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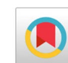

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Neuroprotective effects of paeoniflorin against neuronal oxidative stress and neuroinflammation induced by lipopolysaccharide in mice

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Abstract Oxidative stress and neuroinflammation play important roles in the pathogenesis of Alzheimer's disease (AD). This study investigated the protective effects of paeoniflorin (PF) against neuronal oxidative stress and neuroinflammation in lipopolysaccharide (LPS)-induced mice. The brains of LPS-injected control group showed significantly increased neuroinflammation by activating the nuclear factor kappa B (NF-κB) pathway and increasing inflammatory mediators. However, administration of PF significantly attenuated oxidative stress by inhibiting lipid peroxidation, nitric oxide levels, and reactive oxygen species production in the brain; PF at doses of 5 and 10 mg/kg/day downregulated the expression of NF-κB pathway-related proteins and significantly decreased inflammatory mediators including inducible nitric oxide synthase and cyclooxygenase-2. Moreover, the levels of brain-derived neurotrophic factor and its receptor, tropomycin receptor kinase B, were significantly increased in PF-treated mice. Furthermore, acetylcholinesterase activity and the ration of B-cell lymphoma 2 (Bcl-2)/Bcl-2 associated X were significantly reduced by PF in the brains of LPS-induced mice, resulting in the inhibition of cholinergic dysfunction and neuronal apoptosis. Thus, we can conclude that administration of PF to mice prevents the development of LPS-induced AD pathology through the inhibition of neuronal oxidative stress and neuroinflammation, suggesting that PF has a therapeutic potential for AD.

Keywords Alzheimer’s disease · Lipopolysaccharide · Neuroinflammation · Oxidative stress · Paeoniflorin

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Introduction

With an increase in the aging population, Alzheimer’s disease (AD) is increasingly becoming a severe disabling disease among the elderly [1]. Although the underlying mechanism of AD is unclear, neuronal oxidative stress and neuroinflammation are thought to play a critical role in the pathogenesis and exacerbation of AD [2]. Overproduction of reactive oxygen species (ROS) and reactive nitrogen species leads to oxidative stress in the brain [3,4]. Oxidative stress affects lipid peroxidation, modification of proteins, and oxidation of DNA and RNA in the brain [4]. In addition, an increase in inflammatory mediators and cytokines by activation of the nuclear factor kappa B (NF-κB) pathway causes neuronal loss and synaptic damage in the central nervous system [5,6]. Furthermore, an inflammatory response of the brain induces cholinergic dysfunction by upregulating acetylcholinesterase (AChE) activity and inhibition of the brain-derived neurotrophic factor (BDNF) levels [7,8]. Neuroinflammation, in turn, leads to memory loss and cognitive impairment of the brain, resulting in the development of AD [9].

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria [10]. LPS is known to increase ROS production, leading to oxidative stress in the brain [11]. LPS activates the NF-κB pathway by binding to toll-like receptor-4 (TLR4), resulting in the release of inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [12,13]. In addition, previous studies have reported that injection of LPS increases cholinergic

dysfunction and decreases BDNF expression [14,15]. Therefore, LPS has been widely used to develop AD models in many studies.

Paeoniflorin (PF), the main bioactive compound extracted from *Paeonia lactiflora*, has been found to have great potential in the treatment of inflammatory diseases [16]. Previously, several studies have reported the neuroprotective effects of PF [17-20]. For example, PF decreased $A\beta_{25-35}$-induced neurotoxicity by inhibiting ROS production in SH-SY5Y neuronal cells [17]. In addition, treatment with PF significantly inhibited pro-inflammatory cytokines and increased antioxidant enzymes in an Aβ-induced AD mouse model [18,19]. However, the protective effects and mechanisms of PF against neuronal damage induced by LPS in mice have not been investigated till date. Therefore, in this study, we investigated the protective effects and mechanisms of PF on neuronal oxidative stress and neuroinflammation in the brains of LPS-induced mice.

