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Dexibuprofen (*S*(+)-isomer ibuprofen) reduces microglial activation and impairments of spatial working memory induced by chronic lipopolysaccharide infusion

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been proposed as a therapeutics to reduce the risk of Alzheimer's disease (AD). The present study shows that the peripheral administration of dexibuprofen (*S*(+)-isomer ibuprofen), which causes less gastric damage and has better anti-inflammatory effects than ibuprofen, reduces the microglial activation in the cortex and hippocampus, and reduces the phosphorylation of extracellular signal-regulated kinases in the hippocampus, which has been induced by chronic infusion of lipopolysaccharide (LPS) into the fourth ventricle of Wistar rats. The effects of dexibuprofen on impairments of spatial working memory induced by LPS infusions were measured with a trial-unique matching-to-place task in a water maze which assessed memory for place information over varying delays. When performing the water maze task, the rats with the LPS infusions showed spatial working memory impairments relative to the rats with the artificial cerebrospinal fluid. Daily administrations of dexibuprofen reduced the spatial working memory impairment induced by the chronic LPS infusion. The results indicate that NSAID treatments using dexibuprofen significantly attenuate the processes that drive the pathology associated with AD and that this process may involve the suppression of microglial activation.

Keywords: Alzheimer's disease; Anti-inflammatory; Lipopolysaccharide; Microglia; Spatial working memory

1. Introduction

Neuroinflammatory responses are characteristics of pathologically affected tissue in several neurodegenerative disorders, including Alzheimer's disease (AD). Epidemiological studies have shown that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD, delays disease onset, ameliorates symptomatic severity, and slows cognitive decline (McGeer and McGeer, 2003; Tabet and Feldmand, 2003). However, a major limitation of NSAIDs is the gastrointestinal pain and occasional liver and kidney toxicity caused by cyclooxygenase-1 (COX-1) inhibition (Graupera et al., 2003; Stichtenoth and Frolich, 2000; Warner et al., 1999).

Ibuprofen is an NSAID and is widely used to reduce pain, fever, and inflammation. This drug inhibits cyclooxygenases and activates peroxisome proliferators-activated receptors; both of these actions result in reduced inflammation (Kaufmann et al., 1997; Kojo et al., 2003; Lehmann et al., 1997). Ibuprofen also has the smallest gastric damaging effects of all current NSAIDs (Warner et al., 1999). Evidence that ibuprofen treatment is beneficial in preventing of AD have arisen from human studies (Tabet and Feldmand, 2003) and animal models of AD (Frautschy et al., 2001; Lim et al., 2000; Weggen et al., 2001).

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These results have stimulated the development of a new ibuprofen formulation with better efficacies and fewer side effects in long-term treatments. Interestingly, one feature of ibuprofen that has been used in many countries as an equal mixture of *R*- and *S*(+)-ibuprofen is the unidirectional metabolic chiral inversion of the in vitro inactive *R*-enantiomer to the *S*-form. *S*(+)-isomer ibuprofen, or dexibuprofen, is a pharmacologically effective enantiomer and has a slower dissolution rate in the simulated gastric and enteric juices compared with the standard racemic ibuprofen (Walser et al., 1997). A study using rodents showed that dexibuprofen reduced the level of gastric damage and improved the analgesic effects, in comparison with those of the standard racemic ibuprofen (Bonabello et al., 2003). Rodents with dexibuprofen treatments showed smaller swellings in the right hind paw that had been induced by injecting carrageenan, which might suggest that dexibuprofen has better anti-inflammatory effects, even though the biological markers of the inflammation were not measured in this study (Bonabello et al., 2003).

The present study was therefore conducted to assess the effects of dexibuprofen with fewer gastric lesions and better efficacy (Bonabello et al., 2003) on the neuroinflammatory responses and memory impairments induced by chronic LPS infusions. Specifically, the chronic infusion of lipopolysaccharide (LPS) into the fourth ventricle of young Wistar rats produced inflammatory responses in the cortex and hippocampus, such as activated microglia, as reported elsewhere (Hauss-Wegrzyniak et al., 1998). We also measured the phosphorylation of extracellular signal-regulated kinases (pERK) that is a superfamily of the mitogen-activated protein kinases (MAPK), and the pERK emerged as a key factor in the regulation of Tau and β-amyloid precursor proteins (Haddad, 2004).

