Roles of the pyroptosis signaling pathway in a sepsis-associated encephalopathy cell model

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Abstract
Objectives: The inhibition of pyroptosis has a protective effect in sepsis-associated encephalopathy (SAE). However, the mechanisms underlying pyroptosis in SAE remain to be elucidated.
Methods: Here, we investigated the effects of the caspase inhibitors, Belnacasan (Beln) and Wedelolactone (Wede), on an induced model of SAE in P12 cells, using immunofluorescence, ELISA, western blotting, and flow cytometry.
Results: The cell viability decreased, IL-1β and IL-18 secretion increased, and the levels of the caspase cleavage products, N-terminal gasdermin D, cleaved caspase-1, and cleaved caspase-11, increased in P12 cells following combined treatment with lipopolysaccharides (LPS) and adenosine triphosphate (ATP). However, treatment with Beln or Wede ameliorated the effects induced by LPS and ATP. Neither Beln nor Wede notably affected the levels of cell apoptosis-associated proteins but these inhibitors regulated the levels of cell pyroptosis-associated proteins. Further, the combination of Beln and Wede exerted greater inhibitory effects on cell pyroptosis than either Beln or Wede alone.
Conclusions: The results demonstrated that both the canonical and non-canonical signaling pathways of cell pyroptosis are involved in LPS-induced cell damage and that the non-canonical signaling pathway may be involved to a greater extent. This suggests that the inhibition of pyroptosis may exert potential therapeutic effects on SAE.

Keywords
Sepsis-associated encephalopathy, pyroptosis, Belnacasan, Wedelolactone, gasdermin D, apoptosis, lipopolysaccharides, adenosine triphosphate, caspase-1, caspase-11

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Introduction

Sepsis-associated encephalopathy (SAE) is a form of brain injury occurring secondary to sepsis in the absence of a central nervous system infection, and patients exhibit symptoms of acute and long-lasting neurological damage with high morbidity and mortality.\textsuperscript{1–3} Recent studies have demonstrated the pyroptosis of peripheral immune cells in patients with sepsis.\textsuperscript{4,5} Furthermore, pyroptosis has been observed in the brains of mice with sepsis, the inhibition of which reduces the inflammatory factor response and ameliorates brain injury in SAE.\textsuperscript{6} Pyroptosis is a type of cell death mediated by the activation of inflammatory caspases that cleave gasdermin D (GSDMD) to form N-terminal GSDMD (GSDMD-NT), leading to the release of inflammatory cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)) and interleukin-18 (IL-18).\textsuperscript{7,8} GSDMD-NT is a main agent in the induction of cell pyroptosis, and GSDMD-NT binding to the bacterial phospholipid, cardiolipin, is conducive to the timely removal of bacteria. However, GSDMD-NT can form holes on the surface of host cell membranes, leading to cell death and the release of cell contents. GSDMD-NT also acts as a pro-inflammatory signal to further strengthen the immune response, although it can induce immune dysfunction through the cytokine cascade reaction. Notably, this active fragment does not kill the surrounding healthy cells directly when released during pyroptosis.

Cell pyroptosis is induced by both canonical and non-canonical signaling pathways. In the non-canonical signaling pathway, intracellular lipopolysaccharides (LPS) bind to and activate caspase-11, -4, and -5, which then cleave GSDMD to form the active GSDMD-NT.\textsuperscript{9} In turn, GSDMD-NT forms gosdermin holes in the cell membrane to mediate the release of IL-1\(\beta\) and IL-18. In contrast with this non-canonical signaling pathway of pyroptosis, activation of the canonical signaling pathway is dependent on the pre-activation of the inflammasome. This, in turn, activates caspase-1, which can also cleave GSDMD to form GSDMD-NT.

A recent study proposed that the movement of inflammatory factors from the peripheral blood into the brain causes pyroptosis in the brain in SAE.\textsuperscript{6} Levels of proteins that mediate pyroptosis, including NLRP3, GSDMD-NT, and cleaved caspase-1, are abnormally increased in the brain in SAE.\textsuperscript{6,10} The NLRP3/caspase-1 signaling pathway is considered to induce cell pyroptosis and this pathway has been implicated in brain injury in SAE.\textsuperscript{10} Further, the inhibition of caspase-1 signaling can reduce GSDMD-NT levels and ameliorate the brain function impairment induced by sepsis.\textsuperscript{6} Thus, the canonical signaling pathway is known to serve a vital role in SAE. However, the roles of the non-canonical signaling pathway in pyroptosis have not yet been elucidated in SAE.

