

EP Receptors and Production of Amyloid- β Peptides

Positive clones were maintained in the presence of 200 $\mu\text{g/ml}$ G418.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (35). For detection of the C-terminal fragment (CTF) α and CTF β , membrane fractions were prepared as described previously (36). For detection of CTF γ , the membrane fractions were incubated for 2 h at 37 $^{\circ}\text{C}$. The protein concentration of each sample was determined by the Bradford method (37). Samples were applied to polyacrylamide SDS gels (Tris-Tricine gel for the detection of APP and Tris glycine gel for other proteins), and subjected to electrophoresis, after which proteins were immunoblotted with their respective antibodies.

Sandwich Enzyme-linked Immunosorbent Assay (sELISA) for $\text{A}\beta$ and EIA for cAMP—Cells were cultured for 24 h and the conditioned medium was subjected to sELISA using three types of specific monoclonal antibodies, as described previously (29, 34).

The amount of $\text{A}\beta$ in mouse brain was determined as described previously (38). Briefly, the brain hemispheres were homogenized in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl with a homogenizer (Polytron) and centrifuged at 200,000 $\times g$ for 20 min at 4 $^{\circ}\text{C}$. The supernatant, defined as the soluble fraction, was taken and guanidine-HCl added to give a final concentration of 0.5 M before sELISA. The pellet was solubilized by sonication in 6 M guanidine-HCl buffer. The solubilized pellet was centrifuged at 200,000 $\times g$ for 20 min at 4 $^{\circ}\text{C}$, and the resulting supernatant was diluted and termed the insoluble fraction. The amounts of $\text{A}\beta_{40}$ and $\text{A}\beta_{42}$ in each fraction were determined by sELISA.

Cells were pre-treated for 30 min with 0.5 M 3-isobutyl-1-methylxanthine (an inhibitor of phosphodiesterase) and further cultured for 10 min with or without PGE_2 , EP agonists, or EP antagonist. Cells were lysed with ice-cold 100% ethanol and centrifuged. The supernatants were dried, re-suspended in the assay buffer, and applied to the EIA kit for measurement of cAMP, according to the manufacturer's instructions.

RT-PCR Analysis—Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocols. Samples (10 μg of RNA) were reverse transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was amplified by PCR (TAKARA PCR Thermal Cycler) using TAKARA Taq DNA polymerase, and reaction products were analyzed by agarose gel electrophoresis. PCR cycle conditions were 2 min at 50 $^{\circ}\text{C}$, followed by 10 min at 95 $^{\circ}\text{C}$ and finally 35 cycles at each of 95 $^{\circ}\text{C}$ for 20 s, 60 $^{\circ}\text{C}$ for 60 s, and 72 $^{\circ}\text{C}$ for 60 s.

Primers were designed using the Primer3 Web site (www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of the primers (written as target cDNA: forward primer and reverse primer) were: *EP1*, 5'-acctctcttggcgctct-3' and 5'-gcacgacaccaccatgatac-3'; *EP2*, 5'-ccacctcattctcctgcta-3' and 5'-cgacaacagaggactgaacg-3'; *EP3*, 5'-agcttatggggatcatgtgc-3' and 5'-tctgcttccctgtgtgtc-3'; *EP4*, 5'-tgcgagtatcgtaaccag-3' and 5'-ggctagtagtgggttcaca-3'; and *actin*, 5'-ggacttcgagcaagatgg-3' and 5'-agcactgtgtggcgtacag-3'.

γ -Secretase-mediated Peptide Cleavage Assay—We performed the assay as previously reported (39, 40). Solubilized

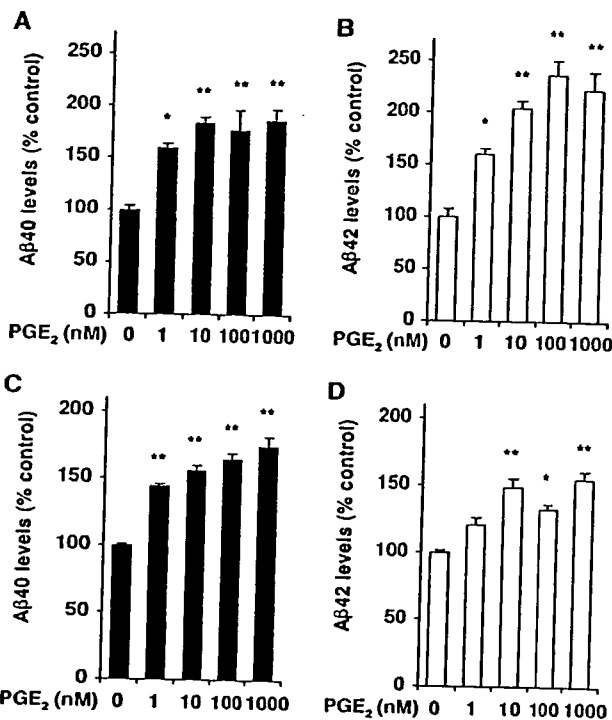


FIGURE 1. Stimulation of production of $\text{A}\beta$ by PGE_2 . HEK293 (A and B) or SH-SY5Y (C and D) cells expressing APPsw were cultured for 24 h with the indicated concentrations of PGE_2 . The amounts of $\text{A}\beta_{40}$ and $\text{A}\beta_{42}$ in the conditioned medium were determined by sELISA and expressed relative to the control (without PGE_2). Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. Similar results in this figure were obtained in two other independent experiments.

membranes were re-suspended and incubated overnight at 37 $^{\circ}\text{C}$ in 200 μl of assay buffer containing 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.25% CHAPSO (w/v), and 10 μM APP-derived fluorescent substrate of γ -secretase. We measured fluorescence using a plate reader (Fluorstar Galaxy) with an excitation wavelength of 355 nm and an emission wavelength of 440 nm.

Statistical Analysis—All values are expressed as the mean \pm S.E. One-way analysis of variance followed by Tukey multiple comparison test or Student's *t* test for unpaired results was used for evaluation of differences among more than three groups or for the evaluation of differences between two groups, respectively. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Stimulation of $\text{A}\beta$ Production by PGE_2 —We used HEK293 cells that stably express a form of APP with double mutations (K651N/M652L), known as the "Swedish" mutations (APPsw) (29). These mutations elevate cellular and secreted levels of $\text{A}\beta$ (29). The amount of these peptides in conditioned medium was determined using a sELISA. Treatment of cells with PGE_2 increased the levels of $\text{A}\beta$ ($\text{A}\beta_{40}$ and $\text{A}\beta_{42}$) in the conditioned medium (Fig. 1, A and B), a similar result to that observed for CHO cells (26). We concluded that this increase is due to stimulation of production of $\text{A}\beta$ because, after treatment of the cells with PGE_2 , not only did the amount of secreted $\text{A}\beta$ increase but the amount of $\text{A}\beta$ in the cells also increased and because pulse label experiments with [^{35}S]methionine showed that treatment

of cells with PGE₂ increased generation of CTFs of APP that are co-generated by γ -secretase (CTF γ) with A β (data not shown). Although it is not clear whether comparison of concentrations of PGE₂ between *in vivo* and *in vitro* is reasonable or not, the concentrations of PGE₂ required for stimulation of A β production (1–10 nM) are within the same range of PGE₂ concentrations that are observed in the human brain (41). However, the fact that production of A β is stimulated by the concentration of PGE₂, which is equivalent to that found physiologically is against the idea that PGE₂-stimulated production of A β seen *in*

vitro is involved in inflammation-stimulated development of AD. We also performed similar experiments in SH-SY5Y cells that stably express APP_{sw}. As shown in Fig. 1, C and D, treatment with PGE₂ also increased the level of A β in SH-SY5Y cells; however, the extent of stimulation of A β production in SH-SY5Y cells was not as dramatic as that seen in HEK293 cells (Fig. 1, A and B). We confirmed that PGE₂ did not affect the cell growth and intracellular lactate dehydrogenase activity at concentrations used in Fig. 1 in both HEK293 and SH-SY5Y cells (supplemental Fig. S1).

