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Interleukin-1β up-regulates TACE to enhance α-cleavage of APP in neurons: resulting decrease in Aβ production

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Abstract

The proinflammatory cytokine interleukin (IL)-1β is up-regulated in microglial cells surrounding amyloid plaques, leading to the hypothesis that IL-1β is a risk factor for Alzheimer's disease. However, we unexpectedly found that IL-1β significantly enhanced α-cleavage, indicated by increases in sAPPα and C83, but reduced β-cleavage, indicated by decreases in sAPPβ and Aβ40/42, in human neuroblastoma SK-N-SH cells. IL-1β did not significantly alter the mRNA levels of BACE1, ADAM-9, and ADAM-10, but up-regulated that of TACE by threefold. The proform and mature form of TACE protein were also significantly up-regulated. A TACE inhibitor (TAPI-2) concomitantly reversed the IL-1β-dependent increase in sAPPα and decrease in sAPPβ, suggesting that APP consumption in the α-cleavage pathway reduced its consumption in the β-cleavage pathway. IL-1Ra, a physiological antagonist for the IL-1 receptor, reversed the effects of IL-1β, suggesting that the IL-1β-dependent up-regulation of α-cleavage is mediated by the IL-1 receptor. IL-1β also induced this concomitant increase in α-cleavage and decrease in β-cleavage in mouse primary cultured neurons. Taken together we conclude that IL-1β is an anti-amyloidogenic factor, and that enhancement of its signaling or inhibition of IL-1Ra activity could represent potential therapeutic strategies against Alzheimer's disease.

Keywords: Alzheimer's disease, Aβ, IL-1Ra, IL-1β, sAPPα, TACE.

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Deposition of amyloid-β peptide (Aβ) in the brain is a hallmark of the pathogenesis of Alzheimer's disease (AD) (Selkoe 2001). Aβ (39–43 amino acids in length) is a proteolytic product derived from amyloid precursor protein (APP). β-secretase (beta-site APP-cleaving enzyme: BACE1) cleaves APP to produce a soluble NH_2-terminal fragment (sAPPβ) and a membrane-bound 12-kDa COOH-terminal fragment (C99). C99 is further cleaved by γ-secretase, resulting in the production of a pathogenic Aβ peptide (De

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Abbreviations used: Aβ, amyloid-β peptide; ADAM, a-disintegrin-and-metalloprotease; AD, Alzheimer's disease; APP, amyloid precursor protein; APPwt, wild-type APP; APPswe, Swedish mutant of APP; BACE, beta-site APP-cleaving enzyme; FL, full-length; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; N, N-terminal side; PS1, presenilin 1; sAPP, soluble extracellular fragment of APP; sAPPα, soluble extracellular fragment of APP generated by α-secretase; sAPPβ, soluble extracellular fragment of APP generated by β-secretase; TACE, tumor necrosis factor (TNF)-α-converting enzyme; TAPI-2, TNF-α-protease inhibitor-2.

Strooper *et al.* 1998; Wolfe *et al.* 1999). As an alternative processing pathway, α-secretase cleaves within the Aβ sequence to produce a soluble NH_2-terminal fragment (sAPPα) and a membrane-bound 10-kDa COOH-terminal fragment (C83) (Buxbaum *et al.* 1998; Lammich *et al.* 1999). C83 is also cleaved by γ-secretase to produce the non-pathogenic p3 peptide. A-disintegrin-and-metalloprotease (ADAM) family proteases, such as tumor necrosis factor (TNF)-α-converting enzyme (TACE/ADAM-17), ADAM-9, and ADAM-10, have been shown to possess α-secretase activity (Allinson *et al.* 2003; Kojro and Fahrenholz 2005). Not only does the action of α-secretase preclude the formation of the intact Aβ peptide, but the large ectodomain of APP released from the cell surface (sAPPα) has neuroprotective and memory-enhancing effects (Furukawa *et al.*, 1996, Meziane *et al.*, 1998; Mattson *et al.*, 1999). Thus, α-cleavage is important for the neuroprotective pathway of APP metabolism.