The chronic LPS infusions impaired the spatial working memory and the impairments were measured with a trial-unique matching-to-place task in a water maze which assessed memory for place information over varying delays. The administration of dexibuprofen attenuated the neuroinflammatory responses and increased the pERK induced by the chronic LPS infusions, and reduced the spatial working memory impairments caused by the chronic LPS infusions.

2. Methods

2.1. Subjects

Twenty-one male Wistar rats, obtained from SLC Inc. (Hamamatsu, Shizuoka, Japan), were used as subjects. The rats resided in a vivarium at Inje University for at least 2 months prior to the experiment and weighed 300–350 g at the beginning of the experiment. The vivarium maintained on a 12 h light/dark cycle (0700–1900) and climate controlled at 22 °C. All efforts were made to minimize the number of animals used and their suffering, and the animal procedures were conducted in accordance with approved institutional animal care procedures. All surgical procedures and behavioral testing took place during the light phase.

2.1.1. Surgery

The rats were anesthetized with isofluorane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) fitted with an isofluorane gas anesthesia system and an incisor bar set 3.3 mm below the ear bars. LPS ($n=14$) or artificial cerebrospinal fluid (aCSF, $n=7$) was chronically infused (0.25 μl/h for 28 days) through a cannula implanted in the fourth ventricle of the brain and was attached to an osmotic minipump (Alzet, Palo Alto, CA, USA, model 2004), as described elsewhere (Rosi et al., 2004). The following three groups ($n=7$) were prepared: (1) aCSF-infused rats that were injected daily (sc) with the drug vehicle; (2) LPS-infused rats that were injected daily (sc) with the drug vehicle; (3) LPS-infused rats that were injected daily (sc) with dexibuprofen (50 mg/kg; obtained from Gebro Pharma, Austria). The drug vehicle for dexibuprofen was deionized water containing NaOH (16.25 mM), Na_2HPO_4 (21.23 mM), and NaCl (12.83 mM).

2.2. Apparatus

The rats were trained in a Morris water maze. The maze was a round tank, 1.83 m in diameter and 58 cm deep, filled to a depth of 35.5 cm with tepid (27 °C) water made opaque by adding white paint (tempera). A moveable circular platform 12 cm in diameter was located 2 cm below the surface of the water. The maze was surrounded by white curtains on which black cloth visual stimuli of various shapes and sizes were placed. A camera was located above the center of the maze and relayed images to a videocassette recorder and an HVS Image Analysis Computer System. Data from the water maze trials were analyzed using software provided by HVS (Hampton, United Kingdom).

2.3. Training procedure for the match-to-place memory task

The behavioral testing consisted of two phases which took place in the following order: initial learning and delayed match-to-place memory testing (Baxter et al., 1995). The animals were handled daily for 5 days before beginning the behavioral testing. The rats received 4 days of initial training (four trials per day, maximum trial duration of 90 s with 30 s on the platform at the end trial, 1 min intertrial interval) at each different platform position.

After the initial training, ten testing sessions took place with one session per day. Two trials were given per session. The trials were otherwise identical to the training sessions given during the initial training, except that a variable delay was imposed between the first trial (the information trial) and the second trial (the retention trial). Five delays were used: 1 min, 0.5 h, 1 h, 2 h, and 24 h. The ten sessions used all five delays in a random order, resulting in two blocks of testing with each delay occurring once in each block.

2.4. Immunohistology

After the behavioral experiments, four rats from each group were euthanized by an overdose of ketamine HCl (30 mg/kg) and

