Prostaglandin I\textsubscript{2} upregulates the expression of anterior pharynx-defective-1\textalpha and anterior pharynx-defective-1\textbeta in amyloid precursor protein/presenilin 1 transgenic mice

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Summary
Cyclooxygenase-2 (COX-2) has been recently identified to be involved in the pathogenesis of Alzheimer's disease (AD). Yet, the role of an important COX-2 metabolic product, prostaglandin (PG) I\textsubscript{2}, in the pathogenesis of AD remains unknown. Using human- and mouse-derived neuronal cells as well as amyloid precursor protein/presenilin 1 (APP/PS1) transgenic mice as model systems, we elucidated the mechanism of anterior pharynx-defective (APH)-1\textalpha and pharynx-defective-1\textbeta induction. In particular, we found that PGI\textsubscript{2} production increases during the course of AD development. Then, PG\textsubscript{2} accumulation in neuronal cells activates PKA/CREB and JNK/c-Jun signaling pathways by phosphorylation, which results in APH-1\textalpha/\textbeta expression. As PG\textsubscript{2} is an important metabolic by-product of COX-2, its suppression by NS398 treatment decreases the expression of APH-1\textalpha/\textbeta in neuronal cells and APP/PS1 mice. More importantly, \beta-amyloid protein (\textalpha\textbeta) oligomers in the cerebrospinal fluid (CSF) of APP/PS1 mice are critical for stimulating the production of APH-1\textalpha/\textbeta, which was blocked by NS398 incubation. Finally, the induction of APH-1\textalpha/\textbeta was confirmed in the brains of patients with AD. Thus, these findings not only provide novel insights into the mechanism of PG\textsubscript{2}-induced AD progression but also are instrumental for improving clinical therapeutics to combat AD.

Key words: \beta-amyloid protein; anterior pharynx-defective-1\textalpha/\textbeta; APP/PS1; cyclooxygenase-2; prostaglandin I\textsubscript{2}.

Introduction
Alzheimer’s diseases (AD) is the most common cause of dementia in aged people and is characterized clinically by cognitive decline and pathologically by the accumulation of amyloid \beta-protein (\textalpha\textbeta) and hyperphosphorylation of tau in the brain (Hoshino et al., 2007; Arnaud et al., 2009). Here, increases in the expression of several potentially toxic secretases, including BACE-1, presenilin 1/2 (PS1/2), anterior pharynx-defective (APH)-1\textalpha/\textbeta, nicastin, and PEN2, result in the formation of \textalpha\textbeta plaques, synapse dysfunction or loss, neuronal loss, and diffuse brain atrophy, thereby leading to the decline of cognitive abilities (De Strooper, 2003). The \gamma-secretases, such as APH-1\textalpha and APH-1\textbeta, are required for notch pathway signaling, for \gamma-secretase cleavage of \textalpha\textbeta-APP, and for \textalpha\textbeta protein accumulation in C. elegans (Francis et al., 2002). Indeed, APH-1 usually interacts with PEN-2, nicastin, and PS to generate an active form of the \gamma-secretase complex, which is responsible for the cleavage of \textalpha\textbeta-APP and the deposition of \textalpha\textbeta (De Strooper, 2003). Once APH-1 was found in C. elegans (Francis et al., 2002), the APH-1 complex was then confirmed in several experimental models (Gu et al., 2003; Luo et al., 2003; Hansson et al., 2004). However, the regulatory mechanism of APH-1\textalpha and APH-1\textbeta are often overlooked during the course of AD progression.

To reveal the possible mechanism of APH-1\textalpha and APH-1\textbeta regulation, it is necessary to identify the molecular pathways that are responsible for the deposition of \textalpha\textbeta. Epidemiological and clinical data suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) are beneficial in the treatment and prevention of AD (Imbimbo et al., 2010). The protective effects of NSAIDs in AD are due to their anti-inflammatory properties that inhibit cyclooxygenase-2 (COX-2) (McGeer, 2000; van Gool et al., 2003). As an important factor in inflammatory reactions of peripheral tissues, COX-2 has a potential role in the pathogenesis of AD. This has been populusly investigated through studies of its metabolic products, the prostaglandins (PGs), including PGE\textsubscript{2}, PGD\textsubscript{2} (and its dehydration product 15-deoxy-\Delta\textsubscript{12,14}-PGE\textsubscript{2} (15d-PGJ\textsubscript{2})), PG\textalpha\textbeta, PG\textalpha\textbeta\textsubscript{2x}, and TX\textalpha\textbeta (Akarasereenont et al., 1999). For example, PGE\textsubscript{2} treatment increases the ratio of APH-1\textalpha/\textbeta against PG\textalpha\textbeta production in SH-SYSY cells and APP23 transgenic mice (Hoshino et al., 2007, 2009). In line with these observations, PGE\textsubscript{2} was further verified to stimulate the production of APH-1\textalpha/\textbeta alone in C57BL/6 mice (Cheverria et al., 2005). In addition to PGE\textsubscript{2}, PG\textalpha\textbeta shows positive effects on stimulating the production of APH-1\textalpha/\textbeta in primary mouse microglia and neuronal cells (Bate et al., 2006). As expected, 15d-PGJ\textalpha is also able to increase fibrillar \textalpha\textbeta in rat cortical neurons (Takata et al., 2003; Yamamoto et al., 2011) while PG\textalpha\textbeta has been shown to be involved in \textalpha\textbeta production in microglial cells (Zhuang et al., 2013). However, the effects of PG\textsubscript{2} on the production of \textalpha\textbeta are not well studied.