Materials and Methods

Reagents

The PF ($C_{23}H_{28}O_{11}$, molecular weight: 480.46, purity: ≥98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and its chemical structure as shown in Fig. 1. The LPS, donepezil, Griess reagent, dichlorofluorescein diacetate (DCF-DA), thiobarbituric acid (TBA), and malondialdehyde (MDA) were purchased from Sigma Chemical Co. NaCl was obtained from LPS solution (Seoul, Republic of Korea). Trichloroacetic acid (TCA) was purchased from Kanto Chemical Co. (Tokyo, Japan). *n*-Butanol was purchased from Duksan Co. (Ansan, Republic of Korea). Pyridine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $NaNO_2$ was purchased from Junsei Chemical Co. (Tokyo, Japan).

Animals and experimental schedule

Male ICR mice (23–29 g, five weeks old) were obtained from Orient Inc. (Seongnam, Republic of Korea). The mice were kept in plastic cages and provided a free diet and water. The housing environment was maintained in a 12-h light-dark cycle laboratory, with standard humidity (50±10%) and temperature (22±2 °C). All experimental procedures were performed by strictly following established animal experimental guidelines and were approved by the Institutional Animal Care and Use Committee of Pusan National University (Approval No.: PNU-2019-2144). The mice were randomly divided into five groups, with eight mice in each group (n =8). The initial body weights of the mice among all the groups were not significantly different. The five groups were treated as follows: (1) Normal: 0.9% NaCl intraperitoneal (*i.p.*) injection + 0.9% NaCl injection (*i.p.*); (2) Control: 5 mg/kg LPS injection (*i.p.*) + 0.9% NaCl injection (*i.p.*); (3) PF5: 5 mg/kg/day LPS injection (*i.p.*) + 5 mg/kg/day of PF injection (*i.p.*); (4) PF10: 5 mg/kg/day LPS injection (*i.p.*) + 10 mg/kg/day PF injection (*i.p.*); (5) donepezil: 5 mg/kg/day LPS injection (*i.p.*) + donepezil 5 mg/kg/day injection (*i.p.*). PF, donepezil, and LPS were dissolved in 0.9% NaCl. PF was injected for three weeks and LPS injected for one week, following one week of acclimatization. Donepezil is a drug used widely for the treatment of AD and was used as a positive control.

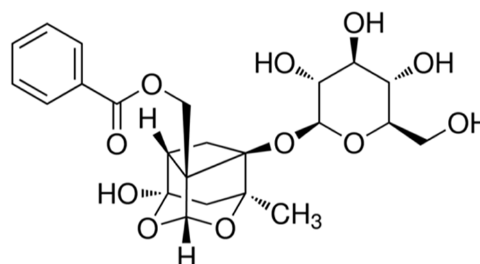

Fig. 1 Chemical structure of paeoniflorin

Measurement of lipid peroxidation

The experimental protocol for lipid peroxidation was established according to the method described by Ohkawa et al. (1979) [21]. Mice were anesthetized using dry ice, and their brain tissues were collected and immediately placed on ice. After homogenizing each tissue with 0.9% NaCl, it was mixed with 20% acetic acid, 46 mM TBA, and 920 mM TCA, and then incubated at 95 °C for 20 min. Next, n-Butanol and pyridine were added and the mixture centrifuged at 3,000 rpm for 20 min. Absorbance of the supernatant was measured at 540 nm using a microplate reader (Thermo Fisher Scientific, Vantaa, Finland), and lipid peroxidation levels were calculated using the MDA standard curve.

Measurement of nitric oxide (NO) levels

Generation of NO was determined using the method of Schmidt et al. (1992) [22]. Each brain tissue was homogenized with 0.9% NaCl, and supernatant of the homogenized tissue was mixed with distilled water. An equal amount of Griess reagent was then added to the mixture. Absorbance was read at 540 nm using a microplate reader, and the NO levels were calculated using the $NaNO_2$ standard curve.

Measurement of reactive oxygen species (ROS) levels

ROS inhibitory activity of PF was measured using the DCF-DA assay [23]. Each brain tissue was homogenized with phosphate buffered saline (PBS), and 50 mM PBS (pH 7.4) and 12.5 mM DCF-DA solution were then added. After 20 min, fluorescence was read at an excitation wavelength of 480 nm and emission wavelength of 530 nm using a fluorescence spectrophotometer (FLUOsatar OPTIMA, BMG Labtech, Ortenberg, Germany).

