cells were treated with 30 μM CA for 24 h. In addition, the inhibition rates of Aβ42 production increased from 50% (30 μM CA treatment for 8 h) to 61% (30 μM CA treatment for 24 h) (Fig 1A).

Similar to Aβ42, Aβ40 secretion was also decreased by CA in a concentration-dependent manner (Fig. 1B) and there was no significant change in Aβ42/Aβ40 ratio depending on the concentration of CA (Fig. 1C). On the other hand, the production of sAPPα (α-cleaved soluble fragment of APP) was enhanced by CA in a concentration-dependent manner (Fig 1D), indicating that CA promotes α-cleavage rather than b-cleavage. The reduction of sAPPb (b-cleaved soluble fragment of APP)

by CA treatment also confirmed the reduction of Aβ42 and Aβ40 by CA (Fig. 1E).

3.2. Influence of CA treatment on the expressions of secretases and APP

The results of the analyses, by real-time quantitative PCR, on the mRNA expressions of *TACE*, *ADAM10*, *BACE1*, *PS1* and *APP* in SH-SY5Y cells are shown in Figs. 2A and 2B. As shown in Fig. 2A, treatment with 30 μM CA for up to 24 h enhanced the mRNA expressions of *TACE*, *ADAM10*, *PS1* and *APP* at different levels (α-secretase *TACE* or *ADAM10* mRNA was increased by 2.1- or 1.5-fold at 6 h, and by 3.6- or 1.1-fold at 24 h, respectively). However, little change was observed in *BACE1* mRNA expression. As shown in Fig. 2B, CA treatment at concentrations up to 30 mM for 6 h enhanced the mRNA expressions of *TACE* (up to 2.8-fold), *ADAM10* (up to 1.7-fold) and *PS1* (up to 2.3-fold) in a concentration-dependent manner. However, CA had little effect on the mRNA expressions of *BACE1* and *APP*. In addition, CA did not significantly enhance the mRNA expression of *MMP-9*, a candidate for alternative α-secretase (data not shown). Western blot analyses revealed that CA treatment for 24 h enhanced the expression of TACE protein in SH-SY5Y cells in a concentration-dependent manner, whereas the levels of full-length APP were insignificantly altered (Fig. 2C).

Pretreatment of the cells with CHX did not decrease the CA-enhanced three genes

(*TACE*, *ADAM10* and *PS1* mRNA), indicating that the effects of CA on these expressions were not due to *de novo* synthesis of other protein mediator(s) (Fig. 2D). CA is known to affect the expression of NGF via the MAPK signaling pathways (Yoshida et al., 2011). However, no treatment with MAPK inhibitors (the p38 MAPK inhibitor SB203580, the MEK1/2 inhibitor U0126 or the JNK inhibitor SP600125) or other signaling inhibitors (the PI3K inhibitor LY294002, the Akt inhibitor, the NF-kappaB inhibitor SN50 or the IkappaB-alpha inhibitor BAY11-7082) inhibited the CA-enhanced mRNA expressions in SH-SY5Y cells (Fig. 2D).

3.3. Influence of TACE siRNA on the secretions of sAPPα and Aβ42

We next investigated whether the CA-induced shedding of APP requires TACE. To test this, SH-SY5Y cells were transfected with control siRNA or siRNA against *TACE* and then treated with or without CA (Fig. 3). The enhanced expressions of *TACE* mRNA (Fig. 3A) and protein (Fig. 3B) in response to CA were suppressed by *TACE* siRNA. The protein levels of APP were not altered by *TACE* siRNA in the cells stimulated with or without CA (Fig. 3B). CA-induced sAPPα secretion was reduced by *TACE* siRNA, implying that CA promoted α-cleavage of APP by activating α-secretase TACE (Fig. 3D). Also, the knockdown of *TACE* by siRNA slightly recovered the CA-suppressed Aβ42 production (Fig.

3C), suggesting that CA suppresed the Aβ42 secretion, at least in part, by promoting the expression of TACE in SH-SY5Y cells.

3.4. Influence of ADAM10 siRNA on the secretions of sAPPα and Aβ42

In order to examine whether CA-suppressed Aβ42 secretion also requires another α-secretase ADAM10, we performed the same experiments as *TACE* knockdown (Fig. 4). Both constitutive (CA-free DMSO) and CA-induced secretions of sAPPα were suppressed down to the same level by *ADAM10* siRNA, suggesting that CA can induce the α-cleavage of APP by not only TACE but also ADAM10 (Fig. 4C). However, *ADAM10* siRNA did not increase the CA-suppressed Aβ42 production (Fig. 4B).

3.5. Influence of Nrf2 siRNA on the CA-suppressed Aβ42 secretion

CA is also known to affect a variety of gene expressions via the transcription factor Nrf2 accumulation (Mimura et al., 2011; Yoshida et al., 2011). However, knockdown of *Nrf2* by siRNA did not affect the CA-suppressed Aβ42 production in SH-SY5Y cells (Fig. 5).