In this study, an intracellular signaling pathway by which PG\textsubscript{2} regulates the expression of APH-1\textalpha/\textbeta has been proposed to contribute to the deposition of \textalpha\textbeta. Specifically, PG\textsubscript{2} treatment increases the expression of APH-1\textalpha/\textbeta via the PKA/CREB and JNK/c-Jun activation pathways in SH-SYSY cells, which facilitates the synthesis of \textalpha\textbeta in neuron cells. More importantly, in AD, the \textalpha\textbeta oligomers were localized to the CSF microenvironment, which contributes to the expression of APH-1\textalpha/\textbeta. Reconstructing the signaling network that regulates PG\textsubscript{2}-mediated APH-1\textalpha/\textbeta expression in neuron cells will facilitate the development of strategies to combat AD.

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Accepted for publication 30 April 2016
Results

**APH-1α/β is highly induced in APP/PS1 transgenic mice**

Due to previous reports of a pivotal role of APH-1α/β in the pathogenesis of AD (Mrak & Griffin, 2001; De Strooper, 2003), we evaluated the expression levels of APH-1α/β in AD brains. As shown in Fig. 1a, the immunoreactivity of APH-1α/β and β-amyloid (Aβ) was highly induced at the patients with AD. In accordance with this observation, the mRNA and protein expression levels of APH-1α/β were elevated at the patients with AD (Fig. 1b). Due to the limited accessibility of human AD samples, we performed similar experiments in APP/PS1 transgenic mice. The results demonstrated that APH-1α/β immunostaining was markedly increased in the cerebral cortex as well as in the dentate gyrus region of the hippocampus of APP/PS1 transgenic mice at 6 months of age, when compared to the WT C57BL/6 mice (Fig. 1d). In accordance with our immunostaining observations, the mRNA and protein expression of APH-1α/β was upregulated in the cerebral cortex and hippocampus of APP/PS1 mice (Fig. 1c,e). When considered together, these results clearly demonstrate that APH-1α/β levels were increased in APP/PS1 transgenic mice. These data agree with previous reports (De Strooper, 2003), suggesting that APH-1α/β are possibly involved in aggravating AD.

**Elevation of PGI2 accelerates the synthesis of APH-1α/β in APP/PS1 transgenic mice**

We next sought to elucidate the mechanism by which APH-1α/β are upregulated in an AD mouse model. Because evidence suggests that PGI2 is a potential mediator of neuroinflammation (Ford-Hutchinson et al., 1978; Honda et al., 2005; Pulchino et al., 2006), we sought to determine the concentration of PGI2 in APP/PS1 transgenic mice at 6 months of age. As shown in Fig. 1f, the synthesis of PGI2 was markedly increased in APP/PS1 transgenic mice. To know the roles of PGI2 in AD development, we first screened the effects of PGI2 on the expression of α-, β- or γ-secretases. The results demonstrated that PGI2 treatment concurrently downregulated the expression of ADAM-10 and upregulated the expression of BACE-1, PS2, and APH-1α/β in n2a cells (Table 1). To further understand the possible role of PGI2 in AD, we then injected (i.c.v.) PGI2 (2 μg/5 μL) or incubated brain slices from WT C57BL/6 mice with PGI2 (10 μM) for 24 h. IHC staining indicated that APH-1α/β expression was induced in both the cerebral cortex and the DG region of APP/PS1 transgenic mice (Fig. 2a,b). In addition, these observations were verified by qRT-PCR and Western blots (Fig. 2c,d). To identify the role of PGI2 in inducing the expression of APH-1α/β, we conducted live animal and two-photon imaging experiments, as described in the 'Materials and Methods'. The results revealed that PGI2 (2 μg/5 μL) injection (i.c.v.) into the ventricles of WT C57BL/6 mice increased the luciferase activity of the APH-1α/β promoters at 12 h following injection. This activity was maximal at 24 h following injection (Fig. 2e). Of note, PBS (−) injection (i.c.v.) does not alter the activity of APH-1α and APH-1β promoters (data not shown). Two-photon imaging results reinforced the notion that PGI2 (2 μg/5 μL) injection (i.c.v.) increased the immunofluorescence of APH-1α/β in the cerebral cortex of WT C57BL/6 mice (Fig. 2f). Accordingly, we then treated the SH-SYSY and n2a neuronal cells with PGI2 (10 μM) for 48 h. Our data demonstrated that PGI2 treatment clearly increases the expression of APH-1α/β in human or mouse neurons (Fig. 2g,h). These results were supported by the immunostaining experiments, and the images obtained using Leica confocal microscopy (Fig. 2i,j). Collectively, our data clearly support the fact that PGI2 elevation increases the synthesis of APH-1α/β in vitro and in vivo.

