Pinocembrin improves cognition and protects the neurovascular unit in Alzheimer related deficits

Rui Liu a, Jin-ze Li a, Jun-ke Song a, Dan Zhou a, Chao Huang a, Xiao-yu Bai a, Tao Xie b, Xue Zhang a, Yong-jie Li a, Cai-xia Wu a,b, Lan Zhang c, Lin Li c, Tian-tai Zhang a,**, Guan-hua Du a,*

a State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, P. R. China
b School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China
c Key Laboratory for Neurodegenerative Diseases of Ministry of Education, Department of Pharmacology, Xuanwu Hospital of Capital Medical University, Beijing Geriatric Medical Research Center, Beijing 100053, PR China

Article info
Article history:
Received 14 May 2013
Received in revised form 13 November 2013
Accepted 25 December 2013
Available online 28 December 2013

Keywords:
Alzheimer’s disease
Amyloid-β peptide
The neurovascular unit
Pinocembrin
The receptor for advanced glycation end products

Abstract
Amyloid-β (Aβ) peptides accumulate in the brain and initiate a cascade of pathologic events in Alzheimer’s disease. The receptor for advanced glycation end products (RAGE) has been implicated to mediate Aβ-induced perturbations in the neurovascular unit (NVU). We demonstrated that pinocembrin exhibits neuroprotection through inhibition of the Aβ and/or RAGE pathway, but the therapeutic role and mechanism involved are not ascertained. Here, we report that a 3-month treatment with pinocembrin prevents the cognition decline in APP/PS1 transgenic mice without altering Aβ burden and oxidative stress. Instead, pinocembrin is effective in conferring neurovascular protection through maintenance of neuropil ultrastructure, reduction of glial activation and levels of inflammatory mediators, preservation of microvascular function, improving the cholinergic system by conserving the ERK-CREB-BDNF pathway, and modulation of RAGE-mediated transduction. Furthermore, in an in vitro model, pinocembrin provides the NVU protection against fibrillar Aβ, accompanied by regulation of neurovascular RAGE pathways. Our findings indicate that pinocembrin improves cognition, at least in part, attributable to the NVU protection, and highlights pinocembrin as a potential therapeutic strategy for the prevention and/or treatment of Alzheimer’s disease.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder, characterized by abnormal accumulation of amyloid plaques and neurofibrillary tangles throughout the cerebrocortical and limbic regions (Coyle et al., 1983). Recent studies have provided a vascular-derived insult hypothesis that AD is not solely caused by abnormal amyloid metabolism, but blood vessel damage must also be involved (Marchesi et al., 2011; Shin et al., 2007). Further evidence suggests that initial vascular damage plays a pivotal role in functional and structural changes of neurons (Bell et al., 2010; Marchesi et al., 2011; Zlokovic, 2005), and accumulation of brain amyloid-β (Aβ) peptides is subsequent to the blood-brain barrier (BBB) dysfunction and reduction in cerebral blood flow (Bell et al., 2010; Zlokovic, 2011). Importantly, vascular clearance of brain Aβ is impaired after BBB damage (Deane et al., 2004, 2003; Zlokovic, 2011) and the β-amyloidogenic process is up-regulated as a result of reduced brain blood perfusion (Cullen et al., 2006; Kumar-Singh et al., 2005; Weller et al., 2008). At subsequent pathophysiological events, Aβ accelerates neurovascular (Deane et al., 2003; Zlokovic, 2005) and neuronal dysfunction (Takuma et al., 2009; Walsh et al., 2002; Yan et al., 1996), causes oxidative stress, and mediates chronic inflammatory processes, which are involved in the neurovascular unit (NVU) pathology.

The NVU comprises of cerebral blood vessels and surrounding astrocytes, neurons, and other supporting cells (e.g., perivascular microglia and pericytes) (ladedola, 2004), which couples local neuronal function to local cerebral blood flow, as well as regulating...
transport of blood-borne molecules across the BBB (Bell and Zlokovic, 2009; Kalaria, 2009; Zlokovic, 2008). The intimate association between astrocytes and brain microvessels is referred to as the major component of the NVU; each cell type is important for the physiological function of the BBB (Hawkins and Davis, 2005), which restricts permeability across brain endothelium (Zlokovic et al., 1985a, 1985b) and regulates the transport of nutrients and peptides using specific transporters expressed in brain endothelium either under physiological or pathologic conditions (Zlokovic and Apuzzo, 1997; Zlokovic et al., 1990, 1989). The BBB is easily disrupted under pathologic conditions (Sagare et al., 2013; Sengillo et al., 2013). Animals receiving a peripheral Aβ injection (Liu et al., 2011a; Su et al., 1999) and transgenic AD mice (Ujie et al., 2003) displayed increased BBB permeability. Further, morphologic alterations of tight junction proteins, suggestive of a leaky BBB, have been observed in AD patients’ brain biopsies (Stewart et al., 1992). As the therapy of reducing neuronal injury alone would be expected to be less successful in slowing the course of AD, the treatment strategy with maintaining the integrity of BBB is recommended as a potential method to prevent degeneration of this disease.