3.6. Influence of CA treatment on the expressions of Aβ-degrading enzymes

Aβ is a physiological peptide, which is constantly anabolized and catabolized in the brain (Seubert et al., 1993). Therefore, we examined whether CA has a function that promotes the degradation of Aβ42 by activating Aβ-degrading enzymes, such as NEP, IDE and ECE1. Although CA slightly enhanced the expression of *IDE* mRNA in time- and concentration-dependent manners (Figs. 6A and 6B), knockdown of *IDE* by siRNA did not affect the CA-suppressed Aβ secretion (Figs. 6C and 6D).

4. Discussion

We revealed that CA treatment clearly suppressed the level of Aβ42 peptide secretion in cultured SH-SY5Y human neuroblastoma cells. This finding is strengthened by more evidence that Aβ40 and sAPPβ (products of amyloidogenic pathway) decreased in parallel with Aβ42 in CA treatment, whereas sAPPα (a product of non-amyloidogenic pathway) increased reciprocally with Aβ42, Aβ40 and sAPPβ. We also found that CA influenced the mRNA expressions of secretases (*TACE, ADAM10*, *BACE1*and *PS1*) and of their substrate (*APP*). To assess their possible mechanisms for the effect of CA, we calculated the integrated values of mRNA expressions for up to 24 h based on their time-courses in Fig. 2A and summarized them in Fig. 7. As a result, the mRNA expressions of *TACE* and *PS1* were drastically increased in cells treated with CA. These result suggest that the production of Aβ42 can be inhibited by the CA enhanced-expression of *TACE*, which is in agreement with the ideas that activating *TACE* by pharmacological manipulation might prove beneficial in Alzheimer's disease (Buxbaum et al., 1998) and that the upregulated component of α-secretase can compete with β-secretase and consequently reduces Aβ42 production (Kuhn et al., 2010).

In order to validate the importance of *TACE*, we analyzed the levels of sAPPα and Aβ42 secretions from the cells transfected with control siRNA or siRNA against *TACE*.

Because knockdown of *TACE* by siRNA reduced the CA-enhanced sAPPα (Fig. 3D) and recovered the CA-suppressed Aβ42 production slightly but significantly (Fig. 3C), we assert that α-secretase TACE is at least partially responsible for the observed Aβ42 suppression in SH-SY5Y cells. Indeed, the synthetic phorbol ester PMA-induced shedding of APP requires TACE activity since this shedding is lost in mouse em bryonic fibroblasts deficient in TACE (Buxbaum et al., 1998; Kuhn et al., 2010). However, the influence on the increase of TACE protein and sAPPα secretion does not fully explain the effect of CA on Aβ42 production because *TACE* siRNA recovered the CA-suppressed Aβ42 production only slightly.

Another α-secretase ADAM10 has been also reported to be involved in the non-amyloidogenic pathway (Xu et al., 2009). In our cell culture experiments, the levels of CA-enhanced *ADAM10* mRNA were low (1.5-fold at 6 h, 1.1-fold at 24 h) and the knockdown of *ADAM10* by siRNA did not attenuate the CA-suppressed Aβ42 production (Figs. 4A and 4B). However, *ADAM10* knockdown reduced both constitutive (CA-free DMSO) and CA-enhanced secretions of sAPPα down to the same level (Fig. 4C). These results suggest that the constitutive activity of ADAM10 is responsible for α-cleavage of APP even though the level of CA-induced *ADAM10* mRNA is low. Indeed, Kuhn et al. (2010) defined ADAM10 as the physiologically relevant, constitutive α-secretase based on their experiments using cultured cells including SH-SY5Y cells. At present, it is unclear why our results

of protein-fragment production using *ADAM10* siRNA did not show the reciprocal relationship between Aβ42 and sAPPα. However, because our *ADAM10* siRNA did not completely downregulate sAPPα production (Fig. 4C), it seems possible that the effect of *ADAM10* knockdown may not be enough for the lower levels of Aβ42 production.

Alternatively, additional metalloproteases of the ADAM or MMP family might also contribute to the upregulation of α-site APP cleavage. Based on over-expression studies, candidates for the upregulated α-secretase are matrix metalloprotease-9 (MMP-9), membrane type-1 MMP (MT1-MMP), MT3-MMP, MT5-MMP and ADAM8 (Ahmad et al., 2006; Amour et al., 2002; Higashi and Miyazaki, 2003; Lichtenthaler, 2011; Naus et al., 2006; Talamagas et al., 2007; Vaisar et al., 2009). These possibilities remain to be clarified in future studies except for MMP-9 whose mRNA was not significantly enhanced by CA treatment in this study.

