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TITLE PAGE

Honokiol ameliorates amyloidosis and neuroinflammation and improves cognitive impairment in Alzheimer's disease transgenic mice

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Effects of honokiol in an AD mouse model

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AD, Alzheimer's disease; Aβ, amyloid-β; BACE1, β-site amyloid precursor protein cleavage enzyme 1; CNS, Central nervous system; DM, diabetes mellitus; PPARγ, Peroxisome proliferator-activated receptor-γ; TZD, thiazolidinedione.

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ABSTRACT

The present study examined the effects of honokiol on amyloid-β (Aβ)-induced cognitive impairment and the underlying mechanisms in APPswe/PS1dE9 transgenic mice. The results showed that honokiol administration (20 mg/kg per day, intraperitoneally) for 6 weeks effectively improved spatial memory deficits in APPswe/PS1dE9 transgenic mice. Honokiol significantly lowered Aβ production and senile plaque deposition by downregulating BACE1 and enhancing Aβ phagocytosis by microglia. Honokiol reduced glial cell activation and the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6). Honokiol increased the transcriptional activity and protein levels of PPARγ. However, all of the beneficial effects of honokiol on pathological changes, including biochemistry and cognitive function, could be blocked by GW9662, a specific PPARγ inhibitor. These findings suggested that honokiol may be a natural PPARγ agonist, acting to attenuate Aβ generation and neuroinflammation. Therefore, honokiol may be a potential therapeutic approach for AD.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease and it is characterized by progressive cognitive dysfunction. Extracellular senile plaques, intracellular neurofibrillary tangles and massive neuronal loss in the brain, are prominent neuropathological hallmarks of AD (Hardy and Selkoe, 2002; Steel, 2010). Although the exact mechanism of AD pathogenesis remains unknown, extensive studies suggest that the accumulation and aggregation of Aβ in brain areas is associated with synaptic loss, neuronal death, and cognitive decline (Selkoe and Hardy, 2016).

Clinical and epidemiological studies indicate a close link between AD and diabetes mellitus (DM). Patients among with type 2 diabetes mellitus have a higher risk of AD (Maher and Schubert, 2009), and AD may be considered "type-3 diabetes" (de la Monte and Wands, 2008) given the evidence for central insulin resistance as well as dysregulated glucose metabolism observed in the brains of AD patients (Talbot et al., 2012). Thiazolidinediones (TZDs) are an important class of insulin sensitizers used in the treatment of type 2 diabetes mellitus (T2DM). These drugs are known to act by increasing the transactivation activity of peroxisome proliferator-activated receptor-γ (PPARγ). Recent studies have shown TZDs to be a potential treatment for AD (Escribano et al., 2010; Perez and Quintanilla, 2015). TZDs downregulate β-site amyloid precursor

protein cleavage enzyme 1 (BACE1) and reduce Aβ generation (Searcy et al., 2012). It has been reported that PPARγ agonists can suppress Aβ-induced glial activation and neuroinflammation, enhance microglia activation into anti-inflammatory phenotypes and facilitate Aβ phagocytosis by microglia (Mandrekar-Colucci et al., 2012). Taken together, this growing body of research indicates that PPARγ agonists may represent new drugs for the treatment of AD. However, current PPARγ-targeting drugs, such as TZDs treatment, have been associated with undesirable side effects, and therefore, it is urgent to seek an alternative drug for AD therapy that possesses the capacity for PPARγ activation with minimal side effects.

Honokiol, isolated from traditional Chinese herbal drug magnolia bark, exhibits broad pharmacological functions, such as anti-inflammatory, antithrombotic, antidepressant, analgesic, and antihypertrophic effects. Honokiol has been shown to have good CNS penetration (X. Wang et al., 2011) and potential effects in the prevention and treatment of neurodegenerative disorders (Jangra et al., 2016; H. Wang et al., 2014). Oral administration of honokiol attenuates age-related cognitive impairment and neuronal injury in senescence-accelerated mice (Matsui et al., 2009). Honokiol improves scopolamine-induced cognitive deficits in mice (Xian et al., 2015). Moreover, honokiol can ameliorate lipopolysaccharide-induced memory deficits via its antiamyloidogenic

and antineuroinflammatory effects (Lee et al., 2013). Research has indicated that honokiol acts as a PPARγ agonist via directly binding to purified PPARγ ligand-binding domain without the side effects of pioglitazone (Atanasov et al., 2013). Whether long-term treatment with honokiol could ameliorate cognitive impairment and whether the activation of PPARγ by honokiol is responsible for its neuroprotective effect in AD remain unclear. In this study, the effect of honokiol consumption on the improvement of cognitive decline, Aβ burden, neuroinflammation, and PPARγ activation was analyzed in Alzheimer's disease transgenic mice.

MATERIALS AND METHODS

Animals and Treatment

Six-month-old male APPswe/PS1dE9 transgenic mice and wild-type littermates were used (n = 15). The generation of mice expressing human mutated forms APPswe and PS1dE9 has already been described (Yuan et al., 2011). Mice received a daily intraperitoneal injection (i.p.) of GW9662 (4 mg/kg), a special PPARγ inhibitor, or its solvent (DMSO) 2 h prior to the daily administration (i.p.) of honokiol (20 mg/kg) or its solvent (DMSO). Treatments were administered to the following groups of mice for 6 weeks: Group 1 (WT), wild-type littermates received a daily injection of honokiol solvent and GW9662 solvent; Group 2 (APPswe/PS1dE9), APPswe/PS1dE9 mice received a daily injection of honokiol solvent and GW9662 solvent; Group 3 (APPswe/PS1dE9+Hon), APPswe/PS1dE9 mice received a daily injection of honokiol and GW9662 solvent; Group 4 (APPswe/PS1dE9+Hon+GW9662), APPswe/PS1dE9 mice received a daily injection of honokiol and GW9662; Group 5 (GW9662), wild-type littermates received a daily injection of honokiol solvent and GW9662; Group 6 (Honokiol), wild-type littermates received a daily injection of honokiol and GW9662 solvent. Honokiol (purity > 98% by high-performance liquid chromatography analysis) and PPARγ inhibitor GW9662 were dissolved

in 10% dedimethyl sulfoxide (DMSO). All experimental protocols and animal usage were approved by the Institutional Animal Experiment Committee of Henan University of Science and Technology, China.

