Hydrophobic dipeptide Leucine-Isoleucine inhibits brain damage caused by hypoxia-ischemia/reperfusion in the newborn rat

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Abstract

The immunosuppressant drug FK506 has been reported to have neuroprotective effects against the hypoxic ischemic encephalopathy in a newborn rat model. However, there are systemic immunosuppressant side effects, which might prevent the use of FK506 for human neonates clinically. Hydrophobic dipeptide Leucine-Isoleucine (Leu-Ile) has a binding site similar to that of FK506, binding immunophilin and protecting against neuronal death without immunosuppressant actions. The aim of this study was to examine the neuroprotective effects of Leu-Ile to hypoxia-ischemia (HI)/reperfusion brain damage in a neonatal rat model. In 7-day-old Wistar rats (n=24), HI/reperfusion was induced by transient left carotid artery occlusion for 120 min, followed by 60 min of 8% O2. The rats were then resuscitated by releasing the carotid artery occlusion and were reoxygenated in room air. After the HI/reperfusion insult, the rats received Leu-Ile (Leu-Ile group, n=13) or only vehicle (vehicle group, n=11) every 24 hrs for 7 days. At 8 days after HI, hemispheric brainweight, and myelin basic protein (MBP) positive area analysis showed that the ratio of the left to the right hemispheres in the Leu-Ile group was significantly higher than that in the vehicle-only group (p<0.01). Following ionized calcium binding adapter molecule 1 (Iba-1) staining, the Leu-Ile group significantly suppressed the increase of activated microglia cells (p<0.01). There were no significant differences in body weight gains and mortality rates between the Leu-Ile and the vehicle groups during the 8-day experiment. We showed the neuroprotective effects of therapy with Leu-Ile to HI/reperfusion brain-damage without side effects in a neonatal rat model.

Introduction

The mortality rate of neonates is decreasing due to improvements in neonatal intensive care. However, hypoxic ischemic encephalopathy (HIE) still remains a major cause of neonatal death and neurological problems. It is considered that hypoxia-ischemia and later cerebral reperfusion induce various molecular biological mechanisms related to neuronal cell death resulting in cerebral disorders associated with HIE. No effective treatment has been established for HIE accompanying neonatal asphyxia.

In 1994, Sharkaly et al. reported the neuroprotective efficacy of FK506 as an immunosuppressive agent in a rat cerebral infarction model. Thereafter, the neuroprotective efficacy of FK506 has been reported in many adult animal cerebral infarction models. FK506 is used clinically to inhibit the rejection of transplanted organs in a wide variety of patients including infants. The term immunophilin is used for receptors of immunosuppressant drugs, and FK506 is one such drug. The immunosuppressive mechanism is thought to derive from the suppression of T lymphocyte activation by a complex formed between FK506 and its binding protein (FKBP12), i.e. the FK506-FKBP12 complex, which targets calcineurin, a phosphatase known to control...
calcium influx into cells under physiological conditions, mediated by the IP3 receptor, NMDA receptor and ion channels\(^{20}\). Furthermore, our group demonstrated for the first time that FK506 has neuroprotective effects in an HIE newborn rat model. However, systemic immunosuppressant side effects such as growth-suppression and death caused by the drug have also been reported, which might be obstacles to the use of FK506 for human neonates clinically\(^{99}\).

Hydrophobic dipeptide Leucine-Isoleucine (Leu-Ile) resembles some part of the binding site on FK506 for immunophilin-FKBP12. Nitta et al. reported that Leu-Ile protected against natural neuronal cell death among cultured mesencephalic cells; Leu-Ile reduced the number of turns methamphetamine induced mice to turn around 360° and; Leu-Ile has no immunosuppressive effect because Leu-Ile is not inhibitory toward calcineurin in cellular activity in cultured neuronal cells. Nitta et al. concluded that Leu-Ile neuroprotective effects induce synthesis of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), which are part of the neuroprotective mechanism\(^{10}\). However, the neuroprotective effects of Leu-Ile for HIE in a newborn rat model has not been demonstrated, and its exact neuroprotective mechanism is unknown. Nevertheless it is possible that Leu-Ile has neuroprotective effects for neonatal HIE without systemic immunosuppressant side effects.

On the other hand, Ito et al. reported that activated microglia increase after transient focal cerebral ischemia in rat brain\(^{11}\). Microglia constitute approximately 5% of glial cells in the central nervous system\(^{12}\). Activation of microglia commonly occurs in the early response in the central nervous system to a wide variety of pathological stimuli, including axotomy, trauma, inflammation, and ischemia\(^{13-19}\). Microglias have been known to exert dual effects, e.g. neurotrophic and neurotoxic, on the central nervous system\(^{10}\). However, the exact neurotrophic or neurotoxic action of microglia is not fully understood among the various kinds of neonatal brain damage caused by HIE. Therefore, we focused on the activation of microglia playing a major role for neurotrophic and neurotoxic action in the central nervous system. Whether Leu-Ile has neuroprotective effect against neonatal brain damage, it is expected that activated microglia are affected by the neuroprotective mechanism of Leu-Ile.