Materials and methods

Cell line

Rat pheochromocytoma PC12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in high-sugar basic Dulbecco’s Modified Eagle’s Medium (DMEM) containing penicillin (1 \(\times\) 10\(^5\) U/L) and streptomycin (100 mg/L) at 37\(^{\circ}\)C with 5% CO\textsubscript{2}. Nerve growth factor (NGF, 50 ng/mL) was added (2.5 mL) to the DMEM medium to induce the differentiation of PC12 cells into sympathetic neuron-like cells. The PC12 cells were differentiated continuously for 7 days and the culture solution was changed every 2 days. When the protrusion lengths of the PC12 cells were more than twice the width of the cell body the cells were considered to be completely differentiated.
Serum-free medium was added to the differentiated PC12 cells, and 2 hours later, the cells were treated with different concentrations (0.1, 1, 10, and 50 μg/mL) of lipopolysaccharides (LPS; Sigma-Aldrich, St. Louis, MO, USA) at different time points (0, 12, and 24 hours). This was followed by treatment with 5 mM adenosine triphosphate (ATP) for 0.5 hours. Cells in the control group were cultured under normal conditions (no LPS or ATP). These cells were then subjected to treatment with 40 μM Belnacasan (Beln) or 2.5 μM Wedelolactone (Wede) purchased from MedChemExpress (Shanghai, China). This study was approved by the ethics committee of Yantai Yuhuangding Hospital, Qingdao University.

**CCK8 assay**

Cells were seeded into 96-well plates (1 × 10⁶/L). After the cells had adhered to the plate surface and entered the logarithmic growth phase, the medium was changed according to the treatment groups and the cells were cultured for 24 hours. Cell Counting Kit-8 (CCK-8; MedChemExpress) solution (10 μL) was added to each well, and the cells were incubated at 37°C for 2 hours. The optical density (OD) value at 450 nm was then measured for each well. Cell viability (%) was calculated using the following formula: (OD of treatment group/OD of control group) × 100.

**Western blotting**

Total protein concentration in the cells was detected using the bicinchoninic acid (BCA) method. The proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was subsequently blocked with 5% defatted milk for 1 hour. The primary antibodies (anti-cleaved caspase-1 p20: 1:500 dilution, AB1871, Merk Millipore, Burlington, MA, USA; anti-cleaved caspase-11 p26: 1:1,000 dilution, ab180673, Abcam, Cambridge, MA, USA; anti-Bax: 1:1,000 dilution, ab32503, Abcam; Bel-2: 1:1,000 dilution, ab32124, Abcam; anti-cleaved caspase-3: 1:500 dilution, ab49822, Abcam; caspase-3: 1:500 dilution, ab13847, Abcam; anti-GADPH: 1:5,000 dilution, ab8245, Abcam; insoluble ASC: 1:1,000 dilution, sc-514414, Santa Cruz Biotechnology, Dallas, TX, USA; GSDMD-NT: 1:1,000, 93709, Cell Signaling Technology, Danvers, MA, USA) were added to the PVDF membrane and incubated at 4°C overnight. After three washes with phosphate-buffered saline (PBS) containing Tween 20 (PBST), horseradish peroxidase-labeled secondary antibodies were added (goat anti-rabbit IgG: 1:10,000 dilution, ab6721, Abcam; rabbit anti-mouse IgG, 1:10,000 dilution, ab6728, Abcam), and the PVDF membrane was incubated for 1 hour. The electrochemiluminescence substrate solution was then added for signal detection (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai China). Analysis was performed using ImageJ software 1.46r (U.S. National Institutes of Health, Bethesda, MA, USA).

