RETRACTED ARTICLE: Pristimerin inhibits neuronal inflammation and protects cognitive function in mice with sepsis-induced brain injuries by regulating PI3K/Akt signalling

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ABSTRACT

Context: Sepsis is a systemic inflammatory disease; pristimerin exhibits strong antibacterial, anti-inflammatory and antioxidant properties.

Objectives: We explored whether pristimerin protected against cognitive dysfunction and neuroinflammation in C57BL/6 J mice with sepsis-induced brain injuries.

Materials and methods: Sepsis was induced by intraperitoneal administration of 2 mg/kg lipopolysaccharide (LPS). C57BL/6 J mice were separated into four groups (n = 10 per group): positive control, negative control, pristimerin 10 mg/kg and pristimerin 100 mg/kg. Pristimerin was administered orally for 28 days prior to LPS administration and for six days thereafter. Behavioural changes were assessed one day after LPS administration using the Morris water maze and via neurological dysfunction scoring. Molecular pathogenesis was explored by measurement of malondialdehyde, superoxide dismutase, reactive oxygen species and inflammatory cytokine levels in mouse brains. Neuronal apoptosis was evaluated using the TUNEL assay. The levels of p-Akt/Akt, p-PI3K/PI3K, mTOR, Bax, Bcl-2 and caspase-3 proteins were determined via Western blotting.

Results: Pristimerin improved cognitive function and reduces the neurological score to 1.15 ± 0.03. Pristimerin significantly reduced all cytokine levels: TNF-α by 18 ± 0.6 pg/mg, IL-1β by 43 ± 1.3 pg/mg and IL-6 by 34 ± 1.12 pg/mg. There was a significant (p < 0.01) improvement in PI3K/Akt signalling and histopathological changes in the brain tissue of sepsis-induced brain injured rats.

Conclusions: Pristimerin ameliorated neuronal injury by regulating PI3K/Akt signalling in mice with sepsis-induced brain injuries. Pristimerin may merit further development for clinical applications.

Introduction

Sepsis is a systemic inflammatory disorder associated with the development of multiple types of organ dysfunction, including brain dysfunction (Caraballo and Jaimes 2019). Clinically, sepsis enhances blood–brain barrier permeability, reduces cerebral perfusion and promotes the entry of neurotoxic substances and neuroinflammation (Nwafor et al. 2019). Sepsis is a major public health problem worldwide, associated with cognitive impairment, morbidity and mortality (Singer et al. 2016). Neutrophils and monocytes produce inflammatory cytokines involved in sepsis pathogenesis. Moreover, sepsis triggers production of reactive oxygen species (ROS). Oxidative stress and inflammatory cytokines promote neuronal cell apoptosis through the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, injuring the brain (Uttara et al. 2009) by triggering haemorrhage and cerebral ischaemia (Tang et al. 2017). A PI3K inhibitor has been shown to reverse sepsis-induced neuronal injury (Shang et al. 2019). Sepsis management remains challenging; existing therapies are ineffective. Lipopolysaccharide (LPS; an endotoxin secreted by Gram-negative bacteria) impairs cognitive function by increasing oxidative stress and inflammatory cytokine levels (Brown 2019).

The LPS-induced mouse sepsis model resembles human sepsis (Poli-de-Figueiredo et al. 2008; Beurel and Jope 2009). Molecules from herbs have been used to treat sepsis. Pristimerin (a triterpenoid antioxidant) is a component of Chinese medicinal plants including Celastrus and Maytenus spp. (Celastraceae) (Costa et al. 2008); it reduces nitric oxide (NO) levels by inhibiting inducible nitric oxide synthase (iNOS) (El-Agamy et al. 2019). Pristimerin reduces the levels of inflammatory cytokines and NF-κB by inhibiting the inflammatory cascade (Kim et al. 2013). Pristimerin reportedly ameliorates sepsis-induced lung injury by modulating the inflammatory and oxidative stress cascades (Wang et al. 2020). Pristimerin exhibits insecticidal, antibacterial, antimalarial and anticancer activities (Figueiredo et al. 1998; Lopez et al. 2011; Kim et al. 2013; Wu et al. 2019). Here, we explored whether pristimerin protects against sepsis-induced brain injury.