Nord et al. (2010) reported that estrogen treatment of human primary neurons and glial cells in culture leads to a decrease in BACE protein expression as well as a downregulation of *TACE* and *PS1* gene expression. Li et al. (2010) demonstrated that salidroside (2-(4-hydroxyphenyl) ethyl β-D-glucopyranoside), a component of traditional Tibetan medicine extracted from the root of *Rhodiola rosea* L., inhibited the hypoxia-induced BACE1 mRNA and protein expression with no effect on the levels of TACE, ADAM10 and APP. Interestingly, our data showed the upregulation of the mRNA

expressions of *TACE* (also protein) and *PS1*; however, *BACE* expression was not promoted, suggesting that CA may also have an advantage in affecting APP processing directly by regulating the expression of these secretases.

PS1, one of the core proteins in γ-secretase complex, is shared in both amyloidogenic and non-amyloidogenic processing of APP. It has been reported that the moderate inhibition of γ-secretase represents an attractive anti-amyloid therapy for AD (Li et al., 2007). However, it is worth emphasizing our result that CA treatment did not downregulate, but rather, upregulated *PS1* expression (up to 2.3-fold). This is particularly intriguing in the light of recent data indicating that PS is not the final point of AD (Rodríguez-Manotas et al., 2012). In this context, the moderate upregulation of *PS1* expression by CA might provide a safer alternative approach that avoids side effects such as the inhibition of the cleavage of Notch (Imbimbo and Giardina, 2011; Panza et al., 2011).

From the results of this study, we conclude that CA significantly reduces the Aβ42 production in SH-SY5Y human neuroblastoma cells, and provides a possible mechanism by which activity of α-site APP-cleavage increases through the induction of TACE protein without inducing BACE1. The use of CA may have a potential in the prevention of Aβ-involved diseases, particularly AD.

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Conflict of interest statement

None declared.

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Figure legends

Fig. 1. Concentration-dependent effect of carnosic acid (CA) on the production of Aβ42, Aβ40, soluble amyloid precursor protein-α (sAPPα) or sAPPβ in cultured SH-SY5Y human neuroblastoma cells. Cells were treated with indicated concentrations of CA for up to 8 and 24 h. After incubation, cell-conditioned media were collected, and the levels of secreted Aβ42 (A), Aβ40 (B), sAPPα (D) and sAPPβ (E) proteins were determined by ELISA. The ratios of Aβ42/Aβ40 secretions are calculated (C). Means ± SD of three experiments are shown. $*P < 0.05$ vs. concurrent control (CA-free), based on Student's *t*-test.

Fig. 2. Time- and concentration-dependent effects of carnosic acid (CA) on the expressions of α-secretases (*TACE* and *ADAM10*), β-secretase (*BACE1*), γ-secretase (*PS1*) and amyloid precursor protein (*APP*) in SH-SY5Y human neuroblastoma cells. Means ± SD of three experiments are shown. (A) Time-course of mRNA expression. Cells were treated with 30 μM CA for up to 24 h. mRNA expression was analyzed by real-time quantitative PCR and normalized to *18S rRNA* expression (0 h control = 1). $*P < 0.05$ vs. concurrent control (CA-free DMSO). (B) Concentration-dependent effect of CA on mRNA expression after 6 h treatment. mRNA expression determined

by quantitative PCR was normalized to *18S rRNA* expression (CA-free control = 1). $*P < 0.05$ vs. CA-free control. (C) Effect of CA on the expressions of TACE protein and APP. Cell extracts were analyzed by western blotting and actin was analyzed as a loading control. (D) Effects of inhibitors on the CA-induced mRNA. Cells were pretreated with 500 ng/mL cycloheximide (CHX), 1 μM SB203580 (SB), 20 μM U0126 (U), 20 μM SP600125 (SP), 50 μM LY294002 (LY), 10 μM Akt inhibitor (Akt), 25 μg/mL SN50 (SN) or 10 μM BAY11-7082 (BAY) for 1 h, and then treated with 30 μM CA for 6 h. mRNA expression was determined by real-time quantitative PCR. $^{\#}P < 0.05$ vs. CA-free control; $*P < 0.05$ vs. CA-treated control (#).

Fig. 3. CA-induced decrease of Aβ42 production partially required TACE. SH-SY5Y human neuroblastoma cells were transfected with either control (Ctrl) siRNA or siRNA against *TACE* (*TACE* siRNA) and then treated with 30 μM CA or CA-free DMSO for 24 h. Means ± SD of three experiments are shown. $*P < 0.05$. (A) Quantitative PCR analysis for *TACE* mRNA expression. (B) Western blot analysis for the expressions of TACE protein and amyloid precursor protein (APP). Cell extracts were subjected to immunoblotting for TACE, APP or actin as described in Fig. 2. (C, D) ELISA for secreted Aβ42 (C) and soluble APP-α (sAPPα) (D). Cell-conditioned media were collected as described in Fig. 1.

Fig. 4. Knockdown of *ADAM10* by siRNA did not affect the CA-suppressed Aβ42 but reduced soluble amyloid precursor protein-α (sAPPα). SH-SY5Y human neuroblastoma cells were transfected with either control (Ctrl) siRNA or siRNA against *ADAM10* (*ADAM10* siRNA) and then treated with 30 μM CA or CA-free DMSO for 24 h. Means ± SD of three experiments are shown. $*P < 0.05$. (A) Quantitative PCR analysis for *ADAM10* mRNA expression. (B, C) ELISA for secreted Aβ42 (B) and sAPPα (C). Cell-conditioned media were collected as described in Fig. 1.