Measurement of protein expression

Protein expression in the brain was determined by western

blotting. Each brain tissue was homogenized with a lysis buffer containing a protease inhibitor cocktail. Proteins were quantitated using the dye-binding method [24]. They were then separated using a 10–13% sodium dodecyl sulfate polyacrylamide gel and later transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated in 5% skim milk for 60 min, washed three times with PBS-Tween® 20 (PBS-T), and then incubated at 4 °C overnight with primary antibodies. The primary antibodies used were as follows: TLR4, β-actin, iNOS (1:1000; Cell Signaling, Beverly, MA, USA); NF-κB p65, BDNF, tropomyosin receptor kinase B (TrkB) (1:1000; Abcam, Cambridge, MA, USA); and B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X (Bax), AChE, COX-2, inhibitor κB (IκB)-α (1:1000; Santa Cruz Biotechnology Inc., Dallas, TX, USA). Next, the membrane was incubated with an appropriate secondary antibody for 1 h (1:1000; Cell Signaling, Beverly, MA, USA). Protein bands were detected using a Davinci-chemiluminescent imaging system (CoreBio, Seoul, Republic of Korea).

Statistical analysis

All results were calculated as mean ± standard deviation (SD). Statistical Product and Service Solutions (SPSS) software was used for statistical analysis. One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to analyze the data. $p < 0.05$ was considered to be statistically significant.

Results

Effects of PF on neuronal oxidative stress in LPS-induced mice

To confirm the level of oxidative stress induced by LPS, we investigated the MDA content in the brains of mice (Fig. 2A). The brains of LPS-injected control group exhibited significantly increased MDA levels (105.09 nmol/mg) than brains of normal group (86.19 nmol/mg). However, in the PF-injected group, the MDA levels in PF5 and PF10 groups were 94.6 and 79.73 nmol/mg protein, respectively, indicating that PF could reduce MDA level in the brain.

The effect of PF on NO production in brain tissues of LPS-induced mice is shown in Fig. 2B. The results of NO production in the brain tissues confirmed that LPS causes excessive production of NO. The NO content in the normal group was 16.72 μmol/L/mg, while that in LPS-injected group was 24.43 μmol/L/mg. In contrast, the PF5 and PF10 groups showed 16.29 μmol/L/mg and 16.07 μmol/L/mg, respectively, indicating that PF has an inhibitory effect on NO production in brain tissue.

The protective effect of PF on ROS production in the brains of LPS-injected mice is shown in Fig. 2C. ROS production in the brains of LPS-induced control group was significantly higher than that in brains of normal group. However, PF5 and PF10 groups showed significantly decreased ROS levels than control group. These results suggest the anti-oxidative effect of PF can be attributed to attenuation of MDA, NO, and ROS production in the brains of LPS-induced mice.

Effects of PF on neuroinflammation in LPS-induced mice

To investigate the effect of PF on LPS-induced neuroinflammation of the brain, the expression of inflammation-related proteins such as TLR4, NF-κB-p65, and p-IκB-α was assessed by western blotting. As shown in Fig. 3, the LPS-injected control group showed higher protein expression of TLR4 and NF-κB-p65, as well as a higher ratio of p-IκB-α/ IκB-α. In contrast, the LPS-non-injected normal group showed lower expression of TLR4 and NF-κB-p65, and a lower ratio of p-IκB-α/ IκB-α than control group. However, injection of PF at doses of 5 and 10 mg/kg/day significantly alleviated the expression of LPS-induced TLR4, NF-κB-p65, and the ratio of p-IκB-α/ IκB-α. To further investigate the effects of PF on inflammatory mediators in the brains of LPS-induced mice, we investigated the protein expression of iNOS and COX-2 (Fig. 4). The expression of iNOS and COX-2 increased significantly in the LPS-injected group than in normal group. In

