Characterization of Bovine Neutrophil Extracellular Traps Induced by Histamine

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Research

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Abstract

Background

Histamine plays an central role in many allergic diseases including allergic asthma and allergic rhinitis, and is also involved in bovine laminitis through regulating immune responses. Neutrophil extracellular traps (NETs) formation is a novel effector mechanism of neutrophils to defend against various stimuli. In the present study, we aimed to investigate the role of histamine on bovine NET formation and examined its fundamental molecular mechanisms.

Results

Firstly, the effects of histamine on neutrophil viability was measured by Cell Counting Kit-8 (CCK-8) and Lactate dehydrogenase (LDH) assays. The results showed that histamine had no significant influence on neutrophil viability. Then we characterized histamine-triggered NET formation by confocal microscopy and PicoGreen-derived NETs quantification. Confocal microscopy analyses illustrated NET structures by co-localizing the main components of NETs, and NET quantification revealed that histamine-triggered NETs were released in a dose-dependent manner. In addition, we found reactive oxygen species (ROS) production, phosphorylated extracellular signal-regulated kinase (ERK) and p38 proteins were significantly elevated in histamine-challenged neutrophils. By applying functional inhibitors of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), ERK and p38, histamine-triggered NETs were markedly reduced, indicating their importance in histamine-triggered NET formation.

Conclusions

Our findings described histamine-triggered NET formation, and revealed its potential molecular mechanisms via NADPH oxidase, ERK and p38 pathways. This is the first study to depict NET formation induced by histamine, which could provide a new insight into histamine-related diseases.

Introduction

Histamine is a natural constituent of the body classified as a biogenic amine[1]. Originally, it is only considered an inflammatory mediator causing anaphylactic reactions[2]. Nowadays, histamine is one of the most extensively studied biomolecules, and has been related to various physiological functions, such as hematopoiesis, cell proliferation and differentiation, exocrine pancreatic secretion, embryonic development, regeneration, and wound healing[3-6]. On the other hand, histamine plays a vital role in the course of many allergic diseases including allergic asthma and allergic rhinitis[7, 8]. In addition, laminitis from which cattle suffer is believed to be associated with histamine intolerance (histaminosis)[9].

Neutrophils are the most abundant leukocytes in blood that play a crucial role in innate immunity. Besides well-known phagocytosis and degranulation, the formation neutrophil extracellular traps (NETs) is a novel effector mechanism of neutrophils to defend against foreign invaders[10, 11] which has
received extensive attention by researchers around the world since it was first reported in 2004. NETs are fine web-like structures composed of DNA as a backbone, decorated with histones, and numerous antimicrobial proteins released by neutrophils[12], which capture, neutralize and kill various microorganisms including bacteria, fungi[13], viruses[14] and parasites[15]. Moreover, some studies revealed that NETs hindered bacteria and fungi from disseminating[16, 17]. However, one sword has two edges, if NETs are dysregulated, they can promote the pathogenesis of some immune-related diseases. Additionally, increasing evidence shows that NETs also occur in noninfectious, sterile diseases, such as lupus nephritis[18], rheumatoid arthritis[19], diabetes[20], atherosclerosis[21], vasculitis[22], thrombosis[23], cancer[24], wound healing[25] and trauma[26]. Therefore, NETs has been considered as potential therapeutic targets in many diseases.

Studies have showed that intranasal administration of recombinant DNase improved lung function in allergic diseases[27] indicating NETs’ importance, and histamine is closely associated with allergic actions and symptoms. We speculated that histamine plays a crucial role in the formation of NETs. In this present study, we aimed to investigate whether histamine could induce the release of NETs by bovine neutrophils, and further examined its fundamental mechanisms.

