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Singlet oxygen is essential for neutrophil extracellular trap formation

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ABSTRACT

Neutrophil extracellular traps (NETs) that bind invading microbes are pivotal for innate host defense. There is a growing body of evidence for the significance of NETs in the pathogenesis of infectious and inflammatory diseases, but the mechanism of NET formation remains unclear. Previous observation in neutrophils of chronic granulomatous disease (CGD) patients, which defect NADPH oxidase (Nox) and fail to produce reactive oxygen species (ROS), revealed that ROS contributed to the formation of NETs. However, the active species were not identified. In this study, we discovered that singlet oxygen, one of the ROS, mediated Nox-dependent NET formation upon stimulation with phorbol myristate acetate. We also revealed that singlet oxygen itself could induce NET formation by a distinct system generating singlet oxygen with porfimer sodium (Photofrin) in CGD neutrophils, as well as healthy neutrophils. This was independent of Nox activation. These results show that singlet oxygen is essential for NET formation, and provide novel insights into the pathogenesis of infectious and inflammatory diseases.

1. Introduction

Recent investigations highlighted a novel killing mechanism of neutrophils, called neutrophil extracellular traps (NETs), which capture microbes in extracellular structures consisting of DNA fibers and antimicrobial granule proteins [1,2]. There have been many reports on the antimicrobial effects and pro-inflammatory roles of NETs [3], but the mechanism of NET formation remains unclear. Previous observation revealed that reactive oxygen species (ROS) generated by activated neutrophils contributed to the formation of NETs [4]. However, the active species have not been identified.

Neutrophils first generate superoxide anion ($O_2^{\cdot-}$) by NADPH oxidase (Nox) activation, and this $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Hypochlorous acid (HOCl) is produced from H_2O_2 by myeloperoxidase (MPO), and reacts with H_2O_2 to form singlet oxygen ($^1O_2$) [5]. The role of $^1O_2$ in microbicidal activity is not fully understood. It was recently reported that the ROS with chemical signature of ozone, which is converted from $^1O_2$ by immunoglobulin or several amino acids, contributes to killing of bacteria [6,7]. A recent study showed that MPO is required for NET formation [8], indicative of the involvement of $^1O_2$ in NET formation.

We previously showed that edaravone, (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, and α-phenyl-*N*-*tert*-butyl nitrone (PBN), a spin trap agent, suppressed $^1O_2$ release from activated neutrophils, but did not affect $O_2^{\cdot-}$ release [9,10]. Furthermore, we demonstrated that PBN neither affects MPO activity, nor reacts with HOCl [10].

In this study, we first examined the effect of edaravone or PBN on Nox-dependent NET formation by phorbol myristate acetate (PMA) stimulation, to elucidate the involvement of $^1O_2$ in NET formation. We next studied whether NETs are formed by a distinct system generating $^1O_2$ with porfimer sodium (Photofrin) in neutrophils of a patient with chronic granulomatous disease (CGD), which is an inherited immunodeficiency with Nox defect, leading to recurrent life-threatening infections. These results should uncover an essential role of $^1O_2$ in NET formation.

2. Materials and methods

2.1. Reagents

Hanks' balanced salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA); trans-1-(2′-methoxyvinyl)pyrene (MVP) and Sytox green were from Molecular Probes (Eugene, OR); and 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (CLA) was from Tokyo Kasei Kogyo (Tokyo, Japan). PBN was obtained from Radical Research Ltd. (Hino, Tokyo, Japan) and dissolved in phosphate-buffered saline (PBS) to a final concentration 100 mM (pH 7.4). Edaravone was a kind gift from Tanabe–Mitsubishi