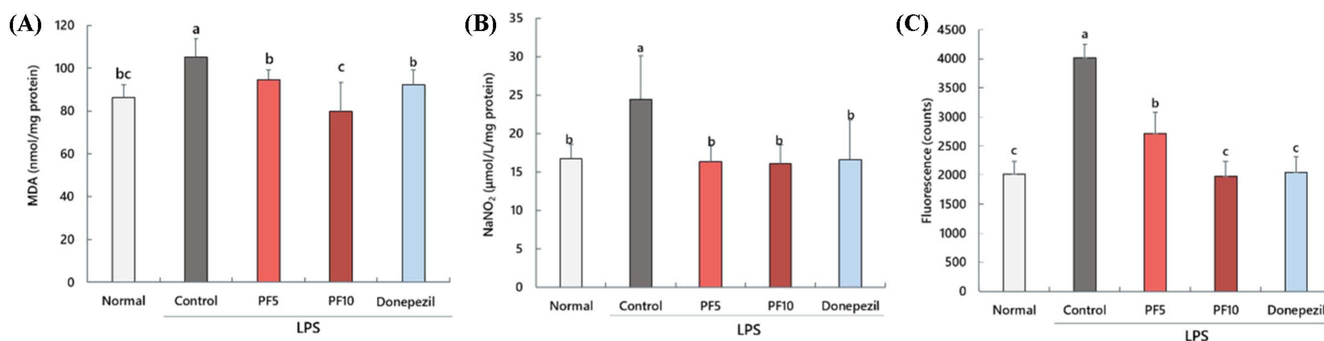

Fig. 2 Effects of paeoniflorin (PF) on lipid peroxidation (A), nitric oxide (NO) generation (B), and reactive oxygen species (ROS) production (C) in the brain of LPS-injected mice. Normal = 0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-c]Means with different letters are significantly different ($p < 0.05$) determined by Duncan's multiple range test

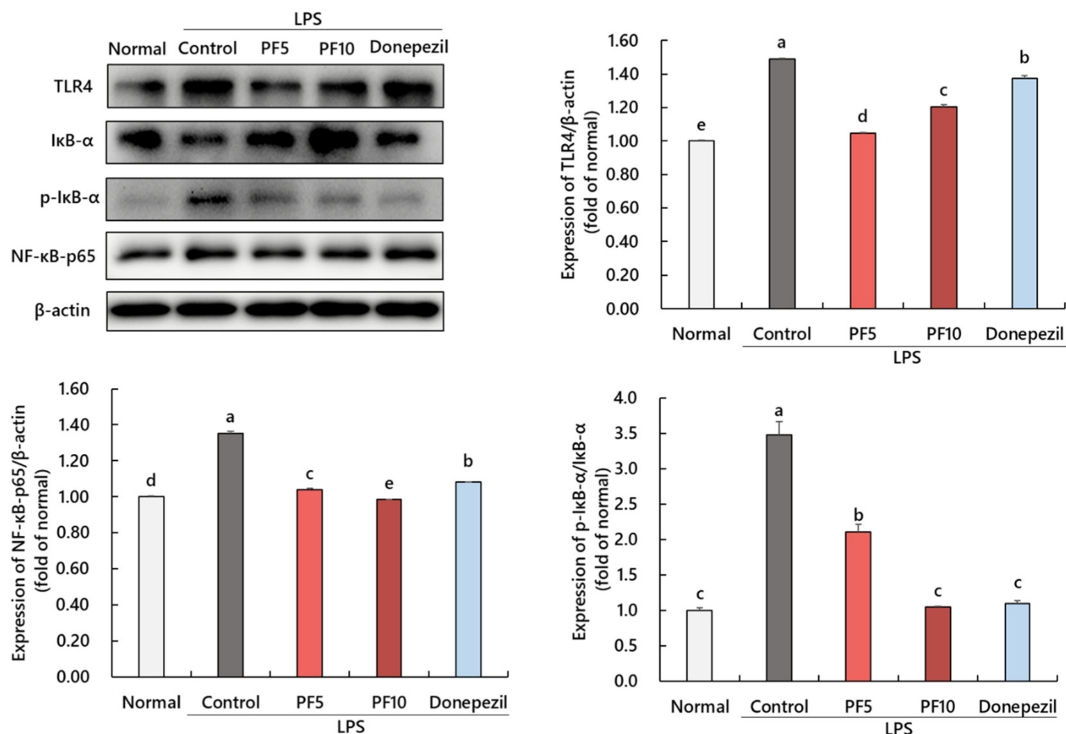

Fig. 3 Effects of paeoniflorin (PF) on protein expression levels of NF-kB pathway in the brain of LPS-injected mice. Normal =0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-e]Means with different letters are significantly different (p <0.05) determined by Duncan's multiple range test

