Effect of 1400W on blocking lipopolysaccharide-induced microglial toxicity to preoligodendrocytes

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Background: **The maternal-fetal infection/inflammation is believed to be the mechanism in the pathogenesis of periventricular leukomalacia (PVL). The activation of microglias (MGs) may contribute to preoligodendroglial damage. The present study was undertaken to explore the effect of N-[3-(aminomethyl) benzyl] acetamidine (1400W), a selective inhibitor of inducible nitric oxide synthase (iNOS), on the blockage of lipopolysaccharide (LPS)-induced microglial toxicity to preoligodendrocytes (preOLs).**

Methods: **The co-cultural MGs and preOLs obtained from two-day-old Sprague-Dawley (SD) neonatal rats were divided into three groups: co-culture control group, co-culture LPS group, and co-culture LPS plus 1400W group. The concentration of nitric oxide (NO) was measured by nitric acid-deoxidize-colorimetry, the level of peroxynitrite ($ONOO^-$) determined by immunocytochemistry, the synthetic level of inducible nitric oxide synthase (iNOS) detected by western blotting, and the apoptotic rate of preOLs assessed by flow cytometry after the co-cultural cells were induced by LPS (100 ng/ml) for 48 hours.**

Results: **Compared with those in the co-culture control group, the levels of NO (82.27±3.41 μmol/L *vs.* 167.86±9.87 μmol/L, *P*<0.01), $ONOO^-$ (6.14±1.27 *vs.* 34.38±7.75, *P*<0.01), and iNOS (0.18±0.027 *vs.* 0.79±0.068, *P*<0.01) induced by LPS increased remarkably in the co-culture LPS group, with a higher apoptotic rate of preOLs (6.73±1.39% *vs.* 24.77±2.05%, *P*<0.01). The levels of NO (69.55±5.07 μmol/L, *P*<0.01), $ONOO^-$ (10.33±3.47, *P*<0.01) and iNOS (0.35±0.042, *P*<0.01) were decreased significantly using 1400W at a dose of 10 μmol/L in the co-culture LPS plus 1400W group, and the apoptotic rate of preOLs (11.80±2.06% *vs.* 24.77±2.05%, *P*<0.01) also decreased compared with the co-culture LPS group.**

Conclusion: **1400W can block effectively the LPS-induced microglial toxicity to preOLs by inhibiting iNOS specifically, resulting in a significant reduction of toxicity parameters investigated and a marked increase of the survival preOLs.**

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Key words: **N-[3-(aminomethyl) benzyl] acetamidine;**
inducible nitric oxide synthase;
nitric oxide;
peroxynitrite;
preoligodendrocyte

Introduction

Periventricular leukomalacia (PVL), a most common neuropathologic form of brain injuries in premature infants, is the major cause of cerebral palsy, cognitive and behavioral deficiency later on. The pathogenesis of PVL is believed to be initiated by two key upstream mechanisms: hypoxia-ischemia and maternal-fetal infection/inflammation,[1] and the role of infection in the pathogenesis of PVL has aroused the interest of researchers in recent years. The studies have confirmed that there is a dramatic increase of nitric oxide (NO) level in the periventricular white matter during the inflammatory reaction of PVL,[2] suggesting that NO plays a pivotal role in the course while inducible nitric oxide synthase (iNOS) is the primary source of NO under pathophysiological condition. N-[3-(aminomethyl) benzyl] acetamidine (1400W), a potent highly selective inhibitor of iNOS, is commonly used in the treatment of cardio-cerebral-vascular diseases. However, 1400W used for PVL in preterm infants has not yet been reported. Based on the infective theory of PVL about lipopolysaccharide (LPS) induced activated microglial toxicity to preoligodendrocytes (preOLs), the present study aimed to determine the effect of 1400W on blocking the death pathway of preOLs induced by LPS-activated microglia. It is expected on the neurobiological basis to elucidate the precise mechanism of LPS-induced activated microglial

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toxicity to preOLs, and to seek an effective method for blocking the death pathway of preOLs induced by infective factors.

