Propofol Ameliorates Ischemic Brain Injury by Blocking Toll-like Receptor 4-dependent Pathway and Suppressing Consequent Inflammatory Cytokine Production

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Abstract

Background

Ischemic stroke is one of the leading causes of mortality and morbidity worldwide. Accumulated evidence suggests that the consequent excessive inflammation plays detrimental roles in the pathogenesis of secondary injury after cerebral infarction and exacerbates the brain tissue damage. Although regulation of the inflammation would be the potential strategy for the novel treatment option, effective methods that control the cerebral inflammation have not yet been established. Recent studies have suggested that propofol, a sedative agent widely used for management of patients with acute stroke, suppresses excessive inflammation and may have neuroprotective effects against ischemic brain injury. However, the available evidence is still limited and controversial, and the underlying mechanism remains unclear. This study aimed to investigate the neuroprotective effects of propofol against ischemic brain injury, with a specific focus on Toll-like receptor 4 (TLR4), the critical mediator of inflammation in the ischemic brain.

Results

Treatment with propofol significantly reduced infarct volume in wild-type mice (7.9 ± 1.4 vs. 12.6 ± 1.1 mm$^3$, n = 10 each, p < 0.05). The propofol-treated mice exhibited lower levels of pro-inflammatory cytokine expressions compared with the control mice (IL-6: 0.57 ± 0.23 vs. 1.00 ± 0.39, p < 0.05, IL-1β: 0.53 ± 0.24 vs. 1.00 ± 0.36, p = 0.087, n = 15 each). The neuroprotective effect of propofol was abrogated by TLR4 gene knockout. Propofol treatment had no significant effects on hemodynamic parameters.

Conclusions

Propofol attenuates brain injury by blocking the TLR4-dependent pathway and suppressing pro-inflammatory cytokine production. This insight into the mechanism underlying the neuroprotective effect of propofol against ischemic brain injury may lead to a new strategy for preventing exacerbation of cerebral infarction.

Background

Ischemic stroke often results in irreversible brain tissue damage and neuronal dysfunction and has become one of the leading causes of death and long-term disability worldwide. Outcomes of ischemic stroke has been improved over the past decades, and the thrombolytic therapy with tissue plasminogen activator (tPA) is currently the most efficacious therapeutic option. However, due to its narrow therapeutic windows and serious complication caused by reperfusion injury, the clinical use of tPA remains limited. Although thrombolytic therapies could reduce the initial volume of brain infarction, the restored blood flow triggers infiltration of inflammatory cells to the peri-infarct area, leading to excessive local inflammation and consequent tissue injury called secondary injury. Regulation of the brain inflammation would be the potential strategy for the novel treatment option. However, effective methods that control...
the cerebral inflammation have not yet been established. Novel pharmacological therapies that target the secondary injury is therefore desperately needed.

Ischemic brain injury is also one of the most serious potential complications during medical intervention under sedation/anesthesia. Surprisingly, in recent studies using magnetic resonance imaging, the incidence of ischemic brain injury associated with noncardiac surgery was as high as 10% in patients with cardiovascular risk factors. There are various causes including hypotension, hypoxia, arrhythmia, systemic inflammation, and blood loss that jeopardize cerebral circulation. In addition, because patients are unconscious during sedation/anesthesia, promptly detecting acute ischemic brain injury is challenging. Consequently, only a limited number of patients that are under sedation/anesthesia receive reperfusion therapy during the acute phase.

Propofol, a γ-aminobutyric acid agonist, provides short-acting sedative effects and has been widely used in various clinical situations, including sedation/anesthesia during medical interventions and management of patients with acute stroke. Some earlier studies using animal models of ischemic stroke have suggested the potential neuroprotective effects of propofol. The evidence is still limited and controversial however, and the mechanisms underlying the possible neuroprotective effects of propofol remain unclear. Several recent studies suggest that propofol blocks Toll-like receptor 4 (TLR4), the key mediator of inflammation, and suppresses inflammatory cytokine production. Given that inflammation is a crucial factor in secondary injury after brain ischemia, and that TLR4 plays a pivotal role in inflammatory response in the ischemic brain, the TLR4-dependent pathway may be involved in the possible neuroprotective effects of propofol reported in the literature.

Thus, in this study we tested the hypothesis that treatment with propofol attenuates ischemic brain injury via inhibition of the TLR4-dependent pathway and suppression of consequent inflammatory cytokine production. The primary outcome of this study was infarct volume; the secondary outcome is pro-inflammatory cytokine mRNA expressions.

**Methods**

**Animals**

Male C57BL/6 mice (Japan SLC, Tokyo, Japan) and male TLR4 knockout mice on a C57BL/6 genetic background (Oriental Bio Service, Kyoto, Japan) were used in the study. All mice were aged 8–12 weeks and weighed 20–25 g, and they were group-housed at 23 °C ± 2 °C with free access to food and water and a 12-hour light/dark cycle.