Fig. 5. Knockdown of *Nrf2* by siRNA did not affect the CA-suppressed Aβ42 production. SH-SY5Y human neuroblastoma cells were transfected with either control (Ctrl) siRNA or siRNA against *Nrf2* (*Nrf2* siRNA) and then treated with 30 μM CA or CA-free DMSO for 24 h. Means ± SD of three experiments are shown. $*P < 0.05$. (A) Quantitative PCR analysis for *HO-1* mRNA expression. *HO-1* is a representative target gene of Nrf2. (B) ELISA for secreted Aβ42 peptide. Cell-conditioned media were collected as described in Fig. 1.

Fig. 6. Effects of carnosic acid (CA) on the expressions of Aβ-degrading enzymes (*NEP*, *IDE* and *ECE1*) in SH-SY5Y human neuroblastoma cells. Means ± SD of three experiments

are shown. (A) Time-course of mRNA expression. Cells were treated with 30 μM CA for up to 24 h. mRNA expression was analyzed by real-time quantitative PCR and normalized to *18S rRNA* expression (0 h control = 1). (A) Time-course of mRNA expression. Cells were treated with 30 μM CA for up to 24 h. mRNA expression was analyzed by real-time quantitative PCR and normalized to *18S rRNA* expression (0 h control = 1). $*P < 0.05$ vs. concurrent control (CA-free DMSO). (B) Concentration-dependent effect of CA on *IDE* mRNA expression after 6 h treatment. mRNA expression determined by quantitative PCR was normalized to *18S rRNA* expression (CA-free control = 1). $*P < 0.05$ vs. CA-free control. (C) Quantitative PCR analysis for *IDE* mRNA expression. Cells were transfected with either control (Ctrl) siRNA or siRNA against *IDE* and then treated with 30 μM CA or CA-free DMSO for 24 h. $*P < 0.05$. (D) ELISA for secreted Aβ42 peptide. Cells were transfected and treated as described in (C). $*P < 0.05$. Knockdown of *IDE* by siRNA did not affect the CA-suppressed Aβ42 secretion.

Fig. 7. Potential mechanisms for the suppressive effect of carnosic acid (CA) on Aβ42 production in human neuroblastoma cells. A number in a parenthesis indicates a relative value calculated by the integration of an mRNA's fold-change curve from 0 to 24 h in Fig. 2A. Secretases cleave amyloid precursor protein (APP) at its α-, β- or γ-site in the plasma membranes of the cells; β-secretase, together

with γ-secretase, generates Aβ42. Overall, CA does not induce β-secretase and may suppress Aβ42 production via the concurrent induction of α-secretase, particularly TACE/ADAM17, which cleaves APP at the inner site of Aβ42.

Figure(s)

Fig. 1 Meng et al.