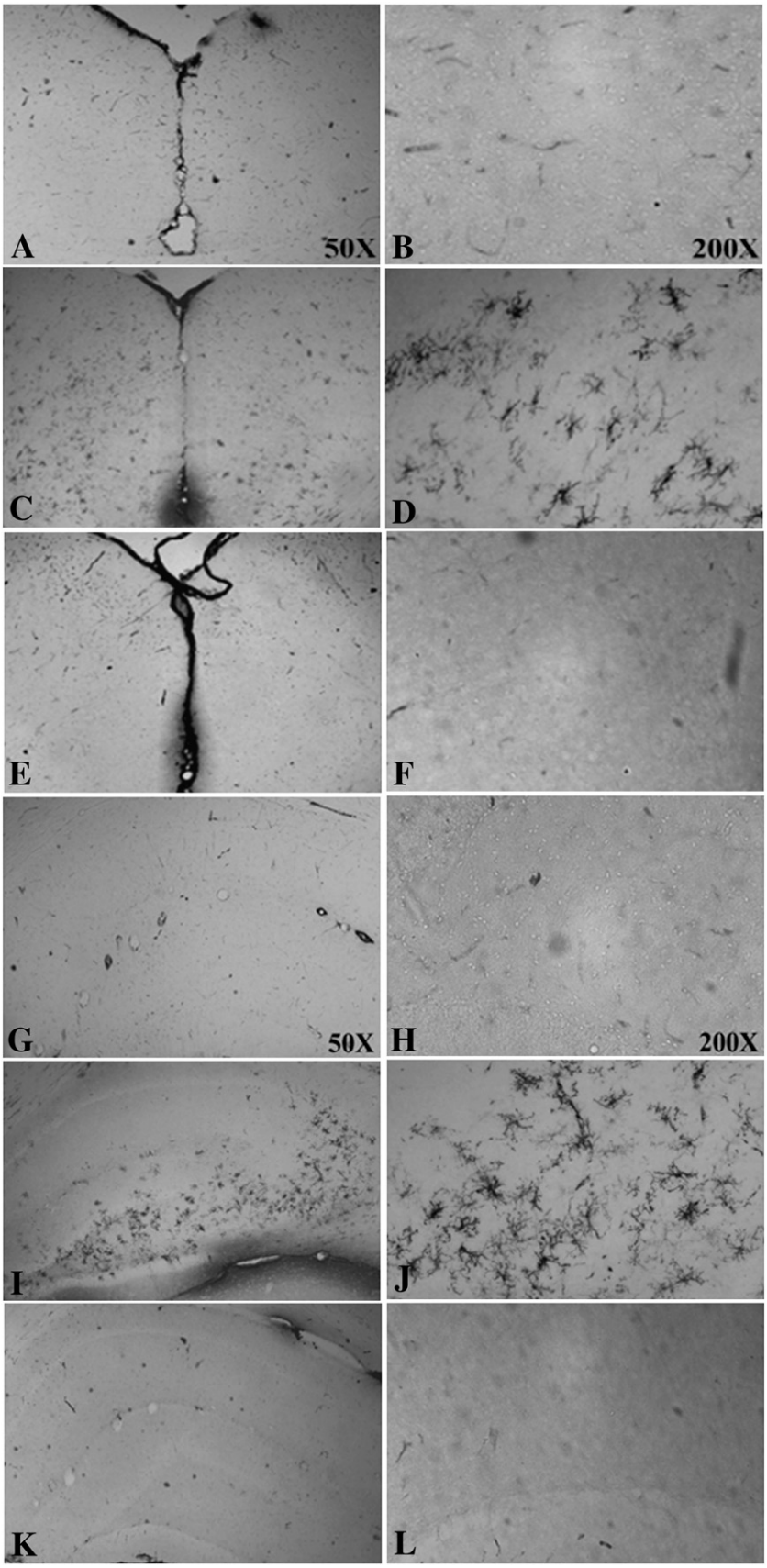

Fig. 1. Immunohistology for activated microglia using the OX-6 antibody in the retrosplenial granular cortex and hippocampus of Wistar rats. The rats with chronic LPS infusion into the fourth ventricle showed highly activated microglia in the retrosplenial granular cortex (C, D) and hippocampus (I, J). Wistar rats infused with aCSF had very few activated microglia (A, B, G, H). Daily administrations of dexibuprofen in the LPS-infused rats reduced microglial activation to an almost identical level to that of the brains of rats with aCSF (E, F, K, L).

xylazine (2.5 mg/kg) and then intracardially perfused with 4% paraformaldehyde in a 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 (verified by the position of the cannula tip) from the hippocampus/cortex-containing brain portion. Following 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 um) on a microtome. The monoclonal antibody OX-6 (1:300, BD Bioscience) was used to visualize the activated microglial cells (Hauss-Wegrzyniak et al., 2002). This antibody is directed against the Class II major histocompatibility complex antigens. The polyclonal antibody pERK (1:250, Cell Signaling) was used to visualize pERK. For OX-6 and pERK immunoreactivity, endogenous peroxidases were blocked by 30 min incubation in 3% H_2O_2/10% MeOH in PBS. The sections were incubated for 1 h (RT) in PBS with 0.3% Triton-X containing 10% serum, followed by 18 h incubation (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 (RT; 1:200), for 1 h in ExtrAvidin peroxidase conjugate (RT; 1:1000), and then reacted using 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.