Morris water maze

The Morris water maze consisted of a circular white tank (100 cm in diameter) filled with nontoxic opaque water (22 ± 1 °C). A removable platform (10 cm in diameter) was submerged 0.5 cm beneath the surface of the water at a constant location in the center of the target quadrants. During the learning phase, each mouse was experimented to four trials per day for 5 consecutive days. The time to find the platform (escape latency) was recorded and calculated as the average of four trials. A probe trial was carried out in the 6^{th} day to evaluate the spatial memory retention. Each mouse was placed in the water tank without the platform and allowed to swim freely for 60 s, and the platform crossings were recorded. Performance was tracked by EthoVision video tracking system (Version XT Noldus. Wageningen, Netherlands).

Histological analysis

Paraformaldehyde-fixed brains were sectioned (30 μm) coronally by using a microtome (Leica, Nussloch, Germany) and stored at 4 °C in PBS. For Thioflavine-S staining, the brain slices were incubated in a solution of

0.015% Thioflavin-S for 3 min at room temperature. Finally, the slices were washed in 50% ethanol and coverslipped with Permount.

Immunohistochemistry of anti-GFAP antibody (1:1000; Proteintech) detecting activated astrocytes and anti-Iba1 antibody (1:2000; Abcam) detecting activated microglia were used to visualize astrocytosis and microgliosis. The brain sections were incubated with the HRP-labeled secondary antibodies for 60 min and visualized by DAB. The stained sections were observed under a light microscopy, and the intensity of the stained area of each group was evaluated quantitatively using Image-ProPlus version 6.0 (Media Cybernetics, MD, USA)

Double immunofluorescence staining of Iba1 and Aβ was performed to evaluate the phagocytic ability of microglia. The brain sections were incubated with the following primary antibodies: Iba1 (1:100; Abcam) and Aβ (1: 200; Cell Signaling Technology) at 4 °C overnight. After 3 washes with PBS, anti-rabbit or mouse secondary antibody conjugated to Alexa Fluor 488 or 594 (Cell Signaling Technology) was added at 37 °C for 30 min. All the fluorescence stainings were captured on a Fluoview FV10i confocal microscope (Olympus, USA). The captured images were viewed and analyzed with MetaMorph® Image Analysis Software (Molecular Devices, CA, USA).

ELISA test for Aβ1–40 and Aβ1–42

Soluble and insoluble Aβ1–40 and Aβ1–42 of the cortex and hippocampus was extracted according to previous study (Handattu et al., 2009). In brief, the frozen mouse cortex and hippocampus were homogenized in ice-cold 20 mM Tris, pH 8.5 (soluble) or 5M guanidine HCl/50 mM Tris-HCl, pH 8.0 (insoluble). The content of Aβ1–40 and Aβ1–42 levels was determined by ELISA kits (Invitrogen, CA, USA).

Estimation of proinflammtory cytokines (TNF-α, IL-1β, and IL-6) levels

After mouse brain tissue was homogenized in lysis buffer and centrifuged, the supernatants were collected and sampled in triplicate to detect the levels of TNF-α, IL-1β, and IL-6 by an ELISA kit (R&D Systems and Invitrogen) according to the manufacturer's instructions.

PPARγ transcriptional activity assay

PPARγ transcription factor activity was measured by using an ELISA-based kit (Cayman Chemicals, Ann Arbor, MI, USA). Nuclear extracts were incubated in a 96-well plate coated with immobilized oligonucleotides containing peroxisome proliferator responsive element. PPARγ contained in nuclear extract was detected by using primary

antibody specific for PPARγ followed by a HRP-conjugated secondary antibody and colorimetric readout at 450 nm.

BACE1 enzymatic activity assays

BACE1-specific enzymatic activity was performed using a fluorometric reaction kit (R&D Systems, Minneapolis, MN, USA). Briefly, brain tissues were lysed with the provided extraction buffer and centrifuged at 25,000 × for 30 min. The supernatant was then incubated with reaction buffer containing β-secretase substrate. Fluorescence intensity was measured with a microplate reader at 320 nm and 420 nm as excitation and emission wavelengths, respectively.

Western blot analysis

Protein isolation and immunoblotting procedures were performed as previously described (Li et al., 2013; Liu et al., 2013). Primary antibodies for immunoblotting included anti-GFAP (1:1000, Proteintech), anti-Iba1 (1:500, Abcam), anti-PPARγ (1:500, Abcam), and anti-BACE1 (1:1000, Abcam). Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the stated antibodies overnight at 4 °C. The membrane was then incubated with HRP-conjugated secondary antibodies. Immunoblots were visualized using a chemiluminescent detection system.

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Statistical analysis

All data were presented as the mean ± SEM. Data were examined by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons using SPSS 13.0. For the Morris water maze test, the group differences of escape latencies were analyzed using two-way ANOVA with repeated measures. Significant differences were determined at $p < 0.05$.

RESULTS

Honokiol alleviates spatial learning and memory deficits in APPswe/PS1dE9 mice

To evaluate the effects of honokiol on spatial reference learning and memory impairments, we tested spatial learning and memory by MWM. In the place navigation test, the APPswe/PS1dE9 mice showed the longer escape latencies compared to the WT group ($p < 0.01$ for day 2-5). Administration of honokiol significantly decreased the escape latencies ($p < 0.05$ for day 2-3; $p < 0.01$ for day 4-5).