Materials And Methods

Reagents

2,7-dichlorodihydrofluorescein diacetate (DCF-DA), Diphenyleneiodonium (DPI), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126), SB202190, and zymosan are purchased from Sigma-Aldrich. Sytox Orange nucleic acid stain (Invitrogen), PicoGreen (Invitrogen), Histone H3 antibody (LSC353149; Life Span BioSciences, Inc), Myeloperoxidase (MPO) antibody (Orb16003; Biorbyt), anti-p38 (Bs3566; Bioworld), anti-ERK (Bs3627), anti-pp38 (Cell Signaling Technology Inc, USA), anti-p-ERK (Cell Signaling Technology Inc, USA) were used in this study.

Isolation of bovine neutrophils

Bovine neutrophils were isolated from peripheral blood of healthy dairy cows by bovine PMN (Polymorphonuclear leukocytes) isolation kit (TianJin HaoYang Biological Manufacture CO. China) according to the manufacturer's instructions. Briefly, blood was diluted 1:1 with cleaning solution, layered on separating solution in centrifugal tubes, and then centrifuged at 700g for 30min. The lower cellular layer was collected, and washed by erythrocyte lysis solution until the cell pellet became white. Finally, isolated neutrophils were re-suspended in RPMI 1640 medium without phenol red, and placed in a incubator until further use.

Cytotoxicity assay by CCK-8 kit

Briefly, neutrophils were seeded into a 96-well plate, confronted with histamine (6.25, 12.5, 25μM) for 2h. Subsequently, 10μL CCK-8 solution was added into each well. After 2h incubation in an incubator, the
plate was read by a plate reader at 450nm.

Lactate dehydrogenase (LDH) assay

Neutrophils were seeded into a 96-well plate and confronted with histamine (6.25, 12.5, 25μM) for 2h. The plate was centrifuged at 400g for 5min, and the supernatants were transferred to a new plate. LDH activity was measured by LDH Cytotoxicity Assay kit (Beyotime Biotechnology, China) according to the manufacturer's protocols.

Confocal microscopy analyses

Isolated neutrophils were seeded on glass coverslips pre-treated with poly L-lysine (0.1 mg/ml) and stimulated with histamine (25μM) for 2h at 37 °C with 5 % CO_{2}. Then, samples were fixed with 4% (w/v) paraformaldehyde for 30min. For immunostaining, paraformaldehyde solution was removed, and samples were washed thrice with PBS (Phosphate buffered saline), followed by 1h incubation with blocking buffer 3% BSA (bovine serum albumin). Labeling of specific proteins (histone and MPO) for NETs was performed with the incubation of anti-histone antibody and anti-myeloperoxidase antibody overnight, and secondary antibody goat-anti-rabbit conjugated to Alexa 488 for 2h. For DNA staining, the samples were incubated with 5μM Sytox Orange (dissolved in PBS) for 10 min in dark. Samples were observed and images were taken using confocal microscope (Olympus FluoView FV1000).

NETs quantification based on PicoGreen® fluorescent dye

In the first set of experiment, we quantified NETs induced by different concentrations of histamine (6.25, 12.5, 25μM). Neutrophils were seeded in a 96-well plate, and then stimulated with histamine for 2h in an incubator at 37 °C with 5 % CO_{2}. Quant-iT™ PicoGreen solution was added into each well, and the fluorescence intensity was measured using an Infiniti M200 fluorescence plate reader (Tecan, Austria).

In the secondary set of experiment, we examined the effects of specific inhibitors on NET formation induced by histamine (25μM) via NETs quantification. Neutrophils were pretreated with NADPH oxidase inhibitor (DPI), the inhibitors of ERK1/2-signaling pathway (U0126) and P38 MAPK-signaling pathway (SB202190) for 30 min in a 96-well plate, and then challenged by histamine for 2h in an incubator with 37 °C and 5 % CO_{2}. After adding PicoGreen solution, the plate was read by an Infiniti M200 fluorescence plate reader (Tecan, Austria) with 485nm length of excitation and 535nm length of emission.