In the NVU, the receptor for advanced glycation end products (RAGE), addressed as a neurovascular coupling molecule (Zlokovic et al., 2008), plays an important role as a cell-surface receptor for different forms of Aβ at the BBB, neurons, and microglia (Deane et al., 2012; Zlokovic, 2011). RAGE mediates the re-entrée of circulating Aβ into the brain at the BBB, followed by pathologic events in the endothelium, such as NF-xB-mediated activation accompanied with inflammatory cytokine production (Liu et al., 2013). RAGE ligation in neurons and glia has been shown to activate multiple signaling pathways, including the Ras-extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinase (p38 MAPK) pathways. This results in oxidative stress, inflammation, cholinergic dysfunction, and neurovascular uncoupling. Because of its pivotal pathologic role in neurovascular signaling and potential cytoprotection toward the NVU through interference, RAGE pathway is increasingly considered as a new therapeutic approach for AD.

Pinocembrin (5, 7-dihydroxyflavonol, Fig. 1A) is a flavonoid abundant in propolis and extracted as a pure compound. Pinocembrin has been shown to be effective in the protection of brain injury from ischemic and Aβ impairment. It was approved by the State Food and Drug Administration of China for treatment of ischemic and Aβ-precursor protein (APP) and in the APP/PS1 double transgenic AD mouse model (Guang and Du, 2006) and intracerebroventricular Aβ-injected model (Liu et al., 2012). In the Aβ1-42-treated RAGE overexpressing cell model, pinocembrin inhibited the overexpression of RAGE (Liu et al., 2012). Moreover, pinocembrin attenuated neuronal apoptosis through down-regulating RAGE expression and inhibiting RAGE downstream pathways, both in SH-SY5Y cells overexpressing the Swedish mutant form of human Aβ-precursor protein (APP) and in the Aβ25–35-induced amnesia model (Liu et al., 2012). These results suggest that pinocembrin has potential therapeutic effects on Aβ-related cognitive deficits and might have prospects as an AD therapeutic agent.

To confirm the cognitive improvement and explore the potential mechanism, we examined the effects of pinocembrin in the APP and presenilin 1 (PS1) double transgenic AD mouse model on cognitive impairment and pathologic changes within the NVU. In addition, we investigated the mechanisms underlying the efficacy on potential signal transduction in an in vitro model.

2. Methods

2.1. Materials

Pinocembrin (purity >99%) was synthesized by Institute of Materia Medica, Chinese Academy of Medical Sciences. Unless otherwise stated, all other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Animals and drug treatment

APP/PS1 double transgenic mice were purchased from The Jackson Laboratory (strain name, B6C3-Tg[APPswe, PSEN1dE9]85Dbo/J; stock number, 004462). Age-matched wild-type (WT) littermates were used as controls. All animals received care according to the Guide for the Care and Use of Laboratory Animals.

Four-month-old APP/PS1 mice and littermate WT mice were divided randomly into 4 groups: WT controls (n = 9, 4 males and 5 females), WT + pinocembrin 40 mg/kg (n = 9, 5 males and 4 females), APP/PS1 controls (n = 9, 5 males and 4 females), and APP/PS1 + pinocembrin 40 mg/kg (n = 9, 5 males and 4 females). Pinocembrin-treated WT and APP/PS1 groups received pinocembrin dissolved in 20% hydroxypropyl-β-cyclodextrin by oral gavage 5 days per week. The vehicle controls received 20% hydroxypropyl-β-cyclodextrin in the same manner. Treatment continued for 12 weeks.

After the behavioral tests were completed, the mice were divided into 3 parallel experiments: (1) one-third of each group was anaesthetized and blood was collected following transcardial perfusion with phosphate buffered saline. The brains were quickly removed. One hemi-brain was snap frozen in liquid nitrogen. The other was fixed and stored in 4% paraformaldehyde in phosphate buffered saline and later dehydrated. The effects of pinocembrin on cholinergic neuronal changes and protein expressions were examined; (2) another one-third mice were used for BBB permeability assessment and neuropil ultrastructure detection; and (3) the remainders were used for the detection of cerebral oxidative stress, inflammatory mediator levels, and Aβ burdens. The division of mice into treatment groups and selection of mice to be sacrificed within each group were both done randomly.

2.3. Behavioral tests

Spatial learning and memory capabilities were assessed by Morris water maze (MWM) test. The acquisition task consisted of 5 consecutive days of training. The duration required to escape onto the hidden platform was recorded as escape latency. In the probe trial, the time the mouse spent in the platform quadrant and numbers of crossings where the platform had been located were recorded. Only mice with swimming speed over 8 cm/s in all trials were included in the analysis to discard occasional floating mice. The passive avoidance task was used to assess the contextual short-term memory of the mice. The time a mouse took to enter the NON-illuminated compartment after opening the door was defined as latency.

