Biochemistry and Biophysics Reports 11 (2017) 147–153

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon

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ARTICLE INFO

Chemical compounds studied in this article:
hemin (PubChem CID: 121225420)
phorbol myristate acetate (PubChem CID: 22833501)
protoporphyrin IX (PubChem CID: 4971)
hydrogen peroxide (PubChem CID: 784)
sytox green (PubChem CID: 46863923)
TAK-242 (PubChem CID: 11703255)
diphenylene iodonium (PubChem CID: 3101)
polymyxin B (PubChem CID: 4868)

Keywords:
Hemin
Neutrophil
Extracellular trap
Quantitative detection
Hydrogen peroxide

ABSTRACT

Formation of neutrophil extracellular traps (NETs) can perpetuate sterile inflammation; thus, it is important to clarify their pathophysiological characteristics. Free heme, derived via hemolysis, is a major contributor to organ damage, and reportedly induces neutrophil activation as well as reactive oxygen species (ROS) production and NET formation. For this study, we examined hemin (Fe^{3+} -protoporphyrin IX)-induced NET formation quantitatively in vitro as well as the effects of oxidative stress.

NETs formed in vitro from cultured neutrophils were quantitatively detected by using nuclease treatment and Sytox Green, a nucleic acid stain. Hemin-induced NET production was found to be in a dose-dependent manner, NADPH oxidase-dependent and toll-like receptor (TLR)-4 independent. Additionally, the iron molecule in the porphyrin ring was considered essential for the formation of NETs. In the presence of low concentrations of hydrogen peroxide, low concentrations of hemin-induced NETs were enhanced, unlike those of phorbol myristate acetate (PMA)-induced NETs.

Quantitative analysis of NET formation may prove to be a useful tool for investigating NET physiology, and hemin could function as a possible therapeutic target for hemolysis-related events.

1. Introduction

Neutrophil extracellular traps (NETs) are actively discharged from activated neutrophils, and are composed of decondensed chromatin fibers coated with antimicrobial granular and cytoplasmic proteins, such as myeloperoxidase (MPO), neutrophil elastase, and alpha-defensin [1,2]. Although NETs form to prevent dissemination of pathogens [1], excessive release of DNA and DNA-associated proteins can also perpetuate sterile inflammation as well as lung injury, thrombosis, sepsis, autoimmune diseases, and metastasis of cancers [2–6].

In addition to damage-associated molecular patterns (DAMPS), free heme is a major contributor to organ damage induced by sepsis [7] or hemolysis [8]. Several studies have postulated that free heme activates human neutrophils and induces reactive oxygen species (ROS) production [9–11] and NET formation [12,13].

The in vitro method to detect NETs is mainly based on morphological observation, but quantitative detection is also important. Released NETs are quantitatively measurable using nucleic acid staining agents such as Sytox Green, fluorescent nucleic acid stain [14] or double strand DNA quantification kit [15], or by means of ELISA detecting myeloperoxidase and DNA complex [16]. In addition, plasma cell-free DNA (cfDNA) is reportedly as useful for several pathological conditions [17,18], while the application of flow cytometric techniques is also being developed [19]. We employed a nucleic acid quantification method using Sytox green which has been used for microscopic observation and identification of NETs [20].

For this study, we evaluated hemin (Fe^{3+} (ferri)-protoporphyrin IX)-induced NET formation quantitatively and confirmed its

Abbreviations: NET, neutrophil extracellular traps; MPO, myeloperoxidase; ROS, reactive oxygen species; ELISA, Enzyme-Linked Immuno-Sorbent Assay; PMA, phorbol myristate acetate; DPI, diphenyleneiodonium; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; TLR, toll-like receptor; PAD4, peptidylarginine deiminases 4; LPS, lipopolysaccharide; HO-1, heme oxygenase-1

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http://dx.doi.org/10.1016/j.bbrep.2017.07.009
Received 24 March 2017; Received in revised form 10 July 2017; Accepted 20 July 2017
Available online 24 July 2017

concentration-dependency. Moreover, hemin-induced NET formation was found to increase in the presence of oxidative stress.

2. Methods

2.1. Preparation of neutrophils

Heparinized peripheral blood was collected from healthy volunteers after obtaining their written informed consent. Neutrophil separation (> 90% purity) was performed at room temperature (RT) using the density gradient method with the Polymorphprep separation medium (Alere Technologies AS, Oslo, Norway). This study was approved by the Ethics Committee of Himeji Dokkyo University (12-01) and by Sysmex Corporation (2014-50).