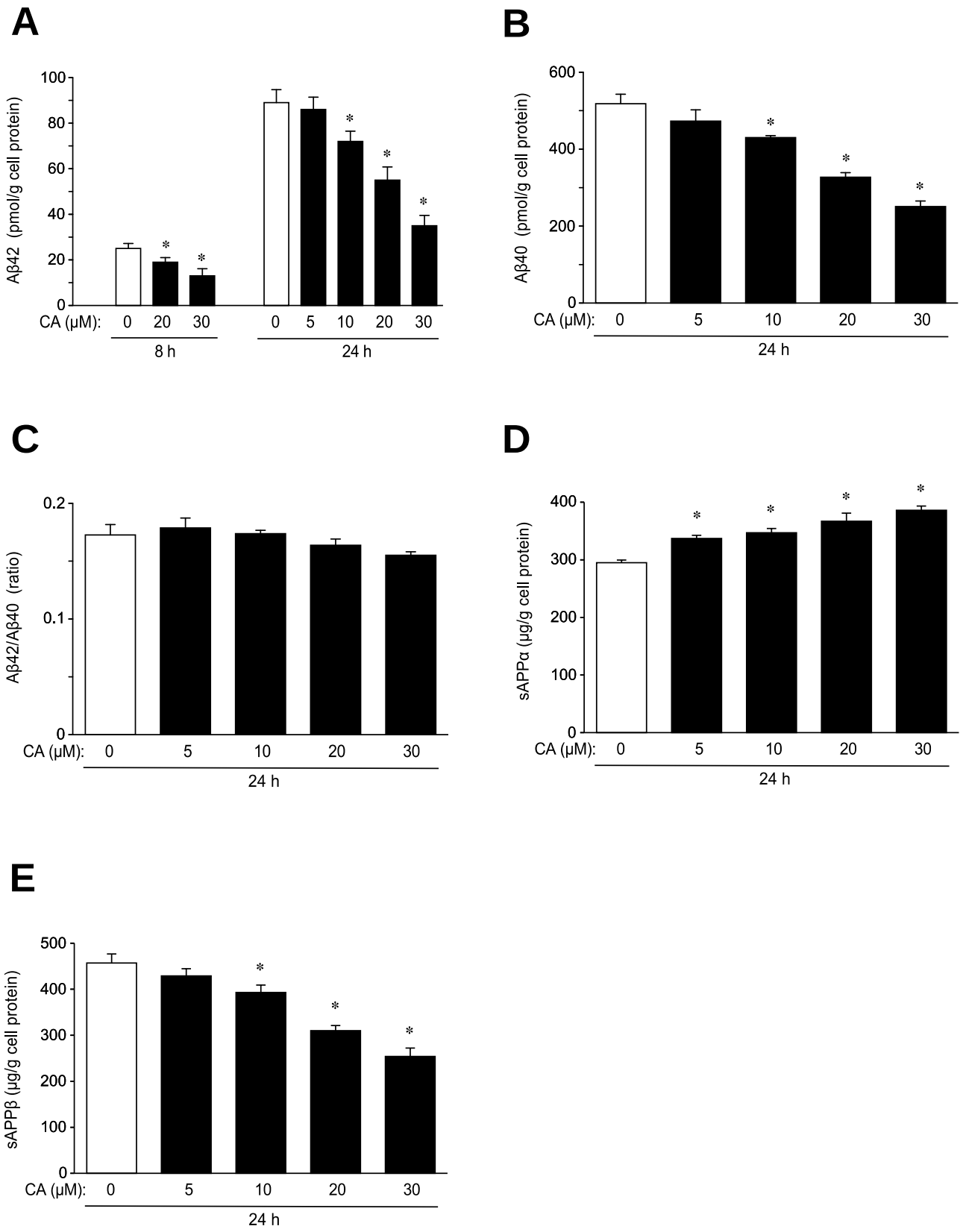
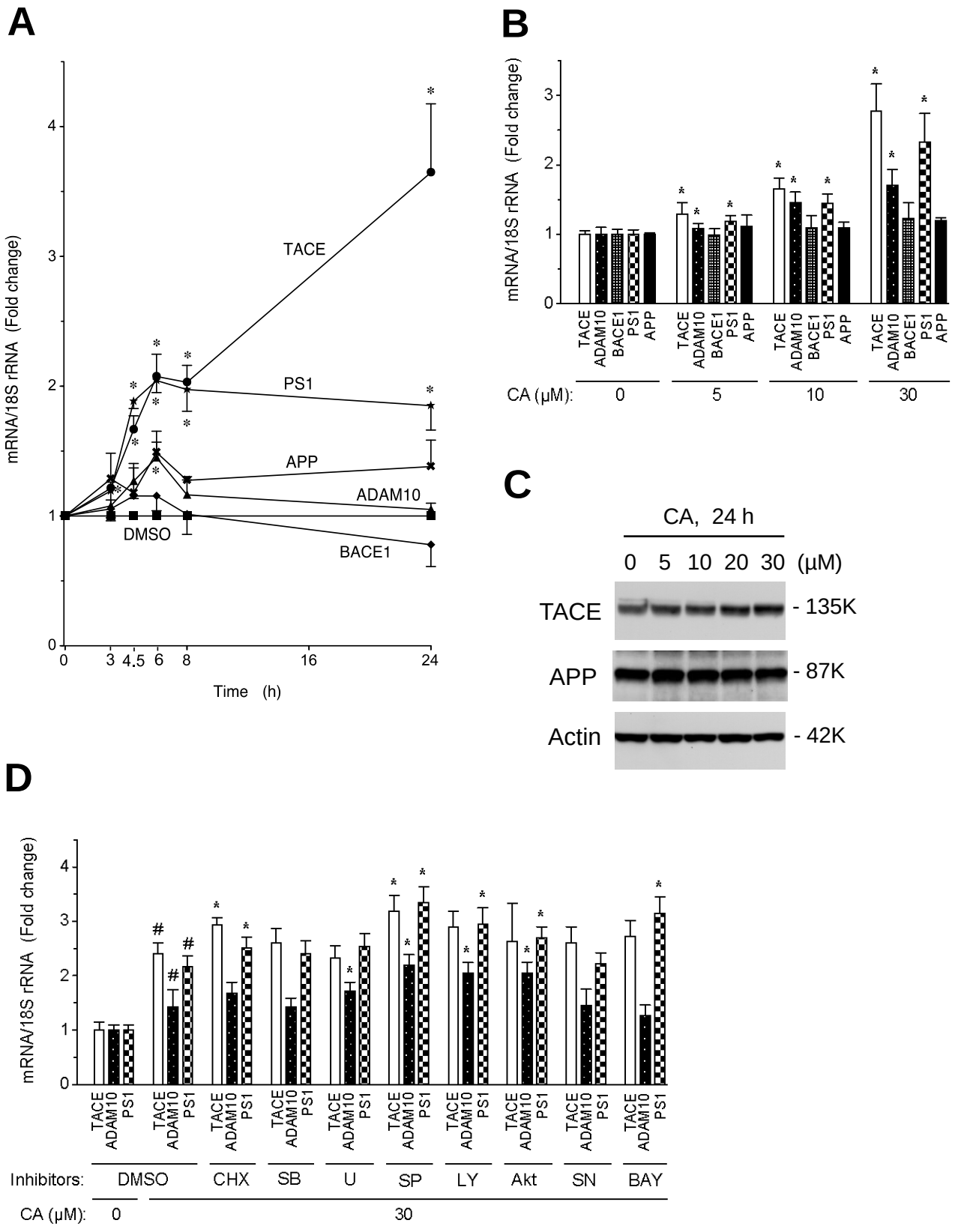


