



Uric acid induces NADPH oxidase-independent neutrophil extracellular trap formation



Yasuyuki Arai ^{a,1}, Yoko Nishinaka ^{b,c,1}, Toshiyuki Arai ^{a,d}, Makiko Morita ^b, Kiyomi Mizugishi ^a, Souichi Adachi ^b, Akifumi Takaori-Kondo ^a, Tomohiro Watanabe ^e, Kouhei Yamashita ^{a,*}

^a Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

^b Human Health Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

^c Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan

^d Department of Anesthesia, Kyoto City Hospital, Kyoto 604-8845, Japan

^e Center for Innovation in Immunoregulatory Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

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ABSTRACT

Neutrophil extracellular traps (NETs) are composed of extracellular DNA fibers with antimicrobial peptides that capture and kill microbes. NETs play a critical role in innate host defense and in autoimmune and inflammatory diseases. While the mechanism of NET formation remains unclear, reactive oxygen species (ROS) produced via activation of NADPH oxidase (Nox) are known to be an important requirement. In this study, we investigated the effect of uric acid (UA) on NET formation. UA, a well-known ROS scavenger, was found to suppress Nox-dependent ROS release in a dose-dependent manner. Low concentrations of UA significantly inhibited Nox-dependent NET formation. However, high concentrations of UA unexpectedly induced, rather than inhibited, NET formation. NETs were directly induced by UA alone in a Nox-independent manner, as revealed by experiments using control neutrophils treated with ROS inhibitors or neutrophils of patients with chronic granulomatous disease who have a congenital defect in ROS production. Furthermore, we found that UA-induced NET formation was partially mediated by NF-κB activation. Our study is the first to demonstrate the novel function of UA in NET formation and may provide insight into the management of patients with hyperuricemia.

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1. Introduction

Neutrophils, the first line of defense against the microbes, play a critical role in innate immunity [1]. In infected sites, they phagocytose microbes, degranulate enzymes, and produce reactive oxygen species (ROS) such as superoxide and hydrogen peroxide generated by the NADPH oxidase (Nox) complex. Recently, a novel killing mechanism known as neutrophil extracellular traps (NETs) has been reported. NETs capture microbes with their extracellular structures consisting of DNA fibers and antimicrobial granule proteins [2,3]. Many physiological stimuli are known to induce NETs. Notably, NET formation is generally ROS-dependent. Patients with chronic granulomatous disease (CGD), who are defective in Nox

Abbreviations: CGD, chronic granulomatous disease; DHR, dihydrorhodamine 123; DPI, diphenyleneiodonium; HBSS, Hanks' balanced salt solution; MSU, monosodium urate; MVP, trans-1-(2'-methoxyvinyl)pyrene; Nox, NADPH oxidase; NET, neutrophil extracellular trap; PBN, α-phenyl-N-tert-butyl nitron; PMA, phorbol myristate acetate; ROS, reactive oxygen species; ¹O₂, singlet oxygen; UA, uric acid.

* Corresponding author. Fax: +81 75 751 4963.

E-mail address: kouhei@kuhp.kyoto-u.ac.jp (K. Yamashita).

¹ These authors contributed equally to this work.

activity, fail to generate ROS and to make NETs [4]. In a recent study, we demonstrated that singlet oxygen (¹O₂), one species of ROS, is required for Nox-dependent NET formation on stimulation with phorbol myristate acetate (PMA) [5]. Interestingly, neutrophils of CGD patients treated with ¹O₂ *in vitro* produced NETs, revealing that the pathway could be rescued downstream of Nox [5].

Uric acid (UA), a product of purine metabolism, is a scavenger of ¹O₂ that regulates oxidative stress in humans [6]. Since ¹O₂ is produced by Nox [7], UA is expected to suppress Nox-dependent NET formation. However, a recent study showed that peripheral and synovial fluid neutrophils derived from patients with acute gout, whose UA levels in serum were mostly high, formed NETs [8]. Acute gout is an inflammatory arthritis that is triggered by the deposition of monosodium urate (MSU) crystals, uric acid precipitates in sodium, into the joint space. The inflammatory cascade results in the secretion of inflammatory cytokines, especially interleukin (IL)-1β and neutrophil recruitment into the joint [9]. Thus, it is still a matter of debate what effect UA directly exerts on NET formation.

