Purple sweet potato color suppresses lipopolysaccharide-induced acute inflammatory response in mouse brain

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1. Introduction

Anthocyanins, which are natural plant pigments from the flavonoid family, widely exist in most flowers and fruits of the high plants. Emerging evidence reveals that anthocyanins possess several pharmacological properties such as anti-oxidative (Shih et al., 2007), anti-inflammatory (Karlsen et al., 2007) and anti-neurodegenerative (Joseph et al., 2003). Recently, much attention is being paid to purple sweet potato due to its unique color, nutrition and health benefit (Lila, 2004; Mano et al., 2007). Purple sweet potato has been regarded as a good source of stable anthocyanins and purple sweet potato color (PSPC) has been demonstrated to exhibit multiple physiological functions including antimutagenicity (Yoshimoto et al., 2001), antihyperglycemic effect (Matsui et al., 2002) and free radical-scavenging activity (Kano et al., 2005).

Inflammation has been proposed as a critical factor of the pathogenesis for many neurological diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and multiple sclerosis (MS). Systemic administration of Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, may cause inflammatory responses in the brain. Many reports have demonstrated that LPS may lead to a collection of behavioral changes (Dunn and Swiergiel, 2005) and produce alterations in cognitive processes, including learning and memory (Sparkman et al., 2005). The acute administration of LPS can induce expression of a large spectrum of proinflammatory molecules. The two key inducible enzymes of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthases (iNOS), which are barely detectable in healthy brain, can be induced by LPS and play fundamental roles in the process of inflammation. Increased expression of COX-2 and iNOS has been demonstrated to correlate with many inflammatory brain disorders (Choi et al., 2003). Furthermore, peripheral infection can induce a large release of cytokines in the brain including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These inflammatory cytokines may mediate sickness behavior syndrome (Konsman et al., 2002) and affect the functions of the brain (Pollmacher et al., 2002). Studies have also shown the involvement of cytokines in the chronic neurodegenerative diseases such as MS and AD (Holmes et al., 2003). In addition, many papers have suggested that the production of several proinflammatory molecules is associated with the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB). Both MAPKs and NF-κB can be stimulated

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by LPS and are tightly involved in the pathological conditions in the brain (Zhu et al., 2001; Munhoz et al., 2006). Collectively, suppressing the release of proinflammatory mediators such as iNOS, COX-2, IL-1β, IL-6, and TNF-α is an important approach to prevent inflammation-associated brain diseases.

As previously described, many reports indicated that PSPC possesses a variety of biological activities. However, little work has been done to explore the effect of PSPC on neuroinflammation stimulated by LPS. This study was to investigate whether PSPC protected mouse brain from LPS-induced injury and to provide novel insight into the mechanism of action.

2. Materials and methods

2.1. Subjects
Male C57BL/6 mice (22 ± 1 g) were purchased from the Shanghai Slac Laboratory Animal Ltd. (Shanghai, China). Before experiments, the mice had free access to food and water and were kept under constant conditions of temperature (23 ± 1°C) and humidity (60%). Three mice were housed per cage on a 12 h light/12 h dark schedule.

Animals, and were approved by the respective university committees for animal experiments, and were performed in compliance with Chinese legislation on the use and care of laboratory animals, and were approved by the respective university committees for animal experiments.

2.2. Behavioral tests

2.2.1. Open field assessment
The first cohort of animals (n = 7) was used to examine the effect of PSPC on LPS-induced behavioral changes in open field tests. Mice received either an intraperitoneal injection of LPS (sigma L-2880 from Escherichia coli 055:B5) (0.5 mg/kg) or vehicle 2 h before open field testing. The dose and time chosen are those at which LPS had produced the most reliable sickness behavior studies involving behavioral measures in LPS-treated rodents (Lacosta et al., 1999; Cunningham et al., 2009; Painsipp et al., 2008). The open field, which was located in a dimly lit room, consisted of a square, opaque acrylic container (60 cm × 60 cm × 40 cm) which was painted white to offer best contrast to the black mice. A video camera fixed 1 m above the arena tracked the animals’ movements. A computerised tracking system analyzed the images and measured speed and distance of movements. Tests were performed in the breeding room from 8:30 to 16:00. Mice were placed in the dimly lit room 1 h before testing to acclimatize to the new environment. The individual mice were placed in the middle of the chamber for each trial. After 1 min adaptation, the behavior of each mouse was recorded for 5 min. Between trials, the mice were returned to their home cage in the same room and the open field was wiped clean with a slightly damp cloth. The number of rearing events, the grooming sessions, the total distance and speed traveled, and the time spent in the central area were assessed.