2.4. Histochemistry

Slices from frontal cortex were used for staining with anti-glial fibrillary acidic protein (GFAP) (1:1000, Abcam, Cambridge, UK, USA), anti-ionized calcium-binding adaptor molecule 1 (Iba-1) (1:500, Wako, Osaka, Japan), and anti-p38 (1:200, Cell Signaling Technology, Beverly, MA, USA). Image analysis was performed using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).
2.5. BBB permeability assessment

The tracer lanthanum was used to assess the restoration of the BBB function by reported methods (Liu et al., 2011a, 2013). The evaluation was performed by counting at least 10 randomly selected microvessels in 1 ultrathin section ($\text{mm}^2$) of cortex per animal. It was calculated as a percentage by dividing the number of affected vessels by the total number of vessels examined in the sections.

2.6. Estimation of amyloid plaques by transmission electron microscope

To estimate amyloid plaques, photographs were taken randomly. Amyloid plaques were quantified in the neuropil (i.e., avoiding the neuronal and glial somata, and blood vessels) (Alonso–Nanclares et al., 2013). To obtain an adequate sample size, at least 30 micrographs were obtained per cortical ultrathin section at a magnification of 20,000. It was calculated as the mean total number of amyloid plaques examined in different ultrathin sections of each group. There are 3 parallel samples in each section.

2.7. Acetylcholinesterase assay

The cerebral tissues of the hemi-brain were weighed and homogenized in 5 vol/wt of Tris-buffered saline containing a cocktail of protease inhibitors. One aliquot of homogenate of each sample was used to determine acetylcholinesterase (AChE) activity by the spectrophotometric method (Ellman et al., 1961).
2.8. Enzyme linked immunosorbent assay analysis

Cerebral acetylcholine (ACh), interleukin-1, interleukin-6, and tumor necrosis factor alpha levels were determined using the respective immunoassay kits (R&D Inc, Minneapolis, MN, USA) in strict accordance with the manufacturer’s instructions. The levels of soluble and insoluble Aβ in the brain were quantified according to the procedures by the human Aβ1-40/42 enzyme linked immunosorbent assay kit (BioSource, Camarillo, CA, USA).

2.9. Cerebral oxidative stress measurement

The activities of superoxide dismutase (SOD) and glutathione peroxidase in the cerebral homogenates were measured using commercial assay kits (Nanjing Jiancheng Company, Nanjing, China) according to the manufacturer’s instructions.

2.10. Cell culture and exposure to Aβ

Establishment of the NVU coculture was previously reported (Liu et al., 2011b). Briefly, newborn WT mouse neuronal parenchymal cultures were cultured on the bottom of transwell filter in advance. Mouse cerebral microvascular endothelial cells (CMECs) from 1-month-old WT mouse were seeded on the upper side of the inserts after 72 hours. The day when the CMECs were plated was defined as day 0. On day 7, the experiments were performed. The exposure to 10 μM Aβ1-42 (Sangon Biotech Company, Shanghai, China) started from the upper compartment for 72 hours. Pinocembrin was administrated from the upper compartment following the 24 hour-exposure to Aβ1-42, and then combined with Aβ1-42 for a subsequent 48-hour. These cocultures were randomly divided into: (1) coculture group; (2) coculture group treated with 1 μM; (3) 10 μM pinocembrin for 48 hours; (4) Aβ1-42 group exposed to 10 μM Aβ1-42 for 72 hours; (5) 1 μM pinocembrin; and (6) 10 μM pinocembrin-treated groups.

2.11. Transendothelial electrical resistance detection

Transendothelial electrical resistance (TEER) value was expressed as Ω·cm² by multiplying the surface area of the transwell insert. The final value was calculated from the displayed readings by subtraction of the resistance values attributed to both the blank insert and the insert cultured with parenchymal cells.

2.12. Immunofluorescence

In the coculture, the neuronal growth, and neurite lengths were stained with a mouse monoclonal anti-βIII-tubulin (1:200, CST, Beverly, MA, USA). The secondary antibody was Dylight 488 goat anti-mouse antibody. Determination of neurite length was done with Micro-Color Image Analysis System (No.T20021107-XSHOW-LIU-Z, China).

2.13. Brain-derived neurotrophic factor content analysis

Neuronal parenchyma of coculture was homogenized with ultrasonication and centrifuged at 4 °C for 10 minutes, 13,000 rpm. The supernatants were used for detection by enzyme linked immunosorbent assay.

2.14. Western blot analysis

The expression of RAGE, ERK1/2, CREB, p38, and NF-κB p65 in neuronal parenchymal homogenate, RAGE, ERK1/2, and NF-κB p65 in CMEC homogenate, and RAGE in mouse cortex was performed with commercial antibodies as described, using β-actin as loading controls.