In the present study, we first examined the effect of UA on Nox-dependent NET formation by control neutrophils stimulated with PMA. Thereafter, we investigated how UA directly affected NET

formation by using control neutrophils treated with ROS inhibitors or CGD neutrophils. Finally, we demonstrate that UA may induce NET formation in a manner distinct from that of PMA.

2. Materials and methods

2.1. Reagents

Hanks' balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA, USA); trans-1-(2'-methoxyvinyl)pyrene (MVP), Sytox green and orange (for double-strand DNA staining), and dihydrorhodamine 123 (DHR) were ordered from Molecular Probes (Eugene, OR, USA). α -Phenyl-*N*-tert-butyl nitron (PBN) was acquired from Radical Research Ltd. (Hino, Tokyo, Japan) and was dissolved in phosphate-buffered saline (PBS) at a final concentration of 100 mM (pH 7.4). Anti-myeloperoxidase (MPO) antibody and matched secondary antibody (anti-rabbit IgG-Alexa Fluor 488) were obtained from Abcam (Eugene, OR, USA) and Life Technologies (Carlsbad, CA, USA), respectively. Other chemicals, including UA, PMA, diphenyleneiodonium (DPI), and apocynin, were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). (E)-3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile (BAY 11-7082) was obtained from Merck (Darmstadt, Germany).

2.2. Human CGD patients

We studied two CGD patients, a 29-year-old man with gp91-phox deficiency with a G-to-A point mutation at nucleotide 252 in exon 3, and a 24-year-old man with gp91-phox deficiency with a G-to-A point mutation at nucleotide 389 in exon 10.

2.3. Isolation of human neutrophils

Human neutrophils were isolated from peripheral blood by sedimentation through two-step Percoll (GE Healthcare, Tokyo, Japan) gradients. The experiments were conducted with the understanding and the consent of each participant. The ethical committee of Kyoto University approved the experiments.

2.4. Chemiluminescence assay

Neutrophils (2×10^6 cells) were mounted on a luminescence reader (Aloka BLR-310; Aloka, Tokyo, Japan) in the presence of 40 μ M MVP, a $^1\text{O}_2$ -specific probe [10]. After that, neutrophils were stimulated with 100 ng/ml PMA in the presence of 0–5 mg/dl UA, or 8 mg/dl UA alone, and MVP luminescence was monitored every 30 s for 30 min.

2.5. Flow cytometric DHR assay

Neutrophils (1×10^5 cells) treated with 2 μ M DHR were stimulated with 100 ng/ml PMA or 8 mg/dl UA for 30 min at 37 °C and analyzed by flow cytometry using a FACSCanto II (Becton Dickinson, Durham, NC, USA).

2.6. Immunofluorescence stainings of NET-forming neutrophils

Purified neutrophils (1×10^5 cells) were incubated with 100 ng/ml PMA or 8 mg/dl UA in HBSS without serum for 3 h on culture slides (BD Biosciences, San Jose, CA, USA). After fixation with 2% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 15 min and