Identification of EP Receptors Involved in PGE₂-stimulated Production of A β —We used agonists specific for each EP receptor (Table 1) to identify EP receptors involved in the PGE₂-stimulated production of A β . Initially, we used HEK293 cells and examined the mRNA expression of each EP receptor by RT-PCR. As shown in Fig. 2A, mRNA for each of the EP receptors was detected, although the level of expression varied between them. We also confirmed the expression of EP₂ receptor by immunoblotting experiments (Fig. 5A). Then, we examined the effect of agonists specific for each EP receptor on the production of A β in HEK293 cells. Treatment of cells with AE1-329 (an EP₄ agonist) increased the level of A β in conditioned medium (Fig. 2, H and I). The amplitude of this increase

TABLE 1
Compounds used in this study

Compound	Primary action
DI-004	EP ₁ agonist
AE1-259	EP ₂ agonist
AE-248	EP ₃ agonist
AE1-329	EP ₄ agonist
8713	EP ₁ antagonist
AE3-240	EP ₁ antagonist
AE3-208	EP ₁ antagonist
pCPT-cAMP	cAMP analogue
LY294002	PI3K inhibitor
H-89	PKA inhibitor
pCPT-O-Me-cAMP	Epac activator
DAPT	γ -Secretase inhibitor

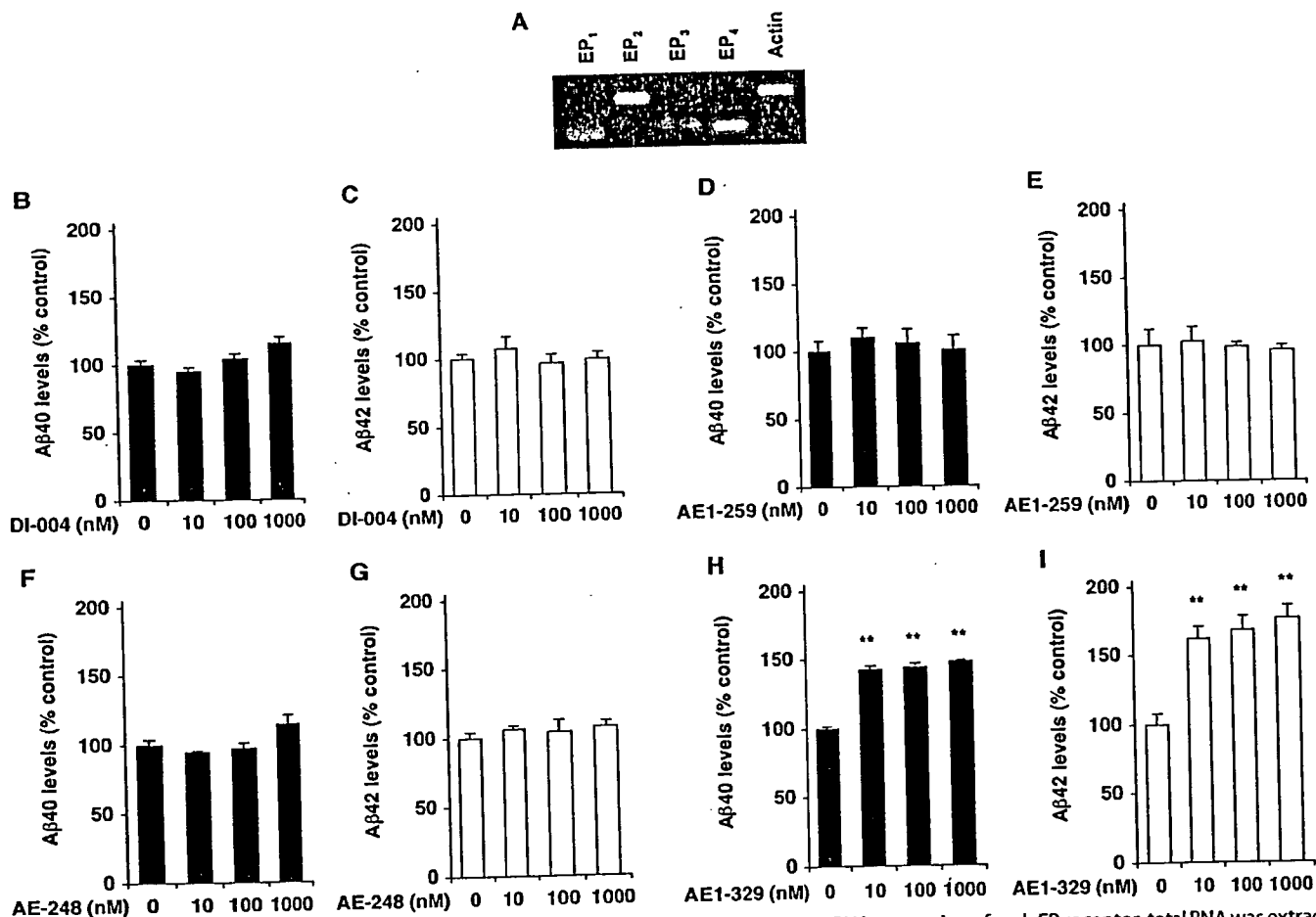


FIGURE 2. Effect of EP receptor agonists on production of A β in HEK293 cells. To determine mRNA expression of each EP receptor, total RNA was extracted from HEK293 cells expressing APP_{sw} and subjected to RT-PCR by use of a specific primer set for each gene. Actin was used as a control. Reaction products were analyzed by agarose (2%) gel electrophoresis (A). HEK293 cells expressing APP_{sw} were cultured for 24 h with the indicated concentrations of DI-004 (EP₁ agonist) (B and C), AE1-259 (EP₂ agonist) (D and E), AE-248 (EP₃ agonist) (F and G), or AE1-329 (EP₄ agonist) (H and I). The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$. Similar results in this figure were obtained in two other independent experiments.

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was similar to that achieved with PGE₂ (Fig. 1, A and B). Based on previously reported findings using AE1-329, it is reasonable to postulate that, for the concentrations used in the experiment described in Fig. 2, H and I, it acts as a specific agonist for the EP₄ receptor (42). In contrast, none of the other specific agonists, including DI-004 (an EP₁ agonist), AE1-259 (an EP₂ agonist), and AE-248 (an EP₃ agonist), significantly affected the level of A β (Fig. 2, B–G). Based on previous reports, the concentrations of EP agonists employed in the experiments described in Fig. 2 should have been sufficient to activate their respective EP receptor (42). We confirmed that each agonist did not affect the cell growth at these concentrations used in Fig. 2 (data not shown). Consequently, the results in Fig. 2 suggest that EP₄ is responsible for the PGE₂-stimulated production of A β in HEK293 cells. As described below, we suggested that EP₂ receptor is not functional in HEK293 cells (Fig. 5A). Furthermore, we found that none of PGE₂, DI-004, and AE-248 increased the intracellular Ca²⁺ levels (supplemental Fig. S2), suggesting that neither EP₁ nor EP₃ receptor is functional in HEK293 cells (both EP₁ and EP₃ receptors are coupled to Ca²⁺ mobilization (27, 43)). Thus, we could not conclude that activation of these receptors (EP₁, EP₂, and EP₃) does not affect the level of A β in general, based on the inability of DI-004, AE1-259, and AE-248 to affect the level of A β in HEK293 cells.