Methods

Preparation, culture and identification of cells

The mixed glias obtained from the cerebral cortex of two-day-old Sprague-Dawley (SD) neonatal rats (Shanghai SIPPR/BK Experimental Animal Co., China) were prepared using a modified selective detachment procedure. The care and use of laboratory animals were according to the institutional and national guide. The microglias (MGs) were isolated from the upper layer of mixed glias by shaking the flask for 2 hours at 200 rpm on an orbital shaker, maintained in Dulbecco's modified Eagle's medium-F12 mixture (DMEM/F12, 1:1) containing 10% fetal bovine serum (FBS) and cultured for 2-3 days. preOLs were isolated from the astrocytic layer of mixed glias in which MGs had been stripped by shaking the flask for 18-22 hours at 220 rpm on the orbital shaker. The purified preOLs were plated on poly D-lysine-coated culture plates and cultured for 5-7 days in serum-free medium consisting of DMEM/F12, 10 mmol/L HEPES, 0.1% bovine serum albumin, 5 μg/ml apo-transferrin, 20 nmol/L hydrocortisone, 10 nmol/L biotin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nmol/L selenium, 10 ng/mL platelet-derived growth factor (Protech systems, USA), and 10 ng/mL basic fibroblast growth factor (Protech Systems, USA). Cultures were identified using specific markers for MGs and preOLs. The MGs were stained with FITC-labelled isolection-B4 monoclonal antibody (Victor, USA) and the preOLs were labeled with O4 monoclonal antibody (R&D System, USA) for assessing the purity of MGs and preOLs.

Treatment and grouping

Co-cultural MGs and preOLs were divided into three groups: co-culture control group, co-culture LPS (SIGMA, USA) group, and co-culture LPS plus 1400W group. LPS (100 ng/ml) was added to the upper compartment of the transwell in the co-culture group, and LPS (100 ng/ml) and 1400W (10 μmol/L) were added to the upper compartment of the transwell in the co-culture LPS plus 1400W group. All lower compartments of the transwells in the three groups were fed with serum-free chemically defined medium. After the co-culture cells were incubated for 48 hours in the three groups, the medium supernatant and protein of all cultures were collected for further determination. Besides, preOLs were separately cultured to serve as the single-culture control group.

Determination of NO level by nitrate reductase-colorimetry method

NO level was determined by nitrate reductase-colorimetry method. The level of NO in the cultures was measured using a nitric oxide detection kit (Nanjing Jiancheng Biotechnology Institute, China) following the manufacturer's protocol.

Detection of iNOS level by western blot analysis

After cultures were induced by LPS for 48 hours, the total protein in MGs was extracted. The equal amount of protein (25 μg) was separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membrane. Then the PVDF membrane was blocked with 5% non-fat milk for one hour, and incubated with rabbit polyclonal anti-rat iNOS polyclonal antibodies (Stressgen, USA) at 4°C overnight at a dilution of 1:1000. After several rinses, the PVDF membrane was incubated with secondary antibody goat anti-rabbit IgG (conjugated to horseradish peroxidase, Jacson, USA) (1:2000) for 2 hours at room temperature. Specific protein was detected using ECL protein detection kit (Pierce Biotechnology, Inc, USA) and image analysis was performed using Image Pro (Carlsbad, CA). The stable expressive β-tubulin was used as a control.

Detection of 3-nitrotyrosine by immunocytochemistry

Based on the theory that the tyrosine residues of proteins could be nitrified by peroxynitrite ($ONOO^-$), resulted in the formation of 3-nitrotyrosine (3-NT), a stable biochemical marker of peroxynitrite. The immune staining method was used to detect the level of 3-NT. PreOLs were stained with 3-NT, a marker of peroxynitrite. Cultures were fixed with 4% paraformaldehyde for 30 minutes, and then incubated with 1% bovine serum albumin (BSA) and 0.3% triton X-100 for one hour at room temperature. Afterward the cultures were incubated with anti-nitrotyrosine polyclonal antibody for 2 hours. The primary antibody was detected using a FITC-labelled secondary antibody (Upstate, USA). 3-NT positive cells were visualized and counted at 200 × magnification under a fluorescence microscope. Cell survival was expressed as mean ± SD.