**Immunofluorescence (IF) assays**

Cells were washed with PBS twice for 5 minutes each and fixed with 4% paraformaldehyde for 10 minutes. Subsequently, cells were washed with PBS three times for 5 minutes each. Then, 10% normal goat serum (Gibco Cell Culture, Carlsbad, CA, USA) was added for blocking for 1 hour at room temperature. The primary anti-GSDMD-NT antibody (Amyjet Scientific, Inc., Wuhan, China) was added to the cells, and the cells were then incubated at 4°C overnight. Subsequently,
a fluorescein isothiocyanate (FITC)-labeled secondary antibody (Thermo Fisher Scientific, Inc., Rockford, IL, USA) was incubated with the cells for 30 minutes at room temperature. After washing the cells in PBS three times for 5 minutes each, 4’,6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei at room temperature for 15 minutes. The residual DAPI was removed using three 5-minute washes with PBS. Images were immediately captured with a fluorescence microscope (Olympus, Tokyo, Japan).

**Flow cytometry**

Cells were seeded into 6-well plates. Following experimental treatment, suspended cells and adherent cells were collected and resuspended in 490 μL of Binding Buffer. Subsequently, Annexin V (5 μL) and propidium iodide (PI, 5 μL) were added to each cell suspension. Cell apoptosis was analyzed by flow cytometry within 1 hour (Epics-XL II flow cytometer; Beckman Coulter, Inc., Brea, CA, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

The cell supernatants were collected and the levels of inflammatory factors (IL-1β and IL-18) were measured using ELISA kits (IL-1β: PI303, Beyotime Institute of Biotechnology, Jiangsu, China; IL-18: KRC2341, Thermo Fisher Scientific). After the color reaction was terminated, the OD values of each well were measured at 450 nm using a microplate reader (Biotek, Winooski, VT, USA). The IL-1β and IL-18 concentrations in each well were calculated based on standard curves.

**Statistical analysis**

All experiments in the present study were repeated independently in triplicate and the data obtained were presented as the mean ± standard deviation (mean ± SD). One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A P-value < 0.05 was considered statistically significant.

**Results**

**LPS and ATP activate pyroptosis signaling pathway in PC12 cells**

PC12 cells treated with different concentrations of LPS (0.1, 1, 10, and 50 μg/mL) were used to establish an in vitro model of sepsis. LPS significantly reduced cell viability in a dose- and time-dependent manner (P < 0.05 to P < 0.01 in comparison with earlier time points; Figure 1a). The levels of cleaved caspase-1 and caspase-11 were significantly increased when PC12 cells were treated with different concentrations of LPS for 24 hours (P < 0.05 to P < 0.001 in comparison with lower LPS concentrations; Figure 1b). LPS-induced caspase-11 activation is a vital marker of non-canonical pyroptosis during sepsis. Therefore, it is likely that LPS activated the non-canonical pyroptosis signaling pathway in these PC12 cells.

ATP is a common activator of the NLRP3 inflammasome. Therefore, ATP (5 mM) was used to stimulate cells following LPS induction. Combined treatment with LPS and ATP significantly decreased cell viability in a dose- and time-dependent manner (P < 0.05 to P < 0.001 in comparison with earlier time points; Figure 1c). Additionally, the levels of cleaved caspase-1 and caspase-11 were further and significantly increased relative to cells treated with LPS alone (P < 0.05 to P < 0.001 in comparison with lower LPS concentrations; Figure 1d), which implied that caspase-11-mediated non-canonical pyroptosis was activated in these cells. LPS (1 μg/mL) and
ATP (5 mM) were used in all subsequent experiments.

**Belnacasan and Wedelolactone suppress PC12 cell pyroptosis induced by LPS and ATP**

Belnacasan and Wedelolactone are specific inhibitors of caspase-1 and -11, respectively. Therefore, Belnacasan (Beln) and Wedelolactone (Wede) were used, separately and in combination, to treat cells following LPS and ATP induction. The results demonstrated that by 24 hours, both Beln and Wede treatment had significantly increased the cell viability in comparison with the model group ($P < 0.05$ to $P < 0.001$, Figure 2), and the combination of Beln and Wede further increased the cell viability compared with either Beln or Wede treatment alone.

**Belnacasan and Wedelolactone reduce GSDMD-NT levels**

The effects of Beln and Wede on GSDMD-NT levels in PC12 cells were next evaluated.
GSDMD-NT was observed in the cell nucleus and cytoplasm, and treatment with Beln and Wede separately and in combination markedly reduced the levels of GSDMD-NT compared with the model group ($P < 0.001$ for all; Figure 3). Furthermore, the effect of Wede was greater than that of Beln.