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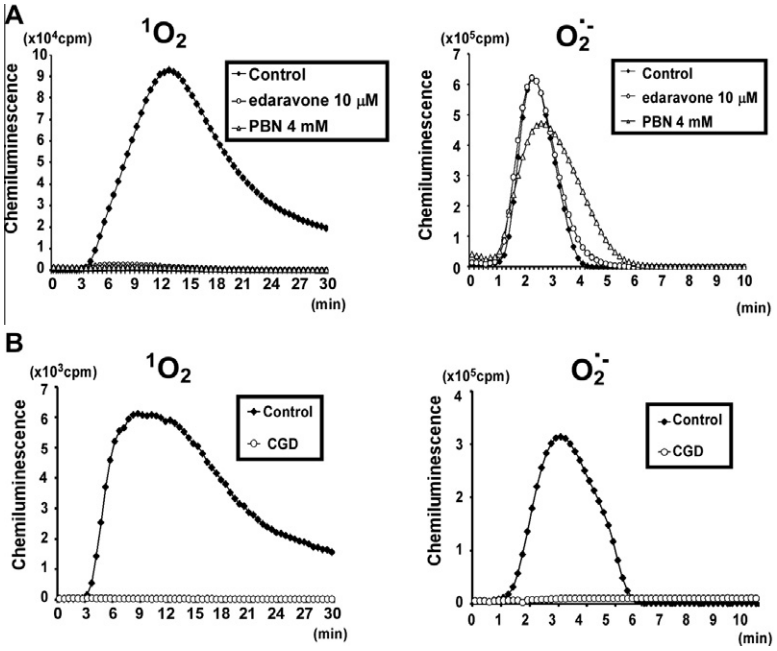

Fig. 1. ROS generation by PMA stimulation. (A) The effect of edaravone or PBN on PMA-stimulated ROS release of healthy neutrophils. ROS generation was examined by chemiluminescence using $^1O_2$- and $O_2^{\cdot-}$-specific probes, MVP (left panel) and CLA (right panel) respectively. Healthy neutrophils (2×10^6 cells) by PMA stimulation (100 ng/ml) with 40 μM MVP (left panel) or 2.5 μM CLA (right panel) in the treatment of 10 μM edaravone or 4 mM PBN mounted on a luminescence reader. The luminescence of MVP or CLA was monitored every 30 s for 30 min or every 10 s for 10 min, respectively. The inhibitory effect on $^1O_2$-release of edaravone or PBN was observed. In contrast, edaravone or PBN did not exhibit the inhibitory effect on $O_2^{\cdot-}$ release. The experiments were performed at least 3 times, and representative data are shown. (B) ROS generation in neutrophils of a healthy volunteer (control) and a CGD patient (CGD). Healthy or CGD neutrophils (2×10^6 cells) by PMA stimulation (100 ng/ml) with 40 μM MVP (left panel) or 2.5 μM CLA (right panel) mounted on a luminescence reader. CGD neutrophils did not produce $^1O_2$- and $O_2^{\cdot-}$.