Materials and methods

Animals

Forty male C57BL/6 mice weighing 25–30 g were housed under controlled conditions (temperature 21–22 ± 3°C and 60%±5%
humidity) with a 12-h light/dark cycle. All animal experiments were approved by the Animal Ethical Committee of the First Affiliated Hospital of Anhui University of Science and Technology, Huainan, China (approval no. IAECFAH-AUS&T/02/2019).

**Experimental**

C57BL/6 J mice were divided into four groups (n = 10/group): positive control (saline only), negative control (LPS 10 mg/kg and pristimerin 100 mg/kg), Group 1 (Positive control, saline only); Group 2 (LPS 10 mg/kg), and Group 3 (LPS + pristimerin 10 mg/kg). Sepsis was induced through intraperitoneal injection of 2 mg/kg LPS. Pristimerin was administered orally for 28 days prior to LPS injection and for six days thereafter. Behavioural changes were assessed one day after LPS administration; biochemical data were collected after the last pristimerin dose.

**Morris water maze test**

The Morris water maze test was used to assess cognitive function (Vorhees and Williams 2006). The water maze pool was divided into four quadrants: a target quadrant (T), quadrants on the right (R) and left (L) of T, and a quadrant opposite T (O). The platform was maintained in a constant position in T on day 1; it was submerged in water on days 2–5. Mice were released into the pool at various positions and the escape latencies (times required to locate the platform (within 2 min)) were measured. Each mouse underwent four daily trials at intervals of 5 min on five successive days. On day 6, the platform was removed from the pool and the T retention time (total time spent in T; within 2 min) was measured using a DigBehav system.

**Neurological dysfunction**

Neurological scores were obtained using the Longa method (Sonneville et al. 2013): 0, no deficit; 1, forelimbs did not completely extend and were weak; 2, circling to the contralateral side; 3, weight-bearing capacity reduced on the injured side; and 4, no spontaneous locomotor activity.

**Cytokine measurements**

The levels of interleukin (IL)-1β, IL-6, NF-κB and TNF-α in brain homogenates were measured using enzyme-linked immunosorbert assay kits (catalogue nos. MLB00C and D6050, R&D Systems (Shanghai, China); catalogue no. 85-86083-11, Thermo Fisher Scientific Ltd., Waltham, MA; MTA00B, R&D Systems, Shanghai, China), in accordance with the manufacturers’ protocols.

**Estimation of ROS levels and other parameters of oxidative stress**

The MitoSOX Red mitochondrial superoxide indicator was used to estimate ROS levels in brain tissues. Briefly, tissue homogenates were stained at 37 °C in the dark for 30 min in a 5 μM solution of the dye. A fluorescence reader was used to estimate intracellular ROS levels at excitation and emission wavelengths of 510 and 580 nm, respectively. The brain levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were estimated using enzyme-linked immunosorbent assay kits (catalogue no. ab238537, Abcam Ltd., Changzhou, China; EIASODC, Thermo Fisher Scientific Ltd., Waltham, MA), in accordance with the manufacturers’ instructions.

**Western blotting**

Protein levels were assessed by Western blotting. Antibodies used to detect the following proteins are shown as catalogue numbers in parentheses: Bax (33-6400, Thermo Fisher Scientific Ltd., Waltham, MA); Bcl-2 (MA5-11757, Thermo Fisher Scientific Ltd., Waltham, MA); caspase-3 (MA1-91637, Thermo Fisher Scientific Ltd., Waltham, MA); p-Akt (9271, Cell Signaling Technology, Shanghai, China); Akt (9272, Cell Signaling Technology, Shanghai, China); and PI3K (4292, Cell Signaling Technology, Shanghai, China). A bicinchoninic acid assay kit (7780, Cell Signaling Technology, Shanghai, China) was used to measure protein concentrations prior to gel electrophoresis. Proteins were separated via 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrobotted onto nitrocellulose membranes; the membranes were blocked with 5% (w/v) blocking buffer with the primary antibodies overnight at 4 °C. Goat secondary antibodies conjugated with horseradish peroxidase were added, and a chemiluminescence kit was used to detect proteins.