**PGI2 inhibition impaired the expression of APH-1α/β following intranasal administration of NS398 in APP/PS1 transgenic mice**

Because PGI2 is an important metabolic product of COX-2, we treated APP/PS1 transgenic mice with a COX-2-specific inhibitor, NS398.
The results demonstrated that the injection (i.c.v.) of NS398 (25 µM) or SP600125 (10 µM) or Bafilomycin A1 (10 nM) in APP/PS1 transgenic mice (Fig. 3f) reversed the concurrent downregulation of sAPPα and a decrease in the production of sAPPβ in APP/PS1 transgenic mice (Fig. 5d–f). In addition, the elevated induction of APP/PS1 transgenic mice was injected into WT C57BL/6 mice in the absence or presence of Aβ antibody for 2 weeks prior to sacrifice. When compared to control animals, the expression of APP-1α/1β in both the cerebral cortex and hippocampus was markedly increased by the injection (i.c.v.) of APP/PS1 CSF (Fig. 5a–c). In addition, the injection of APP-1α/1β was suppressed by the injection (i.c.v.) of the Aβ antibody (1 mg/5 µL) (Fig. 5a–c). Therefore, these observations demonstrate that the confinement of the secreted form of Aβ to AD-related microenvironments might induce the expression of APP-1α/1β in a PGI2-dependent manner.

To confirm these observations, we performed experiments to directly evaluate the involvement of Aβ oligomers in the progression of AD. Aβ oligomer (2 µg/5 µL) injection (i.c.v.) clearly increases the expression of APP-1α/1β in both the cerebral cortex and hippocampus of WT C57BL/6 mice (Fig. 5d–f). The upregulation of APP-1α/1β was further suppressed by NS398 (25 µg/5 µL) injection (i.c.v.) in Aβ1–42-stimulated C57BL/6 mice (Fig. 5d–f). These in vivo observations were reinforced by in vitro experiments that demonstrated that Aβ (1 µM) treatment stimulates the expression of APP-1α/1β in neuron cells by organotypic slice or cell culture (Fig. 5g–i). Thus, Aβ oligomers are critical for worsening AD.

Table 1 The effects of PGI2 on the expression of α-, β-, or γ-secretases in n2a cells

| Gene Name | Control | PGI2 (10 µM) |
|-----------|---------|-------------|
| ADAM-10   | 1       | 0.59        |
| BACE-1    | 1       | 4.59        |
| PS1       | 1       | 0.99        |
| PS2       | 1       | 1.92        |
| APH-1α    | 1       | 3.25        |
| APH-1β    | 1       | 1.99        |
| Nicastrin  | 1      | 0.94        |
| PEN2      | 1       | 1.08        |

(50 µg kg⁻¹ day⁻¹), for 5 months. Our results revealed that PGI2 was significantly suppressed by NS398 (50 µg kg⁻¹ day⁻¹) administration in APP/PS1 transgenic mice (Fig. 3a). More interestingly, the inhibition of PGI2 by NS398 resulted in a decrease in the levels of APP-1α/1β in APP/PS1 transgenic mice by IHC staining (Fig. 3c). Similar results were verified by qRT-PCR or Western blot experiments (Fig. 3e,f). Of note, NS398 treatment does not affect the body weight of mice and induces wound healing to the mice as previously indicated (Wang et al., 2011a). We then injected APP/PS1 transgenic mice with NS398 (2 µg/5 µL) for 24 h. The results demonstrated that the injection (i.c.v.) of NS398 (2 µg/5 µL) shows a suppressive effect on the production of PGI2 in APP/PS1 transgenic mice (Fig. 3b). Of note, our results reinforce the hypothesis that NS398 treatment (2 µg/5 µL) suppressed the expression of APP-1α/1β (Fig. 3d, g,h) by inhibiting the production of PGI2. In addition, the production of sAPPα was restored to the control level (i.e. the basal level of WT mice), whereas the increased production of sAPPβ was attenuated at the basal level of WT mice following intranasal administration of NS398 for 5 months in APP/PS1 mice (Fig. 3i,j). Intranasal administration of NS398 also decreased the production of APH-1α (Fig. 3i,j), which potentially decelerates the pathogenesis of AD. To further verify the role of NS398 in suppressing the expression of APP-1α/1β, we performed intracerebroventricular injections of the inhibitor. Similar to nasal administration, NS398 injection (i.c.v., 2 µg/5 µL) reversed the concurrent downregulation of sAPPα and upregulation of sAPPβ in APP/PS1 mice (Fig. 3k,l). Therefore, our results concretely support the hypothesis that NS398 has the ability to suppress the expression of APP-1α/1β by decreasing the production of PGI2 in APP/PS1 transgenic mice.