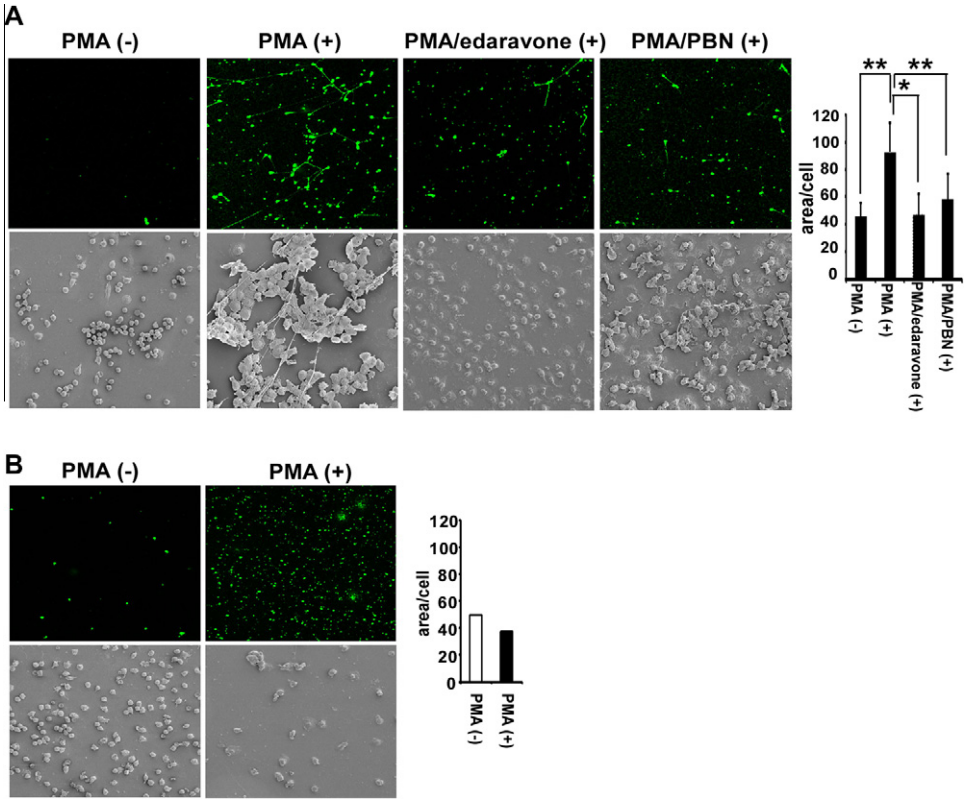

Fig. 2. NET formation by PMA stimulation. (A) NET formation upon stimulation with PMA by fluorescence confocal microscopy and scanning electron microscopy (SEM). Healthy neutrophils stained with 500 nM Sytox green were observed after 3 h of stimulation with PMA (100 ng/ml) by laser-scanning confocal microscopy (upper panels, 100×) and SEM (lower panels, 250×). Representative data are shown. Quantitative analysis was performed using ImageJ software (right graph). The inhibitory effect of edaravone (10 μM) or PBN (4 mM) on PMA-stimulated NET formation was observed. Values are the means ± SD ($n = 5$). $*$ and $**$ indicate $P < 0.05$ and $P < 0.01$, respectively. (B) NET formation of CGD neutrophils by PMA stimulation by fluorescence confocal microscopy (upper panels, 100×) and SEM (lower panels, 250×). Quantitative analysis was also performed (right graph). Note that NET formation was not observed in a CGD patient.

Pharma Corporation (Tokyo, Japan) and directly dissolved in PBS or D_2O to a final concentration of 4 mM (pH 7.4). Porfimer sodium (Photofrin) was obtained from Pfizer Japan Inc. (Tokyo, Japan). Other chemicals, such as phorbol myristate acetate (PMA), were purchased from Sigma Chemicals (St. Louis, MO).

2.2. Preparation of neutrophils

Human neutrophils were isolated from peripheral blood by sedimentation through two-step Percoll (GE Healthcare Japan, Tokyo, Japan) gradients. The CGD patient was a 24-year-old male with *gp91-phox* deficiency with a G-to-A point mutation at nucleotide 389 in exon 10. Healthy volunteers and the patient provided written informed consent for participation in an institutional review board-approved protocol at Kyoto University Hospital.

2.3. Chemiluminescence assay

The productions of $^1O_2$ and $O_2^{\cdot-}$ of neutrophils stimulated with 100 ng/ml PMA were examined by chemiluminescence using $^1O_2$- and $O_2^{\cdot-}$-specific probes, MVP [11] and CLA [12], respectively. The effect of edaravone and PBN on ROS production was examined. Healthy or CGD neutrophils (2×10^6 cells) were mounted on a luminescence reader (Aloka BLR-310; Aloka, Tokyo, Japan) in the presence of 40 μM MVP or 2.5 μM CLA. After the start of measurement, neutrophils were stimulated with 100 ng/ml PMA. The luminescence of MVP was monitored every 30 s for 30 min and that of CLA was monitored every 10 s for 10 min.