**TUNEL assay**

The TUNEL assay was used to detect neuronal apoptosis. Paraffin-cleared brain slices were sealed with 0.1% (v/v) Triton X-100 at 37 °C for 15 min. After slices had been washed with phosphate-buffered saline, they were incubated with the TUNEL solution at 37 °C for 1 h in the dark. After slices had been washed with phosphate-buffered saline, they were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) at 37 °C for 15 min. The slices were again washed with phosphate-buffered saline, then cover slipped prior to observation using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). TUNEL-positive neurons were counted in five random hippocampal regions. The apoptosis index was: (number of TUNEL-positive neurons/total number of DAPI-positive neurons) × 100%.

**Histopathology**

Brain tissue specimens were fixed in 10% (v/v) formaldehyde and embedded in paraffin. For morphological examination, 6-mm-thick coronal sections were stained with 3% (w/v) haematoxylin and eosin. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohol baths, then rinsed in tap and distilled water. The sections were stained with haematoxylin for 3–5 min and treated with 1% (v/v) HCl in 70% (v/v) alcohol. After sections had been washed in tap water for 15 min, they were stained in eosin for 1–4 min, then dehydrated and exposed to HCl treatment as described above. The sections were then cleared with xylene, mounted with Permount (Fisher Chemical, Pittsburgh, PA), and observed under a light microscope (BX41; Olympus, Tokyo, Japan) at ×400 magnification.

**Statistical analysis**

All data are expressed as means ± standard errors of the means (SEMs; n = 10). Statistical analyses were performed in GraphPad Prism software (ver. 6.1; La Jolla, CA) using one-way analysis of
variance, followed by post hoc comparisons using the Dunnett test. Values $p < 0.05$ were considered to indicate statistical significance.

**Results**

**Pristimerin ameliorates cognitive dysfunction**

The effect of pristimerin on cognitive function was studied (Figure 1(A(a–c), B)). Learning and memory were assessed using the water maze. In the negative control group, the percentage of time spent in the target quadrant (26%±0.25%) and the number of crossings (2.7±0.14) were significantly lower, while escape latency was enhanced (to $42 ± 1.4$ s) on day 5 of training, compared with the results in the positive control group. Pristimerin ameliorated the day 5 escape latency (to $21 ± 1.1$ s), the number of crossings (to $11 ± 0.46$) and the percentage of time in the target quadrant (to $63% ± 1.3%$) in mice with sepsis-induced brain injuries (Figure 1(A(a–c))). The neurological score was...
significantly higher \((p < 0.01)\) in the negative control group \((3.7 \pm 0.09)\) than in the positive control group \((0.1 \pm 0.01)\); pristimerin reduced the neurological score to \(1.15 \pm 0.03\) (Figure 1(B)).

**Pristimerin ameliorates cytokine synthesis**

Brain cytokine levels are shown in Figure 2. The levels of TNF-\(\alpha\) \((43 \pm 1.6 \text{ pg/mg})\), IL-1\(\beta\) \((89 \pm 1.3 \text{ pg/mg})\) and IL-6 \((64 \pm 2.8 \text{ pg/mg})\) were significantly (all \(p < 0.01\)) higher in the negative control than in the positive control group. Pristimerin significantly reduced all cytokine levels: TNF-\(\alpha\) by \(18 \pm 0.6 \text{ pg/mg}\), IL-1\(\beta\) by \(43 \pm 1.3 \text{ pg/mg}\) and IL-6 by \(34 \pm 1.12 \text{ pg/mg}\) (all \(p < 0.01\)).