ROS detection

Neutrophils were seeded into a 96-well plate, and stimulated with histamine (6.25, 12.5, 25μM) for 2h in an incubator with 37 °C and 5 % CO_{2}. DCF-DA (10μM) was added to each well, and the plate was incubated for 20min. After thrice washing with PBS, fluorescence intensity was measured by an Infiniti M200 plate reader at 485 nm of excitation and 525 nm of emission.

Western blotting
For protein isolation, neutrophils were confronted with histamine (6.25, 12.5, 25μM) for 2h, and then lysed with M-PER™ mammalian protein extraction reagent (Thermo Fisher Scientific). After centrifugation, the supernatant was collected, and protein concentration was measured by a bicinchoninic acid (BCA) protein assay reagent kit (Pierce).

For western blotting, firstly we separated proteins in samples using gel electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. Next, the membrane was blocked with 3% BSA to prevent any nonspecific binding of antibodies, incubated with primary antibody (anti-p38 monoclonal antibody, 1:1000; anti-phosphor-p38 monoclonal antibody, 1:1000; anti-ERK monoclonal antibody, 1:1000; anti-phosphor-ERK monoclonal antibody, 1:1000) overnight at 4°C, and incubated with HRP-conjugated secondary antibody for 2h at room temperature. In the end, the membrane was detected using enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (ProteinSimple, San Jose, CA, U.S.A.).

Statistical analysis

All Data were illustrated as means ± SEM of at least three biological replicates and two technical replicates. Graphs and statistical analyses were generated by using GrapPadPrism software (v.7.03). One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests were used. Statistical significance was defined by a p value < 0.05.

Results

Histamine had no effect on neutrophil viability

Firstly, we used CCK-8 assay to check the effect of histamine (6.25, 12.5, 25μM) on neutrophil viability. The result showed that no obvious difference in cell viability was observed when neutrophils were confronted with three different concentrations of histamine compared to the control group (Fig. 1).

Secondly, we checked the cellular cytotoxicity of histamine on neutrophils via LDH assay. LDH is a cytosolic enzyme existing in many different types of cell, and extracellular LDH is an indicator of cellular cytotoxicity. Similarly, histamine (6.25, 12.5, 25μM) challenge did not significantly change extracellular LDH level (Fig. 2), indicating no cytotoxicity on neutrophils.

Histamine triggered NET formation visualized by immunofluorescent staining

To visualize NET structures, neutrophils were seeded onto coverslips and confronted with 25μM histamine for 2h, and the samples were performed by immunostaining. NET structures were co-localized by DNA, histone and MPO staining. As shown in Fig. 3, NET formation induced by histamine was obviously observed (white arrows).

Histamine-triggered NET formation was dose-dependent
Here we chose three different concentrations of histamine (6.25, 12.5, 25 μM) to check if there is difference in NET formation. NETs quantification were conducted based on PicoGreen dsDNA reagent-derived fluorescent intensity. Compared to the control group, histamine induced an increasing extracellular DNA with increasing concentrations (Fig. 4), suggesting its dose-dependent manner in NET formation.

**Histamine increased ROS production in a dose-dependent manner**

ROS generation are required for NETs release[28]. Here we measured intracellular ROS production in neutrophils confronted with histamine by DCF-DA. When compared to that of the control group, an increasing rising trend in ROS production induced by histamine was observed (Fig. 5), indicating that histamine dose-dependently increased ROS generation.

**Histamine enhanced the phosphorylation of ERK and p38 proteins**

Mitogen-activated protein kinase (MAPK) pathways regulate a large range of cellular activities, such as gene expression, cell growth, metabolism, survival, apoptosis, and differentiation [29]. In this present study, phosphorylated ERK and p38 proteins were enhanced in histamine-treated neutrophils (Fig. 6), implying a potential role of ERK and p38-related MAPK pathways in NET formation.