Fig. 2 Meng et al.



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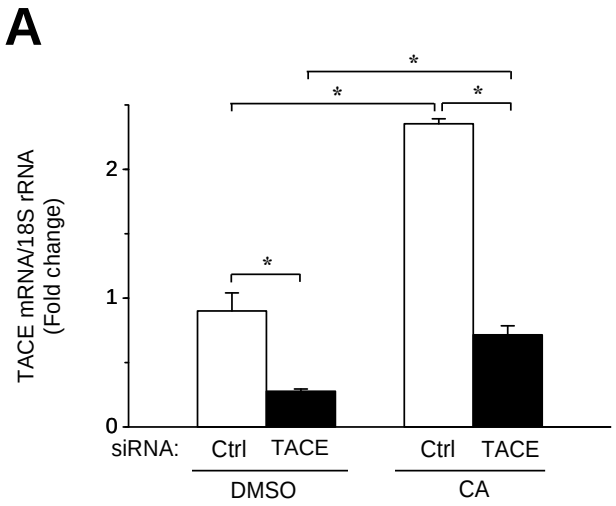
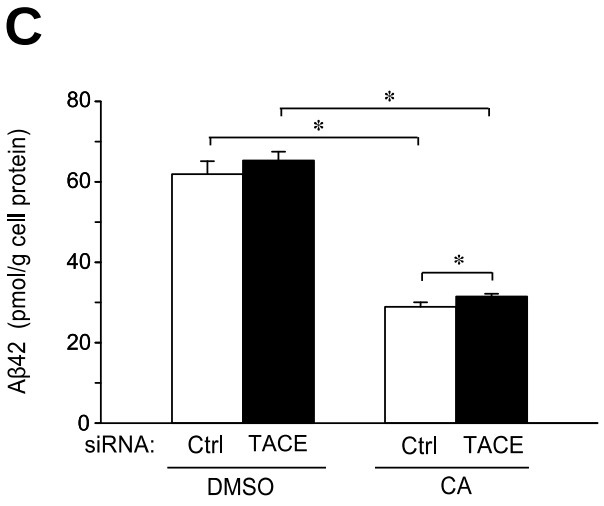
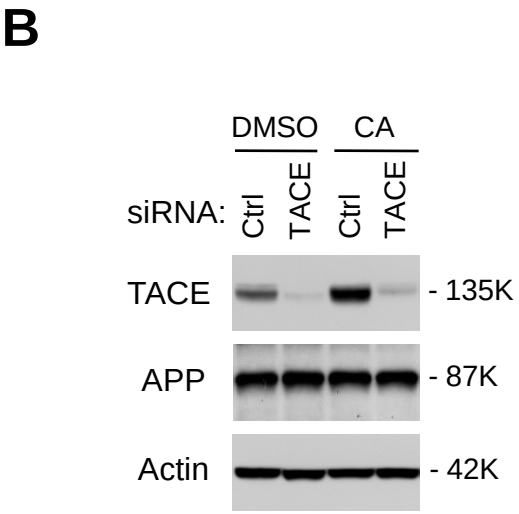