In the subsequent probe test the number of platform crossings decreased by 47.6 % in APPswe/PS1dE9 mice compared with WT mice ($p < 0.01$), indicative of cognitive impairment. Compared to APPswe/PS1dE9 mice, the number of platform crossings of the APPswe/PS1dE9+honokiol mice was significantly increased by 64.3 % ($p < 0.01$). However, the positive effects of honokiol on the cognition were blocked by co-administration of GW9662, an inhibitor of PPARγ. Intraperitoneally injection of GW9662 into WT group did not influence memory function. Mice treated with only honokiol showed no significant differences in the number of platform crossings in comparison to WT mice ($p > 0.05$). In addition, no significant difference in swimming speed (Fig. 1C) or path length (Fig.1D) was found in the probe test between the six groups of mice ($p > 0.05$).

Honokiol treatment reduces Aβ levels and deposition in APPswe/PS1dE9 mice

To investigate whether honokiol treatment would inhibit Aβ deposition in the cortex and hippocampus, sections of mouse brains from different groups were stained with Thioavine-S, which specifically binds amyloid plaques. As shown in Fig. 2, the WT mice had no visible Aβ plaque. However, Thioflavin-S-positive dense-core plaques accumulated in the hippocampus and cortex of the APPswe/PS1dE9 transgenic mice, whereas the APPswe/PS1dE9 mice treated with honokiol exhibited 52.6% and 35.7% fewer Thioavine-S positive compact plaques, and 51.2% and 34.1% less plaque area ($p < 0.05$) in hippocampus ($p < 0.01$) and cortex ($p < 0.05$) when compared with APPswe/PS1dE9 transgenic mice. Furthermore, we measured the levels of soluble Aβ peptide fraction and insoluble Aβ peptide fraction in mouse brains using ELISA. Compared with APPswe/PS1dE9 mice, treatment with honokiol significantly decreased levels of soluble Aβ1–42 by approximately 52.3% in the hippocampus ($p < 0.01$) and 65.5% ($p < 0.01$) in the cortex (Fig. 3C), and lowered the insoluble Aβ1–42 levels by 52.5% in the hippocampus ($p < 0.01$) and 61.8% ($p < 0.01$) in the cortex (Fig. 3D). Neither soluble Aβ1–40 levels (Fig. 3A) nor insoluble Aβ1–40 levels (Fig. 3B) in the hippocampus and cortex were changed by honokiol treatment. However, intraperitoneal injection of GW9662 almost abolished the

effects of honokiol on Aβ generation and deposition.

Honokiol suppresses neuroinflammatory response and enhances Aβ clearance in APPswe/PS1dE9 mice

The inflammatory response was manifested by an elevation in the levels of TNF-α, IL-1β, and IL-6 in the brain of APPswe/PS1dE9 mice. Moreover, glial activation was observed with increased Iba1 and GFAP immunoreactivity as well as the expression of proteins in the hippocampus of APPswe/PS1dE9 mice. Honokiol treatment significantly inhibited neuroinflammatory response, as indicated by decreased levels of TNF-α by 54.7% ($p < 0.01$), IL-1β by 49.5% ($p < 0.01$), IL-6 by 51.4% ($p < 0.01$), reduced number of Iba1 and GFAP positive cells, and lowered expression of proteins compared with those in the APPswe/PS1dE9 mice. Mice treated with only honokiol showed similar neuroinflammatory response as WT mice (Fig. 4). Interestingly, we observed that the microglia around the amyloid plaques was increased in the cortex of honokiol-treated APPswe/PS1dE9 mice, suggesting the enhanced Aβ phagocytosis by microglia (Fig. 5). Addition of GW9662 treatment blocked the inhibitory effects of honokiol on neuroinflammatory response.

Honokiol improves PPARγ function and downregulates BACE1

activity and expression in APPswe/PS1dE9 mice

The above data indicated that PPARγ was associated with the antiamyloid and anti-inflammatory effects of the honokiol in APPswe/PS1dE9 mice. Both the expression and the activity of PPARγ were significantly decreased in the hippocampus of APPswe/PS1dE9 mice compared with the WT mice. Honokiol treatment significantly increased PPARγ protein expression (Fig. 6A) as well as PPARγ transcriptional activity (Fig. 6B). Recent studies showed that honokiol could directly bind to PPARγ and act as a PPARγ agonist, which may explain the effects of honokiol on PPARγ functional improvement.

PPARγ suppresses BACE1 activity and expression by binding to the β-site amyloid precursor protein cleavage enzyme 1 (BACE1) gene promoter and subsequently reduces Aβ generation. To determine whether honokiol affected the BACE1 in APPswe/PS1dE9 mice, we examined the expression and activity of BACE1 in the hippocampus in different groups. The results demonstrated that the expression and activity of BACE1 were elevated in APPswe/PS1dE9 mice compared to those in the WT group and significantly decreased in honokiol-treated mice (Fig. 6C and 6D). Furthermore, GW9662 treatment suppressed the effects of honokiol on the expression and activity of BACE1, suggesting a PPARγ-dependent mechanism. No significant difference was observed in the expression and transcriptional activity of PPARγ or in the expression and activity of

BACE1 between mice treated with only honokiol and WT mice.

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DISSCUSSION

This study demonstrated that honokiol administration ameliorated spatial learning and memory impairment, reduced plaque burden and Aβ levels, and suppressed glial activation and the production of neuroinflammatory cytokines in the APPswe/PS1dE9 transgenic mouse model of AD. Furthermore, honokiol treatment improved PPARγ function and downregulated the expression and activity of BACE1. Importantly, the PPARγ inhibitor could block the beneficial effects of honokiol on biochemistry, pathological changes and cognitive function in mice.