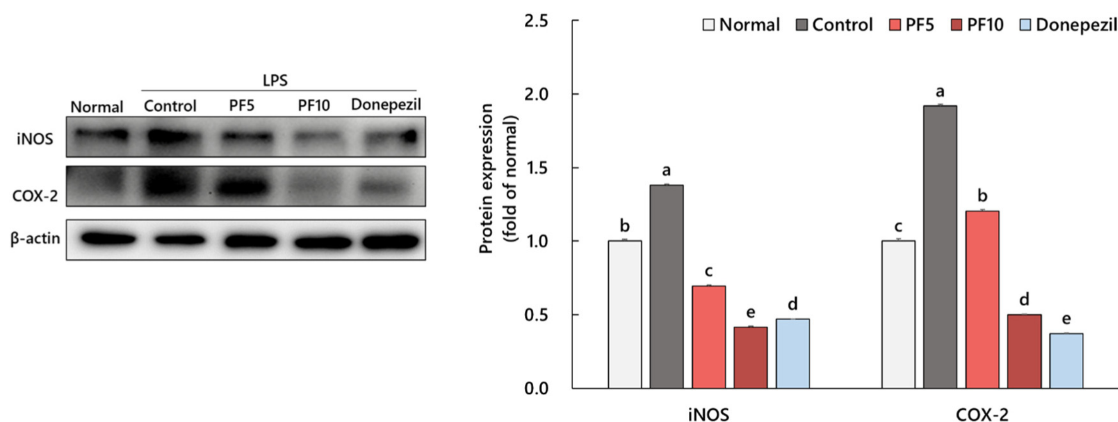

Fig. 4 Effects of paeoniflorin (PF) on protein expression levels of iNOS and COX-2 in the brain of LPS-injected mice. Normal = 0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-e]Means with different letters are significantly different (p <0.05) determined by Duncan's multiple range test

contrast, the expression of iNOS and COX-2 was significantly downregulated in PF5 and PF10 groups than in LPS-injected control group. These results indicate that PF modulates the NF-κB pathway and inflammatory mediators related to LPS-induced neuroinflammation of the brain.

Effects of PF on cholinergic dysfunction in LPS-induced mice

To assess whether PF has an effect on AChE activity in LPS-induced mouse models, AChE protein expression was assessed. As shown in Fig. 5, the level of AChE in the LPS-injected control group was significantly increased compared to that in the normal

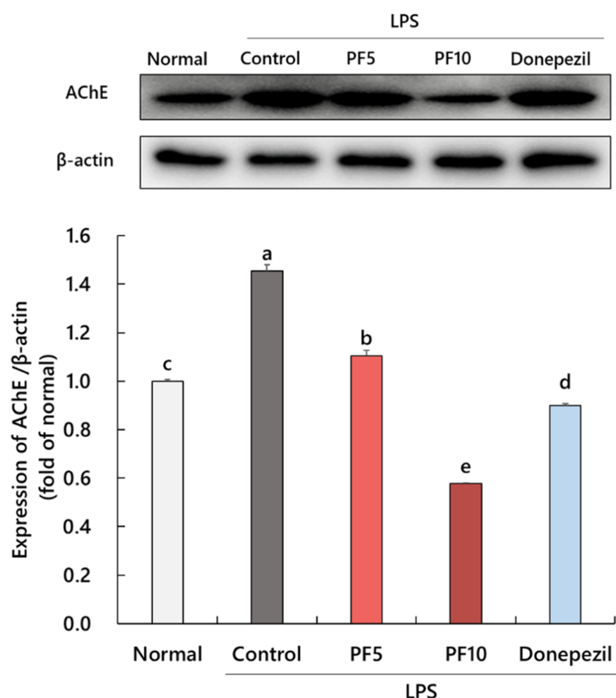

Fig. 5 Effects of paeoniflorin (PF) on protein expression levels of AChE in the brain of LPS-injected mice. Normal = 0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-e]Means with different letters are significantly different (p <0.05) determined by Duncan's multiple range test

group. However, administration of PF at 5 and 10 mg/kg/day significantly reduced AChE expression. In particular, administration of PF at 10 mg/kg/day (PF10) inhibited the AChE activity to a greater extent than that at 5 mg/kg/day (PF5), indicating that PF contributes to a reduction of AChE activity in the brains of LPS-induced mice.