Analysis of apoptotic rate of preOLs

The apoptotic rate of preOLs was analyzed by flow cytometry and Annexin V/FITC kit (Bend Med Systems, USA). After cultures were induced by LPS for 48 hours, the 0.125% trypson was used to digest cells and then be terminated by 10% FBS, all preOLs were collected for further detection. After treatment, the cells were collected by the low speed centrifugation (1200

rpm) and rinsed with ice-cold PBS, and then recollected by the centrifugation. After rinsing with PBS twice, the cells were incubated in 5 μl annexin V-FITC and 10 μl propidium iodine (PI) at room temperature in darkness for 15 minutes. Finally the cells were analyzed by flow cytometry.

Statistical analysis

Differences between obtained values (mean±SD) were determined by one-way analysis of variance (ANOVA) followed by the S-N-K multiple comparison test. *P* value less than 0.05 was considered statistically significant.

Results

Identification of cultural MGs and preOLs

B4-positive MGs were small, round and amebiform, and O4-positive preOLs were bipolar, tripolar and poorly branched under a fluorescence microscope. The immunocytochemical staining of special FITC-labelled IB4 and O4 (anti-oligodendrocytes marker 4) was used to identify MGs (green immunofluorescence) (Fig. 1A) and preOLs (yellow immunofluorescence) (Fig. 1B), respectively. The nuclei of MGs and preOLs were counterstained with DAPI. Cells counting under a fluorescence microscope showed that the purities of cultures were >90% for IB4-positive MGs and >95% for O4-positive preOLs.

1400W decreased the production of NO

There was an obvious increase in the level of NO induced by LPS for 48 hours (167.86±9.87 μmol/L) compared to the co-culture control group (82.27±3.41 μmol/L) ($P<0.01$). 1400W decreased the level of NO significantly (69.55±5.07 μmol/L) with a statistical difference in the level of NO between the co-culture LPS group and the co-culture LPS plus 1400W group ($P<0.01$).

1400W inhibited the synthetic level of iNOS

The relative synthetic concentration of iNOS was 0.18±0.027 in the co-culture control group, 0.79±0.068 in the co-culture LPS group, and 0.35±0.042 in the co-culture LPS plus 1400W group (Fig. 2). There was a significant increase in the synthetic level of iNOS in the co-culture LPS group compared with the co-culture control group ($P<0.01$). However, 1400W decreased the synthetic level of iNOS significantly ($P<0.01$). There was no statistic difference between the co-culture control group and the co-culture LPS plus 1400W group ($P>0.05$).

1400W blocked the formation of peroxynitrite

There was a significant increase of 3-NT positive cells in the co-culture LPS group (34.38±7.75) compared with the co-culture control group (6.14±1.27, $P<0.01$), whereas 3-NT positive cells decreased remarkably in the co-culture LPS plus 1400W group (10.33±3.47) compared with the co-culture LPS group ($P<0.01$) (Fig. 3).

1400W reduced the apoptosis of preOLs

The significant increase of apoptotic preOLs was observed in the co-culture LPS group (24.77±2.05%) compared with either the single-culture control group (3.17±1.11%) or the co-culture control group (6.73±1.39%) ($P<0.001$ for all). However, the death of preOLs was significantly inhibited by the treatment of 1400W (11.80±2.06%, $P=0.002$) (Table).