**Histamine-triggered NET formation was a NADPH oxidase, ERK and p38 pathways mediated process**

To further examine the role of ROS, ERK and p38-mediated MAPK pathways in histamine-triggered NETs, we applied their functional inhibitors (DPI, U0126, SB202190 respectively). In Fig. 7, histamine-induced extracellular DNA were significantly reduced by these inhibitors pretreatment. This finding showed that ROS, ERK and p38-mediated MAPK pathways played a crucial role in histamine-triggered NET formation.

**Discussion**

Histamine, mainly stored in mast cells, is a prominent mediator in the clinical symptoms of many allergic diseases, such as asthma, allergic rhinitis, urticaria, anaphylaxis. NETs as part of the innate immune response have been reported to be involved in diverse allergic and autoimmune diseases[30]. Therefore, in this present study we characterized NET formation induced by histamine.

The process of NET formation is called NETosis, and it has been the most studied area of PMN functions since its first report. NETs are mainly composed of DNA, histones, and many granule proteins such as NE, MPO, cathepsin G, and lactoferrin. These classical components of NETs have been co-localized and visualized often by immunostaining and cofocal microscope to identify the structures of NETs [31-33]. Here we identified histamine-triggered NET structures via co-localizing DNA, histone and MPO, and observed this kind of structures under cofocal microscope, suggesting histamine is a potent inducer of NETs.
Initially, most of studies were focused on microorganisms-triggered NETs. As a vast number of molecules were identified as NETs stimuli such as ionomycin [34], nicotine [35], hydrogen peroxide [11], TNF-α [36], Fc receptors [13], IFN-γ [37], and antimicrobial peptides [38], increasing studies are paying attention on non-infectious and autoimmune diseases [39, 40]. Histamine is a main mediator in allergic diseases, and we found it also can induce NET formation. Moreover, histamine-induced NETs were dose-dependently, which is similar to other stimuli [41, 42].

Two major types of NETosis have been found: NADPH oxidase (NOX)-dependent and NOX-independent NETosis [43]. NADPH oxidase is a crucial enzyme in host defense, and patients with deficiency of NADPH oxidase suffer from life-threatening infections [44]. NADPH oxidase is the major source of ROS that play an important role in various biological processes [45]. Histamine increased ROS production, and inhibition of NADPH oxidase significantly hindered NET formation induced by histamine, indicating that histamine-triggered NETosis is a NOX-dependent process.

ERK and p38 MAPK pathways regulate a variety of cellular functions including NETosis [46]. In this study, ERK and p38 proteins were increased in histamine-confronted neutrophils, and NETs induced by histamine were obviously decreased by functional inhibition of ERK and p38 pathways, showing the key role of this two pathways in histamine-triggered NETosis. ERK and p38 pathways are closely associated with NET formation via activation of NADPH oxidase [47], which is also confirmed in histamine-triggered NETosis by us.

In short, we illustrated histamine-triggered NET structures, and described its basic characters. In addition, the primary mechanism of histamine-triggered NETosis is dependent on NADPH oxidase, ERK and p38 pathways. However, further studies are still required to delineate the detailed mechanisms of histamine-triggered NETosis.

**Abbreviations**

NETs: Neutrophil extracellular traps

CCK-8: Cell Counting Kit-8

LDH: Lactate dehydrogenase

ROS: Reactive oxygen species

ERK: Extracellular signal-regulated kinase

NADPH oxidase: Nicotinamide adenine dinucleotide phosphate-oxidase

DCF-DA: 2,7-dichlorodihydrofluorescein diacetate

U0126: 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
MPO: Myeloperoxidase
PMN: Polymorphonuclear leukocytes
BCA: Bicinchoninic acid
PVDF: Polyvinylidene difluoride
BSA: Bovine serum albumin
PBS: Phosphate buffered saline
ECL: Enhanced chemiluminescence
ANOVA: One-way analysis of variance
MAPK: Mitogen-activated protein kinase

Declarations

Ethics approval and consent to participate

The experimental protocols used in this experiment, including animal care and use, were reviewed and approved by the Ethics Committee of Jilin University on the Care and Use of Laboratory Animals (JLU20170330).