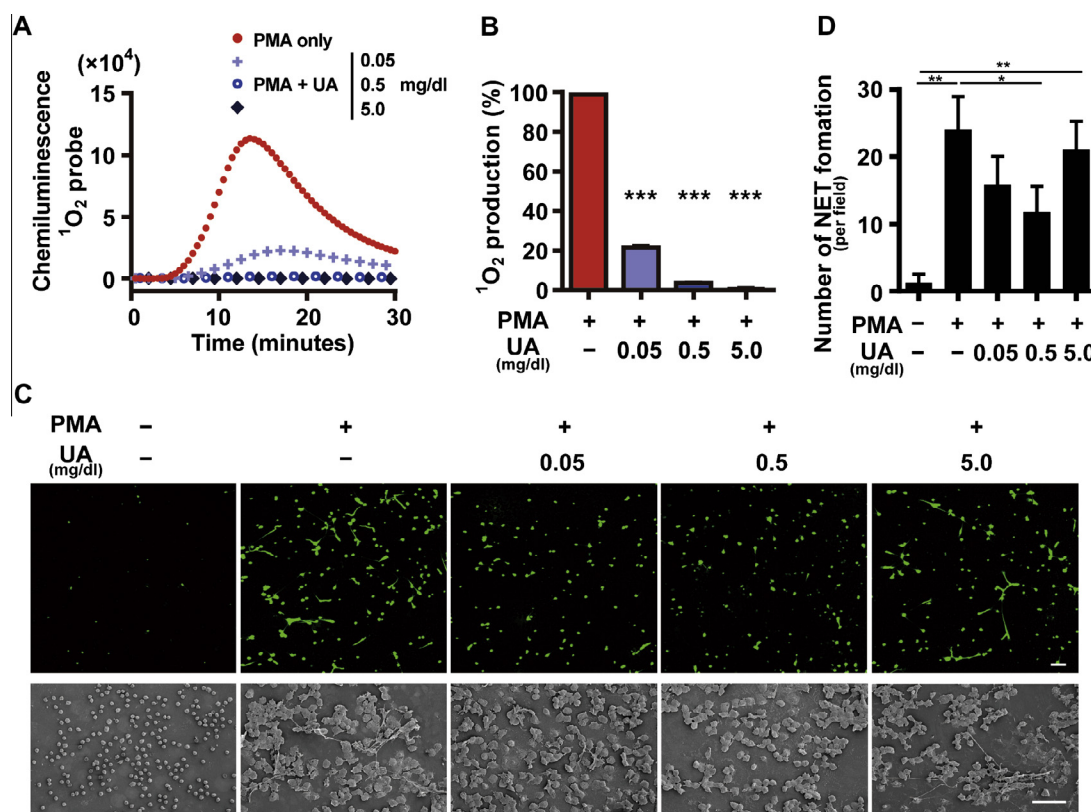


Fig. 1. The effect of UA on PMA-induced ROS production and NET formation. Neutrophils were isolated from the peripheral blood of healthy volunteers. (A) The effect of UA (0–5 mg/dl) on $^1\text{O}_2$ production by PMA-stimulated neutrophils. The $^1\text{O}_2$ production by neutrophils was examined by chemiluminescence using a $^1\text{O}_2$ -specific probe, MVP. (B) Quantitative analysis of $^1\text{O}_2$ production by neutrophils. The $^1\text{O}_2$ production is shown relative to that by PMA-stimulated neutrophils in the absence of UA. The data represent the mean \pm SE ($n = 3$, *** $P < 0.001$, unpaired t -test). (C) The effect of UA on PMA-induced NET formation. Neutrophils stimulated with PMA were incubated with 0–5 mg/dl UA. NET formation was visualized by laser-scanning fluorescence confocal microscopy (upper panels) and SEM (lower panels). Scale bars represent 100 μ m (upper panels) and 30 μ m (lower panels). (D) Quantitative analysis of NET formation. The data represent the mean \pm SE ($n = 4$, * $P < 0.05$, ** $P < 0.01$, unpaired t -test).

permeabilization with 100% methanol (Nacalai Tesque) for 10 min at -20°C , the cells were stained with rabbit anti-MPO antibodies overnight at 4°C , followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG and Sytox orange. The cells were attached to the slides by centrifugation, coverslipped with mounting medium (ProLong Gold Antifade Reagent, Life Technologies), and analyzed by confocal microscopy.

2.7. NET formation by neutrophils

Neutrophils (4×10^6 cells) from healthy volunteers were suspended in HBSS without serum and stimulated with 100 ng/ml PMA in the presence of 0–5 mg/dl UA for 3 h at 37°C under 5% CO_2 in glass base dish (Asahi Glass, Tokyo, Japan). In other experiments, neutrophils from healthy volunteers or CGD patients were stimulated with 1–8 mg/dl UA alone. After incubation, cells were stained with 500 nM Sytox Green, and NET formation was visualized with a laser-scanning fluorescence confocal microscope (Nikon Digital Eclipse C1, Tokyo, Japan). Quantitative analysis was performed by counting the number of NET-forming cells per field (average data of 5 randomly selected fields). NET formation was

also visualized with a scanning electron microscope (SEM, S-4700, Hitachi, Tokyo, Japan).