**Ischemic brain injury**

Focal brain ischemia was induced via a combination of permanent left common carotid artery occlusion and distal left middle cerebral artery (MCA) coagulation. Briefly, mice were anesthetized with 2–3% isoflurane and placed in a dorsal position. The left common carotid artery was isolated and ligated via
ventral middle neck incision. Mice were then placed in the lateral position, and a 2-mm burr-hole craniectomy was performed with a microdrill (Ideal Microdrill; Bio Research, Nagoya, Japan) between the left orbit and the left ear. The distal left MCA was exposed and coagulated using a small vessel cauterizer (Fine Science Tools, Inc., CA, USA) followed by transection of the artery. During the surgery, rectal temperature was maintained at 37 °C ± 0.5 °C with a thermostat-regulated heating pad. Brains were removed 24 hours after the induction of ischemic brain injury for real-time polymerase chain reaction (PCR) and at 7 days for measurement of infarct volume.

**Propofol treatment**

Propofol (10 mg/kg, 1% Diprivan, Aspen Japan, Tokyo, Japan) diluted with fat emulsion by 10 times (10 µL/g, Intralipos, Otsuka Pharmaceutical, Tokyo, Japan) was administered over 30 seconds via the tail vein 10 minutes prior to MCA occlusion. In control mice, an equal volume of the fat emulsion alone was administered in the same manner. The dose was chosen based on previously published data.\(^{14}\)

**Measurement of Infarct Volume**

Seven days after MCA occlusion and after hemodynamic measurements, mice were deeply anesthetized with 5% isoflurane and euthanized via cervical dislocation. Brains were removed and cut into 1-mm-thick coronal sections. The brain slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich, St. Louis, MO, USA) at 37 °C for 15 minutes in a dark room. The infarct area was traced and measured using image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA). To correct for the contribution of edema, the infarct volume was calculated as follows: \[1 – \frac{\text{total ipsilateral hemisphere} – \text{infarct region}}{\text{total contralateral hemisphere}}\] × 100%.\(^{15}\) Total infarct volume was calculated as the sum of all infarct areas multiplied by section thickness. For this assay, each group consisted of 10 mice.

**Hemodynamic measurements**

Heart rate and arterial blood pressure were measured non-invasively using a tail-cuff monitor (Softron, Tokyo, Japan) to evaluate the effects of propofol on hemodynamics. Values were recorded 1 hour before MCA occlusion (baseline), 10 minutes after the injection of either propofol or the control solution, and 1 hour after MCA occlusion.

**Real-time PCR**

Real-time PCR was used to measure mRNA expression levels of interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α). The target mRNAs from the brain slices of propofol-treated wild-type mice were compared with those of control wild-type mice. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for extraction of total mRNA from brain slices. One microgram of mRNA was reverse transcribed into complementary DNA with a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR was performed on a StepOne™ real-time PCR system (Life Technologies, Carlsbad, CA, USA) using the PowerSYBR® Green PCR Master Mix and corresponding primers to quantify target genes. The relative changes were expressed as a ratio to glyceraldehyde-3-phosphate dehydrogenases mRNA of the same sample. The data were analyzed using the \(2^{-\Delta\Delta CT}\) method. The \(2^{-\Delta\Delta CT}\) value of the target transcript from
each mouse was normalized with those of the control group mice as 1.0. The normalized $2^{-\Delta\Delta CT}$ values derived from the two groups were then compared ($n = 15$ each).

**Statistical analysis**

Statistical analysis was performed using Prism 7 software (GraphPad Software, San Diego, CA, USA). Infarct volume was assessed using two-tailed Student’s $t$ test; cytokine expression levels were assessed using Mann-Whitney $U$ test; heart rate and blood pressure were assessed using two-way analysis of variance for repeated measures. For infarct volume and cytokine expression level, the sample size was calculated to detect significance with 95% confidence, assuming alpha of 0.05 and power of 0.8 ($G^*\text{Power 3.1.9.3}$). Values are presented as mean ± standard error of mean for infarct volume, heart rate, and blood pressure, and median ± quantile for cytokine mRNA expression level. All were considered statistically significant for $p$-value of less than 0.05.

