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ONO-5046 suppresses reactive oxidative species-associated formation of neutrophil extracellular traps

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ABSTRACT

Background: Neutrophil extracellular traps (NETs) have been identified as a non-apoptosis cell death pattern that leads to the release of granular contents into the extracellular space and subsequent excessive inflammatory response. The present study aims to investigate whether ONO-5046, a novel neutrophil elastase inhibitor, could affect NETs formation and promote inflammation resolution.

Methods: Neutrophils were separated and identified. Cell survival rate was analyzed using the trypan blue stain-resistance method. Different concentrations of lipopolysaccharide (LPS) (0.01 μg/mL, 0.1 μg/mL, and 1.0 μg/mL) were used to stimulate NETs formation. ONO-5046 (0.1 μg/mL and 1 μg/mL) was administered after high-dose LPS stimulation and NETs formation. Moreover, tBHP was used to further investigate the relationships between the effects of ONO-5046 and reactive oxygen species (ROS) release. ROS was detected by DCFH-DA fluorescent staining and NETs formation was demonstrated *via* immunofluorescence staining for neutrophil elastase and citrullinated histone H3 (H3Cit).

Results: NETs formation was stimulated by LPS in a dose-dependent manner. High doses of LPS induced ROS generation and decreased cellular survival. ONO-5046 reduced LPS-induced NETs formation in a dose-dependent manner, as evidenced by immunofluorescence staining for neutrophil elastase and H3Cit whereby the fluorescence intensity decreased and neutrophil ROS generation was attenuated. However, the effects of ONO-5046 on NETs reduction were reversed by ROS inducer tBHP.

Conclusions: The neutrophil elastase inhibitor ONO-5046 suppresses ROS-associated NETs formation, which may lead to inflammation resolution.

1. Introduction

Excessive and uncontrolled inflammatory response occurs in the process of ischemia/reperfusion disease, sepsis and shock [1–3]. The rapid promotion of inflammation resolution is linked to early prognosis in patients. Neutrophils, the predominant circulating white blood cells in humans, have been identified as the key cell type that contributes to the resolution of inflammation [4]. Mediated by numerous neutrophil chemotactic factors, neutrophils quickly infiltrate injured tissue during excessive inflammation [5]. Removal of apoptotic neutrophils by macrophages after the functional shutdown of neutrophils can lead to timely inflammation resolution [6].

Neutrophil extracellular traps (NETs), which are networks of chromatin fibers, are primarily composed of antimicrobial peptides and enzymes such as myeloperoxidase and neutrophil elastase [7]. NETs allow neutrophils to kill extracellular pathogens while minimizing damage to the host cells [8]. Aggregated NETs have been recently found to limit inflammation by degrading chemokines and cytokines in gout [9]. However, NETs have also been linked to necroptosis of neutrophils, which resulted in neutrophil-associated disorders and excessive release of inflammatory cytokines [10].

During the formation of NETs, neutrophil elastase is translocated from neutrophil granules to the nucleus in a reactive oxygen species (ROS)-dependent manner, which does not involve membrane fusion, but the nuclear translocation mechanism is unknown [11]. Indeed, neutrophil elastase plays a critical role in the formation of NETs and has