The first purpose of the present study was to investigate the neuroprotective effects and efficacy of Leu-Ile administration after HI/reperfusion insult by assessment of the decrease in brain weights in neonatal HIE animal models; secondly we studied the mechanism of the neuroprotective effects of Leu-Ile by evaluating of the myelin and the activated microglia; lastly, we estimated the side effects of Leu-Ile by evaluating the systemic status of experimental animals such as nutrition and mortality rates.

**Materials and Methods**

1. **Animal preparation**

Wistar dams and their litters were purchased from CLEA Japan Inc (Tokyo, Japan) and maintained on a 12-h cycle of light and dark with food and water freely available.

Seven-day-old rats of either sex (n = 24), weighing 13.7-17.7 g, were removed from the litters for preparation and study, and returned to be suckled by their dams at all other times. All experimental procedures were carried out in strict accordance with the guidelines of the Animal Ethical Committee of Tokyo Medical University. We adopted HI/reperfusion brain insult for the neonatal HIE animal models. Rats were anesthetized with isoflurane inhalation, and the left carotid artery was accessed through a ventral midline cervical incision and transiently occluded with Sugita aneurysm clips (Mizuhco co., Ltd, Tokyo, Japan) for 120 min and the neck incision was closed. The entire surgical procedure lasted no longer than 10 min. Rats were then held in an airtight 500 ml plastic chamber through which humidified 8% oxygen/92% nitrogen gas flowed for 60 min. After the HI insult, the rats were then resuscitated by releasing the carotid occlusion and reoxygenation with room air. Rectal temperatures of all animals in the chamber were continuously maintained at 36.5 to 37.0°C automatically by means of a temperature controller system (ATC-101B, Unique Medical Co. Tokyo, Japan) to avoid hypothermia throughout the experiment. The rats were returned to their dams for stabilization for at least 60 min after the surgery.

2. **Experimental protocol**

After the HI insult, the animals (n = 24) were intraperitoneally randomly given either 1.5 mg/kg Leu-Ile (Kokusan Chemical Co. Ltd, Tokyo, Japan) (dissolved in 0.1 ml/kg normal saline, n = 13) or the same amount of only vehicle (0.1 ml/kg normal saline, n = 11). The animals received Leu-Ile or only vehicle every day for 7 days.

After drug administration, all animals were then returned to their dams to be cared for and suckled for 8 days.

3. **Pathology**

**Experiment**

At 8 days after the HI insult, blood was taken transcardially from all animals for histological examination of their brains under deep anesthesia with isoflurane inhalation. The brains were immediately removed intact and then cut into left and right hemispheres and the weight of each was measured. Then the cerebral

hemispheres were placed in 4% paraformaldehyde in 0.1 M phosphatase buffer, pH 7.4, for one week. The brains were then embedded in paraffin and cut coronally into 4-μm-thick slices. Every coronal section was stained with myelin basic protein (MBP), and ionized calcium-binding adapter molecule 1 (Iba1).

**MBP staining**

Sections were deparaffinized and rehydrated, followed by washing twice in distilled water and in PBS for 5 min. To block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H2O2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then anti-MBP polyclonal antibody (Nichirei Co., Ltd., Tokyo, Japan) was added to each section, which was incubated overnight at 4°C. After washing with PBS, sections were processed by N-Histofine® Simple Stain Max PO (M) (Nichirei Co.). The manufacturer’s instructions were followed exactly. Counterstaining was performed with the standard hematoxylin method.

**Iba-1 staining**

After deparaffinization, the sections were placed in the autoclave (105°C, 10 min) with citric acid buffer (pH = 6.0, 0.01 mol). To block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H2O2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then rabbit anti Iba1 polyclonal antibody (Wako Pure Chemicals Co., Ltd., Osaka, Japan) was added to each section, which was incubated overnight at 4°C. After the incubation, the staining was made using the LSAB kit (Dako, Cytomation Co., Ltd., Denmark) as a secondary antibody. The manufacturer’s instructions were followed exactly. Counterstaining was performed with the standard hematoxylin method.

**Ratio of left to right hemispheric brain weight**

In order to assess the severity of the whole brain damage, the ratio of left (with lesion) to right (without lesion) hemispheric brain weight [left hemispheric brain weight ÷ right hemispheric brain weight] was assessed.