Although the exact AD pathogenesis and underlying mechanism remain elusive, the accumulation of Aβ peptides in the brain is considered to be a central event in AD development (Selkoe et al., 2016; Walsh and Selkoe, 2004). We analyzed neuropathological changes after honokiol treatment and observed the decreased intensity of thioflavin-S-positive staining and Aβ1–42 levels in the brains of honokiol-treated APPswe/PS1dE9 mice. There are two major isoforms of Aβ peptides: Aβ1–40 and Aβ1–42. Aβ1–40 accumulates in the AD brain, but the extent of Aβ1–40 accumulation relative to Aβ1–42 is highly variable. It has been shown that Aβ1–40 can promote Aβ42 aggregation in a concentration-dependent manner (Tran et al., 2017). High Aβ1–40 monomeric/Aβ1–42 fibrillar ratios ($\geqslant$ 10) accelerate Aβ fibril formation and the accumulation of fibrillar aggregates in the brains. In contrast, subphysiologic Aβ1–40

monomeric/Aβ1–42 monomeric ratios (0.5–2) facilitate the persistence and accumulation of soluble Aβ aggregates (Jan et al., 2008). In the present study, the ratio of Aβ1–40/Aβ1–42 observed was up to 10, which may promote Aβ1–42 fibril formation. The aggregation of Aβ1–42 into small oligomers and fibrillar plaques is considered as the central initiator in the pathogenesis of AD (Lambert et al., 1998; Selkoe, 2011). Therefore, the lowered Aβ1–42 level after honokiol treatment may explain its attenuating effects on hippocampus-dependent behavioral deficits.

Neuroinflammation plays a crucial role in the development of neurodegenerative diseases, including AD (Minter et al., 2016). Glial cells act as neuronal supportive cells and maintain the health of the neurons. However, glial overactivation in response to Aβ can produce proinflammatory mediators and neurotoxic factors that cause neuronal dysfunction and neurodegeneration, ultimately creating a vicious cycle (Skaper, 2007). Overactivation of microglia and astrocytes and higher levels of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, have been found in the brains of AD patients (Angelopoulos et al., 2008; Forlenza et al., 2009) and AD animal models (Morales et al., 2010). Consistent with this, our results indicated augmented neuroinflammation, as evidenced by increased proinflammatory levels, and Iba1 and GFAP immunoreactivity as well as the expression of proteins in the brains of APPswe/PS1dE9 mice. Honokiol treatment significantly suppressed the

neuroinflammation response.

The important role of the nuclear receptor PPARγ agonists in neuroprotection has been extensively studied in neurodegenerative disorders, including AD (Heneka et al., 2015; Mandrekar-Colucci et al., 2012; Sodhi et al., 2011). Compelling evidence has shown that PPARγ agonists effectively improve cognitive impairment in AD patients (Cheng et al., 2016) and in AD mouse models (Skerrett et al., 2015). PPARγ is a ligand-dependent transcription factor that regulates the transcription of target genes. In recent years, the modulatory role of PPARγ in the transcriptional activity of BACE1 (Chen et al., 2009; Katsouri et al., 2011), a crucial enzyme in APP processing and Aβ production, has been well documented. Previous studies have indicated that PPARγ downregulates BACE1 transcription and exerts effects on Aβ generation through directly binding to the promoter region of BACE1 (Chen et al., 2009). Consistent with this, our results showed that honokiol treatment enhanced PPARγ function and suppressed the expression and activity of BACE1, which in turn led to lowered Aβ production and subsequent senile plaque formation in the brains of APPswe/PS1dE9 mice. However, the effects of honokiol can be effectively blocked by GW9662, a PPARγ antagonist. Therefore, the present data suggested that honokiol represses BACE1 function and Aβ production by inducing the activation of PPARγ. Meanwhile, the anti-inflammatory actions of PPARγ agonists is assumed

to underlie their positive effects on the amelioration of AD pathophysiology (Bright et al., 2008). One striking finding of our study was the extraordinary ability of honokiol to promote microglia to phagocytose Aβ. Although the microglia are competent phagocytes, they fail to effectively phagocytose Aβ and have shown impaired ability to degrade Aβ in the AD brain (Southam et al., 2016). PPARγ activation has been reported to provoke the conversion of microglia from M1 inflammatory (classical) to M2 anti-inflammatory (alternative) phenotypes and promote Aβ clearance, resulting in cognitive improvement in the AD brain (Mandrekar-Colucci et al., 2012; Yamanaka et al., 2012). Thus, in our study, honokiol modulated Aβ metabolism by inhibiting Aβ generation via downregulating BACE1 activity and enhancing the Aβ clearance by microglia in the APPswe/PS1dE9 mice.

Honokiol remarkably improved spatial learning and memory impairment in an APPswe/PS1dE9 transgenic mouse model of AD. The cognitive-enhancing mechanism of honokiol is likely attributable to decreased Aβ generation and enhanced Aβ clearance, along with suppressed neuroinflammation; these effects appear to be due to the activation of PPARγ. Therefore, honokiol might be a potential natural compound candidate against AD.