To further confirm this conclusion, we examined the effect of antagonists specific for each EP receptor (Table 1) on the PGE₂-stimulated production of A β . As shown in Fig. 3, E and F, AE3-208 (an EP₄ antagonist) clearly suppressed the PGE₂-dependent increase in the level of A β in a dose-dependent manner. Based on previous reports, it is reasonable to postulate that, for the concentrations used in the experiment described in Fig. 3, E and F, AE3-208 acts as a specific antagonist for the EP₄ receptor (32). This EP₄ antagonist (1 or 10 μ M) decreased the level of A β to below background (Fig. 3, E and F). This seems to be due to inhibition of the action of endogenous PGE₂, because this antagonist decreased the level of A β even in the absence of exogenously added PGE₂ (data not shown). In contrast, 8713 (an EP₁ antagonist), at concentrations previously reported to antagonize the EP₁ receptor (44), did not significantly affect the level of A β in the presence of PGE₂ (Fig. 3, A and B). AE3-240 (an EP₃ antagonist) weakly inhibited the PGE₂-stimulated production of A β 42 only at the highest concentration tested (10 μ M) (Fig. 3, C and D). This seems to be due to the tendency of AE3-240 to cross-react with the EP₄ receptor at high concentrations, as has been described previously (45). We confirmed that each antagonist did not affect the cell growth at these concentrations used in Fig. 3 (data not shown). Therefore, the results obtained from the experiments with EP antagonists support the idea that EP₄ is responsible for the PGE₂-stimulated production of A β in HEK293 cells.

We also examined the effect of each EP agonist on production of A β in SH-SY5Y cells. Again, mRNA for each of the EP receptors was detected by RT-PCR (Fig. 4A). However, the relative expression of each EP receptor was different from that in HEK293 cells (Fig. 2A). For example, the amount of EP₄ receptor mRNA relative to EP₂ receptor mRNA was less in SH-SY5Y cells than in HEK293 cells (Figs. 2A and 4A). We also confirmed the expression of EP₂ receptor by immunoblotting experiments

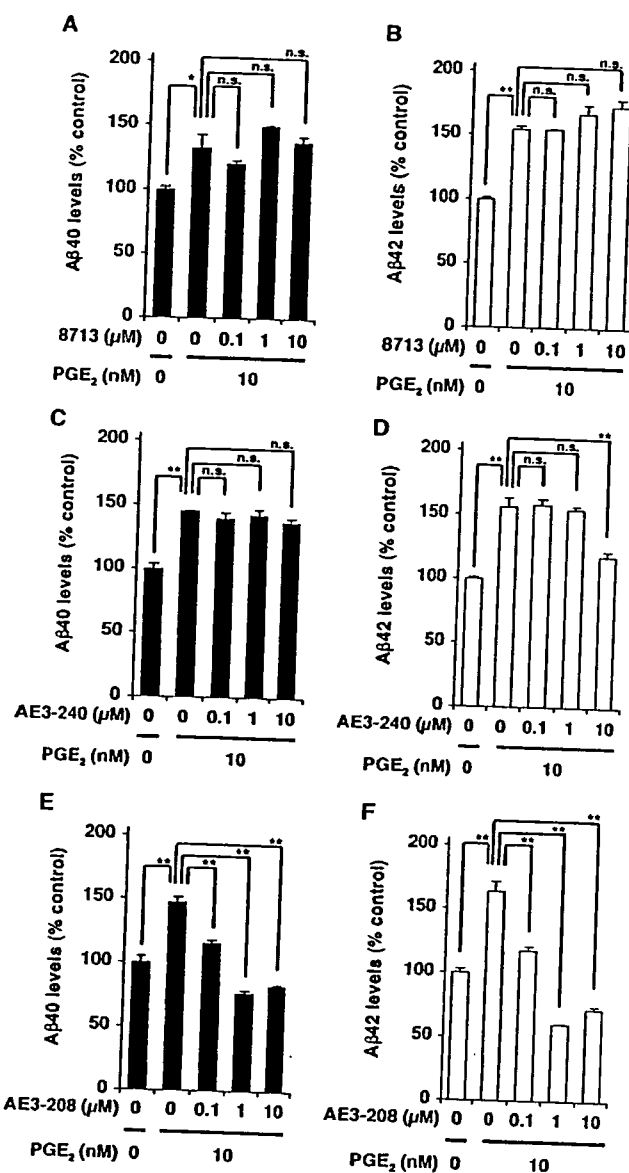


FIGURE 3. Effect of EP receptor antagonists on PGE₂-stimulated production of A β in HEK293 cells. HEK293 cells expressing APP^{sw} were cultured for 24 h with the indicated concentrations of 8713 (EP₁ antagonist) (A and B), AE3-240 (EP₃ antagonist) (C and D), or AE3-208 (EP₄ antagonist) (E and F) in the presence of PGE₂ (10 nM). The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant. Similar results in this figure were obtained in two other independent experiments.

(Fig. 5A). As shown in Fig. 4, B and C, not only the EP₄ agonist but also the EP₂ agonist increased the level of A β in the conditioned medium of SH-SY5Y cells. Again, EP₁ or EP₃ agonists did not significantly affect the level of A β (Fig. 4, B and C). We confirmed that each agonist did not affect the cell growth at these concentrations used in Fig. 4 (data not shown). These results suggest that both EP₂ and EP₄ receptors are involved in PGE₂-stimulated production of A β in SH-SY5Y cells, and therefore differs from that observed for HEK293 cells. As shown in supplemental Fig. S2, each of all PGE₂, DI-004, and AE-248 increased the intracellular Ca²⁺ levels in SH-SY5Y cells, suggesting that both EP₁ and EP₃ receptors are functional

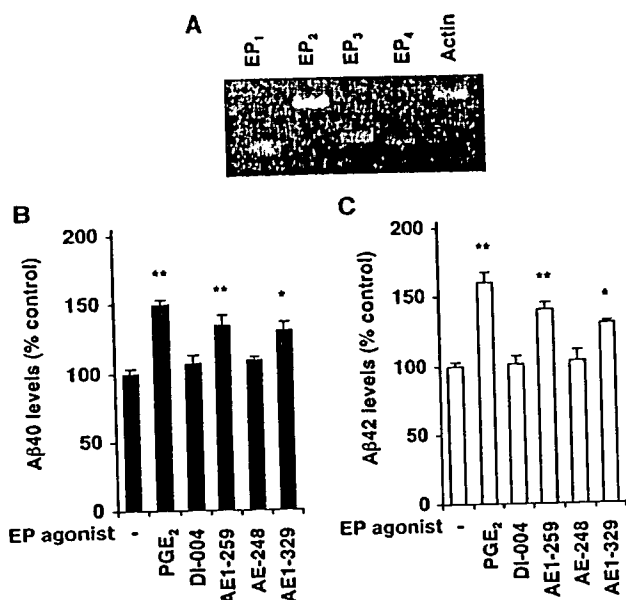


FIGURE 4. Effect of EP receptor agonists on production of A β in SH-SY5Y cells. The mRNA expression of each EP receptor in SH-SY5Y cells expressing APPsw was examined as described in the legend of Fig. 2 (A). SH-SY5Y cells expressing APPsw were cultured for 24 h with 1 μ M DI-004 (EP₁ agonist), AE1-259 (EP₂ agonist), AE-248 (EP₃ agonist), AE1-329 (EP₄ agonist), or PGE₂. The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. B and C, similar results in this figure were obtained in two other independent experiments.

in the cells. Thus, results in Fig. 4 suggest that activation of EP₁ and EP₃ receptors does not affect the level of A β .