2.8. Treatments of neutrophils with inhibitors

Neutrophils (4×10^6 cells) from healthy volunteers were pre-incubated at 37°C for 30 min with Nox inhibitors, DPI (10 μM) and apocynin (10 μM), a $^1\text{O}_2$ inhibitor, PBN (4 mM) [11], or an NF- κB inhibitor, Bay 11-7082 (10 μM), and then stimulated with 100 ng/ml PMA or 8 mg/dl UA for 3 h. NET formation was visualized and analyzed as described above.

2.9. Immunoblotting

Neutrophils (4×10^6 cells) from healthy volunteers were incubated at 37°C for 30 min with 100 ng/ml PMA or 8 mg/dl UA. Lysates were prepared using RIPA lysis buffer (Wako Pure Chemical Industries, Osaka, Japan). Cell debris was separated by centrifugation and equal amounts of proteins in the supernatant were separated by electrophoresis (4–12% SDS-polyacrylamide gels, Life Technologies). Proteins were then electrotransferred onto

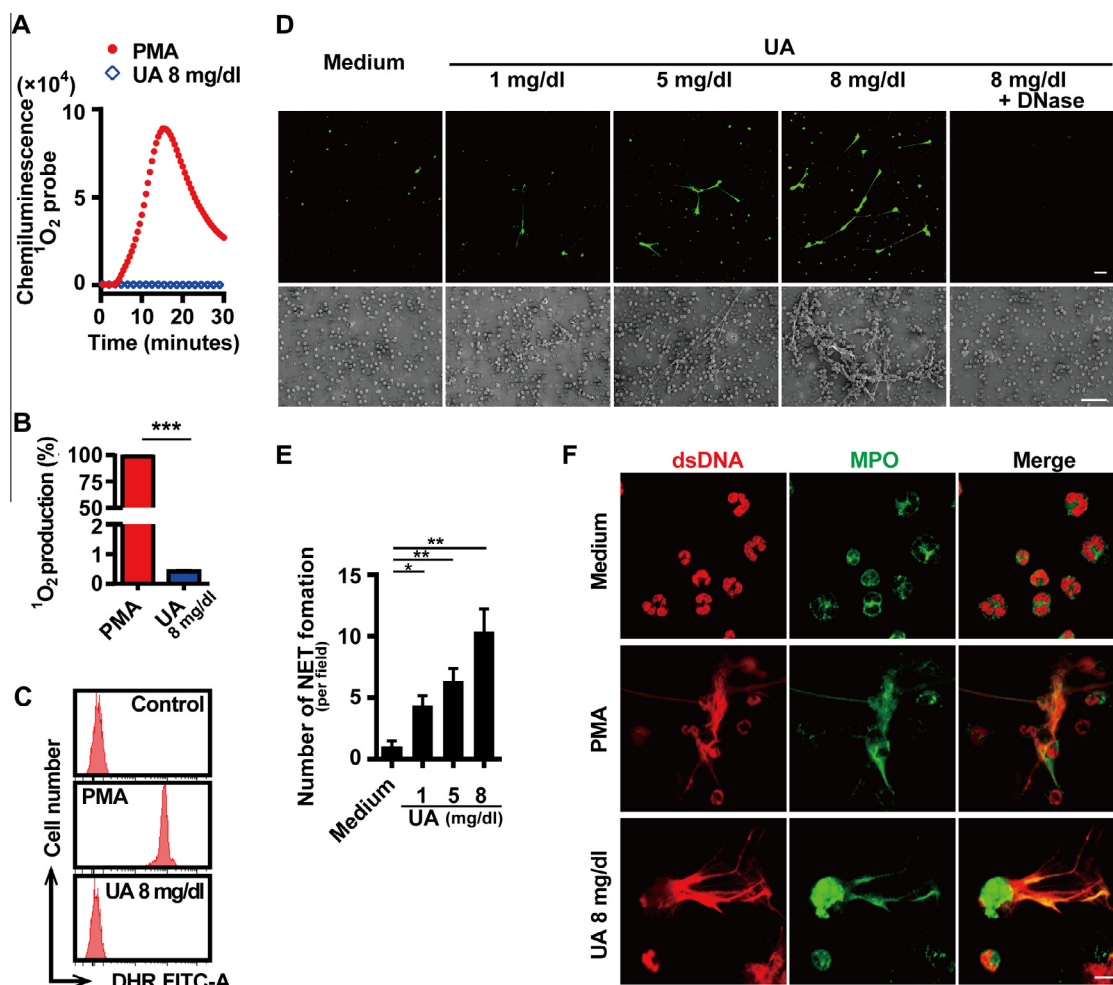


Fig. 2. The effect of UA alone on ROS production and NET formation. (A and B) Effect of UA on $^1\text{O}_2$ production. Neutrophils were stimulated with PMA (100 ng/ml) or UA (8 mg/dl). $^1\text{O}_2$ production was examined by chemiluminescence using a $^1\text{O}_2$ -specific probe, MVP. (A) Representative data. UA-stimulated neutrophils hardly produced any $^1\text{O}_2$. (B) Quantitative analysis of $^1\text{O}_2$ production shown relative to that by PMA-stimulated neutrophils. Data represent mean \pm SE ($n = 3$, *** $P < 0.001$, unpaired t -test). (C) ROS production by DHR assay. The logarithmic fluorescence intensity is shown on the x-axis and the cell count on the y-axis. (D–F) Direct effect of UA on NET formation. (D) Representative micrographs of neutrophils incubated with 1–8 mg/dl of UA. NET formation was visualized by laser-scanning fluorescence confocal microscopy (upper panels) and SEM (lower panels). Scale bars represent 100 μm (upper panels) and 30 μm (lower panels). (E) Quantitative