2.5. Western blot analysis

The remaining three rats from each group after the behavioral experiments were euthanized by rapid decapitation. After they were euthanized, the hippocampi of all rats in the protein study were rapidly dissected and frozen at 80 °C. The remaining section of the brain was used later for verification of the cannula tip. The proteins for the analysis of ERK and pERK were extracted in the following manner. Individual tissue samples were weighed and then homogenized in 5 vol of ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 1% Triton-X 100, 2 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol, 0.234 mM leupeptin, and 1 mM PMSF. The homogenates were centrifuged at 100,000 ×*g* for 60 min at 4 °C. The supernatant was removed from each sample, and an aliquot was taken to determine the total protein concentration using the Bradford Reagent.

The proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was incubated with a primary antibody (Ab) against ERK/pERK (1:1000, Cell Signaling), and then it was incubated with the HRP-conjugated secondary Ab (Santa Cruz). The membranes were visualized using an ECL system and then developed on Hyperfilm (Amersham). The relative expression levels of pERK were determined through a densitometry and normalized by ERK.

2.6. Statistical analysis

A one-way ANOVA and one-way repeated ANOVA were conducted to assess the effects of dexibuprofen on the changes in pERK and the impairment of spatial working memory induced by chronic LPS infusion. Post-hoc analyses (Fisher Test or *T*-test) were subsequently conducted in order to determine the effects of the dexibuprofen treatment. *p* values less than 0.05 were considered significant, unless otherwise specified.

3. Results

3.1. Dexibuprofen reduced LPS-induced microglia activation and increases of pERK in the hippocampus and cortex

The locations of the cannula tips were verified while using a microtome to cut the brain portion containing the fourth ventricle. All cannula tips were located in the fourth ventricle. Four rats per