Involvement of the Cellular Level of cAMP in PGE₂-stimulated Production of A β —Activation of EP₂ and EP₄ receptors causes activation of adenylate cyclase activity and an increase in the cellular level of cAMP (27). Therefore, the results described above suggest that an increase in the cellular level of cAMP is involved in PGE₂-stimulated production of A β . To test this hypothesis, we monitored the cellular level of cAMP under conditions where production of A β is stimulated or suppressed (Figs. 1–4). In HEK293 cells PGE₂ and EP₄ agonist, but not EP₂ agonist, increased the cellular level of cAMP (Fig. 5B). However, PGE₂, EP₂ agonist, and EP₄ agonist all increased the cAMP level in SH-SY5Y cells (Fig. 5C), correlating with the stimulation of A β production shown in Figs. 2 and 4. These data suggest that both EP₂ and EP₄ receptors are functional in SH-SY5Y, whereas only EP₄ receptor is functional in HEK293 cells, which can explain why both EP₂ and EP₄ agonists or only EP₄ agonist stimulated production of A β in SH-SY5Y or HEK293, respectively (Figs. 2 and 4). As shown in Fig. 5A, the expression of EP₂ receptor was relatively higher in SH-SY5Y than HEK293 cells. It is also possible that this low expression of EP₂ receptor in HEK293 cells can explain why this type of cells were inert for the increase in the cellular level of cAMP by EP₂ agonist. Furthermore, EP₄ antagonist clearly suppressed the PGE₂-mediated increase in the cellular level of cAMP, again correlating with the trend in A β production shown in Fig. 3. Therefore, the results in Fig. 5, B–D, suggest that PGE₂-stimulated production of A β is mediated by an increase in the cellular level of cAMP.

To further confirm this conclusion a cAMP analogue, 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP), was used. As shown

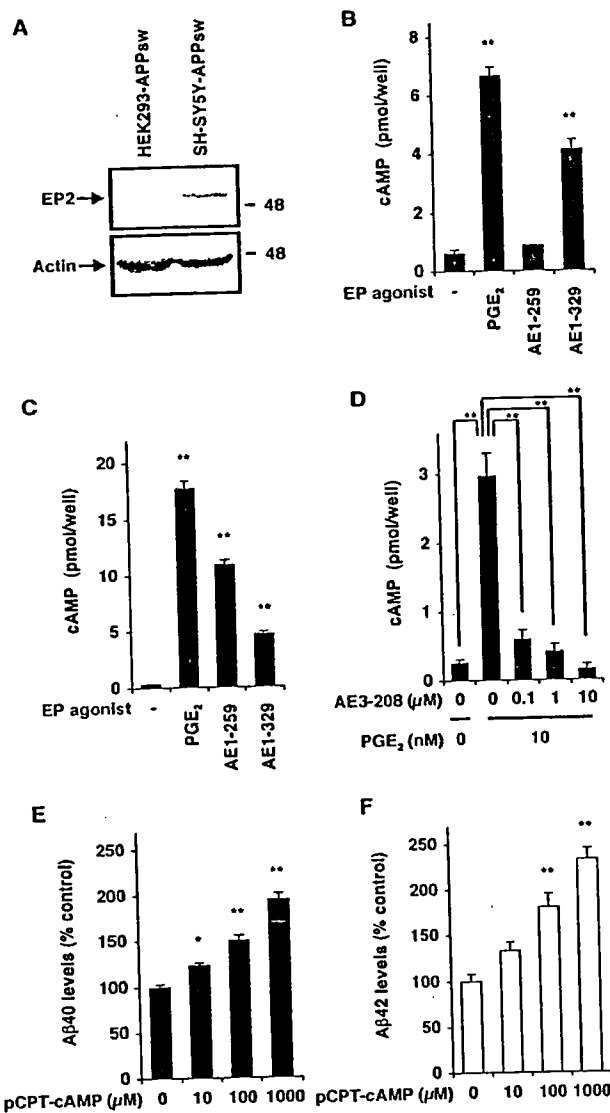


FIGURE 5. Involvement of the cellular level of cAMP in PGE₂-stimulated production of A β . To determine the expression of EP₂ receptor, cell extracts were prepared from HEK293 or SH-SY5Y cells expressing APPsw and subjected to immunoblotting with an antibody against EP₂ receptor or actin (A). HEK293 (B and D) or SH-SY5Y (C) cells expressing APPsw were pre-treated for 30 min with 0.5 μ M 3-isobutyl-1-methylxanthine and further cultured for 10 min with 1 μ M PGE₂, AE1-259 (EP₂ agonist), AE1-329 (EP₄ agonist) (B and C), or with the indicated concentrations of AE3-208 (EP₄ antagonist) in the presence of PGE₂ (10 nM) (D). Cellular cAMP levels were determined by EIA (B–D). HEK293 cells expressing APPsw were cultured for 24 h with the indicated concentrations of pCPT-cAMP (E and F). The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. Similar results in this figure were obtained in two two independent experiments.

in Fig. 5, E and F, pCPT-cAMP increased the level of A β in HEK293 cells and the extent of this increase was similar to that achieved with PGE₂ (Fig. 1, A and B), supporting the idea that an increase in the cellular level of cAMP is responsible for PGE₂-stimulated production of A β . We confirmed that pCPT-cAMP did not affect cell growth at these concentrations as used in Fig. 5 (data not shown).

An increase in the cellular level of cAMP is known to activate two types of kinases, protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K), which are important for cAMP-regu-

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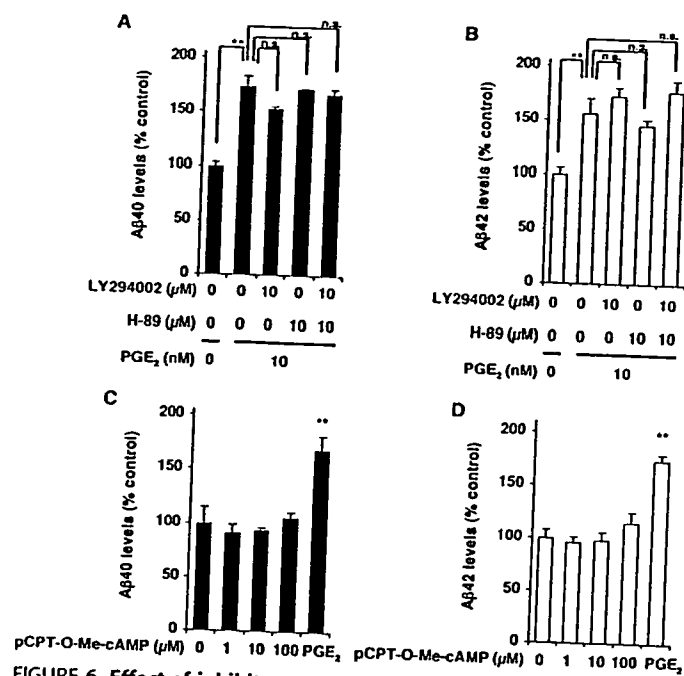


FIGURE 6. Effect of inhibitors or activators of various cAMP-regulated molecules on PGE₂-stimulated production of A β . HEK293 cells expressing APPsw were preincubated for 1 h with the indicated concentrations of LY294002 (PI3K inhibitor) and/or H-89 (PKA inhibitor) and further cultured for 24 h with 10 nM PGE₂ in the presence of the same concentration of each inhibitor as in the preincubation step (A and B). Cells were cultured for 24 h with the indicated concentrations of pCPT-O-Me-cAMP (Epac activator) (C and D). The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; n.s., not significant. Similar results in this figure were obtained in two other independent experiments.

lated intracellular signal transduction (46). On this basis, we next examined the involvement of these kinases in PGE₂-stimulated production of A β in HEK293 cells, using an inhibitor for each kinase. As shown in Fig. 6, A and B, neither an inhibitor of PKA, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide (H-89), nor an inhibitor of PI3K, LY294002, blocked the PGE₂-stimulated production of A β . Higher concentrations of these inhibitors (up to 20 μ M) gave similar results (data not shown). Furthermore, treatment of cells with both of these inhibitors simultaneously also did not block the PGE₂-stimulated production of A β (Fig. 6, A and B). We also examined the effect of H89 and/or LY294002 on the level of A β in the presence of EP₂ agonist and/or EP₄ agonist. As shown in supplemental Fig. S3, neither of these inhibitors affected the level of A β in the presence of EP₂ agonist and/or EP₄ agonist in HEK293 cells. These inhibitors, at the concentrations specified in Fig. 6, A and B, did not affect cell viability (data not shown) and, based on the results of previous publications (47, 48), should have been sufficient to inhibit their target molecules. Therefore, it seems that neither PKA nor PI3K are involved in the PGE₂-stimulated production of A β .