Critical role of PKA/CREB and JNK/c-Jun signaling pathways in mediating PGI2-induced APP-1α/1β expression in n2a cells

We next aimed to elucidate the signaling pathways of PGI2-induced APP-1α/1β synthesis in PGI2-treated n2a cells. First, 48 h of PGI2 (10 µM) treatment activated PKA/CREB and JNK/c-Jun signaling pathways by the phosphorylation of CREB and c-Jun (Fig. 4a–c), which resulted in the synthesis of APP-1α and APP-1β in n2a cells (Fig. 4a,c). To further elucidate the role of PKA/CREB and JNK/c-Jun signaling pathways in regulating the expression of APP-1α/1β, we treated n2a cells with the PKA pharmacological inhibitor H89 (1 µM) or JNK-specific inhibitor SP600125 (10 µM). Treatment of n2a cells with H89 (1 µM) or SP600125 (10 µM) not only suppressed the phosphorylation of CREB and c-Jun (Fig. 4a,c) but also reversed the synthesis of APP-1α/1β in PGI2-treated n2a cells (Fig. 4a,c). To verify these observations and to account for the nonspecificity of the pharmacological inhibitors, we transfected n2a cells with siRNAs that were specific for interfering with the expression of CREB or c-Jun prior to incubating them with PGI2 (10 µM). As shown in Fig. 4d and e, CREB and c-Jun knockdown efficiently decreased the protein levels of CREB and c-Jun. As a consequence, the knockdown of CREB or c-Jun inhibited the effects of PGI2 on inducing the synthesis of APP-1α/1β in n2a cells (Fig. 4d,e). In addition, inhibiting the signaling pathways of PKA/CREB and JNK/c-Jun concurrently results in the restoration of the production of sAPPα and a decrease in the production of sAPPβ at the basal level in PGI2-treated n2a cells (Fig. 4f,g). More importantly, inhibiting the activity of the PKA/CREB or the JNK/c-Jun signaling pathways resulted in the attenuation of APH-1α formation in PGI2-activated n2a cells (Fig. 4f,g). Therefore, these observations support the hypothesis that PKA/CREB and JNK/c-Jun signaling pathways are important in mediating PGI2-induced APP-1α/1β expression, which results in Aβ deposition in neuron cells.

Aβ oligomers in the CSF of APP/PS1 mice have the ability to stimulate the expression of APP-1α/1β

Because NS398 incubation decreases the expression of APP-1α/1β and Aβ oligomers by decreasing the production of PGI2 in vitro and in vivo, we next examined the potential contribution of Aβ1–42 to the pathogenesis of AD. We conducted experiments to determine whether Aβ1–42 is confined to microenvironments that are related to AD in APP/PS1 transgenic mice. In brief, the cerebrospinal fluid (CSF) of APP/PS1 transgenic mice was injected into WT C57BL/6 mice in the absence or presence of Aβ antibody for 2 weeks prior to sacrifice. When compared to control animals, the expression of APP-1α/1β in both the cerebral cortex and hippocampus was markedly increased by the injection (i.c.v.) of APP/PS1 CSF (Fig. 5a–c). In addition, the injection of APP-1α/1β was suppressed by the injection (i.c.v.) of the Aβ antibody (1 µg/5 µL) (Fig. 5a–c). Therefore, these observations demonstrate that the confinement of the secreted form of Aβ1–42 to AD-related microenvironments might induce the expression of APP-1α/1β in a PGI2-dependent manner.