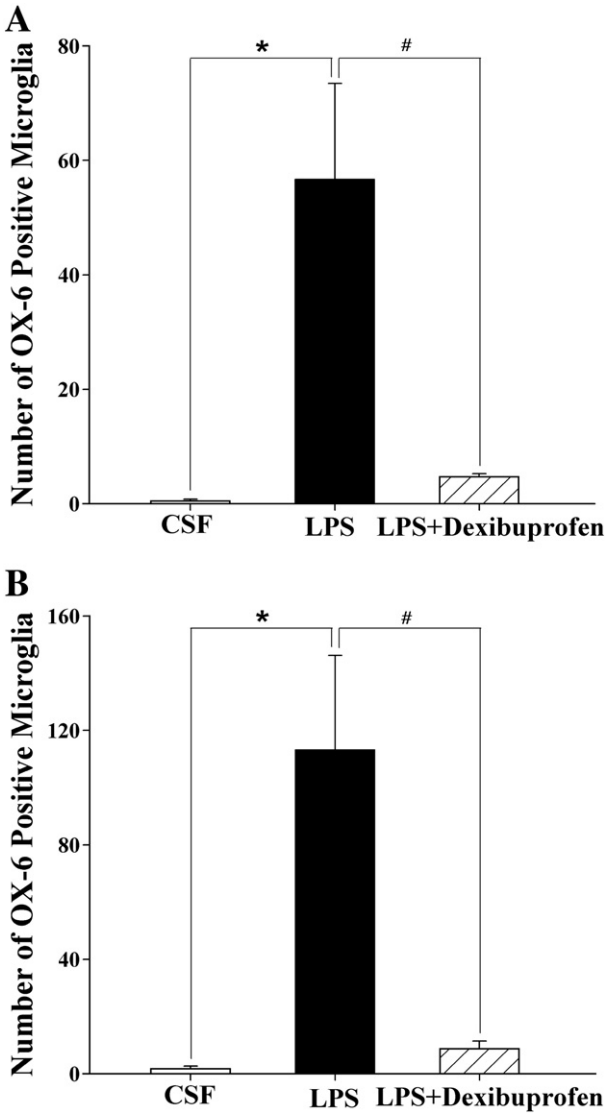

Fig. 2. The number of activated microglia in the (A) retrosplenial granular cortex and (B) hippocampus of Wistar rats. The number of activated microglia in the cortex and hippocampus of the LPS-infused rats was significantly greater than those of the aCSF-infused rats (*, $p<0.05$). The dexibuprofen treatments significantly reduced the number of activated microglia in the retrosplenial granular cortex and hippocampus of the LPS-infused rats (#, $p<0.05$).

group were used for immunohistological observations and the remaining three rats from each group for western blot analyses. Immunostaining for OX-6 revealed numerous highly activated microglias distributed in the cortex and the dentate gyrus (DG) region and around the CA3 of the hippocampus in LPS-infused rats (Fig. 1). The activated microglia had a characteristic bushy morphology with increased cell body size and contracted and ramified processes. Three of the four LPS-infused rats had clear activated microglias. However, one brain showed very mildly activated microglia and did not show any difference compared with the brains of rats with aCSF, but regardless, this brain was included for the behavioral analysis. To quantify the effects of the dexibuprofen treatments, the number of OX-6 positive microglia within the hippocampus and the retrosplenial granular cortex located just above the hippocampus were counted in drawings of the identical sections from every rat. These results are shown in Fig. 2. The ANOVA found significant group effects in the cortex and hippocampus ($F(2,9) < 10.57$, $p < 0.01$). Post-hoc analyses of the group effects revealed that the number of activated microglia in the hippocampus and cortex of the LPS-infused rats was significantly greater than those of the aCSF-infused rats and LPS-infused rats with dexibuprofen ($p < 0.05$); however, those of the LPS-infused rats with dexibuprofen were significantly greater than those of the aCSF-infused rats ($p < 0.05$).

The hippocampal pERK abundances measured using a western blot were highly increased in the brains of the three rats with the LPS infusion when compared with those of brains with the aCSF infusions (Fig. 3; $p < 0.01$). All brains with both LPS