analysis of NET formation. Data represent the mean \pm SE ($n = 5$, * $P < 0.05$, ** $P < 0.01$, unpaired t -test). (F) Colocalization of extracellular dsDNA and MPO in UA-stimulated neutrophils. UA- or PMA-stimulated neutrophils were immunostained with anti-MPO antibody (green). The dsDNAs were counterstained with Sytox-orange (red). Scale bars represent 10 μm .

nitrocellulose membranes. After blocking, membranes were incubated overnight at 4 °C with a rabbit polyclonal anti-phospho-NF- κ B p65 or anti-NF- κ B p65 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) followed by a goat anti-rabbit HRP antibody. Protein bands were visualized by enhanced chemiluminescence, and results were analyzed with ImageJ software.

2.10. Statistical analysis

Data were expressed as mean \pm standard error (SE). Values of $P < 0.05$ determined by the unpaired Student t -test were considered significant.

3. Results and discussion

We examined the effect of UA on $^1\text{O}_2$ production and NET formation by PMA-stimulated neutrophils. First, neutrophils from healthy volunteers were stimulated with PMA with or without

UA, and the $^1\text{O}_2$ production was detected by chemiluminescence. As expected, increasing concentrations of UA (0.05–5 mg/dl) suppressed $^1\text{O}_2$ production by PMA-stimulated neutrophils in a dose-dependent manner, suggesting that UA is a $^1\text{O}_2$ scavenger (Fig. 1A and B). Treatments of less than 0.5 mg/dl of UA suppressed PMA-induced NET formation in confocal microscopy (Fig. 1C, upper panels, and Fig. 1D) and SEM (Fig. 1C, lower panels). Surprisingly, treatment of PMA-stimulated neutrophils with 5 mg/dl of UA failed to suppress NET formation, suggesting that a high concentration of UA may have a novel function in NET formation (Fig. 1C and D). These results were substantiated by the quantitative analysis of representative micrographs (Fig. 1D).