2.2.2. Passive avoidance performance test: step-through test
The second cohort of animals (n = 7) was used to examine the effect of PSPC on LPS-induced cognitive impairment in passive avoidance performance test. The step-through test was performed as described previously (Lu et al., 2006). The step-through passive avoidance apparatus consisted of an illuminated chamber (11.5 cm × 9.5 cm × 11 cm) attached to a darkened chamber (23.5 cm × 9.5 cm × 11 cm) containing a metal floor that could deliver footshocks. The two compartments were separated by a guillotine door. The illuminated chamber was lit with a 25 W lamp. The test was conducted on 2 consecutive days at the same time of day. On the first day (learning trial) mice were placed in the dimly lit room containing the apparatus 1 h before training to acclimatize to the new environment. Each mouse was then placed individually into the illuminated chamber, facing away from the door to the dark chamber, and allowed to acclimatize for 1 min. While the mouse was observed to turn its body fully away from the dark chamber, the door was raised, while the mouse next turned fully toward the dark chamber, the timer was started. An initial time measure was from the time that the mouse faced the opened darkened chamber to the time that the mouse fully entered, with all four paws, the dark chamber. As soon as the mouse entered the dark chamber the door was slid back into place, triggering a mild footshock (0.3 mA, 50 Hz, 5 s). Just after the learning trial, mice were administered LPS (0.5 mg/kg, i.p.) or saline and then immediately returned to their home cages. The retention test was conducted 24 h later with the mouse again being placed in the illuminated chamber and subjected to the same protocol described above in the absence of footshock. The upper time limit was set at 5 min.

2.3. Protein and Western blot analysis

2.3.1. Preparation of tissue samples
The last cohort of animals (n = 3) was subjected to protein assay. Two groups that received PSPC at a dose of 350 mg/kg (kg day) or 700 mg/kg (kg day) and LPS group were given with a single injection of LPS (80 mg/kg, i.p.), and the other three groups with a single injection of saline (0.9%) only. Mice were killed 6 h later and the whole brains were immediately collected for experiment or stored at −70°C. Tissues were homogenized in ice-cold lysis buffer (TBS, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 10 μg/ml Apoprotein, 10 μg/ml Leupeptin, 1 mM PMSF, 10 mM b-glycerophosphate, 1 mM Na3VO4, 1 mM NaF). Homogenates were centrifuged at 10000 g for 10 min at 4°C. The supernatants were collected and centrifuged again the final supernatants were collected. Nuclear and cytoplasmic extracts for Western blotting were obtained by using a nuclear/cyttoplasmic isolation kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein levels were determined using the BCA assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Samples were separated by denaturing SDS-PAGE and transferred to a PVDF membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) by electrophoretic transfer (Bio-Rad Laboratories, Inc., USA). The membrane was pre-blocked with 5% BSA and incubated overnight with the primary antibody (in TBST, with 5% BSA). Each membrane was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked antibodies (Santa Cruz Biotechnology, CA and Cell Signaling Technology, Beverly, MA, respectively). Quantification of bands was performed with Scion Image analysis software (Scion Corp, Frederick, MD, USA). To prove equal loading, the blots were analyzed for β-actin or β-tubulin expression. Each density was normalized using each corresponding β-actin or β-tubulin density as an internal control and averaged from three samples. We set the density of vehicle control as 1.0 for comparison with the other groups.

2.4. Statistic analysis

All statistical analyses were performed using the SPSS software, version 11.5. Western blotting data were analyzed with Newman–Keuls or Tukey’s HSD post hoc test. Data were expressed as mean ± S.E.M. Statistical significance was set at p < 0.05.

3. Results

3.1. Effect of PSPC on the behavior of LPS-treated mice

3.1.1. Open field
Open field activity was used to evaluate locomotor activity and exploratory behavior in mice with various treatments. Data presented in Fig. 1 indicate that the group of mice received a single injection of LPS at a dose of 0.5 mg/kg (LPS model) significantly reduced their motor activities [distance: F(5,36) = 8.332, p < 0.001; speed: F(5,36) = 7.51, p < 0.001; rearing/leaning F(5,36) = 5.78, p < 0.01] and grooming activity [F(5,36) = 7.31, p < 0.01] as compared with the vehicle control group with injection of saline (0.9%) only. LPS-treated mice spent more time in the center than near the walls of the arena, as compared to the control group [F(5,36) = 0.56, p > 0.05]. These results suggest the impairment of the motor and exploration activities in mice treated with LPS. When two groups of LPS-induced mice were given 350 and 700 mg/ (kg day) of PSPC, respectively, for 4 weeks before injection, the distance (LPS + PSPC350: p > 0.01; LPS + PSPC700: p < 0.001) and the speed (LPS + PSPC350: p < 0.001; LPS + PSPC700: p < 0.001) traveled, their activities of rearing/leaning (LPS + PSPC350: p < 0.05; LPS + PSPC700: p < 0.01) and grooming (LPS + PSPC350: p > 0.05; LPS + PSPC700: p < 0.05) were significantly increased. But the difference of the time in the central area did not reach statistically significant level (p > 0.05).