Discussion

β-amyloid protein (Aβ) deposition and hyperphosphorylation of tau are pathological characteristics of AD (1). As the role of PGI2 in AD development is presently unknown, we designed a study to identify the aggravating effects of PGI2 on AD. The major findings of this study are as follows: (i) APP-1α/1β expression was markedly upregulated during the course of AD development; (ii) the accumulation of PGI2 in neuron cells induced the mRNA and protein expression of APP-1α/1β in APP/PS1 mice; (iii) the PKA/CREB and JNK/c-Jun signaling pathways are critical for mediating the effects of PGI2 on stimulating the expression of APP-1α/1β, which is critical for γ-cleavage of β-APP and producing Aβ oligomers; and (iv) Aβ1–42 oligomers in the CSF of APP/PS1 mice are responsible for augmenting the activity APP-1α/1β, which potentially aggravates the pathogenesis of AD (Fig. 6).
Substantial evidence indicates that prostaglandins, such as PGE₂ and 15d-PGJ₂, are important for Aβ deposition and tau tangle, which contribute to the role of COX-2 in the pathophysiology of AD (Hoshino et al., 2007; Arnaud et al., 2009). However, knowledge concerning the specific function of PGI₂ in the human brain is limited. Indeed, the assumption that PGI₂ is synthesized in brain tissue in vivo is based on observations in primary culture of astrocytes and meningeal cells (Murphy et al., 1985) as well as of the expression of PGI₂ receptor (PI) in the rodent brain (Oida et al., 1995; Takechi et al., 1996). Siegle et al. (Siegle et al., 2000) supported this hypothesis by demonstrating, via IHC...
and in situ hybridization, that glia and neuron cells in the human brain express PGi2 synthase (PGIS). Moreover, the expression of PGIS in the human brain was supported by the detection of 6-keto-PGF1α, a stable degradation product of PGi2, in human brain homogenates by enzyme immunoassay kits (Siegle et al., 2000). Our data agree with this prior work (Siegle et al., 2000) by demonstrating the presence of PGi2 in the brains of C57BL/6 mice. PGi2 production was elevated in the brain of APP/PS1 transgenic mice when compared with that of the WT control. PGi2 synthesis may result from COX-2 upregulation in APP/PS1 transgenic mice (data not shown). Yosojima et al. (Yosojima et al., 1999) reported that COX-2 was substantially upregulated in the affected areas of AD brains. In addition, COX-2 is responsible for the systemic synthesis of PGi2 (McAdam et al., 1999). Therefore, PGi2 synthesis was markedly increased during the course of AD progression.
PGI2 elevation stimulates the expression of APH-1α/β via the PKA/CREB and JNK/c-Jun signaling pathways in cultured neuronal cells. n2a cells were treated with PGI2 (10 μM) in the absence or presence of H89 (1 μM) (a, b, f) or SP600125 (5 μM) (c, g) cells for 48 h. In distinct experiments, n2a cells were transfected with CREB (d) or c-Jun siRNA (e) before treating the cells with PGI2 (10 μM) for 48 h. APH-1α/β mRNA and protein levels were determined by qRT–PCR and Western blots, respectively (a, c–e). Phosphorylated CREB and c-Jun as well as total CREB and c-Jun were detected by immunoblotting using specific antibodies (a–e). The production of sAPPα and sAPPβ was determined by Western blots (f, g). The data represent the means ± SE of three independent experiments. *P < 0.05 with respect to the vehicle-treated or vector-transfected control. #P < 0.05 compared to PGI2-treated alone.

Fig. 4 PGI2 elevation stimulates the expression of APH-1α/β via the PKA/CREB and JNK/c-Jun signaling pathways in cultured neuronal cells. n2a cells were treated with PGI2 (10 μM) in the absence or presence of H89 (1 μM) (a, b, f) or SP600125 (5 μM) (c, g) cells for 48 h. In distinct experiments, n2a cells were transfected with CREB (d) or c-Jun siRNA (e) before treating the cells with PGI2 (10 μM) for 48 h. APH-1α/β mRNA and protein levels were determined by qRT–PCR and Western blots, respectively (a, c–e). Phosphorylated CREB and c-Jun as well as total CREB and c-Jun were detected by immunoblotting using specific antibodies (a–e). The production of sAPPα and sAPPβ was determined by Western blots (f, g). The data represent the means ± SE of three independent experiments. *P < 0.05 with respect to the vehicle-treated or vector-transfected control. #P < 0.05 compared to PGI2-treated alone.

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PGI2 elevation was initially found to be involved in the actions of inflammation (Ford-Hutchinson et al., 1978). In addition, treatment with PGI2 analogs, including iloprost and treprostinil, suppressed TNF-α expression in human myeloid dendritic cells (Kuo et al., 2012). Schuh et al. (2014) also reported that the early induction of PGI2 at the site of traumatic injury resulted in the aggregation of IL-1β-expressing macrophages as a critical reason for neuropathic pain. Although the effects of these cytokines on Aβ production are still in debating, these prior works have indicated that PGI2 might play its roles in AD via inducing the production of cytokines. Apart from the inflammatory effects of PGI2, the ability of neuron cells to express elevated amounts of PGI2 in APP/PS1 transgenic mice suggests that PGI2 may be important to the pathogenesis of AD. Because there is no report that demonstrates the functional significance of PGI2 in regulating Aβ deposition, we assayed for the synthesis of α-, β-, and γ-secretases in PGI2-treated neuronal cells. The results demonstrated that the expression of BACE-1 and APP-1α/1β was upregulated while ADAM-10 expression was downregulated in PGI2-treated n2a cells. Therefore, PGI2 elevation could possibly accelerate the deposition of Aβ₁₋₄₂ by decreasing the expression of β- and γ-secretases.