Effects of PF on the expression of a neurotrophic factor in LPS-induced mice

To study whether PF exerts neuronal protection by activating the BDNF and TrkB signaling pathways, we evaluated the expression of BDNF and TrkB (Fig. 6). Compared with those in the brains of normal group, the levels of BDNF and TrkB were significantly reduced in the brains of LPS-injected control group. However, the PF-treated groups showed significantly improved BDNF and TrkB levels. In particular, the PF10 group displayed better protection against BDNF and TrkB signaling. These results demonstrate the neuroprotective effect of PF through the regulation of BDNF/TrkB signaling in the brains of LPS-induced mice.

Effects of PF on neuronal apoptosis in LPS-induced mice

To investigate the neuroprotective effect of PF on LPS-induced apoptosis, the ratio of expression of Bax to Bcl-2 was evaluated by western blotting. As shown in Fig. 7, the ratio of expression of Bax/Bcl-2 was higher in the LPS-treated control group than in normal group. However, treatment with PF at concentrations of 5 and 10 mg/kg/day resulted in increased expression of Bcl-2 and decreased expression of Bax protein, resulting in a decreased Bax/Bcl-2 ratio. These results suggest that PF has a beneficial effect on neuronal apoptosis in LPS-induced brains of mice.

Discussion

Treatment of LPS is widely known to increase inflammation, leading to neurodegenerative disease [25]. Previous studies have shown that high doses (5-10 mg/kg) of *i.p.* injected LPS in mice induced neurodegenerative diseases by increasing neuro-inflammation, neuronal oxidative stress, and synaptic dysfunction [26,27]. PF is an active compound of *Paeonia lactiflora*, it has

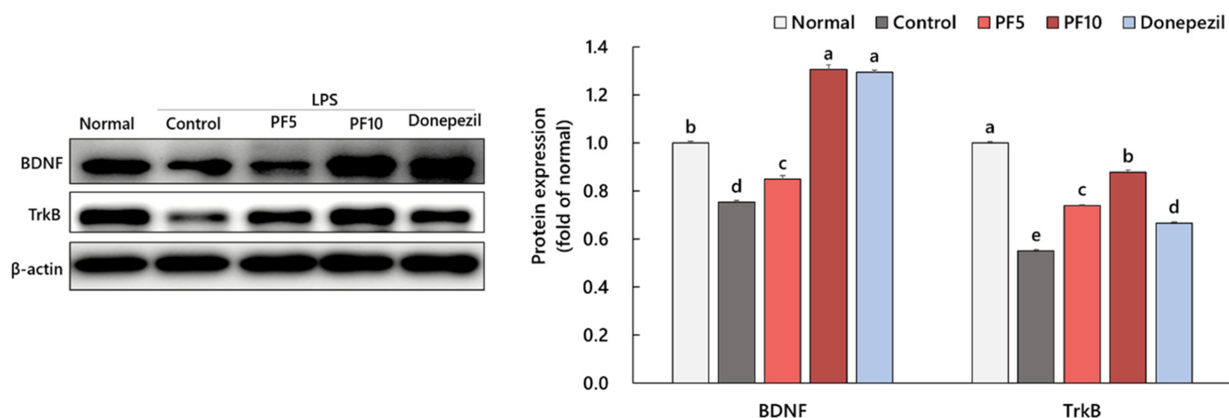

Fig. 6 Effects of paeoniflorin (PF) on protein expression levels of BDNF and TrkB in the brain of LPS-injected mice. Normal = 0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-e]Means with different letters are significantly different (p <0.05) determined by Duncan's multiple range test