Next, we investigated the direct effect of UA on $^1\text{O}_2$ production and NET formation. UA treatment alone did not produce any detectable levels of $^1\text{O}_2$ (Fig. 2A and B). We used the fluorescent dye DHR in a flow cytometric assay to detect ROS. UA-stimulated neutrophils from healthy volunteers did not exhibit any increase in DHR fluorescence, in contrast to a significant increase in PMA-stimulated neutrophils (Fig. 2C). Unexpectedly, UA alone

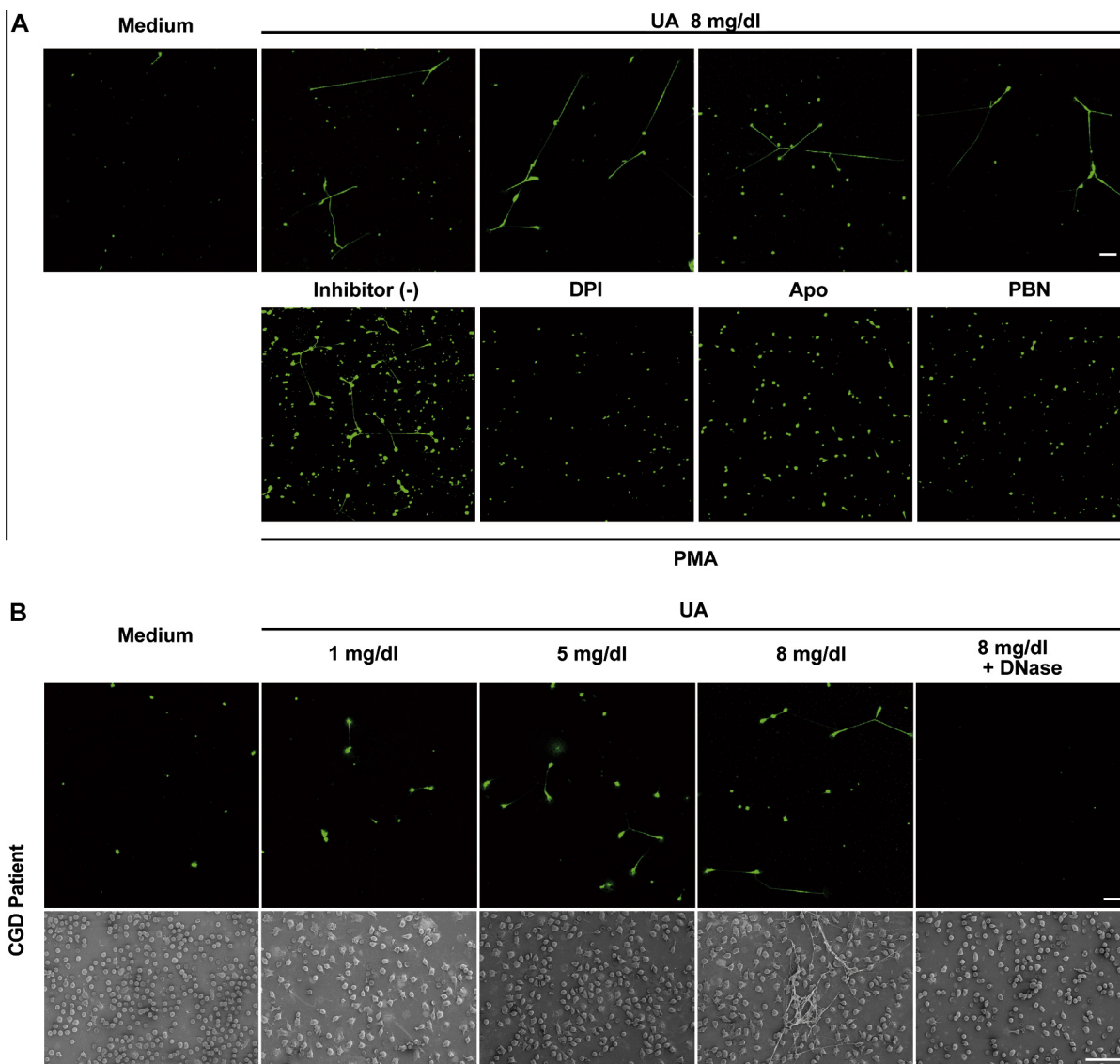


Fig. 3. UA-induced NET formation is independent of ROS. (A) The effect of ROS inhibitors on UA- (upper panels) or PMA- (lower panels) induced NET formation. Neutrophils from healthy volunteers were incubated with 8 mg/dl UA or 100 ng/ml PMA in the presence or absence of Nox inhibitors, DPI and apocynin (Apo), or a $^1\text{O}_2$ inhibitor, PBN. NET formation was visualized by laser-scanning fluorescence confocal microscopy. Scale bars represent 100 μm . (B) The effect of UA on NET formation. Neutrophils from CGD patients were incubated with 1–8 mg/dl of UA. Representative micrographs are shown. NET formation was visualized by laser-scanning fluorescence confocal microscopy (upper panels) and SEM (lower panels). Scale bars represent 100 μm (upper panels) and 30 μm (lower panels).

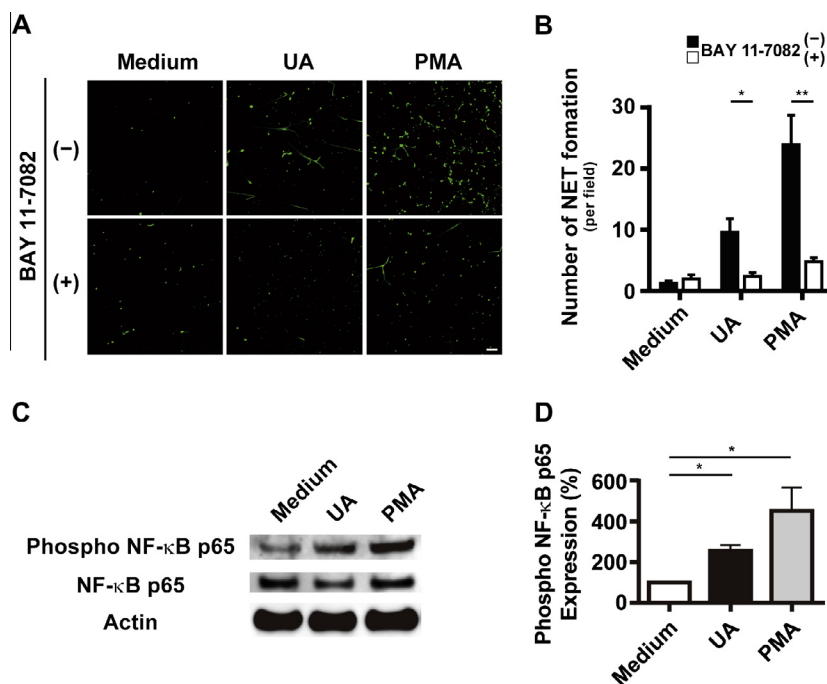


