Macelignan attenuates LPS-induced inflammation and reduces LPS-induced spatial learning impairments in rats

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\textbf{A B S T R A C T}

Previous studies have shown that macelignan has anti-inflammatory and neuroprotective effects. Subsequently, in the current study, we demonstrate that oral administrations of macelignan reduce the hippocampal microglial activation induced by chronic infusions of lipopolysaccharide (LPS) into the fourth ventricle of Fisher-344 rat brains. A Morris water maze was used to evaluate the status of the hippocampal-dependent spatial learning in control rats with an artificial cerebrospinal fluid infusion, rats with chronic LPS infusions, and rats with chronic LPS infusions and oral administrations of macelignan. The rats with chronic LPS infusions showed spatial memory impairments relative to the control rats in the performance of the memory task. Daily administration of macelignan reduced the spatial memory impairments induced by the chronic LPS infusions. The results indicate that macelignan may possess therapeutic potential for the prevention of Alzheimer’s disease.

A chronic neuroinflammatory response is characteristic of pathologically affected tissue in Alzheimer’s disease (AD). Epidemiological studies suggest that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD [17,26]. In agreement with these epidemiological studies, NSAIDs have been shown to suppress inflammation in a transgenic mouse with AD [16]. However, a harmful side effect of long-term NSAID treatment to prevent AD is the gastrointestinal and occasional liver and kidney toxicity associated with the inhibition of cyclooxygenase 1 [2,25,27]. These side effects have stimulated the research for alternative anti-inflammatory drugs that are safe in long-term treatments. One alternative, for example, is curcumin, a component of the yellow curry spice derived from turmeric [13]. Substantial in vitro studies indicate that curcumin has antioxidant, anti-inflammatory, and anti-amyloid activities [14,18–20]. Consistent with the in vitro studies, curcumin reduces the oxidative damage and microglial activation in APPsw mice [15].

Macelignan, a natural compound isolated from \textit{Myristica fragrans}, is reported to have free-radical scavenging and prostaglandin inhibitory activities [22]. Our previous research which involved the use of hippocampal neuron cell lines and LPS-activated primary culture of rat microglial cells demonstrated that macelignan has antioxidant, neuroprotective, and anti-inflammatory effects [10].

The present study, which uses animal model of inflammation, was conducted to assess the anti-inflammatory effects of macelignan. The results show that the neuroinflammation induced by chronic LPS infusions in rats reproduces components of the neuropathology of AD [5]. Specifically, chronic infusions of LPS into the fourth ventricle of young Fisher-344 rat brains produce inflammatory responses in the hippocampus, such as activated microglia [28]. Chronic LPS infusions have been shown to impair spatial memory as measured in a Morris water maze [8]. We demonstrate that oral administration of macelignan attenuates the neuroinflammatory responses induced by chronic LPS infusions and also reduces the spatial memory impairments caused by the chronic LPS infusions.

Houtt (Myristicaceae) was collected from the Biofarmaka Research Center of Bogor Agricultural University (Indonesia). The plant material was shade dried and ground to powder. One hundred grams of dried seed kernels of \textit{M. fragrans} was ground and extracted twice with 75% aqueous methanol (400 ml, v/v) for 24 h at room
(85 mg/kg) was determined on the basis of its permeability of blood
vehicle once a day; and (3) LPS-infused rats (LPS + Macelignan)
infused rats (LPS) that were orally administrated with the drug
orally administrated with the drug vehicle once a day; (2) LPS-
depth of 35.5 cm with tepid (27°C) water in a round tank, 1.83 m in diameter and 58 cm deep, and filled to a
osmotic infusion pump; it continued for 25 d and during the behav-
istration of macelignan was started 5 d after the surgery of the
rats were assessed for cue learning with a visible platform. The
location of this platform varied from trial to trial in a single session
of six training trials.