Interleukin (IL)-1 has been found to play important roles in the development of inflammatory responses in the brain against acute injury, infection and chronic neurodegenerative diseases, including AD (Rothwell and Luheshi 2000; Allan and Rothwell 2001). IL-1 is secreted by macrophages/microglial cells upon their activation (Dinarello 1996), and triggers both cytokine cascades and self-defense mechanisms. For example, IL-1 acts on hypothalamic sites and raises the body temperature, which is beneficial for host defense mechanisms.

IL-1 receptor antagonist (IL-1Ra) is a naturally occurring inflammatory inhibitor that competes with IL-1β by binding to the IL-1 receptor. Although IL-1Ra binds to the receptor, it does not generate any intracellular signaling (Arend 1993; Arend *et al.* 1998). Indeed, lipopolysaccharide injection into human volunteers increases the plasma concentration of IL-1β (up to 80 pg/mL), followed by the production of a large excess of IL-1Ra (up to 6400 pg/mL) (Arend *et al.* 1990; Granowitz *et al.* 1991), Thus, IL-1Ra appears to be an important regulator of inflammatory responses.

In the AD brain, IL-1 expression is up-regulated around amyloid plaques (Mrak and Griffin 2001). In the present study, we examined the effects of IL-1β on α-cleavage of APP in neuroblastoma cells and primary cultured neurons.

Materials and methods

Materials

The materials and antibodies used in this study were as follows: anti-APP (6E10) monoclonal antibody (Signet Laboratories, Dedham, MA, USA); anti-TACE polyclonal antibody and anti-glyceraldehyde 3-phosphate dehydrogenase monoclonal antibody (Chemicon International, Temecula, CA, USA); anti-sAPPβ antibody (IBL, Fujioka, Japan); recombinant murine IL-1β and human IL-1β, IL-6, and IL-1Ra (PeproTech EC, London, England); TNF-α-protease inhibitor-2 (TAPI-2; Peptides International, Osaka, Japan). An anti-APP (C15) antibody against endogenous membrane-bound (intact) APP was a generous gift from Dr. Kei Maruyama (Saitama Medical University, Saitama, Japan). An anti-sAPPα antibody against a VRHQK peptide was prepared as described previously (Saido *et al.* 1995).

ELISAs for amyloid peptides

The following ELISA kits were used: human amyloid-β (1–40) (FL), human amyloid-β (1–42) (N) and mouse/rat amyloid-β (1–40) (N) assay kits (IBL); human/rat β-amyloid (42) ELISA kit Wako, high-sensitivity (Wako, Tokyo, Japan).

Cell culture

Human neuroblastoma SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. SH-SY5Y cells, a subline of SK-N-SH cells, were also used. Cortical neurons were isolated from C57BL/6CrSlc mice as previously described (Hama *et al.* 2001). Briefly, cerebral cortices were dissected from the brains of embryos at the developmental stage of 16–18 days. After removal of the meninges, the brains were minced in 0.25% trypsin (Difco, Sparks, MD, USA) and 0.05% Dnase I (Roche Diagnostics, Mannheim, Germany). Cortical neurons were filtered through a Cell Strainer (Falcon, Oxford, UK) and seeded into 12-well plates (NUNC, Roskilde, Denmark) coated with 100 mg/mL of poly-L-lysine (Sigma, St. Louis, MO, USA) at a density of 4×10^5 cells/well. Subsequently, the neurons were cultured in Neurobasal medium (Gibco-Invitrogen, Burlington, ON, USA) supplemented with 2% B27 (Gibco-Invitrogen), 0.5 mmol/L glutamine (Gibco-Invitrogen) and penicillin/streptomycin (Gibco-Invitrogen) at 37°C under a humidified 95% air/5% CO_2 atmosphere. The cells were cultured in the presence or absence of IL-1β. The effects of TAPI-2 were examined at final concentrations of 2 and 10 μmol/L. After 18 h of culture, the culture media were collected for analysis of the levels of secreted sAPPα and sAPPβ. During this experimental period, the cell viability was 92–95%, as evaluated by Trypan blue dye exclusion analysis. The cells were harvested for analysis of cellular TACE, intact (membrane-bound) APP and GAPDH. Total RNA fractions from the harvested cells were subjected to real-time PCR analysis.