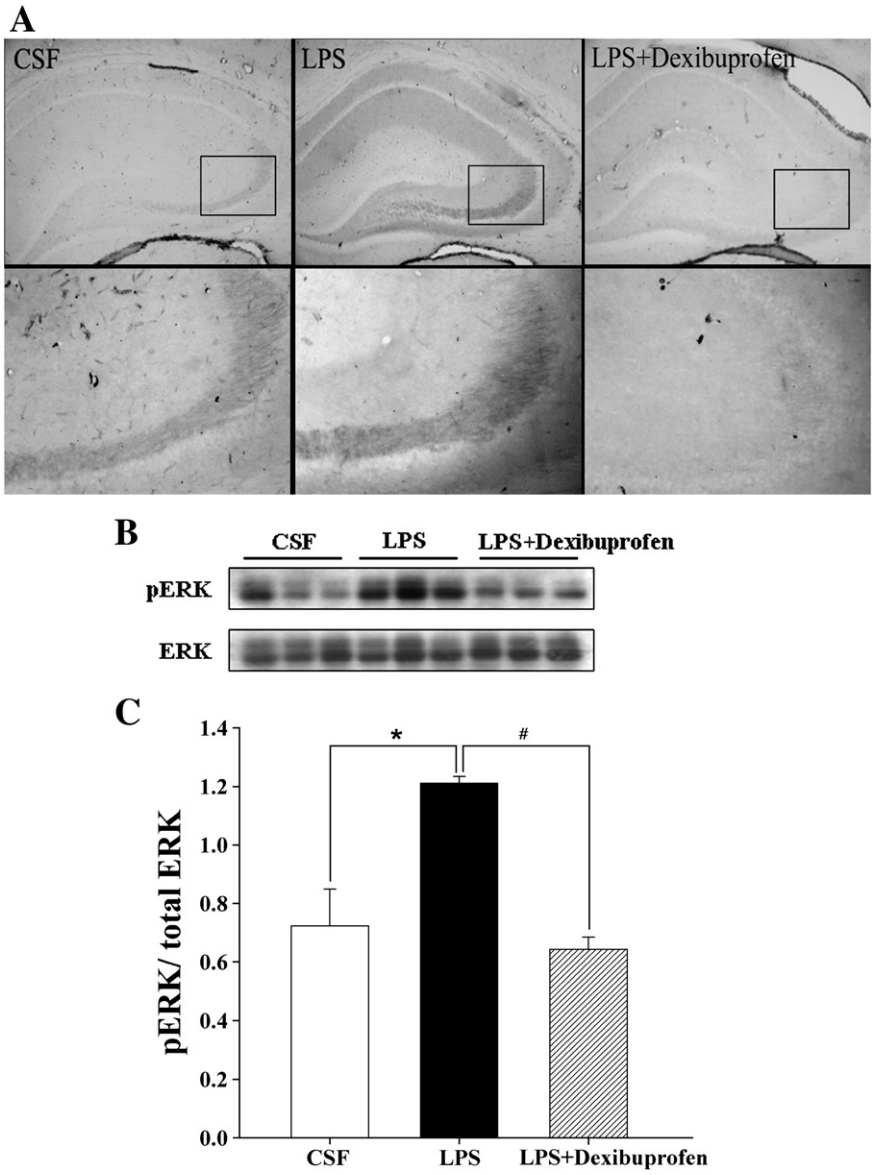

Fig. 3. Dexibuprofen treatment suppresses the hippocampal ERK phosphorylation induced by chronic LPS infusion (A–C). (A) A representative immunohistological staining in the hippocampus of rats with aCSF (left), LPS (middle), and LPS + dexibuprofen (right). The bottom panels are higher magnifications of the boxed areas in the top panels. (B) A representative western blot. (C) Hippocampal pERK abundances greatly increased in the brains with LPS relative to those of the brains with aCSF (*, $p < 0.01$), and the hippocampal pERK abundances in brains with both LPS and daily administrations of dexibuprofen were almost identical to those of brains with aCSF (#). CSF, aCSF-infused rat; LPS, LPS-infused rat; LPS + dexibuprofen, LPS-infused rat with daily administrations of dexibuprofen.

infusions and daily administrations of dexibuprofen did not show any activated microglias in either the cortex or the hippocampus (Fig. 1), and the hippocampal pERK abundances were almost identical to those of the brains with the aCSF infusions (Fig. 3).

3.2. Dexibuprofen reduced LPS-induced spatial working memory impairments

All behavioral data was included for the behavioral analysis. Pathlengths were used to assess the performance accuracy in the water maze and are described in more detail elsewhere (Gallagher et al., 1993). The performances during the memory testing are shown in Fig. 4. Fig. 4(A) shows the initial learning performances. The between group effects (aCSF, LPS, or LPS with dexibuprofen) failed to reach statistical significance in the pathlength of the initial training ($F(2,18)=2.64$, $p=0.10$). The pathlength of the rats in the three groups decreased across the training sessions ($F(3,54)=11.09$, $p<0.01$). The interaction effects between the group and session did not reach statistical significance ($F(6,54)=11.01$, $p=1.07$).