Fig. 4. UA induces NET formation through NF- κ B activation. (A and B) The effect of an NF- κ B inhibitor on UA-induced NET formation. Neutrophils from healthy volunteers were stimulated with 100 ng/ml PMA or 8 mg/dl UA in the presence or absence of an NF- κ B inhibitor. (A) Representative micrographs. NET formation was visualized by laser-scanning fluorescence confocal microscopy. Scale bars represent 100 μ m. (B) Quantitative analysis of NET formation. The data represent the mean \pm SE ($n = 5$, * $P < 0.05$, ** $P < 0.01$, unpaired t -test). (C) Immunoblot analysis of PMA- or UA-stimulated neutrophils. Cell lysates were subjected to immunoblotting using an anti-phospho-NF- κ B p65 or anti-NF- κ B p65 antibody. Membranes were reprobated with an anti-actin antibody. (D) Quantification of proteins on immunoblots. The expression levels are shown relative to that in neutrophils without stimuli. The data represent the mean \pm SE ($n = 5$, * $P < 0.05$, unpaired t -test).

significantly induced NET formation in healthy neutrophils, irrespective of the absence of ROS (Fig. 2D and E). NET formation was abrogated by DNase treatment, which degrades DNA fibers of NETs (Fig. 2D). NET formation by UA was further verified by immunostaining, in which extracellular dsDNA colocalized with a granule protein MPO, an important structural component of NETs (Fig. 2F). These results suggest that NET formation by UA may not be mediated by ROS.