Exchange protein directly activated by cAMP (Epac) was recently identified as another target of cAMP for signal transduction (49). We tested the involvement of Epac in PGE₂-stimulated production of A β by using a specific activator for Epac, 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-3'-5'-cyclic monophosphate (pCPT-*O*-Me-cAMP) (49). As shown in Fig. 6, C and D, pCPT-*O*-Me-cAMP (1–100 μ M) did not affect

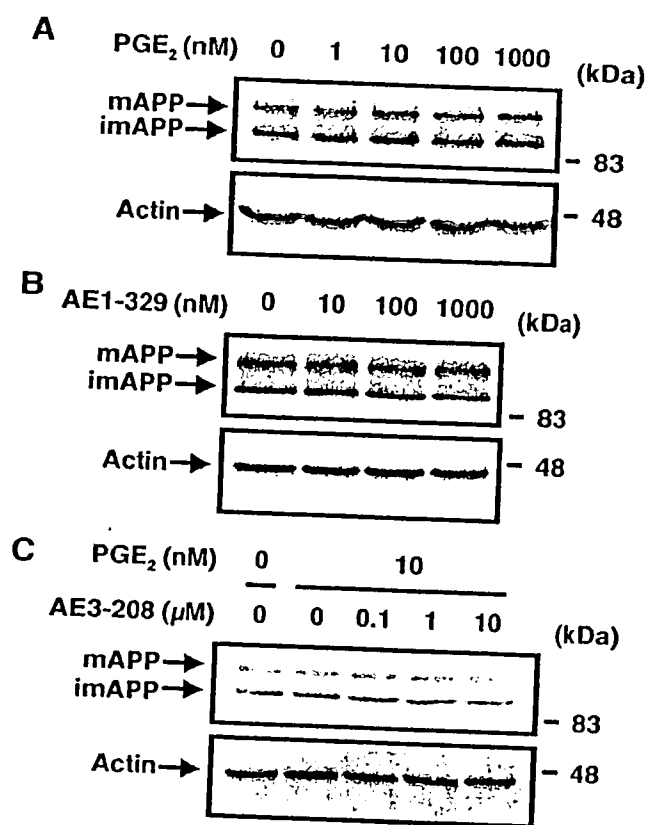


FIGURE 7. Effect of PGE₂, EP₄ agonist, and EP₄ antagonist on the maturation of APP. HEK293 cells expressing APPsw were cultured for 24 h in the presence of the indicated concentrations of PGE₂ alone (A), AE1-329 (EP₄ agonist) (B), or PGE₂ plus AE3-208 (EP₄ antagonist) (C). Whole cell extracts (10 μ g of protein) were analyzed by immunoblotting with an antibody against the C-terminal fragment of APP or actin. Similar results in this figure were obtained in two other independent experiments.

the level of A β in the conditioned medium. This chemical at the concentrations specified in Fig. 6, C and D, did not affect cell viability (data not shown) and, based on previous results (50), should have been sufficient to activate Epac. Therefore, it seems also that Epac is not involved in the PGE₂-stimulated production of A β . We confirmed that pCPT-*O*-Me-cAMP did not affect the cell growth at these concentrations used in Fig. 6 (data not shown).

Involvement of APP Phosphorylation and γ -Secretase Activation in PGE₂-stimulated Production of A β —We subsequently examined the downstream mechanism for PGE₂-stimulated production of A β in HEK293 cells. In general, production of A β is regulated by either modification of APP or by secretase activity. At first, we examined the maturation of APP, an essential step in the production of A β . The mature (*N*- and *O*-glycosylated) and immature (*N*-glycosylated alone) forms of APP (mAPP and imAPP, respectively) can be separated by SDS-PAGE on the basis of molecular weight (51). As shown in Fig. 7, PGE₂, EP₄ agonist, and EP₄ antagonist (in the presence of PGE₂) did not significantly affect the amounts or ratios of mAPP and imAPP. Consistent with a previous report (44), under similar experimental conditions to those described in Fig. 7, overexpression of endoplasmic reticulum chaperones increased or decreased the levels of imAPP or mAPP, respectively (data not shown). The results shown in Fig. 7 suggest that the PGE₂-

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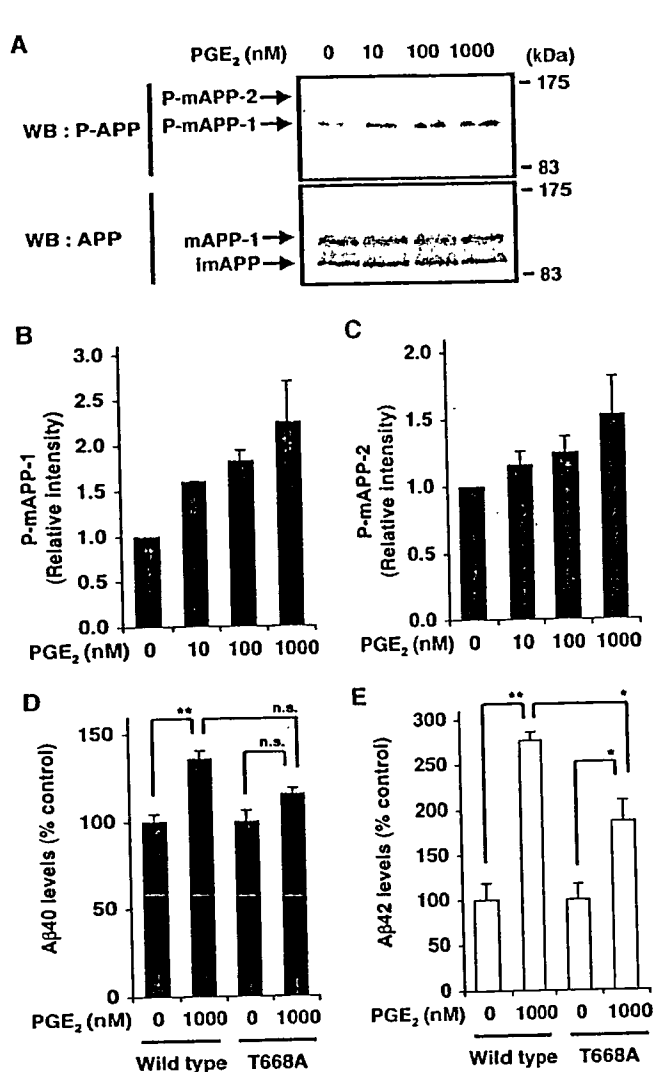


FIGURE 8. Involvement of the phosphorylation of APP in PGE₂-stimulated production of A β . HEK293 cells expressing APP_{sw} were cultured for 24 h in the presence of the indicated concentrations of PGE₂. Whole cell extracts were analyzed by immunoblotting (WB) with an antibody against the C-terminal fragment of APP (APP) or APP phosphorylated on Thr-668 (P-APP) (A). The relative intensity of bands of P-mAPP-1 and P-mAPP-2 in three independent immunoblotting experiments was determined by densitometer (B and C). HEK293 cells were transiently transfected with expression plasmid for either APP695 (wild type) (pcDNA3/APP695) or APP695 T668A (T668A) (pcDNA3/APP695 T668A). After culture for 24 h, cells were incubated with or without 1000 nM PGE₂ for 24 h. The amounts of A β 40 and A β 42 in the conditioned medium were determined and expressed as described in the legend of Fig. 1. Similar results were obtained in two other independent experiments (D and E). Values are given as mean \pm S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant (B–E).

stimulated production of A β is not due to any alteration in the maturation of APP. The results also showed that PGE₂ does not increase the total amount of APP (imAPP plus mAPP), thus PGE₂-stimulated production of A β cannot be explained by alteration in the amount (expression) of APP by PGE₂.