**Pristimerin ameliorates oxidative stress**

The mitochondrial ROS level \((3.64 \pm 0.09 \text{ relative fluorescence units})\) and the MDA level \((2.74 \pm 0.06 \text{ nmol/mg})\) were enhanced, while SOD activity \((5.82 \pm 0.13 \text{ U/mg})\) was significantly reduced \((p < 0.01)\), in the brains of the negative control group, compared with the positive control group (Figure 3). Pristimerin significantly reduced the MDA level \((to 0.71 \pm 0.03 \text{ nmol/mg})\) and ROS production \((to 1.32 \pm 0.06 \text{ relative fluorescence units})\) (both \(p < 0.01)\), while increasing SOD activity \((to 11.2 \pm 0.29 \text{ U/mg})\) (Figure 3).

**Pristimerin ameliorates neuronal apoptosis**

The levels of Bax, Bcl-2 and caspase-3 were determined by Western blotting (Figure 4(A)). Bax expression and caspase-3 expression levels were higher \((2.4 \pm 0.05\text{-fold} \ and \ 2.74 \pm 0.05\text{-fold}, \ respectively)\), while Bcl-2 expression was lower \((0.26 \pm 0.01\text{-fold})\), in the negative control group than in the positive control group. Pristimerin reduced Bax and caspase-3 expression levels \((1.32 \pm 0.02\text{-fold} \ and \ 1.27 \pm 0.02\text{-fold}, \ respectively)\), while increasing Bcl-2 expression \((0.82 \pm 0.04\text{-fold})\). Figure 4(B) shows that the apoptotic index was higher in the negative control group \((27.2\% \pm 0.8\%)\) than in the positive control \((5\% \pm 0.2\%)\) group. Moreover, pristimerin partially restored the apoptotic index to \(8.5\% \pm 0.3\%\).

**Pristimerin ameliorates PI3K/Akt signalling**

Figure 5 shows the effects of pristimerin on expression levels of PI3K, Akt and mTOR. Reductions were observed in the expression levels of p-Akt/Akt \((0.2 \pm 0.012\text{-fold})\), p-PI3K/PI3K \((0.23 \pm 0.007\text{-fold})\) and mTOR \((0.19 \pm 0.01\text{-fold})\) in the negative control group, compared with the positive control group. Pristimerin increased the expression levels of p-Akt/Akt \((0.84 \pm 0.09\text{-fold})\), p-PI3K/PI3K \((0.85 \pm 0.02\text{-fold})\) and mTOR \((0.82 \pm 0.008\text{-fold})\).

**Pristimerin ameliorates pathophysiological changes**

Histopathological changes in brain tissue are shown in Figure 6. Multifocal moderate reductions in neuronal cell layers, more neuronal degeneration, and more pyknotic nuclei were observed in hippocampi in the negative control group, compared with the positive control group. However, pristimerin reversed these changes.

**Discussion**

Sepsis is associated with multiple types of organ dysfunction, including brain injury; mortality can attain 90\% (Hajj et al. 2018). Brain injury caused by hypoxia and ischaemia enhances inflammatory mediator synthesis and oxidative stress (Kalogeris et al. 2012); these processes trigger neuronal inflammation and oxidative damage that stimulate neuronal apoptosis (Klein and Ackerman 2003), leading to hippocampal injury (Chi et al. 2018). Sepsis management is not yet acceptable; we thus evaluated the effects of pristimerin on neuronal inflammation and apoptosis in mice with sepsis-induced brain injuries.

Neuronal injury contributes to the development of cognitive dysfunction and sepsis reported to induce neuronal injury, which causes loss of cognitive functions (Sonneville et al. 2013). Neurological function and Morris water maze are commonly used to determine the cognitive function, which resembles clinically (Vorhees and Williams 2006). Data of the investigation reveal that cognitive dysfunction occurs in sepsis group and treatment with pristimerin reverse it.
Neuronal inflammation is triggered by cytokines such as TNF-α, IL-1β and IL-6, which are associated with brain oedema (Kany et al. 2019) that increases mitochondrial dysfunction and elevates ROS levels. Production of ROS has involved in the development of several inflammatory disorders; Cells produce ROS for host defence response (Nita and Grzybowski 2016). ROS enhances the endothelial dysfunction by oxidation of cellular signalling proteins such as tyrosine phosphatases (Ray et al. 2012). Reactive nitrogen species (RNS) form due to ROS combine with NO, which dismutase superoxide through SOD (Beckman 1996). ROS and SOD also act on mediator of inflammation and promotes the neuronal inflammation and neuronal injury. Reductions in inflammatory cytokines ameliorate neuronal inflammation; pristimerin reduces cytokine levels and ROS production.