2.15. Statistical analysis

Data are represented as the mean ± S.E.M. p < 0.05 was considered significant. Treatment differences of escape latency in MWM task were analyzed using 2-factor analysis of variance with repeated measures on 1 factor. Tukey post hoc test was used if the treatment was significant in analysis of variance (ANOVA). The other studies were analyzed using 1-way ANOVA followed by an appropriate post hoc test to analyze the difference.

3. Results

3.1. Pinocembrin treatment improves cognitive deficits in APP/PS1 mice

Fig. 1B shows the results of all mice during acquisition training in MWM test. Repeated-measures of ANOVA revealed a significant day effect on escape latency ([F(14,128) = 71.634, p < 0.001]) within the groups. There is also a significant treatment effect ([F(3,122) = 13.300; p < 0.001]) on the escape latency, and subsequent comparisons further suggest that 40 mg/kg pinocembrin treatment is effective for rescuing spatial learning deficits in APP/PS1 mice (p < 0.05). In the probe trial, APP/PS1 control mice spent less time searching for the platform and showed reduced crossings in the target quadrant compared with the WT vehicle control (p < 0.01, p < 0.05, Fig. 1C and D). Pinocembrin-treated APP/PS1 mice spent more time searching in the target quadrant, and also showed displayed increased crossings where the platform was located (p < 0.01, p < 0.05).

To exclude the possibility that the improvement of pinocembrin in APP/PS1 mice was not owing to sensorimotor abnormality, we analyzed their swimming ability. Results showed that there was no significance on swimming speed amongst the groups across the 5-day training (Fig. 1E).

The effect of pinocembrin on memory performance was also evaluated in the passive avoidance task. During the retention trials, WT control mice hesitated to reenter the ILLUMINATED compartment, however, the APP/PS1 vehicle control reentered the ILLUMINATED compartment more frequently, showing the shorter step-through latency (p < 0.01, Fig. 1F). APP/PS1 mice receiving pinocembrin treatment showed the longer step-through latency (p < 0.05).

In addition, pinocembrin treatment in WT mice did not show any significant effect on learning and memory capabilities, which indicates that long-term oral administration of pinocembrin has no effect on performance in normal mice, but rescues Aβ-dependent behavioral deficits.

3.2. Pinocembrin treatment protects cortical capillaries and the surrounding neuropil in APP/PS1 mice

The major cortical capillaries and the surrounding neuropil consist of major components of microcosmic NVU. In the slices of WT control mice and pinocembrin-treated WT mice, lanthanum ions confines into the vessel lumen (Fig. 1G [a and b]). The endothelial cell layer shows distinct tight junctions, and the surrounding astrocytes don’t exhibit apparent signs of perivascular edema. The neuron shows a smooth surface and its nucleus is not condensed. In vessels of APP/PS1 control mice, the cortex presented leakage of lanthanum ions from the lumen of vessels (Fig. 1G [c and d]). Lanthanum ions fill the endocytotic vesicles of endothelial cells and leak out from the endothelial basement membrane, and there is a
significant increase of vessels with tracer extravasation \( p < 0.001, \text{Fig. 1G [d] and H}. \) In addition, water fills the astrocytes and extracellular space, and adjacent neurons become pyknotic \( \text{Fig. 1G [c]}. \) Microvessels fall into amyloid plaques, most of which belong to mature plaques represented by an epicenter of compacted \( \text{Aβ} \) fibrils \( \text{Fig. 1G [e and f]}. \) Pinocembrin treatment alleviates the leakage of lanthanum ions from the lumen of vessels \( \text{Fig. 1G [g]}. \) and the number of affected vessels shows a significant decrease in comparison with the APP/PS1 control ones \( p < 0.01, \text{Fig. 1H}. \) Astrocytes do not show apparent sign of end-foot swelling as neuronal pycnosis is relieved \( \text{Fig. 1G [g]}. \) accompanied by the absence of mature amyloid plaques around the microvessels \( \text{Fig. 1G [g and h]} \).

3.4. Pinocembrin treatment increases the level of \( \text{ACh}, \) but does not alter the \( \text{AChE} \) activity

A consistent finding in AD is a decrease on the \( \text{AChE} \) activity in most brain regions because of an early and severe depletion of cholinergic innervations \( \text{Carvajal et al., 2013; Dumas and Newhouse, 2011}. \) There is a slight decrease of \( \text{AChE} \) activity in APP/PS1 mice \( \text{Fig. 2D [b]}. \) Pinocembrin treatment does not alter \( \text{AChE} \) activity in the cortices of APP/PS1 mice. In the case of the cerebral \( \text{ACh} \) levels, the content increases in APP/PS1 mice treated with pinocembrin \( p < 0.05, \text{Fig. 2D [a]}. \) suggesting a neuroprotective effect on cholinergic neurons.