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The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: D.M. Wang, Dong, and C.Y. Wang

Conducted experiments: D.M. Wang, Dong, and C.Y. Wang

Performed data analysis: D.M. Wang, and Dong

Contributed to the writing of manuscript: D.M. Wang, and C.Y. Wang

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Honokiol ameliorated spatial learning and memory impairment in APPswe/PS1dE9 transgenic mice as tested using the Morris water maze. Non-transgenic littermates and APPswe/PS1dE9 mice received a daily injection (i.p.) of GW9662 or its solvent (DMSO) 2 h prior to daily administration (i.p.) of honokiol or its solvent (DMSO) for 6 weeks. After treatment, WT mice, APPswe/PS1dE9 mice, APPswe/PS1dE9+Hon mice, APPswe/PS1dE9+Hon+GW9662 mice, GW9662 mice, and Honokiol mice underwent the Morris water maze test. (A) The escape latency during 5 days of hidden platform tests. APPswe/PS1dE9+Hon mice showed shorter escape latencies compared to APPswe/PS1dE9 mice. (B) The number of platform crossings. The crossings were significantly increased in APPswe/PS1dE9+Hon mice compared with APPswe/PS1dE9 mice. However, the positive effects of honokiol on cognition were blocked by co-administration of GW9662. (C) The swimming speed. (D) The path length in the probe test. All of the data are presented as mean ± S.E.M. (n = 15 per group) and analyzed by a two-way ANOVA on ranks for repeated measures, followed by a Bonferroni post-hoc test for multiple comparisons. $^{**}p < 0.01$ vs. WT mice; $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

FIGURE 2. Honokiol treatment reduced Aβ deposition in

APPswe/PS1dE9 transgenic mice. Brain tissues from all of the mice were utilized in standard pathological procedures, and sections were stained with Thioflavin-S to visualize the deposition of Aβ. (A) Representative brain sections showing that honokiol treatment significantly decreased Thioflavin-S immunoreactivity in the brains of APPswe/PS1dE9 mice. (scale bars, 1 cm). However, the inhibitory effects of honokiol on Aβ deposition were blocked by co-administration of GW9662. (B) Graphs showing plaque count and the percentage of area occupied by plaques in the cortex and hippocampus. All of the data are presented as mean ± S.E.M. (n = 4 per group) and analyzed by a one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

FIGURE 3. Honokiol treatment decreased Aβ1–42 levels in APPswe/PS1dE9 transgenic mice. Soluble and insoluble Aβ1–40 and Aβ1–42 from the APPswe/PS1dE9 mice, APPswe/PS1dE9+Hon mice and APPswe/PS1dE9+Hon+GW9662 mice were extracted and measured by ELISA. (A) The soluble Aβ1–40 levels. (B) The insoluble Aβ1–40 levels. No significant difference was observed in soluble Aβ1–40 levels or insoluble Aβ1–40 levels among the three groups. (C) The soluble Aβ1–42 levels. (D) The insoluble Aβ1–42 levels in the hippocampus and cortex. Honokiol treatment significantly reduced Aβ1–42 levels in the

brains of APPswe/PS1dE9 mice. However, the inhibitory effects of honokiol on Aβ1–42 levels were blocked by co-administration of GW9662. All of the data are presented as mean ± S.E.M. (n = 4 per group) and analyzed by a one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

FIGURE 4. Honokiol suppressed neuroinflammation in APPswe/PS1dE9 transgenic mice. (A) The levels of TNF, IL-1β, and IL-6 in mouse brain homogenates of all of the groups (n = 3 per group). Honokiol treatment significantly lowered the levels of TNF, IL-1β, and IL-6 in the brains of APPswe/PS1dE9 mice. (B) Representative photomicrographs of immunohistochemistry for Iba1 in the hippocampal CA1 region of all of the groups (scale bars, 100 μm), and quantitative analysis of Iba1 staining intensity (n = 4 per group). Inserts indicate higher magnification of Iba1-positive cells from the CA1 area. APPswe/PS1dE9+Hon mice exhibited a smaller number of Iba1 positive cells than APPswe/PS1dE9 mice. (C) Representative photomicrographs of immunohistochemistry for GFAP in the hippocampal CA1 region of all of the groups (scale bars, 100 μm), and quantitative analysis of GFAP staining intensity (n = 4 per group). Inserts indicate higher magnification of GFAP positive cells from the CA1 area. APPswe/PS1dE9+Hon mice showed a smaller number of

GFAP-positive cells than APPswe/PS1dE9 mice. (D) Western blot of Iba1 and GFAP in the hippocampus (n = 3 per group). The expressions of Iba1 and GFAP were decreased in APPswe/PS1dE9+Hon mice compared to APPswe/PS1dE9 mice. However, the inhibitory effects of honokiol on neuroinflammation were blocked by co-administration of GW9662. All of the data are presented as mean ± S.E.M. and analyzed by a one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. $^{**}p < 0.01$ vs. WT mice; $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

FIGURE 5. Honokiol treatment increased microglia around the plaque and enhanced Aβ phagocytosis in APPswe/PS1dE9 transgenic mice. (A) Representative images of microglia and Aβ plaques in the cortex stained with anti-Iba1 (green) and anti-Aβ (red) immunofluorescence (scale bars, 10μm). Significantly increased Iba1 immunoreactivity around the plaque was found in APPswe/PS1dE9+Hon mice compared to APPswe/PS1dE9 mice. However, the positive effects of honokiol on Aβ phagocytosis were reversed by co-administration of GW9662. (B) Ratio of fluorescence intensity of microglial marker Iba1 (green) to amyloid plaque (red). All of the data are presented as mean ± S.E.M. (n = 4 per group) and analyzed by a one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

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FIGURE 6. Honokiol improved PPARγ function and decreased BACE1 activity and expression in APPswe/PS1dE9 mice. (A) Western blot of PPARγ expression in the hippocampus. (B) PPARγ transcriptional activity. Honokiol treatment significantly increased PPARγ expression and restored the activity of PPARγ in the brains of APPswe/PS1dE9 mice. However, the protective effects of honokiol on PPARγ function were blocked by co-administration of GW9662. (C) Western blot of BACE1 expression in the hippocampus. (D) β-secretase activity assay. Honokiol treatment significantly suppressed BACE1 activity and expression in the brains of APPswe/PS1dE9 mice. However, the effects of honokiol on BACE1 activity and expression were also blocked by co-administration of GW9662. All of the data are presented as mean ± S.E.M. (n = 3 per group) and analyzed by a one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. $^{**}p < 0.01$ vs. WT mice; $^{\#\#}p < 0.01$ vs. APPswe/PS1dE9 mice.

