Int J Clin Exp Med 2018;11(3):2128-2134
www.ijcem.com /ISSN:1940-5901/IJCEM0069133

Original Article

Inhibitory effect of protectin D1 on NALP3 inflammasomes

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Received November 14, 2017; Accepted December 20, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Objective: To clarify the effects of protectin D1 (PD1) on NALP3 inflammasomes and downstream IL-18 and IL-1β levels in rats with sepsis and the relevant mechanisms of reactive oxygen species (ROS). Methods: Twenty-four male Wistar rats in total were randomly assigned to the sham group, the model group or the PD1 group. The rats in the model group underwent cecal ligation and puncture, those in the sham group did not undergo cecal ligation and puncture, and those in the PD1 group were intraperitoneally injected with PD1 before cecal ligation and puncture. At 24 h after operation, the mean fluorescence intensity ROS in hepatocytes was measured using the flow cytometry, whereas qRT-PCR and Western blot were applied to measure the mRNA and protein levels of NALP3, ASC and Caspase-1 in hepatocytes; the enzyme-linked immunosorbent assay (ELISA) was employed to examine serum IL-18 and IL-1β concentrations in rats. Results: The levels of NALP3, ASC, Caspase-1, ROS, IL-18 and IL-1β in the model group and the PD1 group were remarkably higher than those in the sham group (All $P<0.001$); but the PD1 group had strikingly lower levels than the model group (All $P<0.001$). Conclusion: PD1 reduced the assembly and activation of NALP3 inflammasomes, as well as the levels of inflammatory cytokines in septic rats, which may be related to the reduction in ROS concentrations.

Keywords: Protectin D1, NALP3, inflammasome, inflammatory cytokine, sepsis

Introduction

Sepsis, an infection-induced systemic inflammatory response syndrome, is the response to infectious factors [1, 2]. If not treated in time, the disease is prone to progress into multiple organ dysfunction syndromes [3]. However, its pathogenesis remains uncertain. Studies have shown that activation of a wide range of inflammatory cytokines and oxidative stress play essential roles in the onset and development of sepsis [4]. Mitochondrial dysfunction exerts a great effect on the initial sepsis, mitochondria have been reported to be susceptible to damage exacted by oxidative stress, and damaged mitochondria induce inflammation by promoting NALP3 inflammasome activity [5]. Reactive oxygen species (ROS) act decisively in the course of NALP3 inflammasome activation [6]. ROS directly activate NALPL3 inflammasomes. Besides, ROS can activate the signaling pathways of ERK, NF-κB and p38MAPK, thereby activating NALP3 inflammasomes [7-9].

NALP3 inflammasome, a member of the NLRs inflammasome family, initiates inflammatory response by recruiting apoptosis-associated speck-like protein (ASC) and caspase-1 and cutting the IL-1 family into active IL-18 and IL-1β proinflammatory cytokines. Therefore, effective reduction in oxidative stress and inflammatory response is of significance in alleviating the progression of sepsis.

Additionally, previous studies have substantiated ω-3 unsaturated fatty acids inhibit acute and chronic inflammation [10]. Protectin D1 (PD1) is produced by ω-3 polyunsaturated fatty acids through transcellular pathway with the action of lipoxygenase [11]. PD1 protects the liver and the kidney, the lung and nerve tissues, functions anti-inflammation, alleviates disease progression and reduces the incidence of complications [12]. Nevertheless, the effects of PD1 on NALP3 inflammasomes and their mechanisms are still unclear. In the present study, an inflammatory model of septic rats was established to study the effects of PD1 on the activity of NALP3 inflammasome, and delves into the mechanism of PD1 governing inflammatory response.

Materials and methods

Experimental animals

Twenty-four clean male Wistar rats at an age of 8 weeks and with a mean body weight of 200±10 g were provided by the experimental center of animal in Central South University.

Main reagents

PD1 (Cayman, USA), rabbit anti-mouse NALP3 polyclonal antibody (Millipore, USA), rabbit anti-mouse ASC polyclonal antibody (Life-Span Biosciences, USA); rabbit anti-mouse Caspase-1 polyclonal antibody (Abcam, USA), rabbit anti-mouse β-actin polyclonal antibody (Abcam, USA), ELISA kits for measurement of serum IL-18 and IL-1β8 concentrations (Biolegend, USA), and ROS Kits (Genmed Scientfics, USA) were prepared for this study.