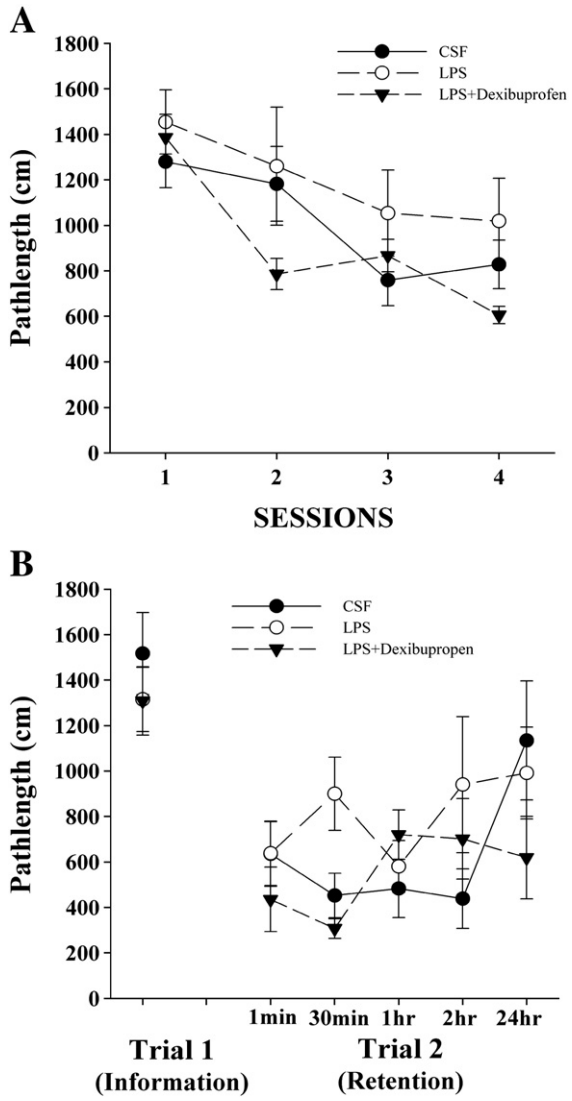

Fig. 4. Match-to-place memory testing performance (pathlength) for CSF (aCSF-infused rat), LPS (LPS-infused rat), and LPS+dexibuprofen (LPS-infused rat with daily administrations of dexibuprofen). (A) No differences in the pathlength between groups (CSF, LPS, and LPS with dexibuprofen) in the initial training. (B) Data shown are the means of Trial 1 (information) performances across all ten sessions of testing and the means of Trial 2 (retention) performances across the two sessions at each delay. There were no differences between the groups in the information trials. The performances of the aCSF-infused rats and LPS-infused rats with dexibuprofen were statistically different from those of the LPS-infused rats in the retention trials.

Fig. 4(B) shows the performances of two trials with varied delays. The performance in the information trial was similar for all three groups ($F(2,18)=0.50$, ns). In the retention trial, rats with the aCSF-infused exhibited a substantial and relatively stable retention at 1 min, 0.5 h, 1 h, and 2 h, and poor retention at 24 h. Relative to the aCSF-infused rats, the LPS-infused rats were impaired at the 0.5 h delay. The daily administrations of dexibuprofen attenuated the spatial working memory impairments induced by chronic LPS infusion (Fig. 4(B)).

The overall repeated ANOVAs showed that the between group effects (CSF, LPS, or LPS with dexibuprofen) reached statistical differences ($F(2,18)=3.60$, $p<0.05$). As the delay increased, the pathlength in the retention trial appeared to increase over the delays, but it was statistically insignificant ($F(4,72)=2.32$, $p=0.07$). Furthermore, the interaction effects between the group and session were not statistically significant ($F(3,54)=2.54$, $p=0.07$). Post-hoc analyses of the between group effects revealed that the performances of the aCSF-infused rats and LPS-infused rats with dexibuprofen were different from the LPS-infused rats ($p<0.05$). These results demonstrate the positive effect of the administration of dexibuprofen upon memory performance of the LPS-infused rats.

4. Discussion

LPS is widely used to experimentally stimulate the production of endogenous cytokines and other inflammatory proteins. Chronic infusion of LPS into rat brains produces many of the inflammatory symptoms, pathological changes, and memory impairments associated with AD (Hauss-Wegrzyniak et al., 1998). Specifically, chronic LPS infusions increase the number and density of OX-6-positive reactive microglia, which are the immune competent cells of the central nervous system (Hauss-Wegrzyniak et al., 2000) and increase the number and density of astrocytes, observed by glial fibrillary acid proteins (Hauss-Wegrzyniak et al., 1998). Using Wistar rats, the present study shows that the number and density of OX-6-positive reactive microglia was remarkably increased in the hippocampus and cortex by chronic LPS infusion and this has also been reported elsewhere (Richardson et al., 2005). It is also reported that the increase of pERK is induced by LPS treatments in culture and by a spinal cord injury or TNF-alpha application in animals (Cheng et al., 2003; Takahashi et al., 2006). This is the first report in which hippocampal pERK has been increased by intraventrically chronic intraventrical LPS infusion in a rat.