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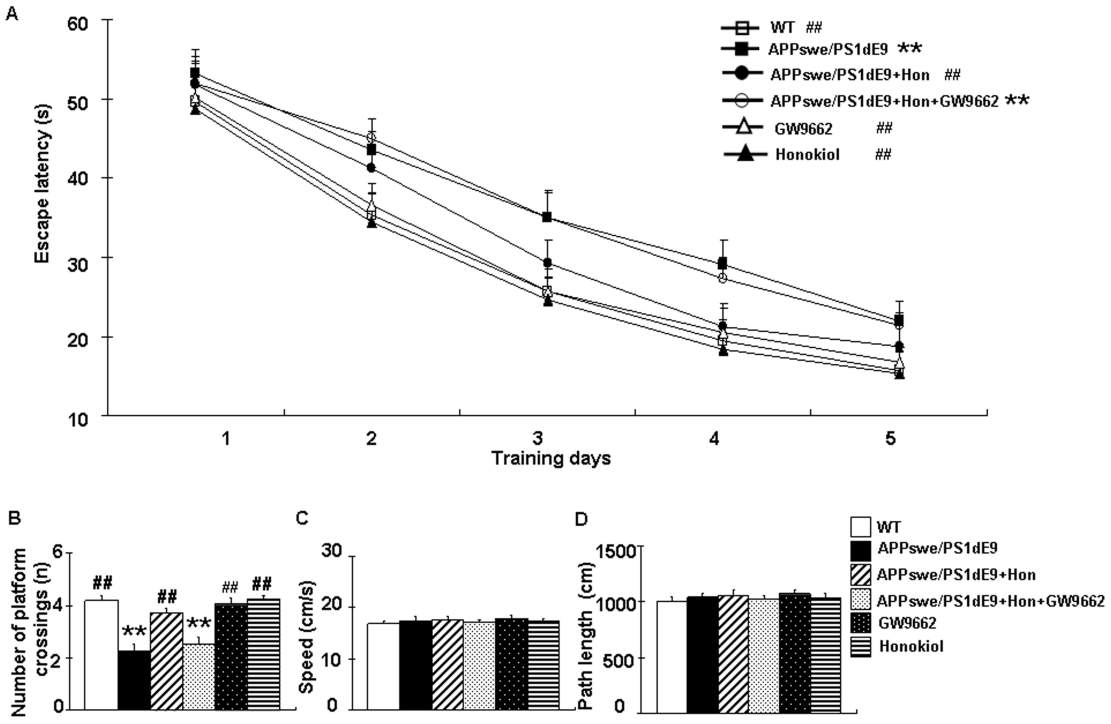

FIGURE 1

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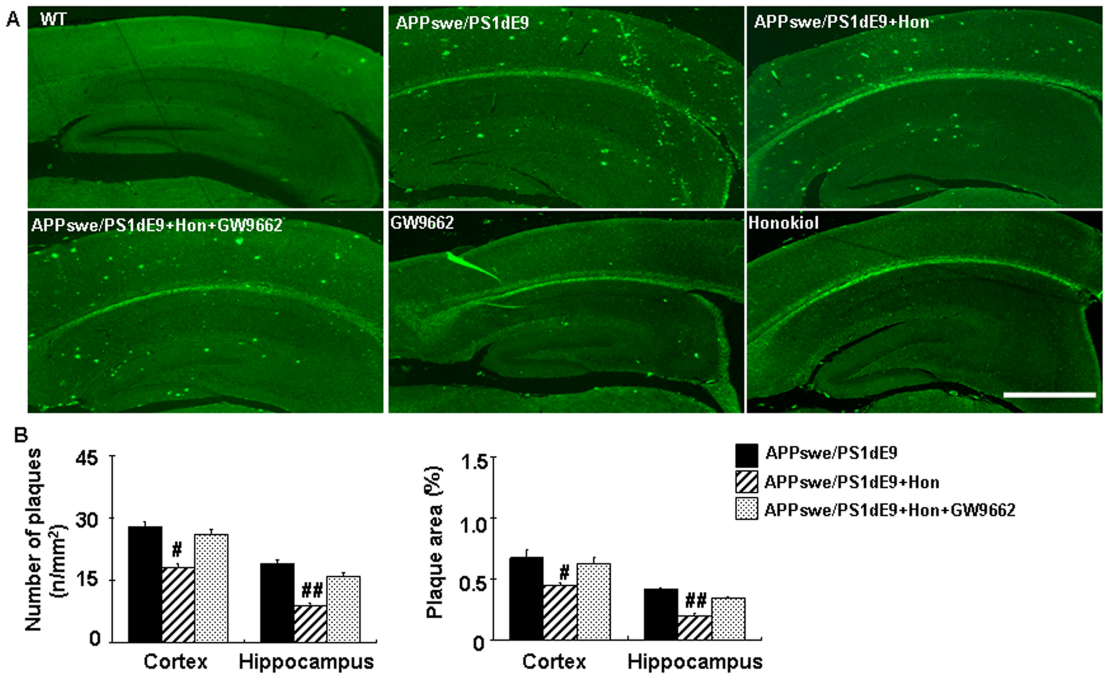