Methods

Establishment of an inflammatory model of rats with sepsis: Twenty-four male Wistar rats were randomly assigned to the model group (n=8), the sham group (n=8) or the PD1 group (n=8). An inflammatory model of septic rats was established with the application of cecal ligation and puncture in the following procedures: After the rats had been anesthetized by intraperitoneal injection of 3% amylobarbitone solution (100 mg/kg), their heads and limbs were fixed. Then a midline incision was made in the abdomen of the rats to expose the cecum. Half of the cecum at the end of the hemal arch was ligated, and then the cecal leakage was made by puncturing the cecum with a needle. After that, the cecum was put back into the abdominal cavity, followed by layer-suture of the incision [13]. As soo as the operation completed, normal saline was subcutaneously injected at a dose of 10 ml/kg to prevent shock. In contrast, the rats in the sham group only underwent laparotomy, abdominal closure and resuscitation, without ligation or perforation, while those in the PD1 group received intraperitoneal injection of PD1 at a dose of 0.08 mg/kg within 12 h before operation [14].

The rats were sacrificed at 24 h after operation. The blood samples were drawn from the heart, and centrifuged at 3000 r/min for 15 min. The supernatant was collected for storage at -20°C. In addition, some hepatic tissues were homogenized to make hepatocyte suspension specimen.

mRNA levels of NALP3, ASC and Caspase-1 detected by RT-qPCR method: The overall RNA was extracted from the hepatocyte specimen by Trizol reagents in each group and then synthesized into cDNA under the action of reverse transcriptase, with β-actin as internal control, the β-actin forward primer was 5'-CATCC-TGCGTCTGGACCTGG-3', and reverse primer was 5'-TAATGTCACGCACGATTTCC-3'; the NALP3 forward primer was 5'-CCAAGCCAGGGCAGCC-TTCA-3', and reverse primer was 5'-GGAAG-AAGACGTACACCG-3'; the ASC forward primer was 5'-CAGGCCCCTCCTCAGTCGGCA-3', and reverse primer was 5'-CCACTCAACGTTTGTGA-CCCT-3'; the Caspase-1 forward primer was 5'-ATTATTACAGACAAGGGTG-3', and reverse primer was 5'-CCCAGCGTCCCTGCCAGGT-3'. The reaction system 20 uL included 2 uL of cDNA, forward and reverse primers of 0.6 uL each, 10 uL of SYBR Premix Ex TaqTM II (2*), 0.4 uL of ROX Reference Dye (50*), and 6.4 uL of dH_2O. Applied Biosystems 7500 quantitative PCR instrument was centrifugally inserted for amplification. The reaction conditions included initial denaturation at 95°C for 30 sec, denaturation at 95°C for 15 sec, renaturation at 60°C for 50 sec, extension at 72°C for 1 min, with a total of 40 cycles. With β-actin as the reference gene, the mRNA relative levels of NALP3, ASC and Caspase-1 in each group were calculated with the use of the $2^{-\Delta\Delta Ct}$ method.

Protein levels of NALP3, ASC and Caspase-1 tested by the Western blot: The total protein was extracted from the lysate-added hepatocyte suspension and then put into the wells of gel electrophoresis in each group. The samples were isolated by SDS-PAGE gel electrophoresis, and then protein was transferred to PVDF membranes by electroblotting. After that, the membranes were clipped based on the required target electrophoretic bands and then blocked in TBS-T solution with 5% skimmed milk power at room temperature for 1 h. After skimmed milk blocking, the membranes were incubated by addition of primary antibodies of respective rabbit anti-mouse NALP3 polyclonal antibody, rabbit anti-mouse ASC polyclonal antibody, rabbit anti-mouse Caspase-1 polyclonal antibody, and rabbit anti-mouse β-actin polyclonal antibody at 4°C overnight. After that, the membranes washed with TBS-T solution at room temperature, they were incubated by adding secondary antibodies of HRP-labeled Goat Anti-Rabbit for 30 min. After washed with TBS-T solution again, the membranes were reacted

Table 1. Comparison of mRNA levels of NALP3 inflammasomes in hepatocytes

Variable	NALP3	ASC	Caspase-1
Sham group	0.191±0.02	0.242±0.03	0.213±0.03
Model group	1.275±0.06	1.153±0.05	0.928±0.04
PD1 group	0.618±0.05	0.576±0.04	0.515±0.03
F value	412.854	424.802	445.960
P value	<0.001	<0.001	<0.001