2.4. Analysis of NET formation by PMA stimulation

NET formation was visualized with a laser-scanning fluorescence confocal microscope (Nikon Digital Eclipse C1, Tokyo, Japan) after stimulation of 2×10^6 neutrophils for 3 h with 100 ng/ml PMA and staining of the NET DNA with 500 nM Sytox green. Quantitative analysis was performed using ImageJ software. Briefly, the Sytox-positive area of each micrograph was measured and divided by the total number of Sytox-positive cells, which showed the mean size of nuclear area per cell, whereby a large mean size was suggestive of NETs. NET formation was also visualized with a Hitachi S-4700 scanning electron microscope.

2.5. Detection of $^1O_2$ in a cell-free system

The direct analysis of near-infrared luminescence was performed in a cell-free system using Raman spectroscopy (LabRAM HR-800, HORIBA, Kyoto, Japan), to detect the $^1O_2$ generation by irradiation of porfimer sodium (Photofrin) solution. D_2O was used as the solvent since the lifetime of $^1O_2$ is much longer (62 μs in D_2O than in H_2O (3.8 μs). The quantum yields of $^1O_2$ production were determined by measurement of the $^1O_2$ luminescence at 1270 nm, which originated from continuous irradiation of 100 μg/ml Photofrin solutions using a laser beam (wavelength 632.8 nm, power 6 mW). The luminescence of $^1O_2$ was measured with a liquid nitrogen-cooled InGaAs photodiode in conjunction with a 1270 nm interference filter.

2.6. Analysis of accumulation of Photofrin in neutrophils

Neutrophils (2×10^7/ml) were visualized by fluorescence microscopy using Cy5 filter (Nikon) after incubation with 10 μg/ml Photofrin for 1 h.

2.7. Analysis of NET formation by photochemically generated $^1O_2$

NET formation was analyzed by fluorescence confocal microscopy and scanning electron microscopy (SEM). Neutrophils (2×10^7/ml) were incubated for 1 h after cells were treated with 10 μg/ml Photofrin for 1 h and subjected to irradiation for 5 min using an LED lamp (λ_{max} = 660 nm, CCS Inc., Kyoto, Japan).

2.8. Statistical analysis

Data are expressed as the mean ± SD. $P < 0.05$ by the paired Student *t*-test was considered significant.

3. Results and discussion

ROS production of healthy or CGD neutrophils upon stimulation with PMA was detected by chemiluminescence. The production of both $^1O_2$ and $O_2^{\cdot-}$ of healthy neutrophils was observed. The administration of edaravone (10 μM) or PBN (4 mM), a scavenger of singlet oxygen [9,10], suppressed $^1O_2$, but not $O_2^{\cdot-}$ production (Fig. 1A). Neither $^1O_2$ nor $O_2^{\cdot-}$ release from CGD neutrophils was observed (Fig. 1B).

NET formation by PMA was visualized by fluorescence microscopy and SEM. NET formation was observed at 3 h after PMA stimulation in healthy neutrophils. The treatment of $^1O_2$ scavenger, edaravone (10 μM) or PBN (4 mM) significantly suppressed NET formation by fluorescence microscopy (Fig. 2A, upper panel) and by SEM (Fig. 2A, lower panel). Quantitative analysis was performed using ImageJ software (Fig. 2A, right graph). In contrast, CGD neutrophils did not exhibit NET formation (Fig. 2B), which is consistent