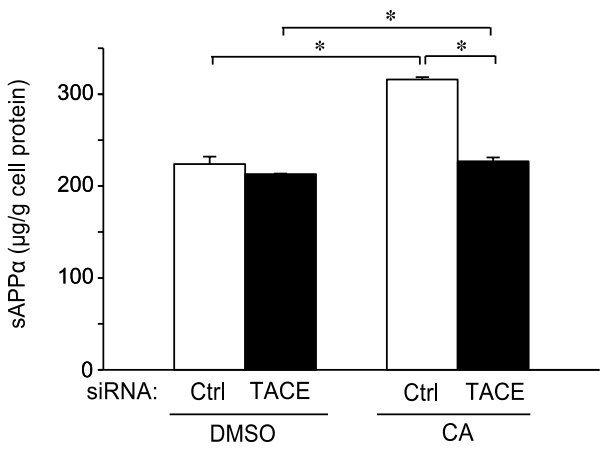
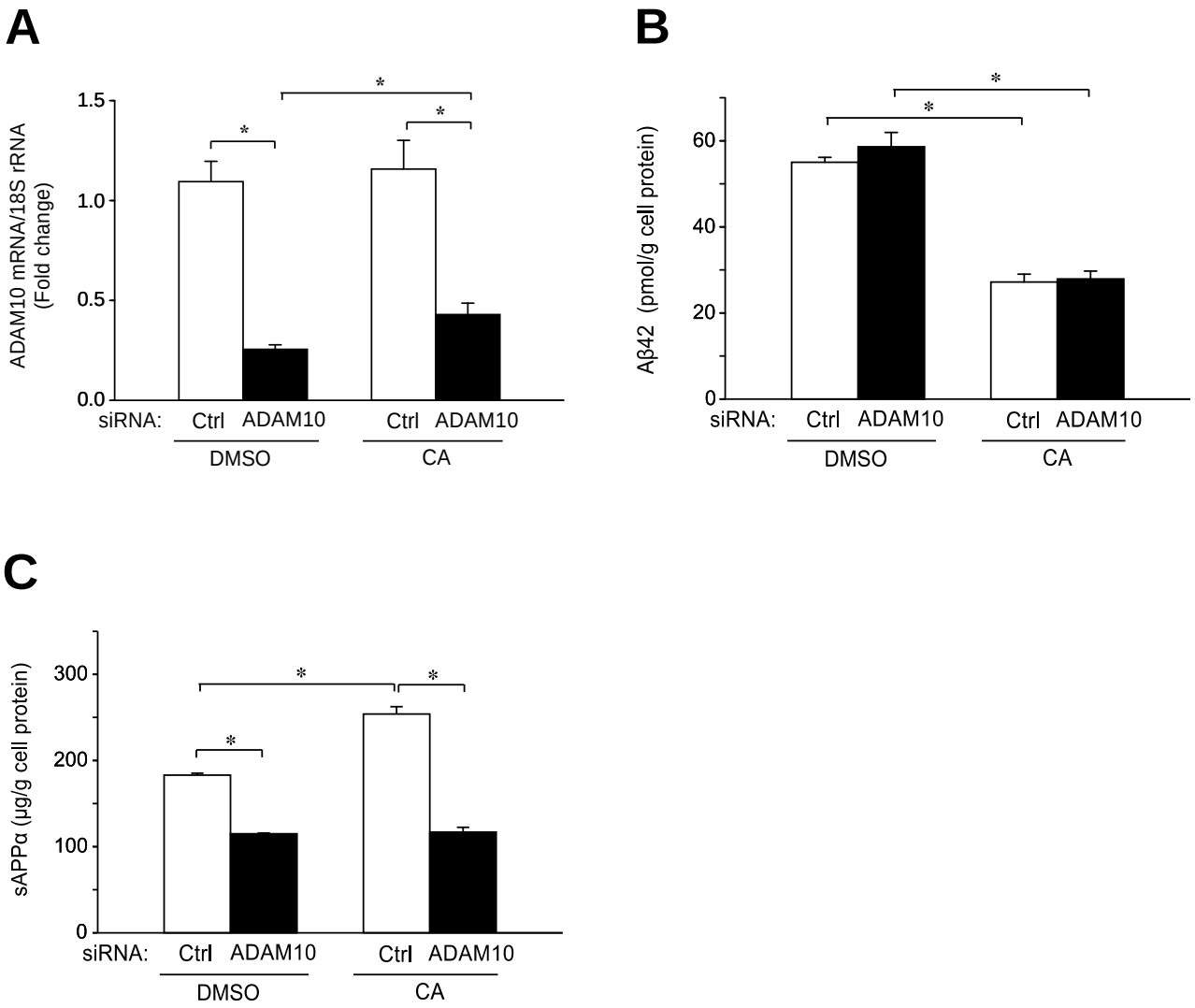


Fig. 4 Meng et al.



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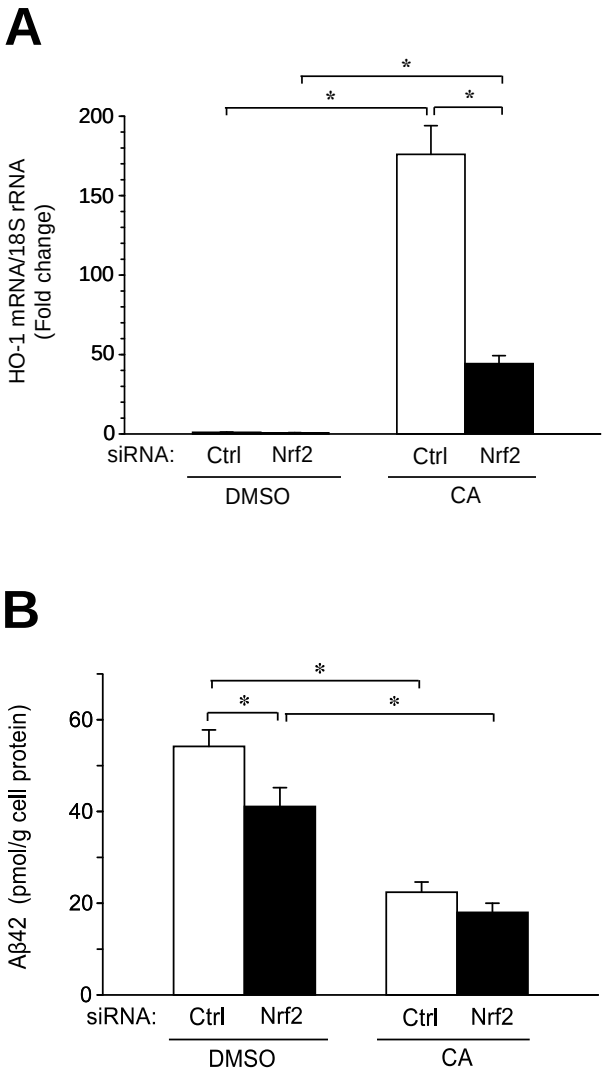