3.5. Pinocembrin treatment inhibits glia activation, attenuates \( \text{RAGE} \)-induced \( \text{p38 MAPK} \) pathway, and reduces inflammatory mediator levels in APP/PS1 mice

In the brains of AD patients and transgenic AD mice, activation of astrocytes and microglia is observed in the area of \( \text{Aβ} \) plaques \( \text{Matsuoka et al., 2001}. \) contributing to an inflammatory process that develops around the injury in the brain \( \text{McGeer and McGeer, 1999}. \) Here, both markers, \( \text{GFAP} \) and \( \text{Iba-1} \), show clear elevations of inflammatory mediator levels in APP/PS1 mice

**Fig. 2.** Pinocembrin treatment shows no effect on \( \text{Aβ} \) burden or markers of oxidative stress and \( \text{AChE} \) activity, but increases \( \text{ACh} \) level in APP/PS1 mice. \( \text{A} \) \( \text{Aβ} \) plaque burden in vehicle-treated and pinocembrin-treated APP/PS1 mouse cortex. There was still mature \( \text{Aβ} \) plaque represented by an epicenter of compacted \( \text{Aβ} \) fibrils \( \text{Fig. 1G [d] and H}. \) Pinocembrin treatment alleviates the leakage of lanthanum ions from the lumen of vessels \( \text{Fig. 1G [g]}. \) and the number of affected vessels shows a significant decrease in comparison with the APP/PS1 control ones \( p < 0.01, \text{Fig. 1H}. \) Astrocytes do not show apparent sign of end-foot swelling as neuronal pycnosis is relieved \( \text{Fig. 1G [g]}. \) accompanied by the absence of mature amyloid plaques around the microvessels \( \text{Fig. 1G [g and h]} \).
through dependent up-regulation of inflammatory cytokines (Rong et al., 2005). According to present results, we observe an obvious up-regulation of RAGE by 65%, and the remarkable immunoreactivity of downstream phosphor-p38 by 44.73% in 7-month-old APP/PS1 mice compared with vehicle-treated WT (p < 0.001, Fig. 4B and C). Oral pinocembrin treatment inhibits the increase of cerebral RAGE expression, and prevents the active immunostaining of phosphor-p38 in APP/PS1 mice (p < 0.05, p < 0.05). These results illustrate that pinocembrin treatment corroborates significant inhibition in RAGE and its downstream signaling, in accordance with our previous AD cell model study.

As a consequence of RAGE signaling activation, the levels of well-known inflammatory markers, tumor necrosis factor alpha, interleukin-1, and interleukin-6 levels are increased in APP/PS1 mice (p < 0.001, p < 0.001, p < 0.01, Fig. 3D), and decreased in pinocembrin-treated APP/PS1 mice (p < 0.01, p < 0.05, p < 0.05). Noteworthy, combined with the significantly less GFAP and Iba-1 immunoreactivity shown in pinocembrin-treated APP/PS1 mice in the same manner, we suggest that cerebral inflammation is modulated by pinocembrin.

Furthermore, as a putative receptor for Aβ in the extracellular space, RAGE transduces pathologic inflammatory responses when bound to excessive Aβ, and in a similar way, its protein level might

![Fig. 3. Pinocembrin treatment inhibits glia activation, attenuates RAGE-induced p38 MAPK pathway, and reduces inflammatory mediator levels in APP/PS1 mice. (A) Immunohistochemistry revealed significant glial activity reduction by pinocembrin treatment as analyzed by an astrocytic marker GFAP and a microglial markers Iba-1 in cortex in age-matched WT and APP/PS1 mice. The optical fields were scanned with a 20× objective lens. b, quantitative image analysis of immunoreactivity demonstrates that reactive astrocytes and microglia were significantly decreased in pinocembrin-treated APP/PS1 mice. *** p < 0.001 versus vehicle-treated WT mice, ## p < 0.01, ### p < 0.001 versus vehicle-treated APP/PS1 mice. (B) Western blot analysis for RAGE protein in cerebral cortex. a, representative immunoblot for RAGE in cerebral cortex extracts; b, quantified results are normalized to β-actin expression and values are expressed as ratios compared to vehicle-treated WT mice (set to 1), demonstrating that RAGE expression is significantly decreased in pinocembrin-treated APP/PS1 mice. *** p < 0.001 versus vehicle-treated WT mice, * p < 0.05 versus vehicle-treated APP/PS1 mice. (C) Immunohistochemical analysis of cortex reveals pinocembrin treatment reduces p38 MAPK levels in APP/PS1 mice. a, representative images of p38 MAPK in cortex in WT and APP/PS1 mice; b, quantitative analysis of immunoreactivity. *** p < 0.001 versus vehicle-treated WT mice, * p < 0.05 versus vehicle-treated APP/PS1 mice. (D) Pinocembrin treatment demonstrates inflammatory mediator lowering effects in cerebral homogenates of APP/PS1 mice using ELISAs. a, TNF-α; b, IL-1β; and c, IL-6 levels. ** p < 0.01, *** p < 0.001 versus vehicle-treated WT mice, * p < 0.05, ** p < 0.01 versus vehicle-treated APP/PS1 mice. All data are presented as mean ± S.E.M., n = 3. Abbreviations: ELISA, enzyme linked immunosorbent assay; IL-1β, interleukin-1; IL-6, interleukin-6; RAGE, receptor for advanced glycation end products; SEM, standard error of the mean; TNF-α, tumor necrosis factor alpha; WT, wild type.]
be modulated. In this study, pinocembrin treatment did not reduce RAGE level and modulate inflammatory cytokine content in WT control mice, indicating that pinocembrin at an oral dose of 40 mg/kg does not suppress baseline levels of RAGE and its subsequent transduction in normal brains, in contrast to AD conditions.