Phosphorylation of APP, in particular of threonine 668, is another protein modification of APP that may stimulate the production of A β (52). We examined the effect of PGE₂ on the level of Thr-668-phosphorylated APP by immunoblotting with an antibody that specifically recognizes this phosphorylated form of APP (Fig. 8A). As described previously (52), two bands (P-mAPP-1 and P-mAPP-2) were detected with this antibody

and the migration of the lower band (P-mAPP-1) was the same as the band detected by antibody against APP (mAPP-1) (Fig. 8A). Although the total amount of the mature or immature forms of APP was not affected by PGE₂, as described in Fig. 7, treatment of cells with PGE₂ increased the amount of Thr-668-phosphorylated APP in a dose-dependent manner but did not affect the total amount of APP (Fig. 8, A–C), suggesting that phosphorylation of the Thr-668 of APP is involved in PGE₂-stimulated production of A β . To test this possibility, we compared the PGE₂-stimulated production of A β in cells expressing wild type APP to those expressing mutant APP (Thr-668 to alanine, T668A). This mutation has been reported to completely suppress the phosphorylation of APP amino acid residue 668 (53). As shown in Fig. 8, D and E, PGE₂-stimulated production of A β was not so distinct in cells expressing mutant APP (T668A) as that observed in cells expressing wild-type APP. Thus, we consider that phosphorylation of APP at Thr-668 is involved in PGE₂-stimulated production of A β .

Next, we tested the notion that PGE₂-stimulated production of A β is mediated by an alteration of secretase activity. To investigate this possibility, we examined the amount of the CTFs of APP that are generated by α -, β -, or γ -secretase (CTF α , CTF β , or CTF γ , respectively) (known as an indirect index of secretase activity). As shown in Fig. 9A, the amounts of CTF α and CTF β in cells were not significantly affected by treatment with PGE₂. Furthermore, PGE₂ did not affect the amounts of CTF α and CTF β even in cells treated with an inhibitor of γ -secretase, DAPT. Treatment of cells with DAPT increased the amount of CTF α and CTF β (Fig. 9), but did not affect cell growth at the concentration (data not shown). These results suggest that PGE₂ does not affect the activity of α - or β -secretases. CTF γ could not be detected under the same experimental conditions as used for CTF α and CTF β , as previously described (34). Therefore, we incubated the membrane fractions *in vitro* to stimulate the γ -secretase-mediated proteolysis, as previously described (36). As shown in Fig. 9B, a band corresponding to CTF γ was able to be detected and its intensity increased according to the dose of PGE₂, suggesting that PGE₂ activates γ -secretase.

We also examined the effect of EP₄ agonist and EP₄ antagonist on the amounts of the CTFs. As was the case for PGE₂, the EP₄ agonist caused an increase in the intensity of the CTF γ band but not of the bands for CTF α or β (Fig. 9, C and D). Furthermore, EP₄ antagonist suppressed the PGE₂-dependent increase in the intensity of the CTF γ band but did not affect the levels of CTF α or β in the presence of PGE₂ (Fig. 9, E and F). The results shown in Fig. 9 strongly suggest that PGE₂-stimulated production of A β is mediated by activation of γ -secretase but not of α - or β -secretase.

We then directly measured the γ -secretase activity by using an APP-derived fluorescent substrate of γ -secretase (39, 40). As shown in Fig. 10A, γ -secretase activity in the solubilized membrane fraction prepared from cells treated with 1000 nM PGE₂ was significantly higher than that from non-treated cells. We confirmed that the addition of 1 μ M DAPT or PGE₂ to the assay system inhibited or had no effect, respectively, on γ -secretase activity (data not shown). We also examined the effect of EP₄ agonist and EP₄ antagonist on γ -secretase activity. As was the

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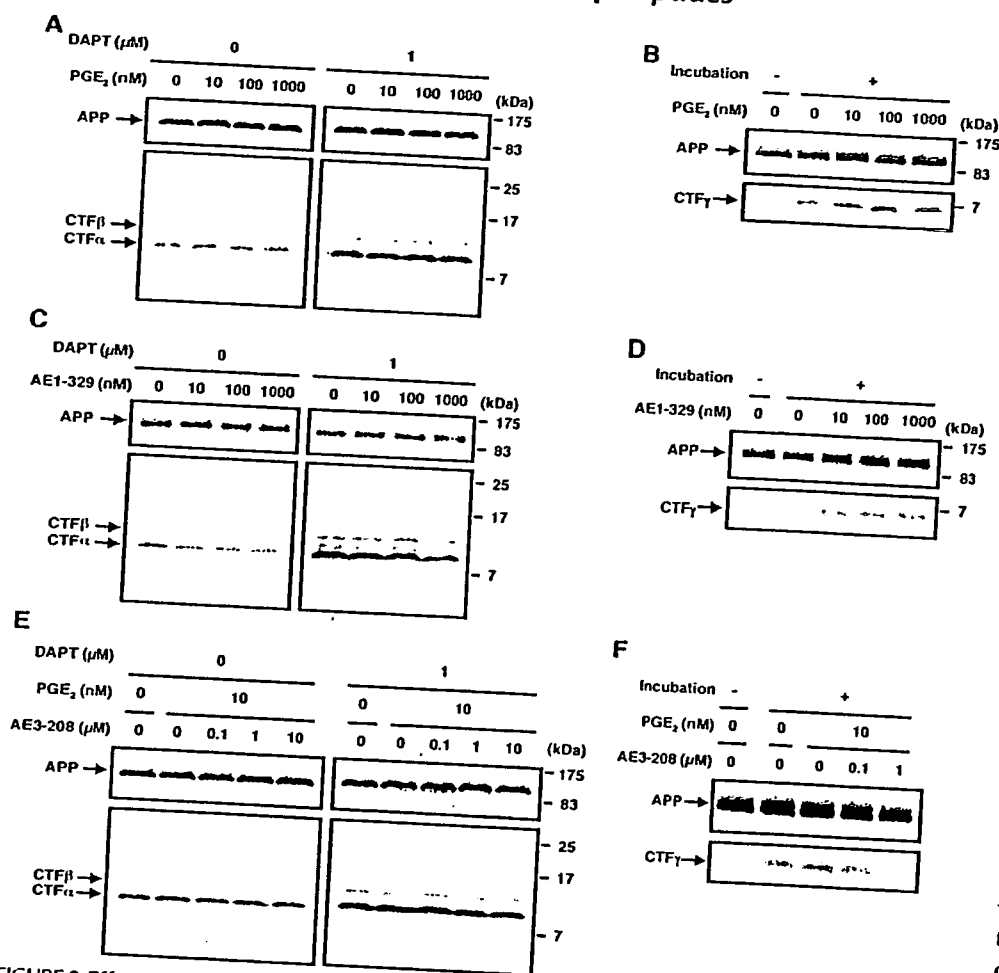


FIGURE 9. Effect of PGE₂, EP₄ agonist, and EP₄ antagonist on the cellular levels of CTF α , CTF β , and CTF γ . HEK293 cells expressing APPsw were cultured for 24 h in the presence of the indicated concentrations of PGE₂ (A and B), AE1-329 (EP₄ agonist) (C and D), or PGE₂ plus AE3-208 (EP₄ antagonist) (E and F) with or without 1 μ M DAPT as indicated. Membrane fractions were prepared and half of each was incubated for 2 h at 37 °C. Membrane fractions before (A, C, and E) or after (B, D, and F) the incubation were analyzed by immunoblotting with an antibody against the C-terminal fragment of APP. Similar results in this figure were obtained in two other independent experiments.