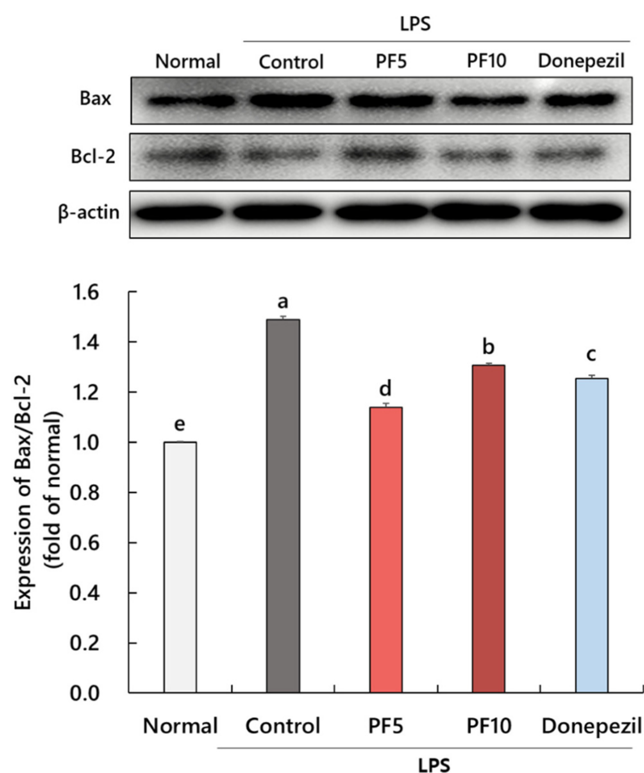

Fig. 7 Effects of paeoniflorin (PF) on protein expression levels of Bax and Bcl-2 in the brain of LPS-injected mice. Normal = 0.9% NaCl injection + 0.9% NaCl; Control = 5 mg/kg/day LPS + 0.9% NaCl; PF5 = 5 mg/kg/day LPS + 5 mg/kg/day PF; PF10 = 5 mg/kg/day LPS + 10 mg/kg/day PF; donepezil = 5 mg/kg/day LPS + donepezil 5 mg/kg/day. Data represent means ± SD (n = 8/group). [a-e]Means with different letters are significantly different (p <0.05) determined by Duncan's multiple range test

been several neuroprotective effects. The treatment of PF at a dose of 5-30 mg/kg/day attenuated cognitive dysfunction in Aβ-induced AD mouse model [17,18]. In addition, the maximum tolerance dose of PF solution to mice is 5 g/kg [28]. Therefore, this study evaluated the underlying neuroprotective effects and mechanisms of PF at doses of 5 and 10 mg/kg/day in mice *i.p.* injected with LPS.

Injection of LPS stimulates ROS generation and oxidative damage [29]. Over-production of ROS is intimately associated with neuroinflammation in brains of individuals affected by AD [30]. It has also been reported that LPS causes a large amount of ROS accumulation, as well as extracellular release of NO and lipid peroxidation in cells, which promotes the development of AD [31]. Studies have shown that inflammation caused by LPS can cause mitochondrial damage and lead to the production of ROS [32,33]. Lipid peroxidation is one of the main causes of oxidative stress. Lipids present on the cell membrane are damaged by free radicals and toxic lipid aldehydes, such as MDA [34]. Studies have shown that the level of lipid peroxidation is higher in an AD brain than in a healthy aging brain [35, 36]. NO damages cells due to its reactive oxidative properties via neurotoxic mechanisms. The presence of stimuli that induce excessive NO production may cause neuronal damage [37]. A pathological feature of AD is oxidative damage induced by excessive NO production [38]. LPS stimulates microglia to mediate the production of iNOS, which is a catalytic enzyme that leads to NO synthesis [39]. In our study, LPS caused an increase in ROS, NO, and lipid peroxidation levels in the mouse brain; these results were similar to other studies, which showed that LPS activated microglia and induced ROS production [40,41]. However, PF injection significantly reduced the levels of ROS, NO, and lipid peroxidation in the brain. These findings indicate that PF exhibits an antioxidative ability against LPS-induced ROS levels. A recent study revealed that PF could significantly reduce lipid peroxidation in the brain of a subarachnoid hemorrhage model [20]. These results confirmed that administration of PF can protect against LPS-induced oxidative stress caused by lipid peroxidation in the AD mouse model.