Bax, Bcl-2 and caspase-3 are involved in neuronal apoptosis (Wang and Youle 2009). Imbalances between anti- and pro-apoptotic protein levels promote apoptosis. The Bcl-2 anti-apoptotic protein reduces activation of mitochondrial-dependent apoptosis (Papailiakas et al. 2007). Neuronal apoptosis is enhanced by activation of the caspase pathway through increased caspase-3 activity. Sepsis-induced neuronal injury promotes neuronal apoptosis; reduced apoptosis prevents neuronal injury (Sun et al. 2019). We found that Bax, Bcl-2 and caspase-3 levels changed in injured brain tissue; pristimerin ameliorated these changes.

LPS induces both sepsis and neuronal injury caused by various cytokines. Benfotamine, a derivative of thiamine monophosphate, reportedly protects BV-2 microglia from the effects of LPS by blocking PI3K/Akt signalling, thus reducing NO production; iNOS, Cox-2 and Hsp70 expression; and TNF-α and IL-6 synthesis. Phosphoinositide 3-kinases (PI3Ks) regulate several key steps in the inflammatory response to external insults (Redzadutordoir and Averill-Bates 2016). PI3Ks transduce signals from growth factors, cytokines, hormones and LPS to intracellular messages, thereby generating phospholipids. These phospholipids activate the serine/threonine kinase Akt and other downstream effector pathways. The eight PI3Ks of mammalian cells are divided into three families (Liu et al. 2014) that differ in terms of regulation and the preferred lipid substrate. PI3K lipid kinases are involved in many cellular functions, including signal transduction and intracellular vesicular trafficking; PI3K pathway dysregulation is a feature of several pathological conditions, including neurological diseases. PI3K/Akt pathway inhibition in LPS-activated microglia reduced the levels of proinflammatory factors (Zhou et al. 2020). However, another study found that PI3K/Akt activation attenuated brain damage by upregulating the anti-oxidative response and inhibiting inflammation and

Figure 3. Pristimerin ameliorates oxidative stress in brain tissue homogenates of mice with sepsis-induced brain injuries. (A) Mitochondrial ROS levels. (B) SOD levels. (C) MDA levels. MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase. Means ± SEMs (n = 10); **p < 0.01 compared with positive controls; ***p < 0.01 compared with negative controls.
Figure 4. Pristimerin ameliorates neuronal cell apoptosis in brain tissue homogenates of mice with sepsis-induced brain injuries. (A) Effects of pristimerin on expression levels of Bax, Bcl-2 and caspase-3, as revealed by Western blotting. (B) Effect of pristimerin on the apoptosis index, as revealed by the TUNEL assay. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end-labelling. Means ± SEMs (n = 10); ##p < 0.01 compared with positive controls; **p < 0.01 compared with negative controls.

Figure 5. Pristimerin ameliorates changes in PI3K, Akt and mTOR expression levels in brain tissue homogenates of mice with sepsis-induced brain injuries, as revealed by Western blotting. Means ± SEMs (n = 10); ##p < 0.01 compared with positive controls; **p < 0.01 compared with negative controls.
apoptosis. In the present study, PI3K/Akt signalling was amelio-
rated by pristimerin in brain-injured mice. Histopathology find-
ings also suggested that pristimerin ameliorated injury.

Conclusions
Pristimerin ameliorates neuronal injury by reducing inflamma-
tion and oxidative stress in mice with sepsis-induced brain inju-
ries. Pristimerin alleviates neuronal apoptosis and restores
 cellular integrity by regulating PI3K/Akt signalling.

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Figure 6. Pristimerin ameliorates histopathological changes in brain tissue of mice with sepsis-induced brain injuries, as revealed by haematoxylin and eosin staining. Means ± SEMs (n = 10).
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