**Ratio of left to right MBP positive hemispheric area**

In order to assess the mechanism of brain damage, the hemispheres with and without lesions of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Ltd., Tokyo, Japan) to measure the MBP-positive area (unit: square pixels) using an image analyzer system (Scion Image, Scion Co.). The ratio of left to right MBP-positive hemispheric area [MBP-positive area of left hemisphere ÷ that of right hemisphere] was assessed.

**Body weight gain and mortality**

We also evaluated mortality rates and nutritional status during the experiment for 8 days [weight gain (g) = weight on the 7th day-weight on the first day].

**4. Statistical analysis**

The Mann-Whitney U-test was used to examine the brain damage between the Leu-Ile and vehicle groups. A value of p<0.05 was considered statistically significant. The results were expressed as means and SD.

**Results**

There was no significant difference in body weight between the vehicle group (15.7±0.3 g) and Leu-Ile (15.6±0.3 g) group before HI/reperfusion insult. The ratio of the left to the right cerebral hemispheric weight in the vehicle group was significantly lower than that in the vehicle group (0.81±0.04) (p=0.0013), which means the severity of the brain damage in the Leu-Ile group was significantly lower than that in the vehicle group (Fig. 1).

**Ratio of left to right MBP positive hemispheric area**

Figure 2-a, b, c, d shows typical histological examples of anti-MBP antibody stain in the vehicle group (Fig. 2-a, b), and the Leu-Ile group (Fig. 2-c, d) rats. In the vehicle group, there was a significant decrease in the intensity of staining as well as the number of MBP-positive axons in the left hemisphere (Fig. 2-b). On the
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<th>No.</th>
<th>Weight of Left Brain Hemisphere (g)</th>
<th>Weight of Right Brain Hemisphere (g)</th>
<th>Left MBP Positive Hemispheric Area (square pixels)</th>
<th>Right MBP Positive Hemispheric Area (square pixels)</th>
<th>Left Iba1 Positive Hemispheric Area (square pixels)</th>
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| Mean | 0.392±0.020 | 0.484±0.005 | 4,266,899±452,012 | 8,900,835±951,453 | 5,041,673±1,040,788 | 1,963,622±204,696 | 14.1±0.6 |

<table>
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<tr>
<th>No.</th>
<th>Weight of Left Brain Hemisphere (g)</th>
<th>Weight of Right Brain Hemisphere (g)</th>
<th>Left MBP Positive Hemispheric Area (square pixels)</th>
<th>Right MBP Positive Hemispheric Area (square pixels)</th>
<th>Left Iba1 Positive Hemispheric Area (square pixels)</th>
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| Mean | 0.469±0.009 | 0.485±0.006 | 6,721,753±766,857 | 6,478,401±839,072 | 2,078,247±196,306 | 1,734,104±169,405 | 16.4±0.4 |
Fig. 2 Typical histological photomicrographs of MBP stain (a, b, c, d) and Iba1 stain (e, f, g, h) in both cerebral hemispheres (a, c, e, and g are right hemispheres, b, d, f, and h are left hemispheres) including the hippocampus, cerebral cortex and basal ganglia in the vehicle-only-treated (a, b, c, f), the Leu-Ile-treated (c, d, g, h), of 8 days after HI insult.

The ratio of the left to the right MBP-positive hemispheric area in the Leu-Ile group (1.10±0.07) was significantly higher than that in the vehicle group (0.55±0.27) (p=0.00148) (Fig. 3).

**Ratio of left to right Iba1 positive hemispheric area**

Figure 2-e, f, g, h shows typical histological examples of anti-Iba1 antibody stain in the vehicle group (Fig. 2-e, f), and the Leu-Ile group (Fig. 2-g, h) rats. In the vehicle group, there was a significant increase in the intensity of staining as well as in the number of activated microglia in the left hemisphere (Fig. 2-f). On the contrary, in the Leu-Ile group, there was a very slight increase in the left hemisphere (Fig. 2-h).

In the vehicle group, the left Iba1-positive hemispheric area and right were 5,041,673±1,040,788 square pixels and 1,963,622±204,696 square pixels. In the Leu-Ile group, the left Iba1-positive hemispheric area and right were 2,078,247±196,306 square pixels and 1,734,104±169,405 square pixels (Table 1).

The ratio of the left to the right Iba1-positive hemispheric area in the Leu-Ile group (1.21±0.06) was significantly lower than that in the vehicle group (2.45±0.40) (p=0.001938) (Fig. 4).
Leu-Ile administration significantly attenuated the decrease of brain weight in the present experiment, suggesting that Leu-Ile suppressed neuronal cell death in neonatal HIE animals. The neuroprotective mechanism of Leu-Ile has been suggested to be induction of GDNF and BDNF synthesis, which results in blocking neuronal apoptosis. The mechanism of GDNF induction by Leu-Ile has been reported to be through Leu-Ile targeting the heat shock cognate protein 70 (Hsc70)/heat shock protein 90 (Hsp90) cochaperone and, thus, triggering the Akt/cAMP response element-binding protein (CREB) signaling, resulting in upregulation of GDNF expression.