**Belnacasan and Wedelolactone reduce cell death**

The effects of Beln or Wede on apoptosis and pyroptosis in PC12 cells induced by LPS and ATP treatment were analyzed. The flow cytometry results revealed that...
the numbers of cells in the FITC$^+$/PI$^+$ region were significantly reduced ($P < 0.001$) in the Beln and Wede groups compared with the model group (Figure 4a, b). FITC$^+$/PI$^+$ cells were considered to be non-viable, apoptotic cells that might have been pyroptotic.

Canonical and non-canonical pyroptosis pathways are involved in cell injury induced by LPS

Cell supernatants were collected to analyze the secretion of IL-1$\beta$ and IL-18 by PC12 cells induced by LPS and ATP (model group). LPS and ATP significantly increased the secretion of IL-1$\beta$ and IL-18 in the model group compared with the control group ($P < 0.001$), while separate treatment with Beln or Wede significantly reduced the secretion of IL-1$\beta$ and IL-18 compared with the model group ($P < 0.001$, Figure 5a). Furthermore, combined treatment with Beln and Wede decreased the secretion of IL-1$\beta$ and IL-18 to a greater extent than that observed for either of these inhibitors alone. Subsequently, we analyzed whether Beln or Wede regulated the expression levels of various apoptosis-related proteins. LPS and ATP treatment significantly induced the expression of two pro-apoptotic proteins, cleaved caspase-3 and Bax ($P < 0.001$ for both), whereas the expression level of the anti-apoptotic protein, Bcl-2, was significantly reduced ($P < 0.001$), compared with the control group (Figure 5b). Beln and Wede treatment significantly reduced the effects of LPS and ATP induction on the expression of Bax relative to the model group ($P < 0.001$); however, Beln and Wede treatment exerted less effect on cleaved caspase-3 expression ($P < 0.05$) and had no effect on Bcl-2 expression.

Figure 4. Flow cytometry analysis of P12 cells induced by lipopolysaccharide and adenosine triphosphate treatment and then exposed to the caspase inhibitors, Belnacasan (Beln) and Wedelolactone (Wede). a) Flow cytometry results. b) Quantification of the results shown in (a). Beln and Wede, both separately and in combination, notably decreased the number of non-viable, apoptotic cells. Data represent the mean ± SD, n = 3 per treatment. **$P < 0.01$, ***$P < 0.001$. 
This result implies that the effects of Beln or Wede treatment are exerted via another pathway to induce cell apoptosis instead of the apoptosis signaling pathway. Subsequently, the effects of Beln or Wede treatment on the expression levels of proteins mediating pyroptosis were analyzed (Figure 5c). ATP has previously been shown to induce ASC oligomerization and the formation of ASC specks, which are considered markers of inflammasomes.14 In the present study, LPS- and ATP-stimulated PC12 cells exhibited significantly higher levels ($P < 0.001$) of insoluble ASC relative to the control group, implying that inflammasome activation might have
been triggered. Compared with the model group, Beln treatment significantly reduced the levels of cleaved caspase-1 and caspase 11 (\(P < 0.001\) and \(P < 0.05\), respectively). Furthermore, Wede treatment significantly decreased the levels of ASC (\(P < 0.05\)), cleaved caspase-11 (\(P < 0.001\)), and GSDMD-NT (\(P < 0.01\)) relative to the model group. Additionally, combined treatment with Beln and Wede significantly reduced ASC, cleaved caspase-1, cleaved caspase-11, and GSDMD-NT levels (\(P < 0.01\) to \(P < 0.001\)) relative to the model group, which indicated that both canonical and non-canonical pyroptosis signaling pathways were involved in the cell injury induced by LPS.