![Fig. 5](image-url) NS398 treatment diminished the effects of Aβ oligomers on inducing the expression of APH-1α/1β. Cerebrospinal fluid (CSF) was obtained from APP/PS1 transgenic mice, which was then injected (i.c.v.), in the absence or presence of Aβ antibody (1 μg/5 μL) into C57BL/6 mice for 2 weeks before sacrifice (a–c). In selected experiments, the WT C57BL/6 mice at 6 months of age were injected (i.c.v.) with Aβ oligomers (2 μg/5 μL) in the absence or presence of NS398 (2 μg/5 μL). The brains were then collected and sectioned after 24 h (d–f). In separate experiments, the brains of WT C57BL/6 mice at 6 months of age were harvested and freshly sectioned (400 μm) before treatment with Aβ (1 μL) in the absence or presence of NS398 (10 μM) for 24 h (g). In distinct experiments, SH-SY5Y or n2a cells were treated with Aβ (1 μM) in the absence or presence of NS398 (10 μM) for 24 h (h, i). The immunoreactivity of APH-1 was determined by IHC using an anti-APH-1 antibody (a, d, g). APH-1α/1β mRNA and protein expression was determined by qRT-PCR and Western blots, respectively (n = 8) (b, c, e, f, h, i). The data represent the means ± SE of three independent experiments. *P < 0.05 with respect to vehicle-treated controls. #P < 0.05 compared to APP/PS1 CSF- or Aβ-treated alone.
Fig. 6 Proposed cascade of the signaling events regulating the pathogenesis of AD by PGI₂. In detail, elevated levels of PGI₂ in APP/PS1 transgenic mice will enhance the expression of APP-1/β via the PKA/CREB and JNK/c-Jun pathways in neuron cells of APP/PS1 transgenic mice, which in turn aggravates the pathogenesis of AD. Moreover, the highly secreted Aβ oligomers from neuron cells are able to reciprocally regulate the expression of APH-1α/β, which further aggravate the pathogenesis of AD in vivo. PGI₂, a metabolic product of COX-2, inhibition by NS398 administration reversed the effects of APP/PS1 overexpression in stimulating the expression of APH-1α/β, which potentially contributes to improvement in study ability and decline in cognitive ability in APP/PS1 transgenic mice.

As the roles of ADAM-10 and BACE-1 in Aβ deposition have been thoroughly investigated (Niemitz, 2013), we sought to determine the effects of PGI₂ in stimulating the expression of APP-1/β. Although there are no reports that demonstrate the effects of PGI₂ in inducing the expression of APP-1/β, APP-1/β are required for notch pathway signaling, for γ-secretase cleavage of β-APP, and for Aβ protein accumulation in C. elegans (Francis et al., 2002). Indeed, APH-1 often combines with PEN-2, nicastrin, and PS to generate an active form of γ-secretase complex, which is responsible for the cleavage of β-APP and for the deposition of Aβ (De Strooper, 2003). Once APH-1 was found in C. elegans (Francis et al., 2002), the APH-1 complex was confirmed in several experimental models (Gu et al., 2003; Luo et al., 2003; Hansson et al., 2004). Along these lines, APP-1/β might also be involved in regulating the deposition of APH-1α/β in response to PGI₂ stimulation. PGI₂ is important for regulating the expression of APP-1/β, which is regulated by the PKA/CREB and JNK/c-Jun signaling pathways and leads to Aβ deposition. Consistent with our observations, Su et al. (2003) reported that H89 treatment suppressed the production of Aβ in cells that have been stably transfected with human APP bearing a ‘Swedish mutation’. They further found that the PKA inhibitor abolishes the mature form of intracellular APP and accumulates the immature form (Su et al., 2003). In addition, Marambaud et al. (Marambaud et al., 1999) found that H89 inhibited the production of Aβ₁-₄₀ and Aβ₁-₄₂ in HEK293 cells that expressed the APP/PS1 genes. However, these studies were not extended to the expression of β- or γ-secretases. Although the PKA inhibitor has shown similar effects in the suppression of the production of Aβ₁-₄₂, the role of H89 in Aβ-induced memory deficit is not conclusively identified (Amini et al., 2015). In addition to the PKA signaling pathway, the JNK/c-Jun signaling pathways have also been suggested to be involved in Aβ deposition. For example, Jung et al. (2015) reported that the c-Jun N-terminal kinase mediates the effects of auraptenon on the production of Aβ by activating γ-secretase. Shen et al. (2008) supported this observation by showing that JNK-dependent activation of γ-secretase is responsible for Aβ deposition in H₂O₂-stimulated SH-SYSY cells. In detail, γ-secretase as well as presenilin nicastrin is involved in mediating the effects of SP600125 on suppressing the production of Aβ₁-₄₂ (Kuo et al., 2008; Rahman et al., 2012). More importantly, the inhibition of c-Jun N-terminal kinase activation reverses the AD phenotype in APP/PS1 mice (Zhou et al., 2015). Along these lines, our data further found that the PKA/CREB and JNK/c-Jun pathways are important for Aβ deposition by activating APH-1α/β in PGI₂-stimulated cells and APP/PS1 mice.