**Results**

**Propofol reduced cerebral infarct volume**

To investigate the effects of propofol on ischemic brain injury, 8–12-week-old wild-type mice were treated with either propofol or 10% fat emulsion (control) 10 minutes before the induction of ischemic brain injury. Propofol-treated mice exhibited significantly smaller infarct volumes than control mice 7 days after ischemic brain injury ($7.9 \pm 1.4$ vs. $12.6 \pm 1.1 \text{ mm}^3$, $n = 10$ each, $p < 0.05$; Fig. 1). Propofol treatment did not affect the hemodynamic parameters significantly (Table 1).
Table 1
Hemodynamic measurements

|                  | wild type + control | wild type + propofol | TLR4KO + control | TLR4KO + propofol |
|------------------|---------------------|----------------------|------------------|------------------|
| HR (bpm) baseline| 443 ± 54            | 423 ± 36             | 445 ± 64         | 465 ± 50         |
| 10 min before MCAO| 428 ± 29            | 448 ± 63             | 388 ± 26         | 367 ± 40         |
| 1 hour after MCAO| 442 ± 77            | 429 ± 84             | 375 ± 27         | 395 ± 20         |
| 7 days after MCAO| 473 ± 74            | 468 ± 64             | 421 ± 37         | 454 ± 41         |
| mean BP (mmHg) baseline| 77 ± 17          | 82 ± 9               | 79 ± 8           | 80 ± 8           |
| 10 min before MCAO| 74 ± 12             | 77 ± 9               | 81 ± 8           | 74 ± 12          |
| 1 hour after MCAO| 87 ± 14             | 76 ± 12              | 83 ± 4           | 80 ± 16          |
| 7 days after MCAO| 86 ± 8              | 80 ± 10              | 85 ± 8           | 83 ± 16          |

There were no significant differences in heart rate and blood pressure among each group.

Data were presented as mean ± standard error of mean. (n = 10 each)

HR, heart rate; MCAO, middle cerebral artery occlusion; TLR4KO, toll like receptor 4 knockout

Propofol treatment and reduced pro-inflammatory cytokine expressions after ischemic brain injury

To investigate the possible involvement of anti-inflammatory action of propofol in the neuroprotective effect observed, mRNA expression of pro-inflammatory cytokines was measured 24 hours after ischemic
brain injury. As shown in Fig. 2, the propofol-treated mice exhibited lower mRNA expression levels of pro-inflammatory cytokines (IL-6: $0.57 \pm 0.23$ vs. $1.00 \pm 0.39$, $p < 0.05$, IL-1$\beta$: $0.53 \pm 0.24$ vs. $1.00 \pm 0.36$, $p = 0.087$, $n = 15$ each) compared with the control mice.

**Neuroprotective effects of propofol were abolished by depletion of the TLR4-dependent pathway**

In experiments testing the effects of propofol treatment against ischemic brain injury in TLR4 knockout mice, there was no significant difference in the infarct volume between the propofol-treated TLR4 knockout mice and control-treated TLR4 knockout mice ($12.4 \pm 1.6 \text{ mm}^3$ vs. $12.1 \pm 2.0$, $n = 10$ each, Fig. 3). Similar to the experiments using wild-type mice, there were no significant differences in hemodynamic parameters between two groups (Table 2).

**Table 1: Hemodynamic measurements**

|                      | wild type   | wild type + propofol | TLR4KO + control | TLR4KO + propofol |
|----------------------|-------------|----------------------|-------------------|-------------------|
| HR (bpm)             |             |                      |                   |                   |
| baseline             | $443 \pm 54$| $423 \pm 36$         | $445 \pm 64$      | $465 \pm 50$      |
| 10 min before MCAO   | $428 \pm 29$| $448 \pm 63$         | $388 \pm 26$      | $367 \pm 40$      |
| 1 hour after MCAO    | $442 \pm 77$| $429 \pm 84$         | $375 \pm 27$      | $395 \pm 20$      |
| 7 days after MCAO    | $473 \pm 74$| $468 \pm 64$         | $421 \pm 37$      | $454 \pm 41$      |
| mean BP (mmHg)       |             |                      |                   |                   |
| baseline             | $77 \pm 17$ | $82 \pm 9$           | $79 \pm 8$        | $80 \pm 8$        |
| 10 min before MCAO   | $74 \pm 12$ | $77 \pm 9$           | $81 \pm 8$        | $74 \pm 12$       |
| 1 hour after MCAO    | $87 \pm 14$ | $76 \pm 12$          | $83 \pm 4$        | $80 \pm 16$       |
| 7 days after MCAO    | $86 \pm 8$  | $80 \pm 10$          | $85 \pm 8$        | $83 \pm 16$       |

There were no significant differences in heart rate and blood pressure among each group.
Data were presented as mean ± standard error of mean. (n = 10 each)

HR, heart rate; MCAO, middle cerebral artery occlusion; TLR4KO, toll like receptor 4 knockout

Discussion

In the present study, treatment with propofol was associated with reduced pro-inflammatory cytokine expressions at 24 hours after ischemic brain injury, the peak timepoint of those mRNA expression levels and attenuates ischemic brain injury, suggesting that propofol exerts neuroprotective effects via its anti-inflammatory capacity. Because propofol at the dose used did not affect the hemodynamic parameters investigated, the effects of propofol on cardiovascular function were not likely to be involved in the neuroprotection observed in the present study.