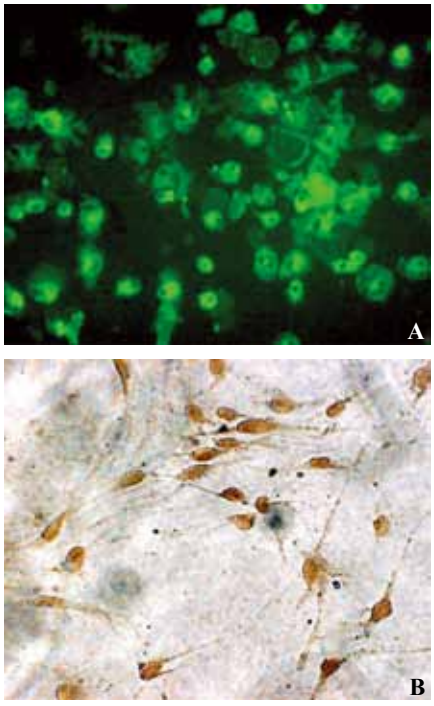

Fig. 1. The immunocytochemistry of cutured preOLs and MGs. **A:** IB4-positive MGs; **B:** O4-positive preOLs. (original magnification × 200)

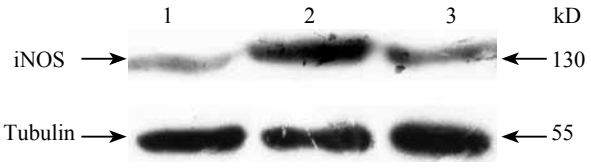

Fig. 2. The synthesis of iNOS detected by western blotting. Tubulin is used as the control. Lane 1: the co-culture group; Lane 2: the co-culture LPS group; Lane 3: the co-culture LPS plus 1400W group. There is a significant decrease in the level of iNOS with the use of 1400W.

Discussion

Recent studies have shown that infection/inflammation is generally involved in the pathogenesis of brain injuries.[3] The underlying mechanism is considered that infective factors can activate MGs to release toxic chemical substances, which are the primary pathway of infection mediating either the damage or the death of preOLs. Among all chemical substances released from MGs, superoxide (O_2^-) and NO play key roles in the death pathway of preOLs.[4-5]

As the resident macrophage-like cells of the central nervous system (CNS), microglias contribute to the innate immune response of the CNS.[6] Under the physiological condition, microglia is at rest and does not play any role. However, it can be soon activated by the infective factors such as LPS, and impels the activation of proteases and the release of serial proinflammatory molecules, especially induces significantly the up-regulative expression of iNOS and NADPH oxidase that results in the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS).[7-8] NADPH oxidase-derived O_2^- reacts with iNOS-derived NO to form more potent byproduct, $ONOO^-$, which is considered to be a primary toxic factor to the death of preOLs. Inhibiting or reducing the production of NO and O_2^- in MGs will probably protect effectively preOLs against infection.[9,10]

NO which produces from L-arginine and oxygen by the catalysis of nitric oxide synthase (NOS) can act as an unique signaling molecule and as an effective molecule.[11] It is well known that there are three types of NOS isoforms consisting of neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Two constitutive eNOS and nNOS exist mainly in the normal neuronal tissue and the endothelial cells, and the activation of eNOS and nNOS must depend on the concentration of intracellular calcium and the combination of calcium with calmodulin. Ischemic hypoxia (HI) can result in intracellular accumulation of calcium, induce the rapid expression of these two