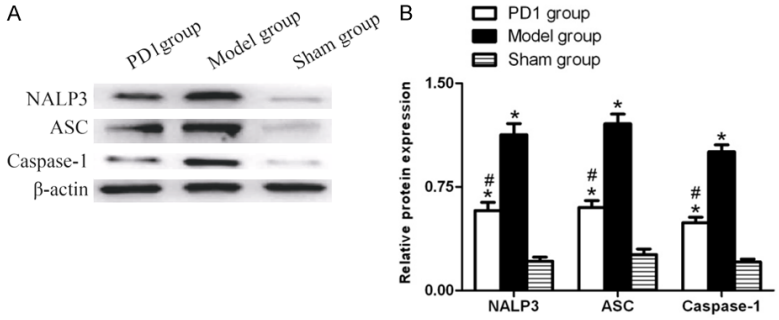

Figure 1. Detection of protein level in NALP3 inflammasomes. A: Western blot images; B: Comparison of protein relative levels of NALP3, ASC and Caspase-1 in hepatocytes. Compared with the sham group, *P<0.05; compared with the model group, #P<0.05.

with chromogenic reagents, followed by exposure and scanning. The target electrophoretic bands were detected on the gel image analyzer, with β-actin as internal control.

ROS in the hepatocytes: In each group, the concentration of hepatocyte suspension was adjusted to $1*10^6$. After addition of 10 μM of DCFH-DA, the hepatocytes were placed in an incubator with an atmosphere of 5% CO_2 at 37 °C for 30 min. The hepatocytes were centrifuged, from which the supernatant was removed, and then the hepatocytes were washed 3 times. The fluorescence F values of each group were measured by flow cytometry. at excitation light of 485 nm and emission light of 530 nm.

Detection of serum IL-18 and IL-1β concentrations: The serum IL-18 and IL-1β concentrations in each group were examined using the enzyme-linked immunosorbent assay (ELISA) in accordance with the specific instructions on the ELISA kits. The values of optical density (OD) at each well at wavelength of 450 nm were then read using a microplate reader, followed by calculation of the concentrations (pg/mL) of all the samples according to the standard curves.

Statistical analysis

All the data analyses were performed with the use of SPSS software, version 20.0. Measurement data were presented as mean ± sd whereas count data were expressed as percentages or rates. The one-way analysis of variance was utilized for multi-group comparisons of measurement data while the post hoc Bonferroni test was applied for inter-group comparisons among the three groups. The chi-square test was employed to compare count data. A *P* value of less than 0.05 was deemed to be significantly diferent.

Results

Comparison of mRNA levels of NALP3, ASC and Caspase-1 in hepatocytes

The mRNA levels of NALP3, ASC and Caspase-1 in hepatocytes were elevated markedly both in the model group and the PD1 group compared with those in the sham group (All P<0.001); the mRNA levels of NALP3, ASC and Caspase-1 in hepatocytes in the PD1 group were strikingly lower than those of the model group (All P<0.001, **Table 1**).

Protein levels of NALP3, ASC and Caspase-1 in hepatocytes

The result of the Western blot analysis demonstrated that protein levels of NALP3, ASC and Caspase-1 in hepatocytes were remarkably higher in both the model group and the PD1 group than in the sham group (All P<0.001), but the corresponding levels were substantially lower in the PD1 group than those in the model group (All P<0.001; **Figures 1**, **2**).

ROS in hepatocytes among the groups

The mean fluorescence intensity of ROS in the hepatocytes was detected with the application of flow cytometry, and the results are shown in **Figure 2.** The ROS concentrations in the hepatocytes in the model group and the PD1 group were markedly higher than that of the sham