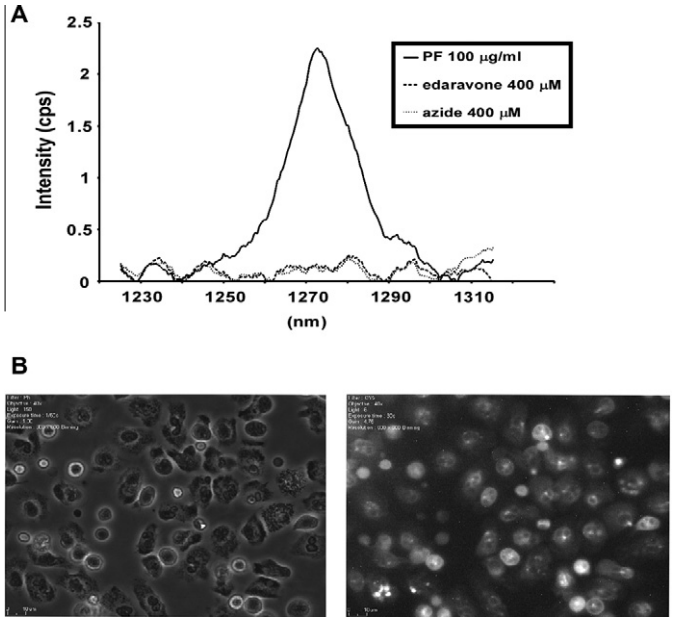

Fig. 3. $^1O_2$ generation by Photofrin in a cell-free system and accumulation of Photofrin in neutrophils. (A) Detection of $^1O_2$ by near-infrared luminescence in a cell-free system. The direct analysis of near-infrared luminescence was performed using Raman spectroscopy, to detect $^1O_2$, which was generated by porfimer sodium (Photofrin (PF), 100 μg/ml) with irradiation using a laser beam (λ = 632.8 nm, power 6 mW) in D_2O solution. The quantum yields of $^1O_2$ production were determined by measurements of the $^1O_2$ luminescence at 1270 nm. The scavenging activity for $^1O_2$ of edaravone (400 μM) or azide (400 μM) was observed. (B) Accumulation of Photofrin in neutrophils. Subcellular localization of Photofrin (10 μg/ml) in neutrophils (2×10^7 cells/ml) was determined by fluorescent microscopy (400×). The phase contrast image (left panel) and the fluorescence image for Photofrin (right panel) are shown. Note that Photofrin was accumulated in the cytosol of neutrophils.