The immune system is promptly activated once ischemic brain injury occurs. Among various inflammatory mediators, TLR is considered crucial in innate immune system as a first-line defense and mediator of inflammation. Exogenous/endogenous TLR ligands such as heat shock proteins, fibrinogen, and components of the extracellular matrix are upregulated by ischemic brain insult. Consequently, those ligands then activate TLR4, the TLR shown to be the pivotal inflammatory mediator in the pathogenesis of ischemic brain injury. It has been reported that while activation of TLR4 exacerbates cerebral infarction, inhibition of the receptor suppresses pro-inflammatory responses and attenuates brain injury.

In the current study, the neuroprotective effect of propofol against ischemic brain injury was abrogated by depletion of TLR4, indicating that the TLR4-dependent pathway was substantially involved in the mechanism underlying that neuroprotective effect. The results of the current study are concordant with recent in vivo studies in which propofol suppressed inflammatory cytokine production via inhibition of TLR4-dependent pathways in various disease models, including lung, liver, and gastric injuries and asthma. Several in vitro studies using lipopolysaccharide-treated microglia, macrophages, spinal astrocytes, and alveolar epithelial cells also suggest that the anti-inflammatory action of propofol involves blocking the TLR4-dependent pathway and consequent pro-inflammatory cytokine production.

Our result showed significantly lower IL-6 mRNA expression levels in the brains of propofol-treated wild-type mice than in the brains of control mice, which is consistent with an earlier study in which there was a correlation between infarct volume and IL-6 mRNA expression in the brain. Among various pro-inflammatory cytokines, IL-6 plays pivotal roles in local inflammation and cytotoxicity after ischemic brain injury, and is involved in the mechanism underlying the expansion of ischemic brain injury. Blockade of IL-6 receptors has been shown to reduce infarct volume and improve cognitive function in an experimental model of ischemic stroke. These earlier studies are concordant with the suppression of IL-6 by propofol observed in the current study after ischemic brain insult, as well as the reduction of infarct volume.
Based on the collective results of the previous studies and the current study, it is reasonable to surmise that propofol exerts neuroprotection against ischemic brain injury by blocking TLR4 and suppressing consequent production of pro-inflammatory cytokines, particularly IL-6. The clinical importance of the present study is that the results indicates that bolus propofol administration prior to ischemic insult can protect against ischemic brain injury, presumably by blocking the TLR4-dependent pathway. This understanding of the mechanism underlying the neuroprotective effect of propofol against ischemic brain injury may lead to a novel strategy to prevent exacerbation of ischemic brain injury.

With regards to study limitations, only relatively young male mice were used. Further studies should test aged mice and female mice at different menopausal stages, because those biological variables can affect the outcomes of ischemic brain injury.

Conclusions

Propofol attenuates brain injury by blocking the TRL4-dependent pathway and suppressing pro-inflammatory cytokine production. This understanding of the mechanism underlying the neuroprotective effect of propofol against ischemic brain injury may lead to a new strategy to prevent exacerbation of cerebral infarction.

Abbreviations

IL-1β, interleukin-1β; IL-6, interleukin-6; MCA, middle cerebral artery; PCR, polymerase chain reaction; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α

Declarations

Ethics approval and consent to participate

Animal experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. The study was approved by the University of Yamanashi Animal Care Committee.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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**Authors’ contributions**

KM conceived the study, acquired, analyzed, and interpreted the data, and drafted the manuscript. MK conceived the study, performed experiments and statistical analysis, and drafted the manuscript. SH, AT and SM performed experiments and helped prepare the manuscript. TI and TM performed statistical analysis and revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

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Figures
Figure 1

(A) Staining for 2,3,5-triphenyltetrazolium chloride in representative 1-mm-thick coronal sections. Arrowheads indicate the infarct area (white). (B) Infarct volumes 7 days after induction of ischemic brain injury. Mice in the propofol group mice exhibited smaller infarct volumes. *p < 0.05.
Inflammatory cytokine expression levels 24 hours after the induction of ischemic brain injury. The 2−ΔΔCT value of the target transcript from each mouse was normalized with the median 2−ΔΔCT value from the control wild-type mice as 1.0. Mice in the propofol group exhibited lower levels of inflammatory cytokine expression. *p < 0.05.
Figure 3

Infarct volumes 7 days after the induction of ischemic brain injury. There was no significant difference in infarction volumes between the propofol-treated Toll-like receptor-4 (TLR4) knockout mice and control-treated TLR4 knockout mice.

Supplementary Files

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