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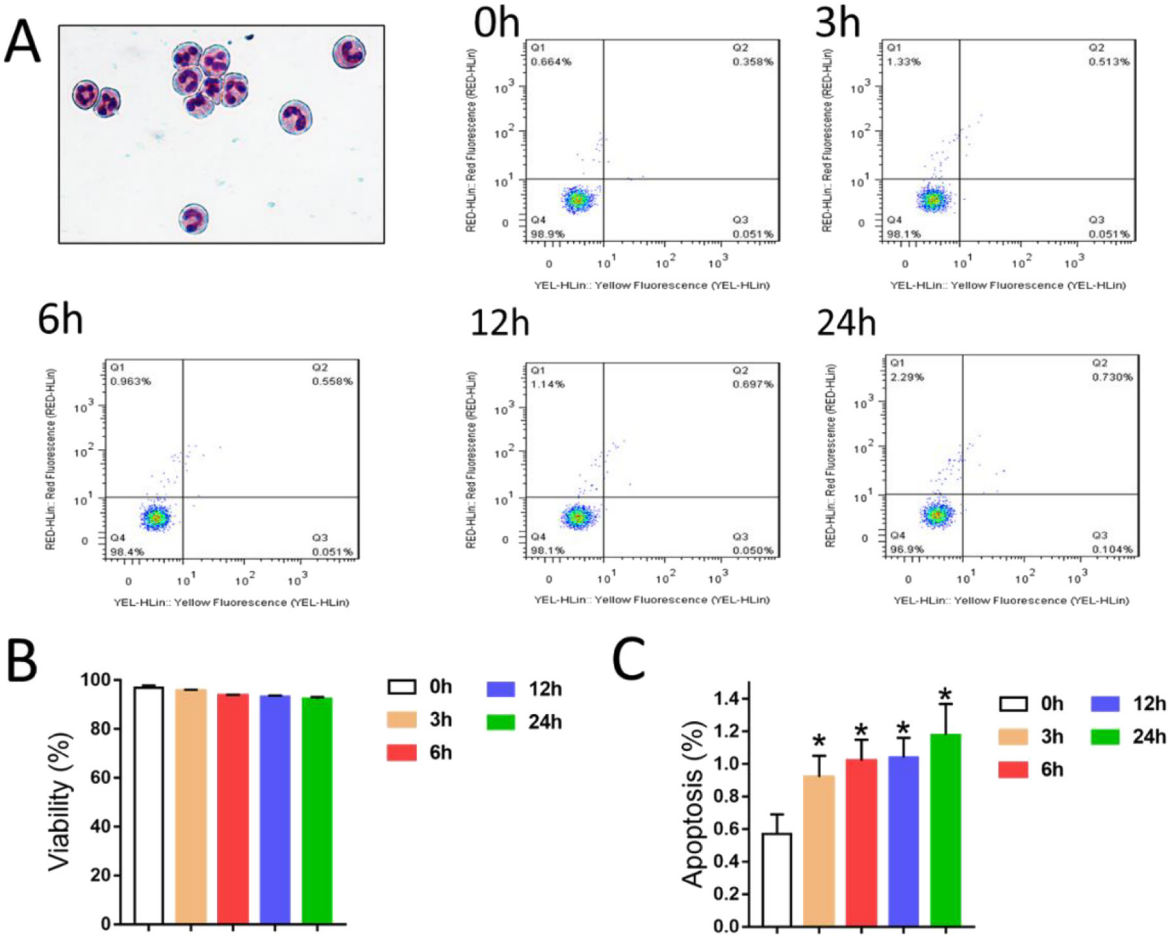

Fig. 1. Human PMN isolation, culture, and identification. (A) PMNs were stained using the Wright-Giemsa Stain method. Cellular apoptosis of PMNs cultured for 0, 3, 6, 12, and 24 h was detected by Annexin V/7-ADD flow cytometry using the Muse™ all-round cytoplasmic examination system. (B) PMN viability was measured by the TBS-resistance method. (C) Positive PMN apoptosis rate was analyzed according to the results in (A). The data are presented as the mean ± SEM, n = 5 independent experiments. *$P < 0.05$ *vs.* 0 h group.

also been tied up with chromatin decondensation in NET formation process by histone cleavage [12]. However, whether inhibition of neutrophil elastase can effectively limit the formation of NETs remains unknown. ONO-5046, a synthetic specific inhibitor of neutrophil elastase, has been verified to directly inhibit neutrophil elastase [13]. Compared with other neutrophil elastase inhibitors, ONO-5046 penetrates tissues and cells sufficiently. It is not affected by ROS and can effectively inhibit the local activity of neutrophil elastase [14].

We hypothesized that inhibition of neutrophil elastase by ONO-5046 could lead to reduction of NETs during acute inflammatory response, which may effectively limit the release of inflammatory cytokines and promote inflammation resolution.

2. Materials and methods

2.1. Isolation and quantification of neutrophils

This study was approved by the medical research ethics committee of The Third Affiliated Hospital of Sun Yat-sen University. Peripheral venous blood was provided by healthy adult volunteers and informed written consent was obtained from all volunteers. Three milliliters of venous blood was collected in tubes containing ethylenediaminetetraacetic acid and gently overlaid onto 5 mL of neutrophil separation liquid (Neutrophils Separation Kit, Tianjin Haoyang, China), followed by centrifugation at 600 ×*g* for 30 min at 22 °C. Six layers of cells appeared in the centrifuge tube, and the fourth layer of neutrophils was moved to another centrifuge tube. The neutrophils were resuspended by lysis buffer (Red Blood Cell Lysis Buffer, Tianjin Haoyang, China) to remove the erythrocytes and subsequently washed in phosphate-buffered saline. The neutrophils were resuspended at a concentration of 1–5 × 10^5 cells/mL in 3 mL of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum. The purity and morphology of the isolated neutrophils were verified with Wright-Giemsa staining, and the cell viability was evaluated by trypan blue dye exclusion assay.

2.2. LPS induction model

Isolated neutrophils (1 × 10^5) were cultured in a coated 24-well plate with lipopolysaccharide (LPS, Sigma-Aldrich) at concentrations of 0.01, 0.1, and 1 μg/mL LPS for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. The control group was cultured in the same medium with no added agent. To identify the optimum concentration of LPS, cell viability was evaluated by trypan blue dye exclusion assay, oxidative burst was quantified by measuring the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich), and NETs were analyzed by fluorescence microscopy.

2.3. ONO-5046 treatment

Isolated neutrophils (1 × 10^5) were plated and allowed to adhere onto a coated 24-well plate for 1 h prior to incubation with LPS,