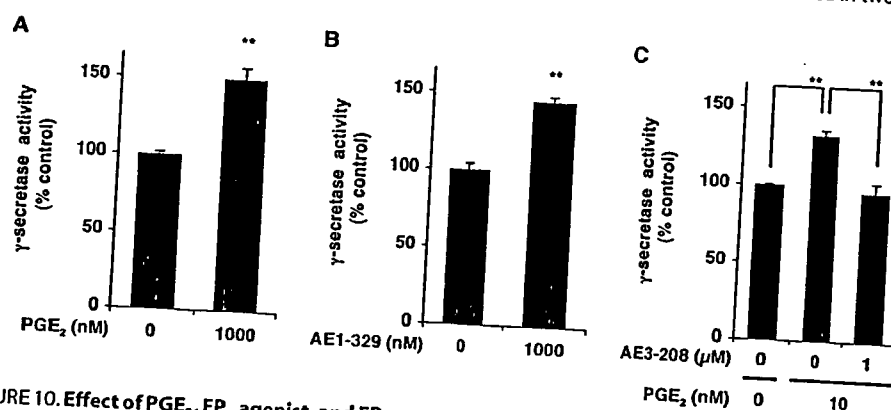


FIGURE 10. Effect of PGE₂, EP₄ agonist, and EP₄ antagonist on γ -secretase activity. HEK293 cells expressing APPsw were cultured for 1 h in the presence of the indicated concentrations of PGE₂ (A), AE1-329 (EP₄ agonist) (B), or PGE₂ plus AE3-208 (EP₄ antagonist) (C). Membrane fractions were prepared and subjected to a γ -secretase-mediated peptide cleavage assay as described under "Experimental Procedures." Values are given as mean \pm S.E. (n = 3). **, p < 0.01. Similar results in this figure were obtained in two other independent experiments.

case for PGE₂, EP₄ agonist activated γ -secretase activity, whereas on the other hand, EP₄ antagonist suppressed the PGE₂-dependent activation of γ -secretase (Fig. 10, B and C). We also showed that treatment of cells with DAPT inhibited the pro-

duction of A β in the presence absence of PGE₂ (supplemental S4). The results presented in Fig. S4 show that treatment of cells with PGE₂ activates γ -secretase in cell

Involvement of EP₂ and EP₄ Receptors in the Production of A β Vivo—To test the *in vivo* relevance of the *in vitro* results of this study we examined the effect of deletion of the EP₂ or EP₄ receptor on the level of A β in mouse brain. For this purpose, we crossed transgenic mice expressing APPsw (APP23 (30) to EP₂^{-/-} mice (31) or EP₄^{-/-} mice (32) to generate APPsw/EP₂^{-/-} or APPsw/EP₄^{-/-} mice respectively. These two types of mice developed normally and gained weight at a rate equal to their controls (APPsw/EP₂^{+/+} mice or APPsw/EP₄^{+/+}).

We measured the amount of A β in both soluble and insoluble fractions prepared from mouse brain by sELISA. As shown in Fig. 11, A–D, the amount of A β in the brains of APPsw/EP₂^{-/-} mice was lower in both fractions than for the APPsw/EP₂^{+/+} mice at the ages of both 3 and 6 months, although the difference in the amounts of A β 42 in the soluble fraction was not statistically significant at the age of 6 months. On the other hand, the amount of A β in the brains of APPsw/EP₄^{-/-} mice was lower in both fractions than that of APPsw/EP₄^{+/+} mice at the age of 6 months, although the difference in the amounts of A β 42 in the insoluble fraction were not statistically significant (Fig. 11, G and H). A difference was not as distinct at the age of 3 months (Fig. 11, E and F). These findings suggest that both EP₂ and EP₄ receptors are also involved in the production of A β *in vivo*; in other words, the *in vitro* results obtained in this article are functionally significant. We also compared the level of PGE₂ and mRNA expression of EP₂ and EP₄ receptors between wild-type and APP23 mice. As shown in supplemental Fig. S5, there was no clear difference between them at both 3 and 6 months, except that the amount of PGE₂ was significantly higher in APP23 mice than wild-type mice at the age of 6 months, which is consistent with previous results (54).

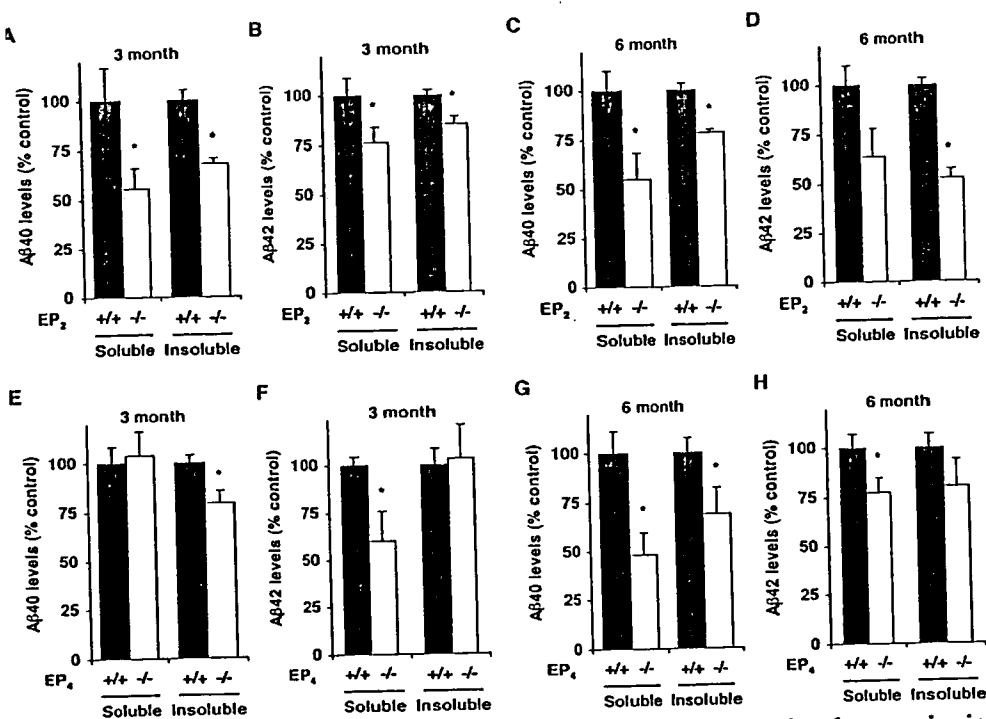


FIGURE 11. Effects of deletion of the EP₂ or EP₄ receptor on the level of A β in the brains of transgenic mice expressing APPsw. The amounts of A β 40 and A β 42 in both the soluble and insoluble fractions prepared from the brains of APPsw/EP₂^{-/-} and APPsw/EP₂^{+/+} mice (A–D) or APPsw/EP₄^{-/-} and APPsw/EP₄^{+/+} mice (E–H) at the age of 3 and 6 months were determined by sELISA and expressed relative to the control. Values are given as mean \pm S.E. ($n = 5–6$). *, $p < 0.05$. The control values used are (pmol/g tissue) (soluble, insoluble): A (0.920 \pm 0.154, 8.320 \pm 0.445); B (0.105 \pm 0.010, 1.630 \pm 0.048); C (1.080 \pm 0.109, 14.360 \pm 0.506); D (0.108 \pm 0.015, 2.700 \pm 0.221); E (1.060 \pm 0.086, 8.810 \pm 0.315); F (0.076 \pm 0.004, 1.820 \pm 0.329); G (1.313 \pm 0.149, 15.597 \pm 1.206); and H (0.142 \pm 0.010, 3.030 \pm 0.216).