After the behavioral experiments, all the rats from each group
were euthanized by an overdose of ketamine HCl (30 mg/kg)
and xylazine (2.5 mg/kg), and then intracardially perfused with 4%
paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, PPB). The
brain was removed and cut coronal at Lambda (rostral to caudal) to
partition the fourth ventricle-containing brain portion (used to ver-
ify the cannula tip) from the hippocampus/cortex-containing brain
portion. After fixation, the brains were removed, postfixed in PPB
(2 h), cryoprotected in PBS containing 20% sucrose (24 h), frozen on
powdered dry ice, and sectioned (coronal plane: 40 μm) on a micro-
tome. The monoclonal antibody OX-6 (1:300, BD Bioscience) was used to visualize the activated microglial cells [6]. This antibody is
directed against Class II major histocompatibility complex antigens.
For OX-6 immunoreactivity, endogenous peroxidases were blocked
by 30 min incubation in 3% H2O2/10% MeOH in PBS. The sections
were incubated for 1 h (RT) in PBS with 0.3% Triton-X containing
10% serum and then incubated for a further 18 h (4°C) in the same
solution with the addition of the appropriate primary antibody.
The sections were incubated for 1 h in the appropriate biotinylated
secondary antibody (RT: 1:200) and for 1 h in ExtrAvidin perox-
idase conjugate (RT: 1:1000); they were then reacted by means of a
Vector SG substrate kit for peroxidase (Vector Laboratories).
The sections were mounted onto Superfrost++ slides, dehydrated through ascending concentrations of alcohol, defatted in Xylene, and coverslipped with Permount.

A one-way ANOVA and one-way repeated ANOVA were con-
ducted to assess the effects of macelignan on the changes of OX-6
and the impairments of the spatial memory induced by chronic
LPS infusion. Post hoc analyses (Duncan) were subsequently con-
ducted to determine the effects of the macelignan treatment. Any p
values that were less than 0.05 were considered significant, unless
otherwise specified.

Two rats died during the oral administration and were excluded from
the behavioral and histological analyses. The locations of the
cannula tips were visually verified while a microtome was used to
cut the brain portion containing the fourth ventricle. All the cannu-
la tips were located in the fourth ventricle. Immunostaining for
OX-6 revealed numerous highly activated microglia distributed in
the dentate gyrus region and around the CA3 of the hippocam-
pus in the LPS-infused rats (Fig. 1). The activated microglia had a
characteristic bushy morphology with an increased cell body
size and ramified and ramified process [5]. The LPS-infused
rats that received macelignan showed a significant reduction in the
number of activated microglial cells within the hippocampus
(Fig. 1 and 2) \( F(2,24) = 112.62, \ p < 0.001 \). According to the post hoc
analyses, the number of activated microglia in the hippocampus of the LPS-infused rats was significantly greater than that of the acSF-infused rats \( ^* \ p > 0.05 \). Moreover, the macelignan treatments significantly reduced the number of
activated microglia in the hippocampus of the LPS-infused rats
\( ^* \ p < 0.05 \).
Fig. 1. Immunohistology for activated microglia using the OX-6 antibody in the hippocampus of Fisher-344 rats. Rats with chronic LPS infusions into the fourth ventricle showed highly activated microglia in the hippocampus (middle). The brains of the aCSF-infused rats had very few activated microglia in the hippocampus (left). Daily administration of macelignan in the LPS-infused rats reduced the microglial activation close to the levels in the brains of the aCSF-infused rats (right). The bottom panels show higher magnifications of the boxed areas in the top panels. aCSF: aCSF-infused rat; LPS: LPS-infused rat; LPS + Macelignan: LPS-infused rat with daily administrations of macelignan. Scale bar (left) denotes 100 μm.

Fig. 2. The number of activated microglia in the hippocampus of Fisher-344 rats. The number of activated microglia in the hippocampus of the LPS-infused rats was significantly greater than those of the aCSF-infused rats (*, p < 0.05). The macelignan treatments significantly reduced the number of activated microglia in the hippocampus of the LPS-infused rats (#, p < 0.05). aCSF: aCSF-infused rat; LPS: LPS-infused rat; LPS + Macelignan: LPS-infused rat with daily administrations of macelignan.