Western blot analysis

We analyzed the protein levels of TACE, intact APP and GAPDH by western blot analysis. SK-N-SH cells or primary cultured neurons were lysed with radio-immunoprecipitation assay buffer (50 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, 0.5% deoxycholate, 1% NP40, 0.1% SDS) containing a protease inhibitor cocktail, and then centrifuged at 17 000 *g* for 10 min. The protein concentrations of the resulting supernatants were determined by a protein assay using the bicinchonic acid reagent (Pierce, Rockford, IL, USA). The sAPPα and sAPPβ secreted into the culture medium were precipitated with heparin-agarose at 4°C overnight, and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% gradient gel). After transfer of the separated proteins to a nitrocellulose membrane, the membrane was incubated

with a primary antibody, followed by incubation with an appropriate secondary antibody, namely horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. Binding of each antibody to its antigen was detected using a chemiluminescent substrate (Pierce), and the signals were quantified using a Luminoimage Analyzer LAS-1000 PLUS (Fuji Film, Tokyo, Japan).

Quantitative real-time PCR analysis

We analyzed the mRNA levels of TACE, ADAM-9, and ADAM-10 by quantitative real-time PCR. Aliquots (2 μg) of total RNA were reverse-transcribed with random hexamers using a Super Script III RT Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The obtained cDNAs were amplified with 900 nmol/L forward primer, 900 nmol/L reverse primer, 250 nmol/L fluorogenic probe and 25 μL of Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The PCR conditions were 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 50°C for 1 min. All the primers and probes were purchased from Applied Biosystems. Regarding the primers and probes for human TACE, ADAM-9, ADAM-10, BACE1, and GAPDH, we used Assays-on-Demand Gene Expression Products, and added the cDNAs to TaqMan Universal PCR Master Mix, which contained all the reagents required for PCR. The probes for TACE, ADAM-9, ADAM-10 and BACE1 were labeled with the fluorescent reporter dye 6-carboxy-fluorescein. The probes for GAPDH were labeled with VIC at their 5′ ends and the quencher dye TAMRA at their 3′ ends. The expression levels of the target genes were measured in duplicate and normalized to the corresponding GAPDH expression levels.

Results

Effects of IL-1β on α-cleavage and TACE expression by human neuroblastoma SK-N-SH cells

We analyzed the effects of the proinflammatory cytokine IL-1β on the secretion of sAPPα by neuroblastoma SK-N-SH cells. Addition of IL-1β (1 ng/mL) to the culture medium significantly enhanced sAPPα secretion and C83 accumulation by threefold, whereas addition of IL-6 had no effect (Fig. 1a–c). IL-1β treatment increased the levels of both the proform and mature form of TACE protein by twofold (Fig. 1d and e). The levels of the cell-associated form of intact APP were not increased by the addition of these cytokines. The dose effects of IL-1β on the enhancement of sAPPα secretion were examined in the range of 0.1–50 ng/mL. The effect was linear for 0.1–1 ng/mL and then reached a plateau (data not shown). Therefore, we used 1 ng/mL of IL-1β as the standard assay condition in the following experiments.

Effect of IL-1β on the mRNA levels of TACE, ADAM-9, and ADAM-10

We examined the mRNA levels of possible α-secretases, such as TACE/ADAM-17, ADAM-9, and ADAM-10, in SK-N-SH cells. Quantitative real-time PCR revealed that IL-1β treatment up-regulated the mRNA level of TACE by threefold (Fig. 2a) but did not affect the levels of ADAM-9 and ADAM-10 (107% and 100% of the control levels,