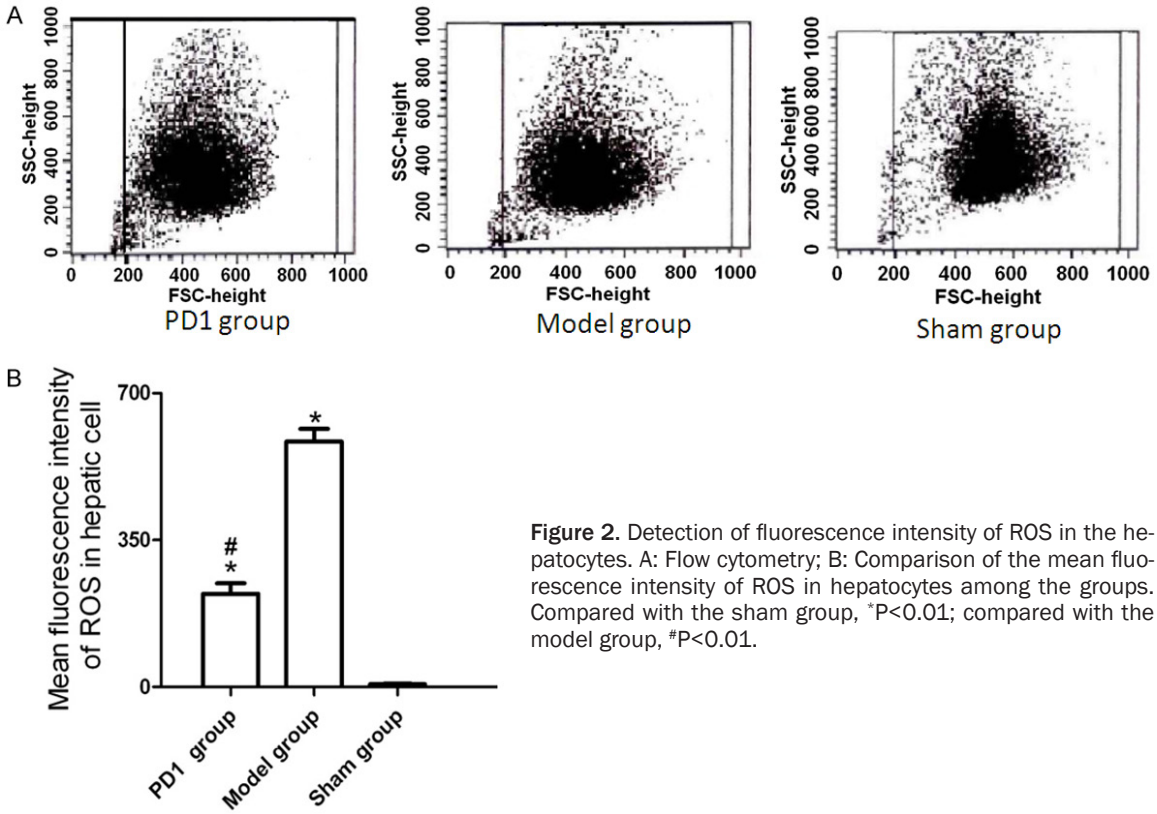

Figure 2. Detection of fluorescence intensity of ROS in the hepatocytes. A: Flow cytometry; B: Comparison of the mean fluorescence intensity of ROS in hepatocytes among the groups. Compared with the sham group, *P<0.01; compared with the model group, #P<0.01.

group (Both P<0.001); the ROS concentration in hepatocytes in the PD1 group was strikingly lower than that in the model group (P<0.001).

Comparison of serum IL-18 and IL-1β concentrations

The serum IL-18 concentrations were increased markedly both in the model group (312.45±20.57 pg/mL vs 78.87±8.65 pg/mL, P<0.001) and in the PD1 group (136.92±22.71 vs 78.87±8.65 pg/mL, P<0.001) when compared with that in the sham group, whereas the serum IL-18 concentration in the model group was substantially higher than that in the PD1 group (P<0.001, **Figure 3**).

The serum IL-1β concentrations were 64.72± 7.84 pg/mL, 247.31±16.57 pg/mL and 118.45±12.25 pg/mL in the sham group, the model group and the PD1 group, respectively. The serum IL-1β concentrations of the model group and the PD1 group increased markedly as compared with that of the sham group, (Both P<0.001), whereas the serum IL-1β concentration was remarkably lower in the PD1 group than in the model group (All P<0.001; **Figure 4**).

Discussion

Sepsis in essence is systemic inflammatory response. Physiologically, the pro- and anti-inflammatory cytokines of the organisms maintain a general equilibrium. When sepsis occurs, the release of a sea of pro-inflammatory cytokines inhibits the activity of anti-inflammatory cytokines and reduces their secretion, resulting in pro-inflammatory/anti-inflammatory disorders and progression of inflammation [15-17]. Intense inflammatory response is the underlining cause of disease progression and multiple organ dysfunctions. In the present study, we employed the cecal ligation and puncture to establish an inflammatory model of septic rats in which the progression of clinical inflammatory response was simulated and PD1 was administered to the rats before operation.

The presence of sepsis leads to pathological hypoxia in the hepatocytes, intracellular mitochondrial dysfunction and oxidative phosphorylation disorders, producing lots of ROS. All these result in insufficient production of adenosine triphosphate (ATP) in mitochondria and energy failure in cells [18, 19]. Other research-

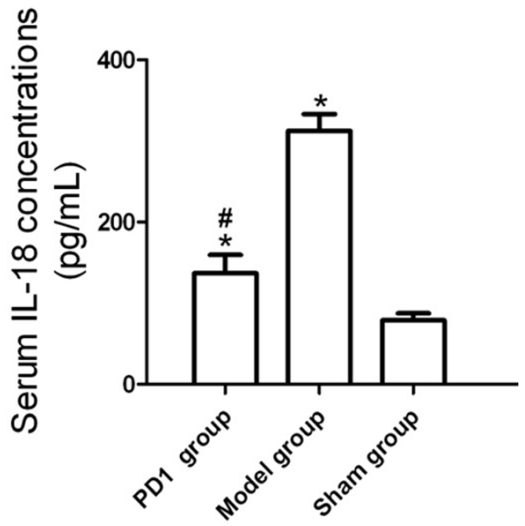

Figure 3. Comparison of serum IL-18 concentrations among the groups. Compared with the sham group, *P<0.05; compared with the model group, #P<0.05.