Fisher-344 rats with chronic LPS infusions failed to learn the spatial reference memory task in a Morris water maze (Hauss-Wegrzyniak et al., 1999). In the present study, the spatial working memory of a Wistar rat was measured with a delayed

matching-to-place task using a Morris water maze to see the effects of chronic LPS and dexibuprofen administrations on the spatial working memory. Our examination of performance in the delayed matching-to-place task revealed deficits in the LPS-infused rats, relative to the aCSF-infused rats. The rats with chronic LPS infusion and dexibuprofen administration showed comparable performances to those of the aCSF-infused rats.

Epidemiological studies have shown that long-term treatment with NSAIDs reduces the risk of AD (Tabet and Feldmand, 2003). In agreement with these epidemiological studies, ibuprofen treatments have suppressed inflammation in AD animal models: the Tg2576, a transgenic mouse of amyloid precursor protein with a Swedish mutant (APPsw), and aged rats intraventrically infused with Aβ (Frautschy et al., 2001; Lim et al., 2000). These findings have heightened interest in the development of therapeutics that target neuroinflammation and considerable clinical attention has focused on NSAIDs and the COX pathway. In animal models of stroke, ibuprofen reduced neuronal injury, improved the cerebral blood flow and neurological outcomes in global ischemia (Grice et al., 1987; Kuhn et al., 1986; Patel et al., 1993), and decreased the infarct size in focal ischemia (Antezana et al., 2003; Cole et al., 1993). However, the use of these drugs to target neuroinflammation has largely failed due to gastrointestinal side effects and occasional liver and kidney toxicity caused by COX-1 inhibition (Graupera et al., 2003; Stichtenoth and Frolich, 2000; Warner et al., 1999). These side effects have stimulated the search for alternative anti-inflammatory drugs and, as an alternative, structural modification of existing NSAIDs to eliminate the COX-1 inhibition effect. NSAIDs derived from 2-arylpropionic acid (profens) exist in two stereoisomeric forms (Evans, 1996): dexibuprofen (*S*(+)-isomer ibuprofen) reduces gastric damage (Bonabello et al., 2003) and human microglial cell neurotoxicities compared with *R*(−)-ibuprofen (Klegeris et al., 2004).

It should also be noted that selective COX-2 inhibitors have similar efficacy without the gastrointestinal effects associated with non-selective NSAIDs, but have an increased thrombotic risk. That is, new platelets lack COX-2, so COX-2 inhibitors show small anti-platelet effects; however, these would inhibit prostaglandin E2 through the impact of COX-2 on endothelial cells, which may exacerbate ischemic damage to the heart (Bouchard et al., 2000; Brophy et al., 2007). In clinical trials, current users of COX-2 inhibitors with a prior myocardinal infarction have a significantly increased risk of a subsequent myocardinal infarction (Brophy et al., 2007; Hermann and Ruschitzka, 2006).

As has been noted elsewhere (Hauss-Wegrzyniak et al., 1998), chronic LPS infusion into the fourth ventricle of young Fischer-344 rats activates microglia to initiate a series of inflammation-induced changes within the hippocampus and results in cell loss within layers II and III of the entorhinal cortex and attenuation of long-term potentiation within the dentate gyrus (Hauss-Wegrzyniak et al., 2002). This inflammation leads to a reduction in the number of NMDA glutamate receptors within the DG and CA3 hippocampal areas without frank evidence of neuronal loss (Rosi et al., 2004) and impairment in the spatial reference memory (Hauss-Wegrzyniak et al., 1998). Thus, chronic LPS infusion reproduces neurobiological components of Alzheimer's disease (Hauss-Wegrzyniak et al., 1998).

Using the chronic LPS infusion in animal model, the present experiments examined in vivo efficacies of the anti-inflammatory agent dexibuprofen. Daily treatments of dexibuprofen attenuated the inflammatory responses in the hippocampus and cortex, and reduced ERK phosphorylation casued by chronic LPS infusions. These treatments also reduced the spatial working memory impairments by chronic LPS infusion. In conclusion, daily administrations of dexibuprofen reduced the brain's responses to inflammation. These results may lead to the development of new anti-inflammatory treatments for AD and CNS diseases.

Acknowledgment

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