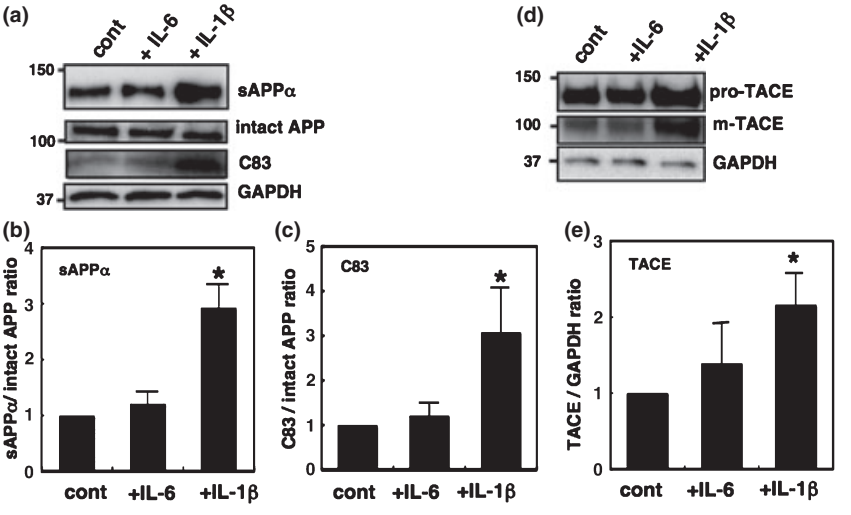

Fig. 1 IL-1β up-regulates TACE to enhance sAPPα production in human neuroblastoma SK-N-SH cells. (a) SK-N-SH cells were incubated with or without IL-6 (10 ng/mL) or IL-1β (1 ng/mL) in OPTI-MEM (serum-free media) for 18 h. Proteins secreted into the conditioned media were subjected to western blot analysis using the 6E10 antibody to detect sAPPα. Cellular protein extracts were subjected to western blot analysis using the C15 antibody to detect the cell-associated forms of intact APP and C83. GAPDH was detected with a specific antibody and used as a loading control. (b) Signal ratios of sAPPα to intact APP are shown as the mean ± SEM ($n = 9$) of three independent experiments (*$p < 0.001$). (c) Signal ratios of C83 to intact APP are shown as the mean ± SEM ($n = 5$) of three independent experiments (*$p < 0.05$). (d) Cellular extracts were subjected to western blot analysis to detect TACE and GAPDH. (e) Signal ratios of TACE to GAPDH are shown as the mean ± SEM ($n = 4$) of three independent experiments (*$p < 0.05$).

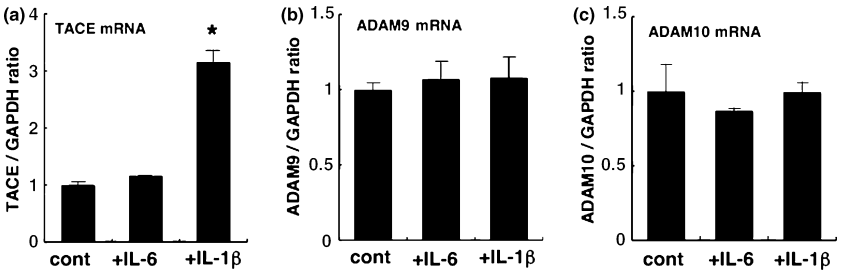

Fig. 2 IL-1β up-regulates mRNA of TACE. The mRNA levels of TACE (a), ADAM-9 (b) and ADAM-10 (c) were determined by real-time PCR, and normalized by the corresponding GAPDH mRNA level. Data represent the mean ± SEM ($n = 3$) of three independent experiments (*$p < 0.01$).

respectively) (Fig. 2b and c), suggesting that IL-1β enhances sAPPα production by up-regulating TACE transcription.

TAPI-2 reverses the IL-1β-dependent increase in α-cleavage

We examined the effect of TAPI-2, a TACE inhibitor, on the IL-1β-dependent increase in α-cleavage in SK-N-SH cells. The increases in sAPPα and C83 were reversed by the addition of 2 or 10 μmol/L TAPI-2 (Fig. 3), suggesting that TACE activity is required for the IL-1β-induced increase in α-cleavage. Furthermore, TAPI-1 was also effective for reversing the increase in α-cleavage.