Place Training: Latencies were used to assess performance accuracy in the water maze; they are described in more detail elsewhere [1]. As shown in Fig. 3 (top), the aCSF-infused rats quickly became proficient at locating the submerged platform during the training trials, as assessed by the latency measure. However, the LPS-infused rats did not show any improvement over the course of the training compared with the control aCSF-infused rats, while the macelignan treatment ameliorated this learning deficit induced by chronic LPS infusions. The overall repeated ANOVAs showed that the between group effects (aCSF, LPS, or LPS + Macelignan) were significant ($F(2,22) = 164.25, p < 0.001$) and the training effects (block) were also significant ($F(3,22) = 55.45, p < 0.001$). There were no interaction effects of the group and training ($F(6,22) = 0.97, ns$).

In addition, there were apparent differences in performance in the probe trials, as assessed by the percentage of time spent in the maze quadrant that contained the platform (target) (Fig. 3B). The overall repeated ANOVAs showed that the between group effects (aCSF, LPS, or LPS + Macelignan) were significant ($F(2,22) = 9.91, p < 0.001$), that the spatial bias (probe) over the course of training were significantly increased ($F(3,22) = 20.53, p < 0.001$), and that the interaction effects of the group and probe were significant ($F(6,22) = 2.66, p < 0.05$).
either LPS group (aCSF, 10.97 trials (mean to reach the platform (in seconds) during the block of cue-training speeds during the training trials (were no significant differences between groups in swimming the memory performance of LPS-infused rats.

These results demonstrate the therapeutic effects of macelignan on LPS: LPS-infused rat; LPS + Macelignan: LPS-infused rat with daily administrations training (

rats showed spatial bias in the probe trial conducted 24 h after 2 blocks of training. The control infused rats (any improvements over the course of the training compared with the control aCSF-submerged platform during the training trials. The LPS-infused rats did not show any spatial bias. As with the results of place learning in the water maze task, the LPS-infused rats seem unable to learn the accurate location of the hidden platform. The chronic LPS-infused rats with daily macelignan administrations showed comparable performances to those of the aCSF-infused rats (Fig. 3).

As previously reported, the LPS-infused rats exhibited deficits in the hidden platform version of the task [8]. In contrast with the aCSF-infused rats, the LPS-infused rats showed apparent differences in latencies during the training trials and percentages during the probe trials; however, the LPS-infused rats were also unpaired in the cued version of the task. The results showed that the LPS-infused rats had an intact performance during cue learning and their speeds were comparable to those of the aCSF-infused rats during training trials; the results also indicate that the deficits are not attributable to general motivation or to sensorimotor deficits [1].

New potent compounds for antioxidant and anti-inflammatory agents are needed to reduce oxidative damage and inflammation in the brain. This need has arisen because, as mentioned, the existing NSAIDs have side effects and oxidative damage and neuroinflammation are closely associated with the hallmark pathologies of AD [17]. As with curcumin, macelignan, which is isolated from M. fragrans Houtt, has both inhibitory reactive oxygen species and anti-inflammatory properties [10,23]. In vitro studies with hippocampal cells (HT22 cell) and microglial cells (culture microglial cell) show that macelignan potently protects the hippocampal neurons against glutamate toxicity and suppresses LPS-induced microglia activation [10]. Using the animal model of brain inflammation, the current experiments also confirm the anti-inflammatory properties of macelignan.

Given that the protecting molecular mechanism of macelignan on activated microglia-induced neuronal dysfunction is still unknown, further study is required. However, the recent studies which show that macelignan has protective effects in the treatment of diabetes mellitus and hepatotoxicity indicate that macelignan is a peroxisome proliferator-activated receptor-α/γ (PPAR-α/γ) agonist and is involved in the mitogen-activated protein kinase (MAPK) signaling pathway [4,24]. It is well documented that PPARγ agonists elicit anti-inflammatory and anti-amyloidogenic effects, and that MAPK has emerged as a key factor in the regulation of Tau and β-amyloid precursor proteins [3,9].