3.6. Pinocembrin protects in vitro NVU model against fibrillar Aβ

TEER is an important indicator to evaluate pathophysiological conditions of the BBB structure and function in the NVU entity (Harhaj et al., 2004; Liu et al., 2011b). Seventy-two hours exposure
of fAβ1-42 caused a serious decrease of TEER in coculture after (p < 0.001, Fig. 4A). Pinocembrin attenuates the decrease of TEER at 1.0 μM and 10.0 μM in a dose-dependent manner (p < 0.05, p < 0.01, Fig. 4A), but does not show any significant effect in normal coculture.

As one of the major components in the NVU, neuronal loss or degeneration is involved in pathophysiology of this disorder. The neurite length shows significant changes after the exposure to fAβ1-42 (p < 0.001, Fig. 4B), indicating that neuronal loss and neurite damage are presented consistently with the damages occurring to the BBB dysfunction. Pinocembrin prevents the loss of neuronal integrity at 1.0 μM and 10.0 μM (p < 0.001, p < 0.001).

Additionally, our findings show that cortical BDNF level, phosphor-ERK1/2, and phosphor-CREB expression in the cerebral cortex were suppressed when subjected to fAβ1-42 for 72 hours (p < 0.01, p < 0.001, p < 0.001, Fig. 4C). Pinocembrin treatments enhance the BDNF-ERK1/2-CREB signal transduction at 1 μM and 10 μM (p < 0.05, p < 0.001, p < 0.001, Fig. 4C).

3.7. Pinocembrin inhibits RAGE-mediated pathways both in microvessels and in parenchyma in vitro NVU model

In CMECs from the coculture, RAGE expression is significantly increased when subjected to fAβ1-42 (p < 0.001, Fig. 4D). Phosphor-ERK1/2 level and the ratio of nuclear to cytoplasmic p65 are also increased in fAβ1-42-treated group (p < 0.001, p < 0.001). In response to fAβ1-42-induced toxicity, pinocembrin treatments at 1 μM and 10 μM reduced RAGE expression, suppressed phosphor-ERK1/2 level, and decreased the ratio of NF-κB p65 in nuclei and in cytoplasm (p < 0.05, p < 0.01, p < 0.001).

In the cerebral parenchymal culture, cerebral RAGE expression is significantly augmented in the fAβ1-42-treated group (p < 0.001, Fig. 4E). Expression of phosphor-p38 and ratio of nuclear to cytoplasmic p65 increased (p < 0.001, p < 0.001). Pinocembrin treatments diminished the expression of RAGE and phosphor-p38 in cerebral cytoplasm, and inhibited the nuclear expression of NF-κB p65 in the cerebral cortex (p < 0.001, p < 0.05, p < 0.01).

4. Discussion

As an extension of previous research, this present study further clarified the beneficial effects of pinocembrin on Aβ-associated pathology and the potential mechanisms with respect to the role of the NVU. Our findings indicate a clear rescue of cognitive deficits in pinocembrin-treated APP/PS1 double-transgenic mice. Cerebral Aβ burden and oxidative stress were unaltered by the treatment. Importantly, pinocembrin was effective in preserving the neuropil ultrastructure and microvascular function, reducing glial activation, decreasing the levels of inflammatory markers, conserving cholinergic function, and restoring RAGE-mediated transduction. These observations are correlated with a prospective neurovascular coupling protection in AD deficits.

Our results showed that pinocembrin treatment, by oral gavage of 40 mg/kg, improved spatial learning and memory capability effectively in MWM tasks in APP/PS1 mice. In the passive avoidance test, pinocembrin treatment showed comparable effects on ameliorating memory impairment in this model. Moreover, we observed the protective effects of pinocembrin on pathologic changes caused by fAβ1-42 indicated by TEER and neuronal integrity in the coculture entity. Thus, combined with the protection of pinocembrin in Aβ-mediated neurotoxicity models (Liu et al., 2012), we further substantiate the effects of pinocembrin on preventing Aβ-related deficits.

As Aβ peptide and its accumulation leading to amyloid plaque deposition are believed to be central in the pathogenesis of AD (Hardy and Higgins, 1992), it is generally thought that the therapeutic effect of pinocembrin on cognitive improvement is likely because of reducing Aβ levels or relieving Aβ burdens in the cortex. However, pinocembrin had no effect on the cerebral soluble or insoluble Aβ1-40 or Aβ1-42 levels in APP/PS1 mice. Mature amyloid plaques still appeared in the cerebral neuropil. Combined with the previous study that pinocembrin did not inhibit Aβ1-42 secretion from neuronal cells, we deduce that pinocembrin does not participate in the amyloidogenic processing of APP or alter the secretion or deposition procedure of Aβ.