Effects of IL-1Ra on the increases in TACE and sAPPα

IL-1Ra is a physiological antagonist of IL-1β that competes with IL-1β by binding to the IL-1β receptor, thereby preventing harmful inflammatory responses. We examined the effect of IL-1Ra on the IL-1β-induced increase in α-cleavage. Addition of IL-1Ra reversed the IL1β-dependent increases in sAPPα, C83, and TACE protein (Fig. 4), suggesting that the effects of IL-1β are mediated by the IL-1β receptor. IL-1Ra addition alone slightly decreased the sAPPα secretion and TACE protein level, but the decreases were not significant.

Effects of inflammatory cytokines on β-cleavage of APP in human neuroblastoma cells

Amyloid precursor protein is processed by two distinct cleavage pathways, namely the α- and β-cleavage pathways. We analyzed the effects of IL-1β on β-cleavage of APP in SK-N-SH cells. Treatment with IL-1β (1 ng/mL) significantly reduced the secretion of sAPPβ (Fig. 5a and b). When TAPI-2 was added together with IL-1β, the reduced secretion of sAPPβ was reversed to the control level (Fig. 5c and d), suggesting that the decrease in β-cleavage is due to up-regulation of TACE activity. We examined the BACE1 mRNA levels after IL-1β treatment. Quantitative real-time PCR analysis revealed that BACE1 mRNA was slightly decreased by IL-1β treatment, but the decrease was not significant (Fig. 5e). These results suggest that the decrease in sAPPβ secretion is not due to down-regulation of BACE1, but possibly due to increased consumption of APP, the common substrate for both cleavage pathways. In other words, the decrease in sAPPβ secretion may be a secondary

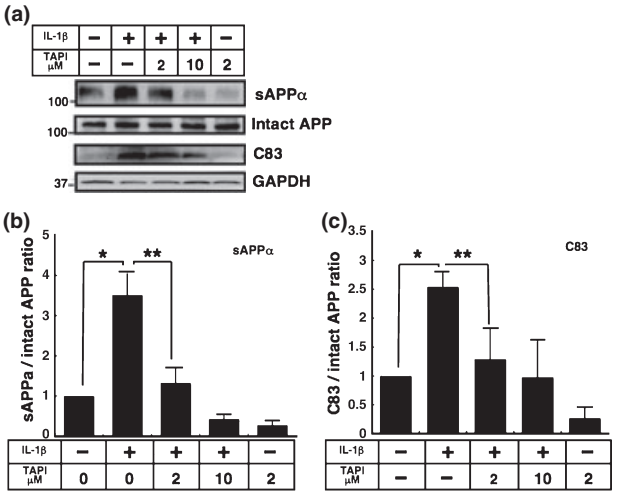

Fig. 3 Effect of TACE inhibition by TAPI-2 on the effect of IL-1β. (a), Proteins secreted into the conditioned media were subjected to western blot analysis using the 6E10 antibody to detect sAPPα. Cellular protein extracts were subjected to western blot analysis using the C15 antibody to detect the cell-associated forms of intact APP and C83. GAPDH was detected with a specific antibody and used as a loading control. (b) Signal ratios of sAPPα to intact APP are shown as the mean ± SEM ($n = 6$) of three independent experiments (*$p < 0.005$; **$p < 0.01$). (c) Signal ratios of C83 to intact APP are shown as the mean ± SEM ($n = 5$) of three independent experiments (*$p < 0.01$; **$p < 0.05$).

effect of the increase in α-cleavage. We also tried to estimate the amounts of Aβ peptides in the culture medium of SK-N-SH cells, but the levels of endogenous Aβ peptides in the medium were too low to be detected. Therefore, we used SH-SY5Y cells (a subline of SK-N-SH cells) stably over-expressing wild-type APP (APPwt; SH-SY5Y-APPwt cells). Following addition of IL-1β (1 ng/mL) to the culture medium, we detected a significant decrease in Aβ40 secretion and a slight decrease in Aβ42 secretion by SH-SY5Y-APPwt cells (Fig. 5f), suggesting that IL-1β is a neuroprotective cytokine that reduces Aβ production by up-regulating α-cleavage of APP.