This hypothesis was substantiated by the use of Nox inhibitors, DPI and apocynin, or a $^1\text{O}_2$ scavenger, PBN. None of the ROS inhibitors suppressed UA-induced NET formation, while PMA-induced NETs were strongly inhibited (Fig. 3A). We next examined NET formation in neutrophils from CGD patients, who have a defect in ROS formation. Regardless of the absence of ROS, CGD neutrophils treated with UA produced NETs, which were suppressed by DNase treatment (Fig. 3B). Taken together, these results suggest that UA may induce NET formation in a Nox-independent manner.

Recently, Lopponi et al. implicated NF- κ B activation in NET formation induced by PMA stimulus or stress signals, such as acidic or hyperthermic conditions [12]. Therefore, we investigated whether NF- κ B blockade affected NET formation by UA-stimulated neutrophils. The treatment of healthy neutrophils with the NF- κ B inhibitor BAY 11-7082 resulted in a marked suppression of UA-induced NET formation (Fig. 4A and B). In addition, immunoblot analysis revealed that phosphorylation of the p65 subunit of NF- κ B was significantly enhanced in neutrophils stimulated with UA (Fig. 4C and D). Taken together, these results suggest that the NF- κ B cascade is important in UA-mediated NET formation, and could be a key regulatory pathway of NET formation. Identification of signaling pathways upstream of NF- κ B in UA-induced NET formation should be a future area of investigation.

In summary, this is the first report to demonstrate the novel function of UA in NET formation. Contrary to expectations, UA, a known $^1\text{O}_2$ scavenger, induced NETs in neutrophils in a Nox-inde-

pendent manner and partially through activation of the NF- κ B pathway. The mechanisms by which UA contributes to NET formation are not yet clear. It has been reported that the Raf-MEK-ERK pathway is involved in a NET formation cascade downstream of Nox activation in PMA-stimulated neutrophils [13]. Furthermore, MSU crystals induced NETs in a ROS-dependent manner as well as PMA [14]. In contrast, NET formation by UA was independent of ROS. In addition, neither the activation of ERK, nor the suppression of NETs by an ERK inhibitor, was observed in UA-stimulated neutrophils in our study (unpublished observation). This suggests that UA induces NETs in a different fashion from that of PMA and MSU crystals. An example of ROS-independent NET formation was reported in a recent study, where the calcium ionophore ionomycin did not require Nox activation to induce NETs [15]. Moreover, *Staphylococcus aureus* and *Candida albicans* have been reported to induce NETs independently of Nox [16,17]. Thus, it is possible that there are several mechanisms of NET formation. The importance of a ROS-independent pathway in NET formation in physiological settings awaits further investigation.

NETs are similar to a double-edged sword; they can either fight disease or cause disease, depending on the situation [18]. Excessive NET formation is associated with the pathogenesis of inflammatory and autoimmune diseases, including preeclampsia [19], cystic fibrosis [20], and systemic lupus erythematosus [21]. Moreover, NETs are relevant to vascular injury, in which extracellular histones released from neutrophils during NET formation injure the endothelium [22], and the injured endothelium, in turn, induces NETs, establishing a vicious cycle leading to severe damage [23]. Clinically, the association of hyperuricemia and gout with other medical conditions such as hypertension, chronic kidney disease, and cardiovascular disease has been recognized [24]. Recent animal and epidemiologic studies support the idea that uric acid elevation in the serum is an independent risk factor for the development of these serious medical problems, by damaging

endothelial cells, although it is still a matter of debate [25]. In light of these findings, we speculate that uric acid elevation may induce NET formation and subsequent vascular endothelial dysfunction, ultimately leading to cardiovascular diseases. Therefore, NETs could be a missing link between uric acid elevation and cardiovascular diseases. Thus, we may need to reappraise the importance of uric acid in human health and disease, and reconsider the management of patients with asymptomatic hyperuricemia in order to decrease the risk of cardiovascular diseases.

Conflict of interest disclosure

The authors declare no conflict of interest.

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