FIGURE 2

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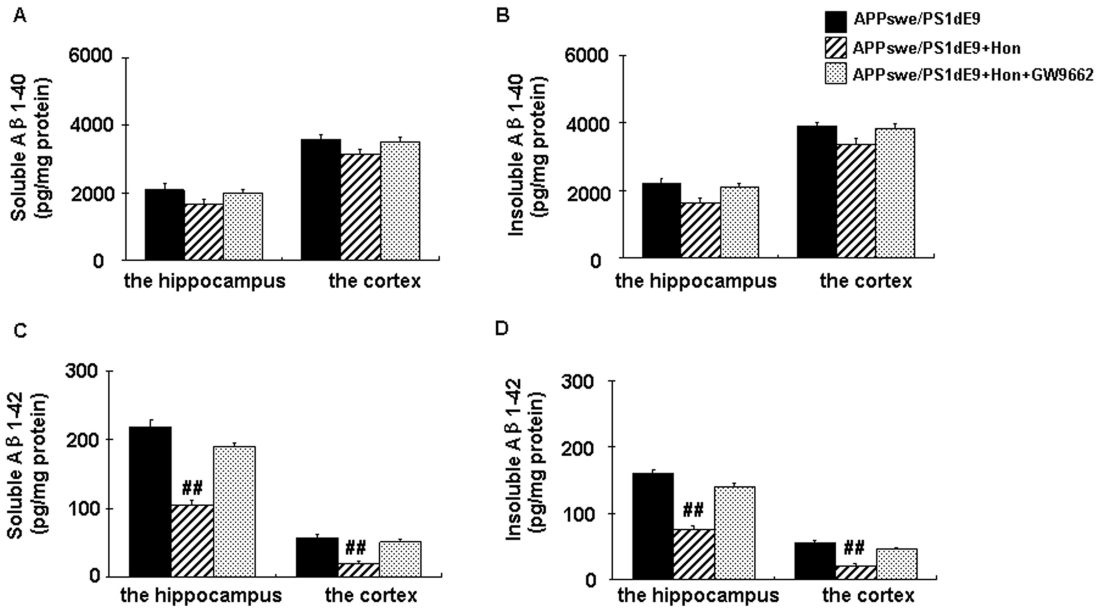

FIGURE 3

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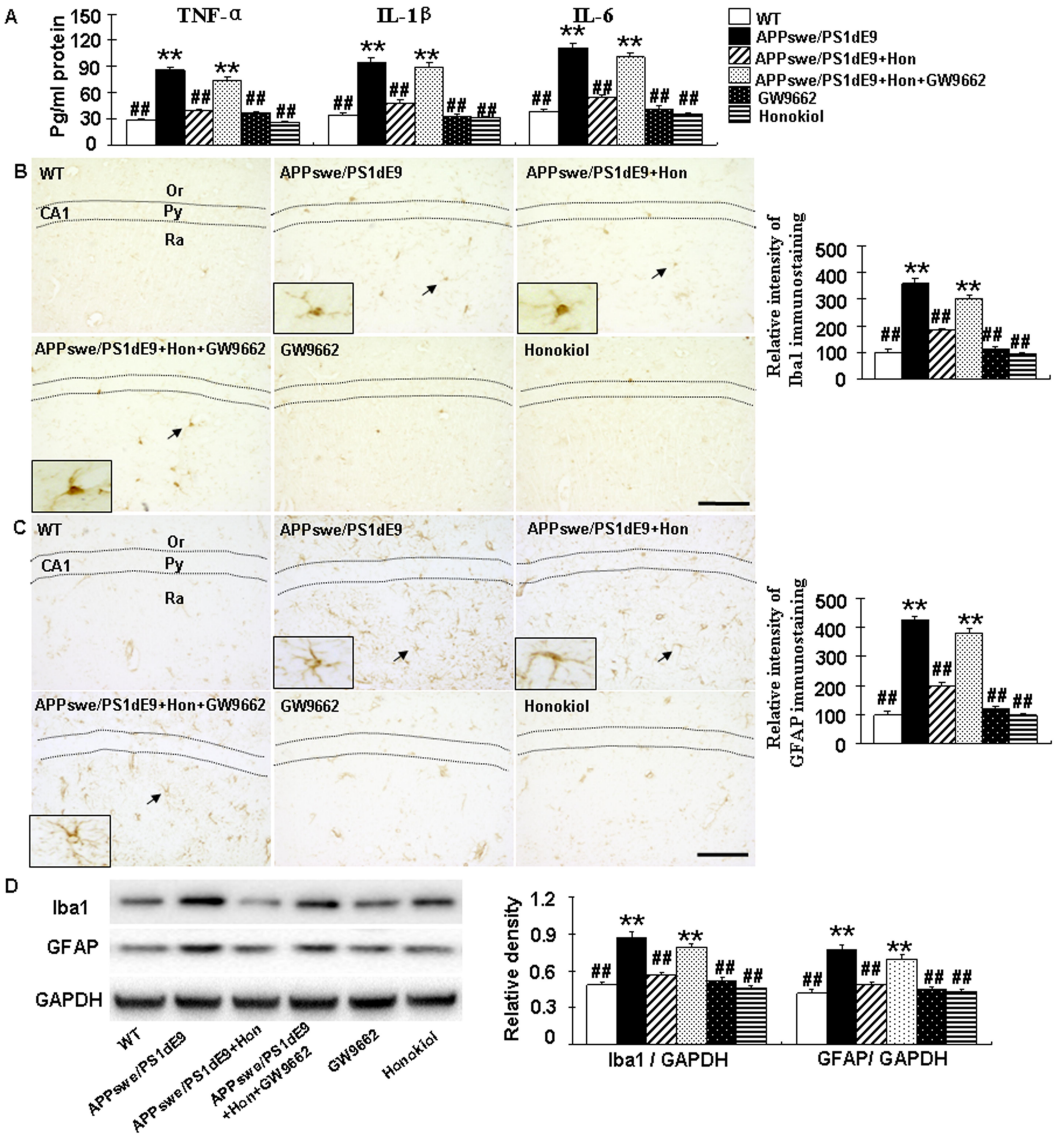