Kitagawa et al. reported that GDNF, a member of the TGF-β superfamily, reduced infarct size after permanent middle cerebral artery occlusion in adult rats, and was associated with the reduction of apoptotic signals predominantly through caspases-1 and -3 cascades. BDNF is also one of the members of the neurophin family of proteins, which includes nerve growth factor (NGF), neurophin-3 (NT-3), NT-4/5, and NT-6. Han et al. reported that BDNF also blocks caspase-3 activation in neonatal HI (permanent occlusion) model. Accordingly, we speculated that the mechanism of the neuroprotection is that increases in both GDNF and BDNF by the Leu-Ile administration may block the activation of the apoptotic pathway. Both GDNF and BDNF themselves are macromolecules that are unable to pass the blood-brain-barrier (BBB), therefore, neither GDNF nor BDNF can reach the brain tissue when they are peripherally administered. In contrast, because of its small hydrophobic molecule, Leu-Ile is thought to reach the brain tissue even when they are administered from the peripheral vein.

Our study also demonstrated that Leu-Ile administration attenuated the decrease of the MBP-positive area, suggesting that Leu-Ile has protective effects for white matter against HIE in newborn rats. In addition, the evaluation of MBP and Iba-1 staining elucidated that the Leu-Ile administrations attenuated the demyelination and the activated microglia, respectively, in the neonatal HIE models. Microglia show a rapid activation in response to even minor pathological changes, i.e. infection, inflammation, trauma, brain tumors and neurodegeneration, in the central nervous system.

Microglia has been known to exert dual neurotrophic and neurotoxic effects on the central nervous system. The activated microglia are involved in neuroprotection by secretion of several neurotrophins and by elimination of microorganisms and deleterious debris. On the other hand, activated microglia induce neuronal cell death by releasing reactive oxygen species, nitric oxide, or...
inflammatory cytokines\textsuperscript{16\textendash}19. In particular, demyelination is caused by activated microglia, because of apoptosis or degeneration of oligodendroglia which are known as the main component of white matter and the only cell that produces myelin\textsuperscript{20}. The neuroprotective mechanism for white matter is presumed to be the inhibition of activated microglia leading to a decrease in the neurotoxic factors, i.e. reactive oxygen species, nitric oxide, or inflammatory cytokines, and in demyelination.

In the present study, Leu-Ile administration had a neuroprotective effect in a neonatal rat HIE model, without any side effects, such as growth-suppression or death, which were observed in animals receiving FK506. Nakata et al. reported that the combination therapy with FK506 and 7-nitronidazol (7-NI) yields neuroprotective effects in HI/reperfusion neonatal rats, but the weight gains of rats given FK506 and 7-NI every 24 hrs for 7 days was lower than rats receiving vehicle alone, and the mortality rate of the combination therapy group was higher than the vehicle-only group\textsuperscript{21}. Furthermore, as the FK506 is dose-dependently neuroprotective, the greater the dose given to obtain a better outcome, the greater the appearance of inconvenient side effects, due to systemic immunosuppression. The inhibition of calcineurin activity, leading to suppression of T lymphocyte activation\textsuperscript{22,23}, causes the immunosuppression of FK506. However, Leu-Ile is not inhibitory of calcineurin activity, and does not induce immunosuppression\textsuperscript{24}. Therefore, administration of Leu-Ile for neonatal HIE may be more useful and safe than FK506 with respect to systemic immunosuppression.

**Conclusion**

In a neonatal rat HI/reperfusion model, we observed neuroprotective effects of Leu-Ile at 8 days after insult. The weight gains and the mortality rates during the experiment for 8 days were not significant between the Leu-Ile and the vehicle-only groups. The findings obtained in the current study indicate the possibility of the clinical applications of Leu-Ile to neonatal HIE.

**Acknowledgments**

The authors thank Prof. Kudo, Mr. Ishikawa, Mr. Fujita, and Ms. Yamada, of the Department of Pathology, Tokyo Medical University, for useful technical advice concerning histology. The authors are indebted to Prof. J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

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**References**

1) Sharkey J, Butcher SP: Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischemia. Nature \textbf{371}: 336\textendash}339, 1994
8) Nakada C, Takei Y, Hoshika A: Combination therapy with FK506 and 7-nitronitrosoimidazol inhibits brain damage due to hypoxia-ischemic/reperfusion in the newborn rat. J Tokyo Med Univ \textbf{64}: 368\textendash}379, 2006

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