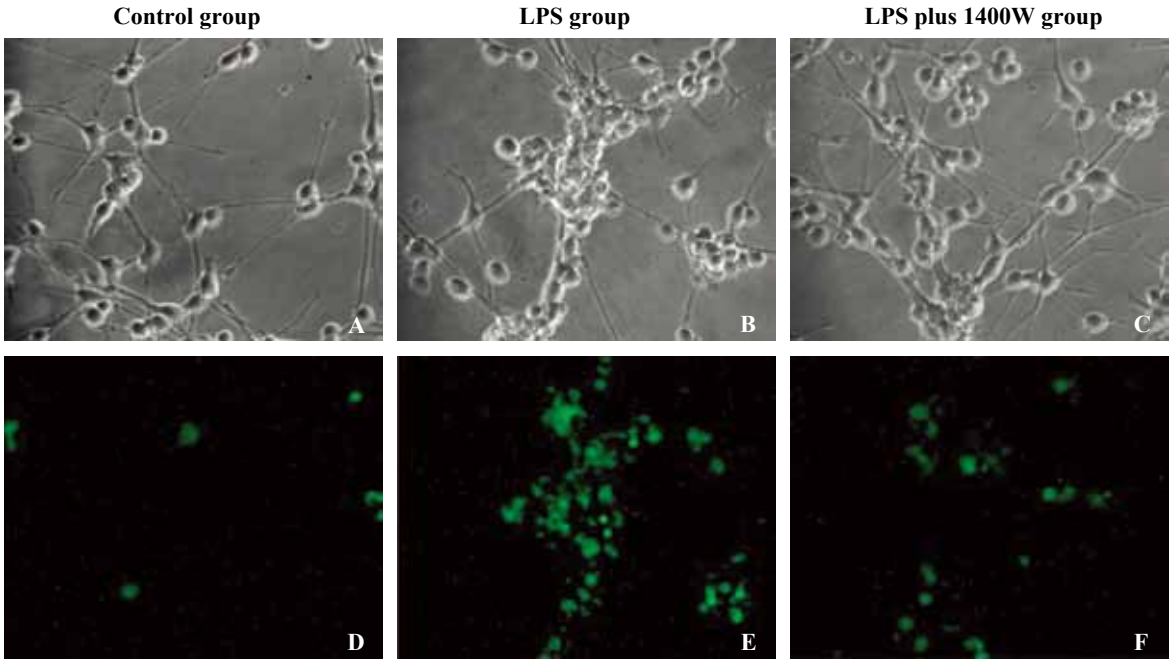

Fig. 3. 3-NT positive immunofluorescence in the three groups (original magnification × 400). **A-C:** cultured preOLs; **D-F:** 3-NT positive preOLs with green fluorescence. The increased 3-NT positive preOLs in the co-culture LPS group are significantly inhibited with the use of 1400W in the co-culture LPS plus 1400W group.

Table. Effect of 1400W on the inhibition of preOLs apoptosis induced by lipopolysaccharide (LPS) (%)

Group	Normal cells	Apoptotic cells	Necrotic cells
Single-culture control	95.47±1.30	3.17±1.11	1.40±0.27
Co-culture control	91.33±1.89	6.73±1.39	1.73±0.51
Co-culture LPS*	72.27±2.71	24.77±2.05	3.30±0.70
Co-culture LPS plus 1400W†	84.87±1.76	11.80±2.06	2.30±1.15

*: $P<0.01$, comparison between the co-culture LPS group and the co-culture control group; †: $P<0.01$, comparison between the co-culture LPS plus 1400W group and the co-culture control group.

calcium-dependent nNOS and eNOS within 15-20 minutes and the excessive production of NO to a toxic level within 4 hours after HI. However, iNOS is different from nNOS and eNOS, which is a soluble synthase at a low or undetected level in the normal resting cells. iNOS has two main features: 1) iNOS is a calcium-independent synthase, and is usually activated by inflammatory factors such as LPS; 2) Unlike nNOS and eNOS with an outbreak production of NO within 4 hours after HI, the generation of NO catalyzed by iNOS is a lasting and slow course. The microglias activated by inflammatory factors are able to initiate the genic transcription and its product synthesis of iNOS.[12] The overproduction of NO catalyzed by iNOS may result in a severe neurotoxicity to nerve cells. The up-regulative expression of iNOS in microglias is the main source of the infection-mediated overproduction of NO. In the present study, LPS was used as an inflammatory factor to activate and impel microglias to induce the expression of iNOS and the production of NO. Based on the feature of iNOS to produce NO with a lasting course, we chose 48 hours after LPS induction as the time point of the research.