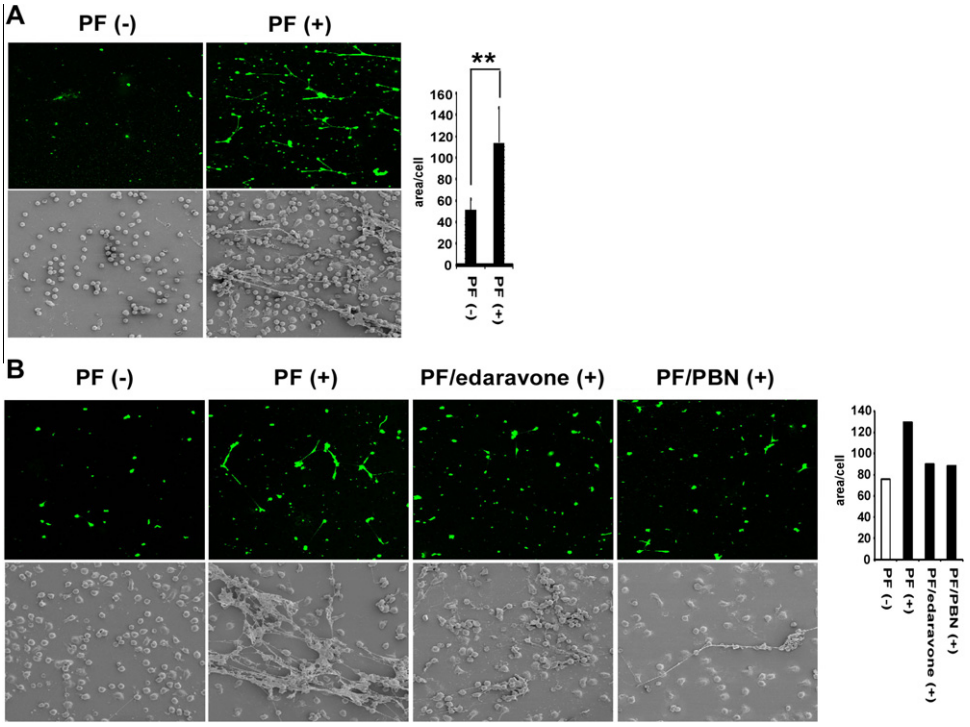

Fig. 4. NET formation of healthy or CGD neutrophils by $^1O_2$-generating system with Photofrin/irradiation. (A) NET formation of healthy neutrophils in the treatment with Photofrin/irradiation by fluorescence confocal microscopy (upper panels, 100×) and SEM (lower panels, 250×). Representative data are shown. Quantitative analysis was performed (right graph). NET formation was observed at 1 h after the treatment with 10 μg/ml Photofrin and 5 min irradiation using an LED lamp (λ_{max} = 660 nm). Values are the means ± SD (n = 6). ** Indicates $P < 0.01$. (B) NET formation of CGD neutrophils in the treatment with Photofrin/irradiation by fluorescence confocal microscopy (upper panels, 100×) and SEM (lower panels, 250×). CGD neutrophils formed NETs by Photofrin/irradiation treatment. The inhibitory effect of edaravone (10 μM) or PBN (4 mM) on NET formation in the treatment with Photofrin/irradiation was observed. Quantitative analysis was also performed (right graph).

with previous reports [4,13]. A recent study showed that myeloperoxidase (MPO) is required for NET formation [8]. It is difficult to identify which ROS is essential for NET formation, since some ROS, such as $^1O_2$ and HOCl, are generated downstream of MPO. We previously reported that edaravone was a $^1O_2$ scavenger [9], but it has been reported that edaravone quenches HOCl and hydroxyl radical as well [14]. In the current study, we used another $^1O_2$ scavenger, PBN, which neither affects MPO activity, nor reacts with HOCl [10]. PBN significantly suppressed NET formation (Fig. 2A), suggesting that $^1O_2$ is involved in NET formation.

We next explored the direct effect of $^1O_2$ in NET formation. First, we examined $^1O_2$ generation by the most specific method, using Photofrin (PF) in a cell-free system [15], which was detected by direct analysis of near-infrared luminescence at 1270 nm (Fig. 3A). It was found that 400 μM edaravone almost completely suppressed the $^1O_2$ spectrum, as well as 400 μM azide, a well-known $^1O_2$ scavenger (Fig. 3A). These findings indicated that $^1O_2$ was generated in this system using Photofrin with light irradiation, and further supported that edaravone is a direct $^1O_2$ scavenger. The accumulation of Photofrin in neutrophils was observed by fluorescence confocal microscopy (Fig. 3B), indicating that $^1O_2$ was generated in neutrophils by Photofrin with light irradiation.

NET formation by Photofrin/irradiation was visualized by fluorescence microscopy and SEM. NET formation was observed in healthy neutrophils (Fig. 4A). Quantitative analysis was performed using ImageJ software (Fig. 4A, right graph). Interestingly, CGD neutrophils made NETs by Photofrin/irradiation as well (Fig. 4B). It is noteworthy that CGD neutrophils did not exhibit NET formation by PMA stimulation, which is dependent on Nox activation (Fig. 2B). This was suppressed by $^1O_2$ scavengers, edaravone (10 μM) or PBN (4 mM) (Fig. 4B). These results suggested that $^1O_2$ directly induced NET formation in both healthy and CGD neutrophils, independent of Nox activation. The modulation of NET formation should be effective in the control of infectious or inflammatory disorders. From the current study, the suppression of $^1O_2$ could be an optimal target for regulating NET formation, minimalizing impairment of innate host defense.

Author contributions

Y.N., T.A., and K.Y. designed and performed experiments, analyzed the data, and prepared the paper. S.A. and A.T.-K. supervised the study.

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