Effects of IL-1β on APP cleavage in mouse primary neurons

We analyzed the effects of IL-1β on APP metabolism in mouse primary cultured neurons. IL-1β treatment significantly increased the TACE mRNA level and sAPPα secretion

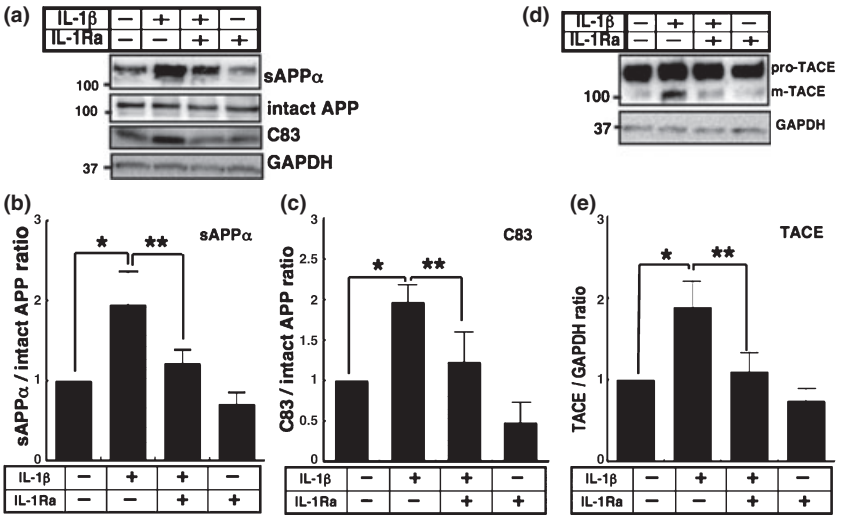

Fig. 4 Effects of IL-1Ra on sAPPα secretion and TACE protein levels. (a) SK-N-SH cells were treated with IL-1β (1 ng/mL) in the presence or absence of IL-1Ra for 18 h. Proteins secreted into the conditioned media were subjected to western blot analysis using the 6E10 antibody to detect sAPPα. Cellular protein extracts were subjected to western blot analysis using the C15 antibody to detect the cell-associated form of intact APP. (b) Signal ratios of sAPPα to intact APP are shown as the mean ± SEM ($n = 5$) of three independent experiments (*$p < 0.05$; **$p < 0.05$). (c) Signal ratios of C83 to intact APP are shown as the mean ± SEM ($n = 4$) of three independent experiments (*$p < 0.05$; **$p < 0.05$). (d) Cellular protein extracts were subjected to western blot analysis to detect TACE and GAPDH. (e) Signal ratios of TACE to GAPDH are shown as the mean ± SEM ($n = 6$) of three independent experiments (*$p < 0.05$; **$p < 0.05$).