Inflammatory reactions induced by LPS have been reported to be related to the pathogenesis of AD [42]. LPS stimulates activation of the NF-κB pathway, thereby releasing inflammatory factors and causing neuronal damage [43]. The NF-κB signaling pathway contributes to the pathogenesis of AD [44]. TLR4 is a pattern recognition receptor that recognizes LPS in microglia of the brain and participates in the release of inflammatory mediators by activating the NF-κB signaling pathway [45]. The activation of NF-κB is triggered by phosphorylation of IκB and subsequent degradation, which results in the translocation of free NF-κB-p65 to the nucleus, thereby promoting the expression of proinflammatory mediators and cytokines [46]. LPS leads to rapid transcription and release of proinflammatory mediators such as iNOS and COX-2 [47]. Proinflammatory enzymes lead to irreversible chronic inflammation, causing oxidative stress and increased NO levels, which are harmful and result in neurodegenerative diseases [48, 49]. In the present study, TLR4 was significantly increased in the brains of mice after LPS injection, the levels of IκB were reduced and those of p-IκB and NF-κB-p65 increased. However, injection of PF significantly reduced the expression of TLR4, p-IκB, and NF-κB-p65, demonstrating the anti-inflammatory effect of PF in LPS-induced mice by controlling the TLR4/NF-κB signaling pathway. In addition, our results indicated that the levels of iNOS and COX-2 in the brains of mice injected with LPS were increased. In contrast, PF injection significantly attenuated the expression of iNOS and COX-2. These findings indicated that PF treatment could effectively prevent neuroinflammation by attenuating NF-kB signaling-mediated inflammatory mediators.

AChE is an enzyme responsible for hydrolysis of the neurotransmitter acetylcholine and has been found to have increased activity in AD [50]. It has been reported that acetylcholine can inhibit the production of pro-inflammatory cytokines induced by LPS. The levels of acetylcholine are continuously regulated by AChE, which rapidly degrade

acetylcholine in the regions of the brain [51]. In the treatment of AD, many studies have focused on AChE inhibitors such as donepezil [52]. In addition, several studies have demonstrated that *i.p.* injection of LPS induced cholinergic dysfunction by elevation of AChE activity in the brain of mice [53,54]. The peripheral injection such as *i.p.* of LPS increased neuroinflammation by over-production of inflammatory cytokines, that leading activation of AChE in the brain of mice [53,55]. In addition, *i.p.* injection of LPS in mice decrease alpha7 nicotinic acetylcholine receptor density in the brain, resulting that cholinergic dysfunction [56]. Our data showed that AChE activity in the brains of LPS-injected mice group was increased compared to that in the brains of normal group. However, PF significantly attenuated the AChE activity. These results suggest that PF ameliorates learning and memory loss caused by LPS.

Growing evidence has suggested that neurotrophins such as BDNF are involved in the pathogenesis of AD [57]. It has been reported that the expression of BDNF is significantly reduced in the brains of AD patients [58]. BDNF/TrkB signaling in the hippocampus plays a vital role in learning and memory [59]. Some studies have demonstrated that LPS can significantly reduce the expression of BDNF/TrkB in the hippocampus; this result is consistent with our results [29,60]. Moreover, in our study, it was observed that injection of PF could significantly prevent the decrease in LPS-induced BDNF levels. These results indicate that the injection of PF could restore the impaired memory caused by LPS.

LPS-induced inflammatory response damages mitochondria and promotes apoptosis, which plays an essential role in the pathogenesis of AD [61]. The anti-apoptotic protein Bcl-2 and pre-apoptotic protein Bax are critical factors in apoptosis [62]. In our study, estimation of Bcl-2 and Bax protein expression revealed that LPS decreased Bcl-2 expression and upregulated Bax expression, while PF reduced the ratio of Bax/Bcl-2. This result suggests that PF may contribute to the upregulation of survival pathways in neuronal cells. Thus, we confirmed that LPS contributes to the pathological development of AD in mice, and that PF could reverse LPS-induced neuronal oxidative stress and neuroinflammation. PF reduced LPS-induced oxidative stress by reducing MDA, NO, and ROS production, and also inhibited LPS-induced neuroinflammation by inhibiting the NF-κB signaling pathway in the brain. In addition, PF showed neuroprotective effects by preventing the production of Aβ, reducing the activity of AChE, enhancing the levels of BDNF/TrkB, and reversing apoptosis.

In conclusion, this study investigated the protective effects of PF against neuronal oxidative stress and neuroinflammation in the brains of LPS-induced mice. These results suggest that PF can be used as a natural medicine for the treatment of AD.

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