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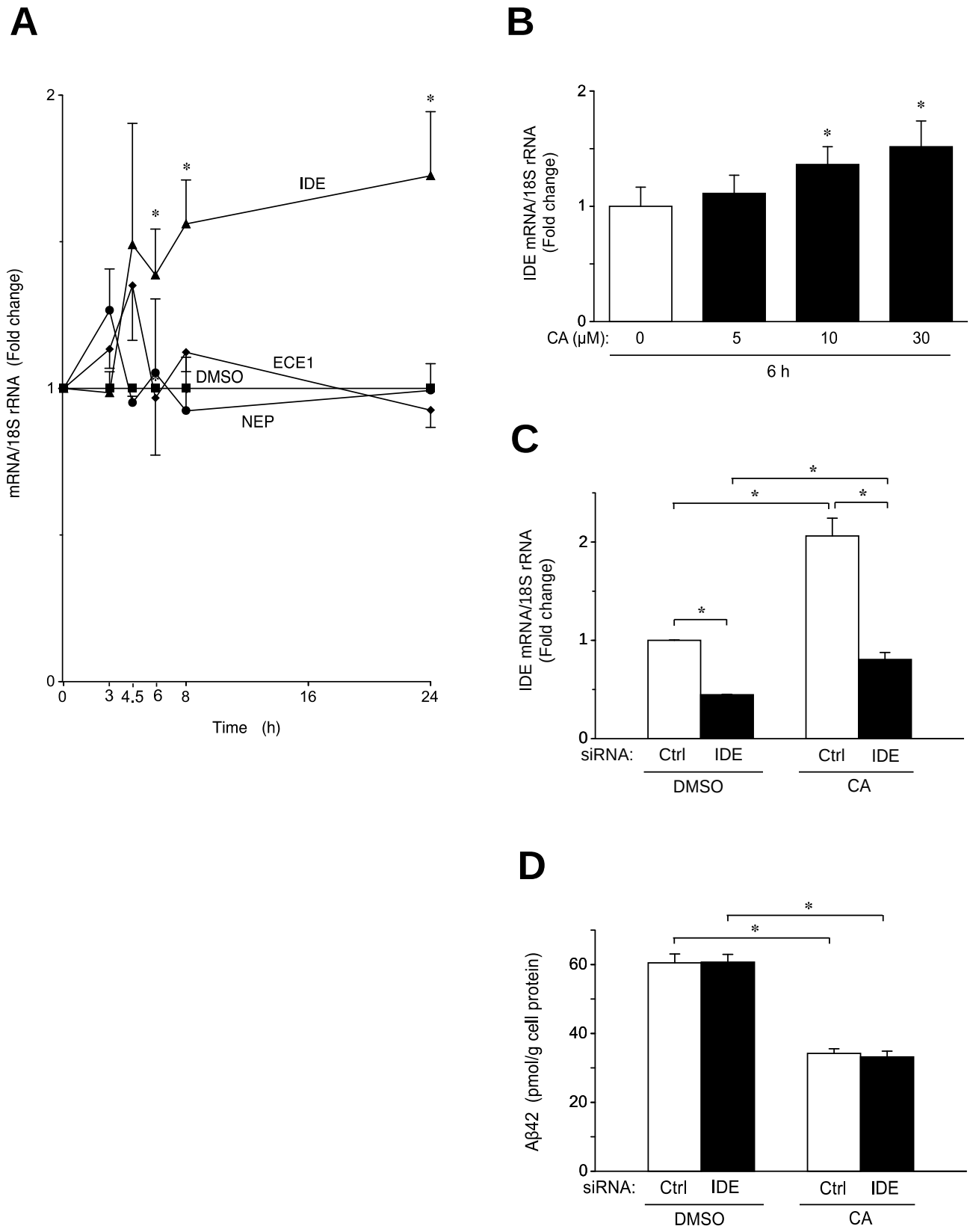

Fig. 7 Meng et al.