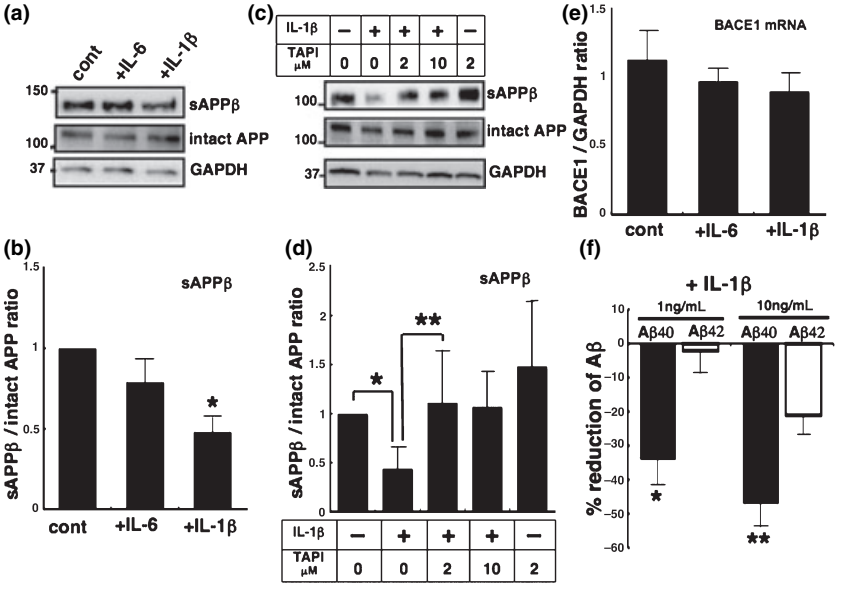

Fig. 5 Effects of IL-6 or IL-1β on the BACE1 mRNA level and secretion of sAPPβ and Aβ by human neuroblastoma cells. (a) Proteins secreted into the conditioned media were subjected to western blot analysis to detect sAPPβ. Cellular extracts were subjected to western blot analysis using the C15 antibody to detect the cell-associated form of intact APP. (b) Signal ratios of sAPPβ to intact APP are shown as the mean ± SEM ($n = 7$) of three independent experiments (*$p < 0.001$). (c) TAPI-2 was added together with IL-1β. sAPPα in the media was subjected to western blot analysis using the sAPPβ antibody. Cellular protein extracts were subjected to western blot analysis using the C15 antibody to detect intact APP. GAPDH was detected with a specific antibody and as a loading control. (d) Signal ratios of sAPPβ to intact APP are shown as the mean ± SEM ($n = 5$) of three independent experiments (*$p < 0.05$; **$p < 0.05$). (e) Quantitative real-time PCR was carried out to detect the BACE1 mRNA levels. (f) SH-SY5Y-APPwt cells over-expressing wild-type APP were treated with IL-1β, and conditioned media were analyzed by ELISA to detect the Aβ levels. Data are shown as the percent change relative to the control level, and represent the mean ± SEM ($n = 4$) of three independent experiments (*$p < 0.05$; **$p < 0.01$).