A direct role of Aβ-mediated oxidation has been observed in APP/PS1 mice with evident increases in protein oxidation and lipid peroxidation (Mohmmad et al., 2004). In this study, pinocembrin nonsignificantly increased the antioxidant defense system in APP/PS1 mouse brain. Although pinocembrin showed antioxidative effects in ischemic models (Liu et al., 2008; Shi et al., 2011), it did not provide sufficient antioxidation against Aβ-mediated neurotoxicity, neither in a cell model using copper to trigger neurotoxicity of Aβ (Liu et al., 2012).

Since neither antioxidation nor the capability of reducing Aβ deposits is involved in the mechanism of preventing Aβ-mediated neurotoxicity, it is suggested that pinocembrin may act synergistically with other crucial mechanisms for the treatment of AD. In this study, there are 3 potentially beneficial effects: (1) protection of cerebral cholinergic neurons by restoring ACh level and preserving the ERK/CREB/BDNF signaling pathway; (2) maintaining the integrity of the BBB via molecular cascades; and (3) alleviating the NVU injury, reducing glial activation, decreasing inflammatory mediator levels, and inhibiting RAGE-mediated transduction.

Altered cognition in AD is associated with the loss of cholinergic input (Bartus, 1982). The degeneration of cholinergic neurons leads to several protein changes of the cholinergic system as AChE and cholineacetyltransferase activities decrease, while Na+-dependent high-affinity choline uptake increases (DeKosky et al., 2002; Potter et al., 2011). According to our results, pinocembrin treatment showed no significant effect on AChE activity; also displayed in an in vitro enzyme test (data not shown). Thus, we conclude that the effect of pinocembrin is not interrelated with primary alteration of AChE activity, however, there are several major signaling pathways implicated in learning and memory at the molecular level (Carew, 1996). The alteration of neural transmitter ACh was observed post pinocembrin treatment as pinocembrin restored ERK-CREB-BDNF pathway in APP/PS1 mice, accompanied by the improvement of cognition in behavioral tests. Taken together, we suggest that pinocembrin might have the effect on the modulation of synaptic transduction of the ERK-CREB-BDNF pathway in cortical cholinergic neurons.

Besides direct neuronal protective effects, pinocembrin may show other beneficial properties to achieve long lasting effects on other cell types in the NVU. Accumulation of Aβ activities in the neurovascular interface plays a critical role in molecular cascades of the BBB disruption (Zlokovic, 2008). Our study demonstrated a serious leakage in BBB permeability and the amyloid plaques fell into the cortex around microvessels, which are consistent with observations in AD patients displaying a leaky BBB that potentially disrupts the CNS homeostatic environment. Previous studies have demonstrated that BBB dysfunction is associated with the accumulation of vasculotoxic and neurotoxic molecules within brain parenchyma (Bell et al., 2010), the reduction in cerebral blood flow (Zlokovic, 2011), the influx transport of Aβ (Deane et al., 2004, 2005; Zlokovic, 2011), and the increase of amyloid-processing (Cullen et al., 2006; Kumar-Singh et al., 2005; Weller et al., 2008). Pinocembrin is probably acting as a multimodal neurovascular agent to exert the beneficial effects on preserving anatomic and functional integrity of the BBB, which can contribute to eliminating part of the vascular-derived insults in the brain.
Additionally, based on the BBB protection from Aβ, pinocembrin may provide protection partly through the regulation of RAGE-mediated pathways. In our previous study, we illustrated the inhibition of the up-regulation of RAGE transcripts in accordance with protein expression in the Aβ-mediated toxicity models by pinocembrin (Liu et al., 2012). Here, oral treatment of pinocembrin for 3 months also decreased RAGE expression accompanied by the subsequent reduced signal transduction both in APP/PS1 mice and in vitro cultured cells. As an increase in Aβ influx to the brain is because of increased expression of RAGE (Deane et al., 2003, 2012; Zlokovic, 2008), it provides the explanation that microvessels showed no surrounding amyloid plaques after pinocembrin treatment. Among the subfamilies of MAPKs, ERK1/2 plays a vital role in Aβ/RAGE-induced toxicity (Sun et al., 2009) via directly reducing the BBB patency caused by activating NF-κB-mediated inflammatory cytokine secretion (Deane et al., 2003), ERK1/2 phosphorylation was down regulated by pinocembrin treatment, and its downstream NF-κB p65 translocation was inhibited in the same manner. The effects of pinocembrin on the BBB have also been reported with an increase in mitochondrial membrane potential (Meng et al., 2011) and a decrease in inflammatory cytokines (Gao et al., 2010), which are partly explained by RAGE signaling. Taken these previous studies into consideration, our study indicates that protection of pinocembrin on the BBB might be mediated through inhibition of RAGE-mediated signaling.