We will focus this discussion on the role of Aβ regulation in the pathogenesis of AD. Interestingly, the expression of APP-1/β was upregulated when we injected (i.c.v.) the CSF of APP/PS1 mice into WT mice. This upregulation was attenuated by the addition of Aβ antibodies. These observations clearly indicate the possible role of CSF-bound Aβ of APP/PS1 mice in upregulating the expression of APP-1/β. However, previous studies have suggested that the CSF-bound Aβ₁-₄₂ level progressively reduced in patients with AD (Mo et al., 2015). These observations indicate that the total level of Aβ₁-₄₂ in the CSF of APP/PS1 mice might not be critical for upregulating the expression of APP-1/β. As noted by Lopez-Gonzalez et al. (2015), the self-aggregated characteristics of Aβ₁-₄₂ result in the Aβ₁-₄₂ oligomers being critical for AD initiation. In agreement with this observation, our data demonstrated that Aβ oligomer injection (i.c.v.) has the ability to stimulate the expression of APP-1/β. More interestingly, NS398 blocked the effects of Aβ oligomers in inducing the expression of APP-1/β. These observations clearly indicated the possible roles of Aβ oligomers in activating COX-2, which potentially further aggravates AD. In agreement with our hypothesis, Kotilinek et al. (2008) also suggested that possible cross talk exists between COX-2 and Aβ.

In conclusion, we elucidated the signaling pathway by which PGI₂ regulates the expression of APP-1/β in neuron cells of APP/PS1 mice. We found that PGI₂ treatment upregulates the synthesis of APP-1/β by activating the PKA/CREB and JNK/c-Jun signaling pathways, which results in Aβ formation in neuronal cells. Aβ injection (i.c.v.) further stimulates the expression of APP-1/β, which potentially contributes to the pathogenesis of AD.

**Experimental procedures**

**Reagents**

Unless otherwise specified, all reagents used for the study were described in the supporting information.

**Transgenic mice and treatments**

The female wild-type (WT) or APP/PS1 transgenic mice [B6C3-Tg (APPSwe, PSEN1dE9) 85Db/1j (Stock Number: 004462)] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Genotyping was performed at 3–4 weeks after birth. In selected experiments, mice at the age of 1 month were treated with NS398 (50 μg kg⁻¹ day⁻¹) for...
5 months before determining the expression of APH-1α/1β. Each group contains 3 mice. The brains of animals in different groups were collected after anesthesia and perfusion as previously described (Yu et al., 2015).

**Cerebrospinal fluid collection**

Cerebral spinal fluid (CSF) was collected according to a published method (Liu et al., 2004) with minor modifications as previously described (Wang et al., 2015; Yu et al., 2015).

**Aβ1-42 preparation**

The methods for preparing Aβ oligomers or fibrils had been described previously (Dahlgren et al., 2002; Moore et al., 2002; Pan et al., 2011). The detailed information was provided in the supportive information.

**Intracerebroventricular injection (i.c.v.)**

NS398, PGI2, Aβ, Aβ antibody, or vehicle (PBS) were injected (i.c.v.) into WT or APP/PS1 transgenic mice as previously described (Yu et al., 2015). Each group contains eight mice. In selected experiments, the WT mice were injected (i.c.v.) with the CSF of APP/PS1 mice. At indicated time intervals, the brains were harvested under anesthesia and perfusion as previously described (Yu et al., 2015).

**Organotypic slice culture of brain tissue**

Brain tissue was freshly collected from WT C57BL/6 or APP/PS1 transgenic mice at 6 months of age. Serial sections (400 μm thick) were cut using a chopper without fixation. Each group contains eight mice. The tissue sections were immediately cultured in DMEM/high-glucose medium with 10% FBS. In a separate set of experiments, the tissues were grown in serum-free medium for an additional 24 h before incubation with PGI2 (10 μM) or Aβ oligomers (1 μM) in the absence or presence of NS398 (10 μM), as previously described (Yu et al., 2015). The tissue sections were fixed and immunostained with APH-1 antibody by IHC staining kit (Invitrogen, Carlsbad, CA, USA).

**Luciferase assays and live animal imaging**

The live animal imaging was performed as previously described (Wang et al., 2015; Yu et al., 2015). In brief, the n2a cells that were transfected with APH-1α/β promoter were preseeded on one side of a cerebral ventricle. PGI2 or vehicle (PBS) solutions were then injected (i.c.v.) into the other cerebral ventricle. At different time intervals, the mice were anesthetized and injected (i.c.v.) with luciferin at the side cerebral ventricle, which was preseeded with n2a cells. Each group contains 6 mice. The scan was performed exactly five minutes after luciferin introduction. All images were analyzed using Bruker in vivo imaging systems (MS FX PRO, Carestream, Billerica, MA, USA).