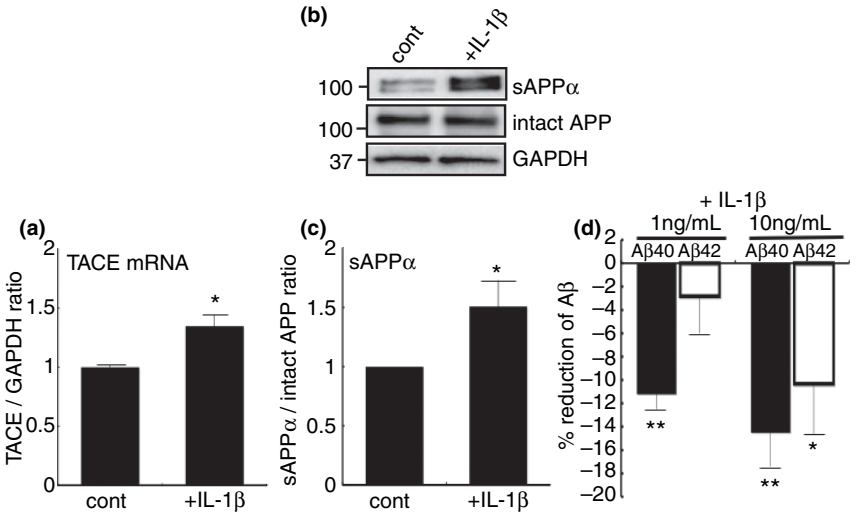

Fig. 6 IL-1β up-regulates the TACE mRNA and protein levels in mouse primary cultured neurons, and the effect of IL-1β on Aβ secretion in mouse primary cultured neurons. (a) Mouse primary neurons were treated with IL-1β (50 ng/mL) for 18 h, and the TACE mRNA levels were determined by quantitative real-time PCR. All values were normalized by the corresponding GAPDH mRNA level. Data represent the mean ± SEM ($n = 3$) of three independent experiments (*$p < 0.05$). (b) Proteins secreted into the conditioned media were subjected to western blot analysis to detect sAPPα. Cellular extracts were subjected to western blot analysis using the C15 antibody to detect the cell-associated form of intact APP. (c) Signal ratios of sAPPα to intact APP are shown as the mean ± SEM ($n = 6$) of three independent experiments (*$p < 0.05$). (d) Conditioned media were analyzed by ELISA to detect the Aβ levels. Data are shown as the percentage change relative to the control level, and represent the mean ± SEM ($n = 6$) of three independent experiments (*$p < 0.05$; **$p < 0.01$).

(Fig. 6a–c). The level of the cell-associated form of intact APP was not increased by the addition of this cytokine. In addition, IL-1β treatment significantly reduced the secretion of Aβ40 and Aβ42 at a final concentration of 50 ng/mL and that of Aβ40 at a final concentration of 1 ng/mL (Fig. 6d), again suggesting that IL-1β could be protective against the pathogenesis of AD.

Discussion

In the present study, we have demonstrated that IL-1β up-regulates TACE expression to enhance α-cleavage of APP in neuroblastoma cells and primary cultured neurons, resulting in increased sAPPα production but decreased sAPPβ and Aβ production.

Up-regulation of TACE activity is a downstream event of IL-1 receptor signaling. Ma *et al.* reported that short-term IL-1β treatment of neuroglioma U251 cells activated intracellular c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinases to up-regulate α-secretase activity (Ma *et al.* 2005). Bandyopadhyay *et al.* extensively investigated the effects of IL-1α on α-cleavage of APP in astrocytic cells and found that it up-regulated ADAM-10 and ADAM-17 (TACE) to increase α-cleavage through the activation of mitogen-activated protein kinases, p38 kinase and PI3 kinase, leading to a concomitant decrease in the Aβ level (Bandyopadhyay *et al.* 2006). Taking their observations together with our present findings, IL-1 appears to be a neuroprotective cytokine for neuronal cells.

IL-1β is secreted by microglia, which phagocytose Aβ peptides around plaques. A recent study on a transgenic (APPswe/PS1) mouse model of AD revealed that many of the microglia near plaques are recruited from the bone marrow and have the ability to eliminate amyloid deposits by phagocytosis (Simard *et al.* 2006). These authors also reported that microglia responded to exogenous as well as endogenous Aβ and subsequently expressed mRNAs for IL-1β, monocyte chemoattractant protein-1 and TLR2. Moreover, these responses appeared to be non-typical innate immunity, since up-regulation of TNF-α was not involved in the recruitment and phagocytosis. Microglia recruitment and up-regulation of IL1β expression gradually increased with age (4–9 months) in the transgenic mouse model, suggesting that up-regulation of IL-1β persists throughout the long-term pathogenic processes.

Very recently, Shaftel *et al.* reported that over-expression of IL1β attenuated plaque formation in an APPswe/PS1 transgenic mouse model, indicating that IL1β is neuroprotective against the pathogenesis of AD (Shaftel *et al.* 2007). Although the involvement of TACE up-regulation was not examined in their transgenic mouse model, our results suggest that TACE up-regulation could be a downstream event of the neuroprotective IL1β signaling. It is notable that only a 30% increase in TACE mRNA significantly reduced sAPPβ and Aβ production in mouse primary cultured

neurons in the present study, indicating that even subtle enhancement of TACE could be sufficient to reduce β-cleavage of APP. Indeed, Skovronsky *et al.* reported the presence of TACE protein in neurons surrounding plaques in the AD brain (Skovronsky *et al.* 2001), supporting our assumption that enhancement of TACE would be a beneficial response induced by IL-1β. In conclusion, IL-1β up-regulates TACE in neurons to ameliorate AD plaque pathology. Thus, enhancement of IL-1β and depletion of IL-1Ra could represent new therapeutic targets for treatment of AD.

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