DISCUSSION

It has previously been reported that PGE₂ stimulates production of A β in CHO cells (26). In this study, we have confirmed that this also occurs in HEK293 cells and have shown that PGE₂-mediated activation of the EP₄ receptor and the resulting increase in the cellular level of cAMP are responsible for this PGE₂-stimulated production of A β . These conclusions have been based on the following observations: EP₄ agonist-stimulated production of A β , EP₄ receptor antagonist suppressed the PGE₂-stimulated production of A β , an increase in the cellular level of cAMP was observed simultaneously with stimulation of the production of A β , and an analogue of cAMP stimulated the production of A β . We have also demonstrated that PGE₂ stimulates production of A β in SH-SY5Y cells. However, in contrast to what was found for HEK293 cells, our data suggest that both EP₂ and EP₄ receptors are responsible for the PGE₂-stimulated production of A β in SH-SY5Y cells. This difference may be due to the fact that the EP₂ receptor is functional in SH-SY5Y but not in HEK293 cells, as suggested previously (55). This suggestion is further supported by our observation that an EP₂ receptor agonist increased the cellular level of cAMP in SH-SY5Y cells but not in HEK293 cells. It is also possible that the low expression of EP₂ receptor in HEK293 cells can explain why this type of cells were inert for the increase in the cellular level of cAMP by the EP₂ agonist. This is the first report of the identification of EP receptors and the intracellular signal transduction pathway for PGE₂-stimulated pro-

duction of A β , which should be important for the development of clinical drugs for AD (see below). Liang *et al.* (56) reported that another EP₂ agonist (butaprost) did not affect the production of A β in CHO cells. This seems to be due to the fact that the EP₂ receptor is not functional in CHO cells, as previously described (57), because others have reported that PGE₂ stimulates the production of A β in CHO cells (26). A number of previous papers reported that both the EP₂ and EP₄ receptors are expressed in rodent brain and in primary cultures of rodent neurons (58–60). Therefore, it is reasonable to speculate that both EP₂ and EP₄ receptors are functional in rodent (maybe in human) neurons and are responsible for PGE₂-stimulated production of A β . Results presented in a previous paper (56) are not consistent with this speculation: they showed that an EP₂ agonist (butaprost) did not affect the production of A β in primary cultures of mouse neurons. We speculate that this is because

they used primary neurons from mice expressing not only APPsw but also the exon 9-deleted human PS-1 (PS1 Δ E9) (see below).

In most of the intracellular signal transduction pathways that are downstream of the increase in the level of cellular cAMP, both PKA and PI3K play important roles (46). However, results from experiments using kinase inhibitors suggest that neither of these kinases are involved in PGE₂-stimulated production of A β . Furthermore, results from experiments using a specific activator for Epac suggested that Epac is also not involved in PGE₂-stimulated production of A β . Therefore, an unknown mechanism, located downstream of the cAMP increase, seems to be involved in the signal transduction pathway for PGE₂-stimulated production of A β .

It was previously reported that PGE₂ increases the amount of CTF γ in CHO cells (26); however, the molecular mechanism governing this increase remained unknown. In this study, we observed that not only PGE₂, but also an EP₄ agonist, increased CTF γ levels in HEK293 cells. Furthermore, we directly measured γ -secretase activity and showed that the activity was higher in cells treated with PGE₂ or EP₄ agonist than in control cells. These results suggest that activation of γ -secretase is responsible for both the PGE₂-mediated increase in the cellular level of CTF γ and for stimulation of the production of A β . Because overexpression of PS1 Δ E9 has been shown to cause activation of γ -secretase (61, 62), we consider that the higher activity of γ -secretase in cells expressing PS1 Δ E9 may mask the EP₂-mediated activation of

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γ -secretase and this could explain why, in the previous report (56), the EP₂ agonist butaprost did not affect the production of A β . We also found that phosphorylation of Thr-668 in APP is stimulated in cells treated with PGE₂ and that cells expressing mutant APP (T668A) showed less of a response than the wild type control against PGE₂-mediated stimulation of A β production. This suggests that Thr-668 phosphorylation of APP is also involved in PGE₂-stimulated production of A β . However, the molecular mechanisms whereby the increase in the cellular level of cAMP affects phosphorylation of Thr-668 of APP remains unknown. A number of previous reports suggested that the γ -secretase activity is modulated by various molecules such as phospholipase D1, CD147, and platelet-derived growth factor (63–65), therefore, it is possible that the increase in the level of cellular cAMP affect γ -secretase activity through modulation of activities of these molecules.

In this paper, both EP₂ and EP₄ receptors have been shown to be involved in the production of A β not only *in vitro* but also *in vivo*: the amount of A β in the brains of APP^{sw}/EP₂^{-/-} and APP^{sw}/EP₄^{-/-} mice was lower than in control mice. This is the first demonstration that deletion of the EP₄ receptor decreases the level of A β *in vivo* and we speculate that this decrease is due to the lack of EP₄-mediated activation of γ -secretase by PGE₂, as was seen *in vitro*. Liang *et al.* (56) recently reported that deletion of the EP₂ receptor, in the genetic background of mice expressing both APP^{sw} and PS1 Δ E9, decreased the level of A β . They suggested that this decrease is due to loss of the EP₂ receptor in microglial cells where they are responsible for the production of reactive oxygen species, which activate β -secretase in neurons. Deletion of the EP₂ receptor decreased the level of A β at the age of 3 months in our mouse model (without expression of PS1 Δ E9) but this effect was observed at ages greater than 8 months in their mouse model (with the expression of PS1 Δ E9). We speculate that the higher activity of γ -secretase caused by the expression of PS1 Δ E9 masks the effect of the deletion of the EP₂ receptor on γ -secretase activity that can be seen at younger ages in mice that do not express PS1 Δ E9. Based on these observations, we consider that the EP₂-mediated direct activation of γ -secretase or activation of β -secretase via reactive oxygen species contributes to the alteration in the level of A β in the brain of EP₂ knock-out mice at younger or older ages, respectively. In other words, the EP₂ receptor is involved in the production of A β through at least two independent mechanisms.

As described above, NSAIDs have attracted much attention as a new class of drugs for the treatment and prevention of AD, although some animal and clinical studies showed negative results for the advantage of NSAIDs as drugs for AD (19, 66, 67). Although some reports suggest that the COX-independent actions of NSAIDs (such as direct binding to γ -secretase, activation of the peroxisome proliferators activated receptor- γ , and resulting inhibition of β -secretase, inhibition of Rho/Rho kinase (Rock) pathway, and activation of nuclear factor- κ B (NF- κ B)) are involved in the inhibitory effect of NSAIDs on the production of A β (19, 21, 22, 68), COX inhibition, and the resulting decrease in the level of

PGE₂ seem to play an important role in the anti-AD activity of NSAIDs (26, 69). COX exists as two subtypes, COX-1 and COX-2, and NSAIDs can be classified into two groups: new developed COX-2-specific NSAIDs (such as celecoxib) and classical NSAIDs without COX-2 specificity (such as indomethacin). Clinical use of classical NSAIDs is associated with gastrointestinal side effects (23), because PGs have strong protective effect on gastrointestinal mucosa (70, 71). Because COX expressed in gastrointestinal mucosa is mainly COX-1, COX-2-specific NSAIDs have less of an effect on the levels of PGs at gastrointestinal mucosa and therefore, would cause less gastrointestinal side effects than classical NSAIDs. However, it was recently shown that clinical use of COX-2 specific NSAIDs is associated with cardiovascular thrombotic side effects (24, 25). This is because prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells whereas thromboxane A₂, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets (72, 73). These side effects of NSAIDs would most likely become problematic when used long-term for the prevention or treatment of AD. On the other hand, results of this study suggest that antagonists for either the EP₂ or EP₄ receptors, or more importantly antagonists for both EP₂ and EP₄ receptors, will be effective for the treatment and prevention of AD through inhibiting the production of A β . EP₁ and EP₃ receptors were reported to be involved in PGE₂-mediated protection of gastrointestinal mucosa through stimulating the production of bicarbonate and gastric mucosal blood flow, respectively (74, 75). Therefore, antagonists specific for both EP₂ and EP₄ would be safer for gastrointestinal mucosa than NSAIDs. At present, there are no reports of specific EP₂ antagonists and it is unclear whether or not the EP₄ antagonist used in this study (AE3-208) is able to cross the blood-brain barrier. Therefore, we predict that blood-brain barrier-permeable antagonists for both EP₂ and EP₄ receptors will be therapeutically beneficial for AD. Of course, it should be noted that this type of drugs may not be as effective as NSAIDs, if the COX-independent actions of NSAIDs (see above) are predominant for their anti-AD actions.

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