No visible difference of the motor and exploration activities could be seen from the mice treated with PSPC only as compared with the controls.

3.1.2. Step-through test
In the acquisition trial, the step-through latencies did not differ among the six groups [F(5,36) = 0.184]. The step-through
Latency in the 24 h-retention trial was significantly decreased \( F(5,36) = 19.96, p < 0.001 \) in the LPS-treated mice, as compared to the vehicle control. In the 24 h-retention trial the latency of the LPS-treated mice received daily 350 or 700 mg/(kg day) PSPC for 4 weeks was significantly increased \( (p < 0.001) \) vs. LPS model (Fig. 2).

No visible difference of the latency could be seen from the mice treated with PSPC only as compared with the controls.

### 3.2. Effect of PSPC on the expression of COX-2 and iNOS proteins in LPS-treated mouse brain

COX-2 and iNOS were extensively studied in terms of their involvement in the inflammation. As shown in Fig. 3, PSPC could significantly suppress LPS-induced upregulation of COX-2 and iNOS. As compared with the vehicle controls, the levels of COX-2 and iNOS were markedly increased in the brains of LPS-treated mice [COX-2: \( F(5,12) = 68.46, p < 0.001 \); iNOS: \( F(5,12) = 115.4, p < 0.001 \)]. However, the upregulation of COX-2 and iNOS expression was largely suppressed in the mice treated with PSPC before a single injection of LPS as compared with the LPS group [PSPC350 + LPS group: COX-2 \((p < 0.001)\), iNOS \((p < 0.001)\); PSPC700 + LPS group: COX-2 \((p < 0.001)\), iNOS \((p < 0.001)\)]. PSPC can almost totally inhibit the upregulation of COX-2 expression at a dose of 700 mg/kg. No marked difference of iNOS expression levels was seen in the brains between the mice treated with PSPC before LPS injection and the controls.

No visible expression changes of COX-2 and iNOS were seen in the brains from the mice treated with PSPC only as compared with the controls.

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Fig. 1. The locomotor and exploratory behavior of LPS-treated mice fed with PSPC at different doses or vehicle \((n = 7)\). All values are expressed as mean \pm S.E.M. Comparison of (A) distance covered, (B) speed traveled, (C) rearing/learning numbers, (D) grooming numbers, and (E) time in center area by all groups \((within 5 \text{ min})\). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) vs. the vehicle control. 

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Fig. 2. The step-through latency of LPS-treated mice fed with PSPC at different doses or vehicle \((n = 7)\). All values are expressed as mean \pm S.E.M. ***\(p < 0.001\) vs. the vehicle control. 

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Fig. 3. The locomotor and exploratory behavior of LPS-treated mice fed with PSPC at different doses or vehicle \((n = 7)\). All values are expressed as mean \pm S.E.M. Comparison of (A) distance covered, (B) speed traveled, (C) rearing/learning numbers, (D) grooming numbers, and (E) time in center area by all groups \((within 5 \text{ min})\). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) vs. the vehicle control.
3.3. Effect of PSPC on LPS-induced IL-1β, IL-6 and TNF-α production in mouse brain

A large part of the response of the brain to LPS has been attributed to the action of cytokines and other acute phase reactants which are involved in the acute and chronic inflammation. The present results showed that PSPC could significantly decrease the upregulation of TNF-α, IL-1β and IL-6 in LPS-treated mouse brain (Fig. 3).

LPS treatment caused significant increase in the protein levels of IL-1β, IL-6 and TNF-α in mouse brains as compared with vehicle controls [IL-1β: \( F(5,12) = 22.78, p < 0.001 \); IL-6: \( F(5,12) = 10.76, p < 0.01 \); TNF-α: \( F(5,12) = 12.44, p < 0.01 \)]. However, the expression of IL-1β, IL-6 and TNF-α in the mice treated with PSPC before LPS injection was significantly reduced as compared with LPS-treated mice [PSPC350 + LPS group: IL-1β (\( p < 0.01 \)), IL-6 (\( p < 0.01 \)), TNF-α (\( p < 0.01 \)); PSPC700 + LPS group: IL-1β (\( p < 0.001 \)), IL-6 (\( p < 0.01 \)), TNF-α (\( p < 0.01 \))]. There was no significant difference with regard to the content of IL-1β, IL-6 and TNF-α between the mice treated with PSPC before LPS injection and the vehicle controls.