So far, Aβ/RAGE is not only a pivotal mediator of neuron damage in AD, but also a contributor to glial activation through the up-regulation of inflammatory cytokines and NF-κB pathway activation (Rong et al., 2005). In this study, increase of inflammatory markers and activation of glia were in parallel with the amplification of Aβ pathology in APP/PS1 mice. As a consequence of Aβ/RAGE transduction, activation of ERK1/2, and downstream NF-κB transduction were evidenced, which are responsible, at least in part, for Aβ-mediated glial activation and the induction of inflammatory mediators in the brain (Fang et al., 2010; Liu et al., 2012).

Herein, one of the possible anti-neuroinflammatory mechanisms of pinocembrin in the NVU is mainly through the regulation of the RAGE signaling. In cerebral parenchymal coculture, pinocembrin inhibited RAGE expression, and attenuated RAGE downstream transduction in parenchymal cells through decreasing the p38 MAPK-NF-κB p65-signaling pathway. These findings correspond with the in vivo pinocembrin treatment study demonstrating a decrease of inflammatory markers and inactivation of astrocytes and microglia. In the CMECs, pinocembrin down-regulated RAGE expression, as well as expression of phosphoryl-ERK1/2 and nuclear NF-κB p65 (Fig. 5). The inhibition of microvascular RAGE signaling correlates with the improvement of the BBB function in the in vivo study. Previous studies have provided evidence of increased expression of RAGE in blood vessels, neurons, and glia in brains of AD-affected individuals, as well as its participation in neurovascular pathogenesis and neuronal dysfunction and death (Deane et al., 2012). It is conceivable that simultaneous inhibition of multiple RAGE-mediated pathways by pinocembrin in cerebral microvessels and parenchyma contributes to neurovascular protection. The interaction mode between pinocembrin and RAGE contributing to the reduction in Aβ-induced NVU lesions needs to be further identified.

Based on the study, our results support the idea that regulation of RAGE-mediated molecular signaling might evoke a beneficial response regarding neuroinflammation linked to neurovascular perturbation. These findings are in concurrent with preclinical data demonstrating the therapeutic role of RAGE in AD. Although a small compound (FP-04494700) used to antagonize ligand binding to RAGE in phase II clinical trials for mild to moderate AD has been terminated, anti-RAGE therapy is still being developed for AD with therapeutic potential. Recently, a second-generation library of small compounds of high-affinity RAGE-specific inhibitors have been built, and in particular a small organic compound called FPS-ZM1, a new next-generation RAGE inhibitor, has been shown to be effective in controlling progression of Aβ-mediated neurovascular and cognitive dysfunction; revealing its potential to be a disease-modifying agent for AD (Deane et al., 2012).

Importantly, pinocembrin regulated the cerebral NVU through modulating the function of cholinergic neurons accompanied by the regulation of microvascular function. This provides evidence for the promising conservation of developed pathophysiological signaling cascades between NVU cells to restore the microenvironment. The hypothesis that cerebral cholinergic fibers originating from the nucleus basalis of Meynert, that project to the small blood vessels in the cortex have a vasodilator function (Roman and Kalaria, 2006; Sato et al., 2002). The basolateral cortical pathway that involves basal forebrain neurons located in the nucleus basalis and...
adjoining substantia innominata is partly in connection with cortical microvessel relaxation. The results described here provide indirect support for this hypothesis. Consistent with cognitive improvement, preservation in the neuronal ERK-CREB-BDNF pathway is accompanied with the regulation of local microvascular endothelial cells by limiting ACh release from cerebral cholinergic fibers. These observations indicate that pinocembrin may exert its effects through sustaining the NVU function by promoting neuron function.

In conclusion, our study demonstrates the therapeutic value of pinocembrin against Aβ-induced toxicity. Although additional investigation is required, the present findings provide preclinical evidence that oral pinocembrin may ameliorate AD-associated deficits through inhibiting the cerebral RAGE-dependent signaling pathway and regulating NVU coupling transduction. Therefore, pinocembrin appears to be a very promising medication for the prevention and/or therapy of AD.

Disclosure statement

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81102830), Peking Union Medical College Youth Fundamental Project (2012J20), the Major Scientific and Technological Special Project for “Significant New Drugs Creation” (2013ZX09J1302-05C), Specialized Research Fund for the Doctoral Program of Higher Education (No. 20111106120023), and Beijing New-star Plan of Science and Technology (xx2013065).

The authors thank Prof Song Wu, the Department of New Drug Development, Institute of Materia Medica, Chinese Academy of Medical Sciences for the provision of pinocembrin.

The authors thank Prof Xin Wang, Principal Investigator of Molecular Cardiology Group, Faculty of Life Sciences, the University of Manchester, and Prof Humphrey Rang, Head of the Department of Pharmacology, University College London, for checking the manuscript and improving the language.

References


1284 R. Liu et al. / Neurobiology of Aging 35 (2014) 1275–1285