In conclusion, using a chronic LPS infusion rat model, the present experiments examined the in vivo efficacies of the anti-

Chronic LPS infusions into the fourth ventricle of Fisher-344 rat brains produce many of the inflammatory symptoms, pathological changes, and memory impairments associated with AD [5,11]. That is, rats with chronic LPS infusions show an increased number and density of OX-6-positive reactive microglia, the immune competent cells of the central nervous system [7]. In the present study, extensive inflammation in the hippocampus was produced by chronic LPS infusions into the fourth ventricle of Fisher-344 rat brains, and the inflammation was attenuated by daily administrations of macelignan (Figs. 1 and 2).

The spatial memory of Fisher-344 rats was measured with a traditional place learning task in a Morris water maze, and the results reveal the effects of chronic LPS infusions and macelignan on spatial memory. Our examination of performance in a traditional place learning task revealed deficits in the LPS-infused rats relative to the aCSF-infused rats, and these deficits are consistent with previous findings [8]. Specifically, in comparison with the performances of the aCSF-infused rats, the LPS-infused rats showed decreased latencies in finding the hidden platform in the early training sessions, but showed no further improvements over the latter sessions training. Whereas the aCSF-infused rats showed increased spatial bias over the course of the place training in the probe trials where the platform was unavailable for escape, the LPS-infused rats did not show any spatial bias. As with the results of place learning in the water maze task, the LPS-infused rats seem unable to learn the accurate location of the hidden platform. The chronic LPS-infused rats with daily macelignan administrations showed comparable performances to those of the aCSF-infused rats (Fig. 3).

Post hoc analyses of the between group effects in both the latency measure of training trials and the percentage measure of the probe trials revealed that the performances of the aCSF rats and LPS rats with macelignan differed from those of the LPS rats (p < 0.05). These results demonstrate the therapeutic effects of macelignan on the memory performance of LPS-infused rats.

Swimming speeds during training trials and cue training: There were no significant differences between groups in swimming speeds during the training trials (F(2,22) = 0.55, ns). The times taken to reach the platform (in seconds) during the block of cue-training trials (mean ± standard error) for the three groups are as follows: aCSF, 10.97 ± 1.30; LPS, 10.12 ± 1.70; LPS + Macelignan, 8.77 ± 0.77). There was no significant difference between the aCSF group and either LPS group (F(2,22) = 0.72, ns).

Fig. 3. Performance of the control aCSF-infused rats, LPS-infused rats, and LPS-infused rats with macelignan treatments in the spatial version of a Morris water maze. (A) Latencies for finding the hidden platform in the spatial learning task during five training trial blocks. The control rats became proficient at locating the submerged platform during the training trials. The LPS-infused rats did not show any improvements over the course of the training compared with the control aCSF-infused rats (p < 0.05), while the macelignan treatment ameliorated this learning deficit by chronic LPS infusions (p < 0.05). (B) The percentage of time spent in the maze quadrant that contained the platform during the 30 s probe trial. The control rats showed spatial bias in the probe trial conducted 24 h after 2 blocks of training trials (10 trials), but the LPS-infused rats show no spatial bias over the course of the training (p < 0.05). The absence of spatial bias induced by the LPS infusions in the rats was reduced with the macelignan treatment (p < 0.05). aCSF: aCSF-infused rat; LPS: LPS-infused rat; LPS + Macelignan: LPS-infused rat with daily administrations of macelignan.
inflammatory agent macelignan. Daily treatment of macelignan attenuates the hippocampal inflammatory responses induced by chronic LPS infusions. These treatments also reduce the spatial learning impairments induced by the chronic LPS infusions. Moreover, the results confirm that macelignan is efficiently transferred to the brain through the blood–brain barrier [10]. These results suggest that macelignan could be a potent alternative for new anti-inflammatory treatments of AD and other CNS diseases.

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