No significant difference of levels of IL-1β, IL-6 and TNF-α could be seen in the brain from the mice treated with PSPC only as compared with vehicle controls.
3.4. Effect of PSPC on LPS-induced MAPK and NF-κB activation in mouse brain

A growing body of evidence showed that the activation of MAPK and NF-κB was tightly associated with inflammation. To further investigate the molecular mechanism of suppression of acute inflammation by PSPC in mouse brain, we observed its inhibitory effect on the expression levels of ERK, JNK, p38 (Fig. 5A) and NF-κB p65 (Fig. 5B).

The phosphorylation of ERK, JNK and p38 was increased in mice treated with LPS alone as compared with vehicle controls [ERK: \( F(5,12) = 112.41, p < 0.001 \); JNK: \( F(5,12) = 31.01, p < 0.001 \); p38: \( F(5,12) = 2.13, p > 0.05 \)]. However, PSPC inhibited phosphorylated ERK, JNK and p38 levels in mice stimulated with LPS as compared with LPS group [PSPC350 + LPS group: ERK \( (p < 0.001) \), JNK \( (p < 0.001) \), p38 \( (p > 0.05) \); PSPC700 + LPS group: ERK \( (p < 0.001) \), JNK \( (p < 0.001) \), p38 \( (p > 0.05) \)]. There was no significant difference of phosphorylated levels of ERK, JNK and p38 between the mice treated with PSPC before LPS injection and vehicle controls (Fig. 5C).

No visible phosphorylated expression changes of ERK, JNK and p38 were seen in the brains from the mice treated with PSPC only as compared with the controls. Furthermore, total-ERK, JNK and p38 kinase expressions were unaffected by LPS or by LPS plus PSPC.

As shown in Fig. 5D, NF-κB p65 levels in nuclear fractions were significantly increased in LPS-treated mice as compared with vehicle controls [\( F(5,12) = 17.65, p < 0.001 \)]. Accordingly, NF-κB p65 levels in cytoplasmic fractions were significantly reduced in LPS-treated mice as compared with vehicle controls [\( F(5,12) = 4.46, p < 0.05 \)]. Pretreatment with PSPC prior to LPS strongly inhibited the translocation of NF-κB p65 as compared with vehicle controls (nuclear: \( p < 0.001 \); cytoplasm: \( p < 0.05 \)). No marked difference of nuclear NF-κB p65 levels could be seen in the brains between the mice treated with PSPC before LPS injection and the controls.

4. Discussion

PSPC, a class of naturally occurring anthocyanins used to color food, has been recently reported to exhibit several pharmacological activities. For example, it could repair D-galactose-induced spatial learning and memory impairment (Wu et al., 2008) and suppress the expression of iNOS and COX-2 in D-galactose treated mouse liver (Zhang et al., 2009). In behavioral tests, we found that LPS could induce remarkable learning and memory impairment in mice. Specifically, PSPC-fed mice showed an enhanced active behavioral response to the open field which could be interpreted either as higher motivation to explore or as higher motor activities compared with the LPS-treated mice (Fig. 1). Moreover, PSPC could also reverse...
the LPS-induced memory impairment in the step-through tasks (Fig. 2). Meanwhile, we also found that PSPC could significantly inhibit LPS-induced COX-2 and iNOS expression in mouse brain (Fig. 3). COX-2 and iNOS are thought to play an important role in the pathogenesis of inflammation. Some researches indicate that iNOS has been implicated in neuronal damage and death and demyelination in a number of central nervous system (CNS) diseases (Giri et al., 2002). Studies have also shown in vivo induction of COX-2 after inflammatory stimuli in the brain (Marcheselli and Bazan, 1996). Our findings are interesting from a pharmacological point of view since suppressor of COX-2 and iNOS can be a promising candidate in the treatment of brain disorders.