**Discussion**

In SAE, the integrity of the blood-brain barrier (BBB) sustains damage, which contributes toward pathogens gaining entry into the central nervous system and directly causing varying degrees of neuron damage.\(^{15}\) The release of inflammatory mediators and immunoactive substances further impairs BBB functions.\(^{16}\) LPS are involved in activating caspase-11 in mice and caspase-4 and -5 in humans,\(^{17-19}\) and LPS and ATP have been shown to markedly upregulate the expression levels of pyroptosis-related genes that activate the NLRP3 inflammasome.\(^{20}\) Additionally, LPS induces caspase-11 in mice to activate the GSDMD protein and trigger non-canonical pyroptosis, while caspase-4 and -5 in humans have similar functions and stimulate the innate cellular immune response.\(^{21-23}\) In mice with sepsis, the continuity of the neural nuclear and mitochondrial membrane suffers from injury, suggesting that neurons undergo pyroptosis.\(^6\) In the present study, the levels of cleaved caspase-1 and caspase-11 were markedly higher in the LPS and ATP treatment group than in the LPS only treatment group. Furthermore, cell viability was markedly decreased. These results implied that ATP activated canonical pyroptosis, thereby aggravating PC12 cell injury.

Pyroptosis is a type of cell death that occurs when a host cell is infected by pathogenic microorganisms or stimulated by endogenous danger signals, and it is characterized by cell swelling, lysis, and the release of a large number of proinflammatory cytokines.\(^{24}\) One such endogenous danger signal, ATP, can induce activation of the NLRP3 inflammasome, thereby promoting pyroptosis.\(^{25}\) Thus, pyroptosis is an important mechanism used by the body to clear pathogens and is involved in the innate immune response of the body.\(^{26}\)

Normally, host cells release inflammatory cytokines via a caspase-1-dependent signaling pathway to control infection, but overactivation of caspase-1 can lead to a cascade of amplified inflammatory responses that cause cell lysis, leading to further exacerbation of the underlying disease.\(^{27}\) Therefore, an excessive pyroptosis-induced inflammatory response can lead to lethal septic shock.

Here, the inhibition of caspase-1 and caspase-11 markedly reduced the levels of GSDMD-NT in PC12 cells induced by LPS and ATP. Therefore, decreased GSDMD-NT levels may have ameliorated cell injury in these cells. Previous studies have demonstrated that the suppression of GSDMD-NT-induced pyroptosis offers therapeutic effects in sepsis treatment.\(^{28,29}\) Caspase-1 activation is a typical marker of canonical inflammasome activation, while caspase-11 activation is a marker of non-canonical inflammasome activation.\(^{21,30}\) In the non-canonical pyroptosis pathway, GSDMD-NT and caspase-11 activate the NLRP3 inflammasome and recruit ASC to induce the maturation and release of IL-1\(\beta\) and IL-18.\(^{9,31,32}\) Therefore, blocking caspase-11 with Wede may have reduced...
IL-1β release in the present study, thereby blocking the pyroptosis signaling pathway.

A previous study has demonstrated that GSDMD-NT and cleaved caspase-1 levels are markedly increased in the cortex of mice with sepsis. GSDMD-NT causes cell pyroptosis by translocating to the plasma membrane to form pores, which induces the release of IL-1β and IL-18, as well as cell swelling. Furthermore, GSDMD-NT is a key factor in both the canonical and non-canonical pyroptosis signaling pathways. The present study revealed that GSDMD levels were increased in the cytoplasm and cell nucleus in the model group (LPS- and ATP-treated), and GSDMD-NT levels were also increased. GSDMD was previously found to cluster close to the nuclear membrane after external stimulation. One possible reason for our observation of GSDMD-NT occurring throughout the whole cell might have been the lysis of the plasma and nuclear membranes. In agreement with this, an earlier study demonstrated that GSDMD-NT is distributed in both the nucleus and cytoplasm after hypoxia induction.

The present study demonstrated that both the canonical and non-canonical pathways of cell pyroptosis are involved in LPS-induced cell apoptosis, and the inhibition of LPS- and ATP-induced pyroptosis in PC12 cells markedly increases cell viability. However, there is still limited knowledge regarding the roles of the canonical and non-canonical pathways in cell pyroptosis in SAE in vivo, and further investigations are required. For example, it would be of great interest to determine the effect of Belnacasan and Wedelolactone on animal SAE models. However, it is clear that the canonical and non-canonical pathways both serve an important role in inducing brain cell injury in SAE, and the inhibition of these pathways may offer therapeutic benefits in SAE.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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