**Two-photon imaging**

*In vivo* two-photon recording was performed as previously described (Wang et al., 2015; Yu et al., 2015). In brief, a custom-built, two-photon microscope that was based on a chameleon excitation laser operating at 690–1064 nm was used. The laser-scanning unit was mounted on an upright microscope that was equipped with a water immersion objective (Zeiss, 20×, Beijing, China). The fluorescence was detected using specific antibody staining. The brain slices were stained and scanned before and after the injection (i.c.v.) of PGI2 or vehicle (PBS) solutions. Each group contains 6 mice.

**Quantitative real-time PCR (qRT–PCR)**

qRT–PCR assays were performed with the MiniOpticon Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using total RNA and the GoTaq one-step Real-Time PCR kit with SYBR green (Promega, Madison, WI, USA) and the appropriate primers as previously described (Wang et al., 2010, 2011b,c, 2014a,b). The GenBank accession number and forward and reverse primers for human or mouse BACE-1, APH-1β, and GAPDH are provided in our previous publications (Wang et al., 2014c; Guan et al., 2015a,b; Yu et al., 2015). Other primers are shown in Table 2, and the gene expression values were normalized to those of GAPDH.

**Immunostaining**

Human SH-SY5Y and mouse n2a cells were immunostained as previously described (Wang et al., 2011c). In brief, cells were permeabilized with 0.1% Triton X-100 for 1 min at 4 °C, fixed with 4% paraformaldehyde for 10 min at 37 °C, washed with PBS (−), and incubated in buffer containing 1% BSA/PBS (−) for 10 min at room temperature. Cells were then incubated with a rabbit antibody to APH-1 for 60 min at room temperature, washed with 1% BSA/PBS (−), and incubated in buffer containing Alexa Fluor 488-labeled goat anti-rabbit IgG for 60 min at room temperature. The cells were then washed five times with 1% BSA/ PBS (−) before incubation in DAPI solution for five min. Finally, the cells were washed five times with 1% BSA/PBS (−) and once with deionized water before observation under confocal microscopy (Leica, TCS-SP8, Liaoning, Shenyang, China).

**Human brain samples**

Human brain samples were obtained from New York Brain Bank, serial numbers P35-00 (normal), T4339, T4304, and 235-95 (patients with AD). Another two normal brain samples were obtained from Fentang Hospital of China (the patients are 59- and 63-year-old men who were diagnosed as cerebral edema, and the normal tissues are collected surrounding the tissues of cerebral edema).

The information for immunohistochemistry (IHC), cell culture, Western blot analysis, measurement of the Aβ1-42 or PGI2 concentration in the culture medium or the brain of mice, Transfection and Animal committee, was described in the supporting information.

**Table 2** The primer sequences for α-, β-, or γ-secretases

| Gene Symbol | GenBank Number | Sequences |
|-------------|----------------|-----------|
| ADAM-10     | NM_007399      | F-ATTGCTGCTCGATGAGCACCAC<br>R-GCAGCGTACGAAACATCAC |
| PS1         | NM_008943      | F-GCTTGAGGCCGCCCTTAGTG<br>R-CATCTGGGCCATTTGGAAGT |
| PS2         | NM_011183      | F-GAAGACCGGGCAGCCTCACTCA<br>R-TCCAGACAGCCAGGGAAGTAGT |
| NCT         | NM_021607      | F-GTGCACTGCCCACAATGAGT<br>R-GGCCACATTCAGAAAAAAGGAC |
| PEN2        | NM_025498      | F-ACTGAAAAGTCCGCGCATCTCA<br>R-ATTTGGCGCAGATGGAAATG |
Statistical analysis
All data are represented as the mean ± SE of at least three independent experiments. The statistical significance of the differences between the means was determined using a Student’s t-test or a one-way ANOVA, where appropriate. If the means were found to be significantly different, multiple pairwise comparisons were performed using the Tukey’s test (Wang et al., 2014a;c; Guan et al., 2015a;b; Yu et al., 2015).

Acknowledgments
This work was supported in part or in whole by the National Natural Science Foundation of China (CN) (31571064, 81500934, 31400777 and 31371091), the Fundamental Research Funds of China (N140204002, N130120002, N120520001, N120320001, N141008001/7 and L1520001), the National Natural Science Foundation of Liaoning, China (CN) (20152020662), and the Liaoning Provincial Talent Support Program (LJQ2013029).

Funding
No funding information provided.

Conflict of interest
The authors declare no competing financial interests.

Author contributions
P. W. and P.P.G conceived and performed all of the experiments, participated in the design of the study, and wrote the manuscript. J.W.G., L.L.C., G.B.X., X.Y, and Y.W. carried out some of the experiments. P.W. and Z.Y.W. conceived the experiments, interpreted the data, and wrote the manuscript.

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