1400W, a potent highly selective iNOS inhibitor, can be slowly and selectively bound with iNOS *in vivo*. It was reported that 1400W was 200- to 500-fold selective for iNOS than eNOS in a rat model of endotoxin-induced vascular injury.[13] As a main component of bacterial outer membrane, LPS has potent immune-induced activity. The present study demonstrated that there was an obvious increase in the levels of NO and iNOS in the co-cultural MGs and preOLs induced by LPS for 48 hours. However, the levels of NO and iNOS all decreased significantly after the use of 1400W, suggesting a remarkable effect of 1400W on blocking iNOS. In this study, the concentration of NO in the co-culture LPS plus 1400W group was lower than that in the co-culture control group. The result may be due to the following reasons: 1) The cell cultural procedure *in vitro* may lead to a partial activation of microglia and induce the expression of iNOS; 2) Apart from potent selective inhibition for iNOS, 1400W can also have a weak or partial inhibition for the other two NOS isoforms. The present study further demonstrated that with the use of 1400W, the decrease of NO concentration was more obvious than the expression of iNOS in MGs. It is presumed that in addition to inhibiting the gene expression and the protein synthesis of iNOS, there may be other pathways for 1400W to restrict the production of NO. 1400W was reported to have interrupted a cycle begetting further inflammation, improved the iNOS-mediated cytotoxicity to cells, blocked the further release of cytokines and the recruitment of inflammatory cells, and inhibited the expression of iNOS.[14] Further studies will be required to clarify the precise mechanisms of action by which 1400W inhibits the production of NO.

Peroxynitrite has been confirmed as a primary toxic factor mediating LPS-induced activated microglia to kill preOLs. It is a highly reactive oxidant reacting with various biomolecules such as protein nitration, lipid peroxidation, and DNA brand breakage, which are complicated as the major pathway leading to the injury, apoptosis and death of preOLs. Thiyagarajan's study[15] showed that activated microglia to preOLs injury could be partially blocked by the inhibitors of the selective iNOS and NADPH oxidase, whereas the decomposition catalysts of peroxynitrite, 5-, 10-, 15-, 20-tetrakis (N-methyl-40-pyridyl) porphyrinato iron (III) (FeTMPyP), could block completely LPS-induced preOLs injury.

One important reaction of $ONOO^-$ is to nitrify the tyrosine residues of proteins, and to form 3-nitrotyrosine, which is found to be a stable specific footprint of $ONOO^-$.[16] The relative level of $ONOO^-$ can hence be estimated either *in vitro*[16] or *in vivo*[17] by detecting the formation of 3-NT. In the present study there was an obvious increase of 3-NT positive cells in the co-culture LPS group after the co-cultural cells were induced by LPS for 48 hours, suggesting that the stimulation of LPS leads to the accumulation of cytoplasmic $ONOO^-$. The result is similar to that reported.[18,19] When a small amount of 3-NT-positive preOLs was observed with the use of 1400W, 1400W could reduce the formation of $ONOO^-$ by inhibiting iNOS and its generation of NO, while blocking the toxicity in the death pathway of preOLs induced by LPS. The present study also confirmed that the use of 1400W decreases significantly the apoptotic rate of preOLs induced by LPS, which further supports the protective effect of 1400W on preOLs.

In summary, 1400W can block effectively the LPS-induced microglial toxicity to preOLs by inhibiting iNOS specifically, resulting in a significant reduction of toxicity parameters and a marked increase of the survived preOLs. The effect was also confirmed in an *in vivo* experiment of our serial studies.[18] However, the long-term neuroprotective effect and suitable therapeutic time of 1400W need further study in the future.

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Contributors: He YF wrote the first draft of this paper. Chen HJ proposed the study and edited the manuscript. All authors contributed to the intellectual content. Chen HJ is the guarantor.

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