PSPC is one of the most powerful agents known to stimulate circulating monocytes and tissue macrophages, which lead to the synthesis and release of a variety of proinflammatory cytokines. IL-1β, IL-6 and TNF-α are among the best characterized early response proinflammatory cytokines. Biological effects of these cytokines could influence the progression of injury in the brain. For example, IL-1β is an important mediator of acute brain injury (Allan et al., 2005) and a key regulator of inflammation during the host defense response. IL-1β elevation is associated with many neurodegenerative diseases (Rothwell and Lukshe, 2000). TNF-α, released during inflammation, serves as a trigger for activation of other cytokines and plays a key role in the development and maintenance of inflammatory and neuropathic pain (Jin and Gereau, 2006). Moreover, although IL-6 expression levels are low under normal conditions, increases occur after brain injury, inflammation, and neuronal disease. Proposed roles for IL-6 include glia proliferation, neuronal survival and differentiation, and proinflammatory activities. Since these cytokines have a pivotal role in the course of inflammation, repression of their production is thought to protect from LPS-induced injury in the brain. As shown in Fig. 4, the upregulation of TNF-α, IL-1β and IL-6 could be inhibited by the pretreatment of PSPC in the brain stimulated with LPS. Our findings further indicated that PSPC has neuroprotective properties.

Because MAPK signaling pathway is known to be critical for the expression of proinflammatory mediators (Koistinaho and Koistinaho, 2002; Ji and Strichartz, 2004), we investigated the effect of PSPC on the phosphorylation and expression of ERK, JNK and p38. Our results showed that a systemic injection of LPS brought about the activation of MAPKs, which could be inhibited by the pretreatment of PSPC in mouse brain (Fig. 5). ERK, which is a major signaling system shared by various cell types, may be neurotoxic if activated by stimuli. For example, the ERK pathway is involved in LPS-induced iNOS expression and TNF-α secretion in addition to phosphorylates and activates transcription factors like NF-κB and activator protein-1 (Guha et al., 2001). Others also demonstrated that activation of ERK can be deleterious after focal ischemic injury (Wang et al., 2001). Our present results indicated that PSPC largely inhibited the ERK phosphorylation stimulated by LPS (Fig. 5C). JNK is another important subgroup of MAPKs. In neurons, activated JNK is involved in the induction of apoptotic responses (Putcha et al., 2003), and as a result, strategies aimed at inhibiting JNK have emerged as a means to reduce cell death. In brain, however, JNK activity is not restricted to neurons and apoptotic pathways, because JNK are also present in microglia where they mediate proinflammatory actions. JNK is now regarded as a pharmacological target for inhibiting brain inflammation and protecting neurons (Borsello and Forloni, 2007; Waetzig and Herdegen, 2004). Our data in the present study that PSPC inhibited LPS-induced JNK phosphorylation verifies the proinflammatory nature of the JNK pathway in mouse brain. It demonstrates, further, an additional anti-inflammatory property of PSPC (Fig. 5C). Like several other members of the MAPK family, p38-MAPK is activated by dual phosphorylation, and activation of p38-MAPK is involved in neuronal responses to various stresses. Our data showed that phosphorylation of p38-MAPK was slightly increased in mouse brain treated with LPS, and pretreatment with PSPC could reduce the phosphorylated level of p38 (Fig. 5C). Based on these results, we ensure that MAPKs, especially ERK and JNK pathways have important roles in inflammatory processes in LPS-stimulated mouse brain. PSPC may exert its anti-inflammatory effect on mouse brain by blocking the phosphorylation of MAPKs, in particular ERK and JNK pathways.

LPS and other inflammatory stimuli activate numerous intracellular signaling pathways, many of which converge on NF-κB and, ultimately, lead to increased cytokine production. NF-κB has been studied most extensively in the immune system in which it plays a key role in regulating the expression of genes involved in immune responses, inflammatory responses, cell survival, and cell proliferation (Li et al., 2004). NF-κB is composed of two proteins: p50 and p65. In resting cells, the NF-κB heterodimer exists in the cytosol. Exposed to proinflammatory stimuli, NF-κB dimmers are liberated and translocate to the nucleus, where the transcription of target genes are induced. In the nervous system, NF-κB is activated by a variety of neurotrophic factors, cytokines, and neurotransmitters. NF-κB has been implicated in regulating neural survival and death, peripheral nerve myelination, and synaptic function (Memet, 2006). Since these proinflammatory mediators are known to be modulated by NF-κB, we examined the effect of PSPC on the activation of NF-κB.

In the present study, we found that the translocation of activated NF-κB to the nucleus was inhibited by PSPC (Fig. 5D), implying that inhibition of NF-κB activation was tightly involved in the anti-inflammatory action of PSPC.

In conclusion, the present study shows that PSPC may exert neuroprotective effect on LPS-treated mouse brain through inhibiting iNOS, COX-2, IL-6 and TNF-α protein expressions, at least in part, by suppressing the ERK and JNK phosphorylation and NF-κB activation. Considering the minimal side effects in the body, PSPC may offer a novel therapeutic strategy for the treatment of inflammatory brain diseases.

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