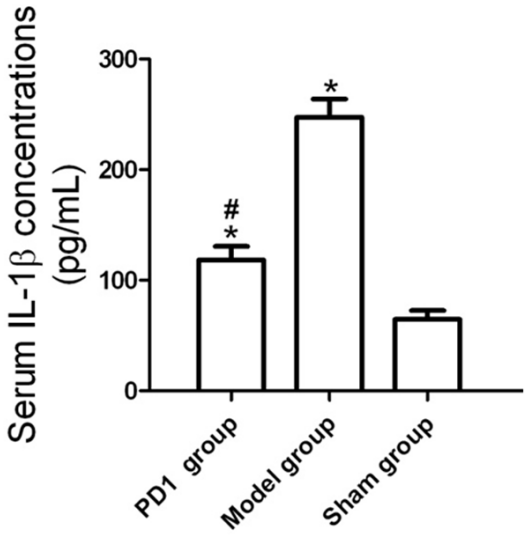

Figure 4. Comparison of serum IL-1β concentrations among the groups. Compared with the sham group, *P<0.05; compared with the model group, #P<0.05.

ers have confirmed that ROS is a key regulator of NALP3 inflammasome activation [20]. A sea of studies have substantiated that NALP3 inflammatory signaling pathway plays a crucial role in inflammatory response and immune regulation [21]. NALP3 is mainly generated by T and B lymphocytes. As an intracellular pattern recognition receptor, NALP3 identifies both risk-associated and pathogen-associated molecular patterns, and is ultimately assembled into NALP3 inflammasomes which activate effector protein Caspase-1 and clip IL-1β precursors into active ones. The activated IL-1β and IL-18 and other inflammatory cytokines are secreted outside the cells, leading to the inflammatory cascade amplification effect of sepsis. It is noted that the key proteins for inflammatory activation are NALP3, ASC and Caspase-1, whereas IL-1β and IL-18 and other inflammatory cytokines are the final products of pro-inflammatory activation. The current study indicated that, the ROS concentration in hepatocytes, the levels of NALP3, ASC and Caspase-1 and the IL-1β and IL-18 concentrations in the peripheral serum were remarkably higher in the model group when compared with those in the sham group. These results suggest that in the inflammatory response of sepsis, the increase in ROS concentration regulated NALP3 inflammasome activation, hence promoting the secretion of inflammatory cytokines IL-1β and IL-18.

Originated from the metabolites of DHA epoxidation, protectins are also known as class D protectins owing to their potent protective effect on the central nervous system [22]. Synthesized and released by glial cells, brain tissues and peripheral blood cells, PD1 is a potent endogenously anti-inflammatory and pro-resolving lipid mediator. It plays a key negative regulatory role in plenty of inflammatory cells and cytokines, and is an important “stop” or “brake” signal for inflammatory response [23-25]. PD1 exerts a great inhibitory effect on exudation or infiltration of neutrophils [26]. It plays its neuroprotective role by reducing the infiltration of leukocytes in the brain, IL-1β-induced NF-κB activity and COX-2 expression levels [27]. Nevertheless, the effect of PD1 on NALP3 inflammsomes in inflammatory response in sepsis and their mechanisms remain uncertain. The present study demonstrated that, intraperitoneal injection of PD1 markedly reduced the ROS concentration in hepatocytes, lowered NALP3 ASC and Caspase-1 levels in cells, and suppressed the serum of IL-1β and IL-18 concentrations in the PD1 group as compared with those in the model group, suggesting that PD1 improved the inflammatory response of sepsis, which might be related to its abilities to reduce ROS concentrations and in turn inhibit NALP3 inflammasome activity.

In conclusion, PD1 reduced the assembly and activation of NALP3 inflammasomes and the serum IL-1β IL-18 concnetrations in septic rats,

which might be associated with the decrease in ROS concentration in cells. This lays an experimental basis for the application of PD1 and analogues with similar chemical structure to prophylaxis and treatment of sepsis and inflammation. However, additional studies are required to delve into the specific mechanisms of PD1 reducing ROS concentrations.

Disclosure of conflict of interest

None.

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