FIGURE 4

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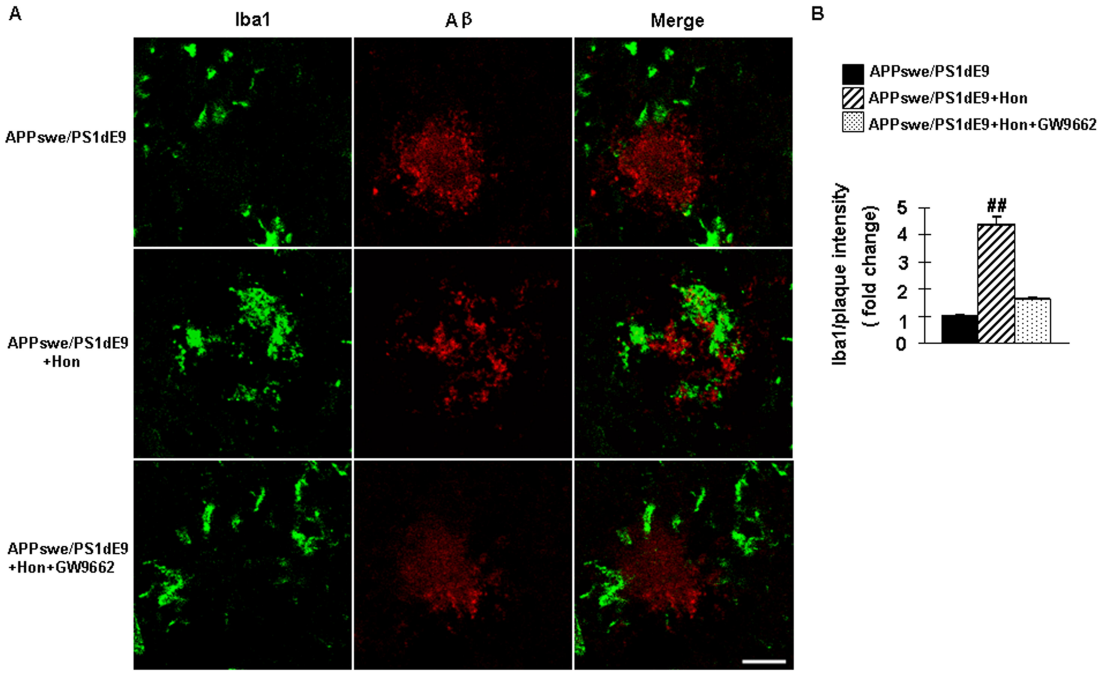

FIGURE 5

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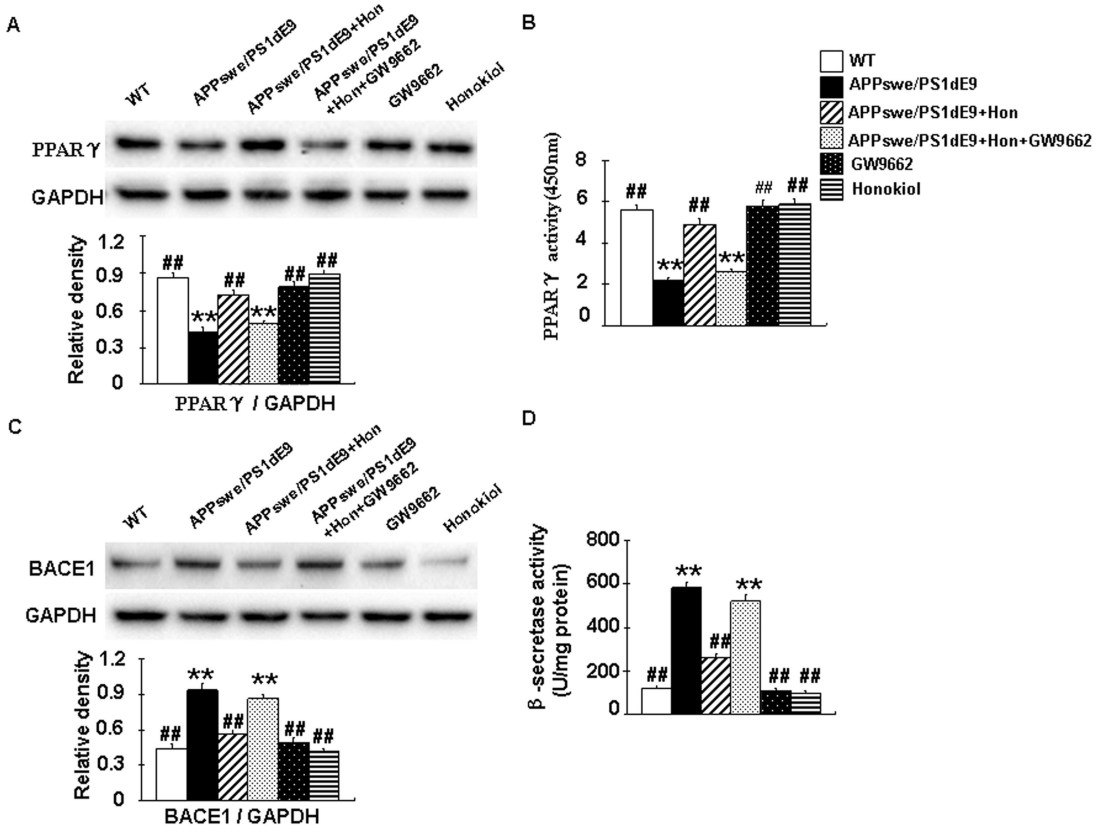

FIGURE 6