Availability of data and materials

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

Contributions

ZY designed this study, EZ directed this study, interpreted the results, and wrote this manuscript. ZW, XY, PL and JW participated in the performance of all experiments. All authors have read and approved the final version of this manuscript.

Competing interest

The authors declare no competing conflicts of interest.

Consent for Publication

All authors have reviewed this manuscript and approved to publish it in this journal.

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Figures
Figure 1

Effects of histamine (His) on neutrophilic viability. Neutrophils were seeded into a 96-well plate and confronted with histamine (6.25, 12.5, 25μM) for 2h, followed by 2h incubation with 10μL CCK-8 solution. At last, the plate was read by a plate reader at 450nm. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01).
Figure 2

Effects of histamine (His) on LDH activity in neutrophils. Neutrophils were seeded into a 96-well plate and confronted with histamine (6.25, 12.5, 25μM) for 2h. The plate was centrifuged at 400g for 5min, and the supernatants were transferred to a new plate. LDH activity was measured by LDH Cytotoxicity Assay kit according to the manufacturer's protocols. Statistical analyses were performed by one-way ANOVA followed by Dunnett’s multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01).
Figure 3

Histamine triggered NET formation visualized via immunostaining. Neutrophils were seeded on glass coverslips pre-treated with poly L-leylsine (0.1 mg/ml) and stimulated with histamine (25μM) for 2h at 37 °C with 5 % CO2. Then, samples were fixed with 4% (w/v) paraformaldehyde for 30min. After labeling specific proteins (histone and myeloperoxidase) for NETs, samples were observed and images were taken under confocal microscope. Panel A: DNA staining in red, histone staining in green. Panel B: DNA staining in red, MPO staining in green. In merged images, NET structures were indicated by white arrows.
Histamine-triggered NETs were dose-dependent. Neutrophils were seeded in a 96-well plate, and then stimulated with histamine (6.25, 12.5, 25μM) for 2h in an incubator at 37 °C with 5 % CO2. Quant-iT™ PicoGreen solution was added into each well, and the fluorescence intensity was measured using a fluorescence plate reader. Zymosan was used as a positive control. Statistical analyses were performed by one-way ANOVA followed by Dunnett’s multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01, *** p<0.0001).
Figure 5

Histamine (His) elevated ROS production in neutrophils. Neutrophils were seeded into a 96-well plate, and stimulated with histamine (6.25, 12.5, 25μM) for 2h in an incubator with 37 °C and 5 % CO2. DCF-DA (10μM) was added to each well, and the plate was incubated for 20min. After thrice washing with PBS, fluorescence intensity was measured by an Infiniti M200 plate reader at 485 nm of excitation and 525 nm of emission. Zymosan was used as a positive control. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01, *** p<0.0001).
Histamine (His) enhanced the expression of ERK and p38 proteins. Neutrophils were confronted with histamine (6.25, 12.5, 25μM) for 2h, and then lysed with M-PER™ mammalian protein extraction reagent (Thermo Fisher Scientific). After centrifugation, the supernatant was collected, and protein concentration was measured by a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). The expression level of ERK and p38 proteins in samples were detected via western blotting. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01).
Histamine-triggered NETosis was dependent on NADPH oxidase, ERK and p38 pathways. Neutrophils were pretreated with NADPH oxidase inhibitor (DPI), the inhibitors of ERK1/2-signaling pathway (U0126) and P38 MAPK-signaling pathway (SB202190) for 30 min in a 96-well plate, and then challenged by histamine for 2h in an incubator with 37 °C and 5 % CO2. After adding PicoGreen solution, the plate was read by an Inniti M200 fluorescence plate reader with 485nm length of excitation and 535nm length of emission. Zymosan was used as a positive control. Statistical analyses were performed by one-way ANOVA followed by Dunnett’s multiple comparisons test. Significant difference was defined by a p-value <0.05 (*p<0.05, ** p<0.01, *** p<0.0001).