2.2. Quantitative method for detection of NETs

Quantitative analysis was performed using Palmer's method [14] with partial modification. A 96-well plate was coated with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) and incubated over night at 4 °C. After addition of 0.2 mL of purified neutrophils in RPMI 1640 to each well at a concentration of 0.5×10^9 cells /L, the cells were stimulated with hemin, protoporphyrin IX (PP IX, lacking iron from hemin), phorbol myristate acetate (PMA) or *Escherichia coli* 0111:B4 lipopolysaccharide (LPS), all from Sigma-Aldrich, for 3 h at 37 °C in humidified air with 5% CO_2. Hydrogen peroxide (Nacalai Tesque, Kyoto, Japan) was added simultaneously to the samples. After incubation, nuclease from *Staphylococcus aureus* (Sigma-Aldrich) treatment at a final concentration of 1 U/mL was performed for 10 min at 37 °C, followed by treatment with 2 mM ethylene glycol tetraacetic acid (EGTA, Sigma-Aldrich) at 4 °C. After being transferred to Eppendorf tubes, the samples were centrifuged at 1800*g* for 10 min at 4 °C. Sytox Green (Molecular Probes, Eugene, OR) was added to 0.17 mL of the supernatants at a final concentration of 2.9 nM, and fluorescence was detected at excitation and emission wavelengths of 488 nm and 520 nm, respectively, using ARVO MX (Perkin Elmer, Waltham, MA, USA). Relative fluorescence was calculated against control.

For the experiments with various inhibitors, 0.01 mM diphenylene iodonium (DPI), a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, 0.01 mM Mito TEMPO (a mitochondria-restricted anti-oxidant), and 1 mg/L polymyxin B (Poly B, an LPS inhibitor), all from Sigma-Aldrich, were added to the samples 10 min before the addition of stimulators.

Following the protocol prescribed by Hussey [21], TAK-242, resatorvid, a small molecular inhibitor of Toll-like receptor (TLR)-4 signaling (Chem Scene, Monmouth Junction, NJ, USA) was added at 0.01 mM or 0.03 mM and incubated for 1 h at 37 °C in humidified air with 5% CO_2.

2.3. Morphological observations

Purified neutrophils (1×10^9 cells/L) were suspended in Hanks' balanced salt solution (HBSS) with 2% heat-inactivated autologous serum at a concentration of 1×10^9/L. The cell suspension (250 μL) was added to 35-mm glass-bottom dishes coated with Poly-L-Lysine (Sigma-Aldrich), and incubated under the same conditions as for quantitative analysis. Next, Sytox Green was added at a concentration of 4 nM, and stained neutrophils were observed under a fluorescence microscope (TH4-100 Cell Sens; Olympus, Tokyo, Japan).

Immunostaining of NETs with antibodies was performed as described previously [15]. Treated neutrophils were fixed with 1% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 1 h at RT. Fixed samples were incubated with anti-MPO mouse monoclonal antibody (ab25989; Abcam plc, Bristol, UK) and anti-citrullinated histone H3 rabbit polyclonal antibody (ab5103; Abcam) at a concentration of 20 mg/L in 1% BSA/PBS for 16 h at 4 °C. Normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Santa Crus, TX, USA) and mouse IgG (x0931; Agilent Technologies, Palo Alto, CA, USA) antibodies were used as negative controls. Following incubation, samples were washed with 1% BSA in PBS, incubated with Alexa Fluor 633 conjugated anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 488 conjugated anti-rabbit IgG (Thermo Fisher Scientific) antibodies, each at a concentration of 2 mg/L in 1% BSA in PBS for 2 h at 4 °C, and observed with a fluorescence microscope system (TCS SP8; Leica Microsystems, Wetzlar, Germany).

2.4. Flow cytometry (FCM)

For flow cytometric detection of CD11b expression after LPS stimulation monoclonal antibodies to CD11b-conjugated with phycoerythrin (anti-CD11b-PE; BD Biosciences, San Jose, CA) were used as previously described [22]. Briefly, 0.1 mL of heparinized whole blood was stimulated with 10 ng/mL LPS for 15 min, followed by the addition of 5 μL of anti-CD11b-PE and incubation for 15 min in the dark at RT. The samples were then fixed and hemolyzed using 1 mL of FACS Lysing Solution (BD Biosciences) for 10 min, after which the washed samples were analyzed by means of FACS Calibur (BD Biosciences) to determine the mean fluorescence intensity (MFI) of the monocytes.

2.5. Statistical analyses

EZR (Easy R) was used for all statistical analyses [23]. A paired *t*-test was employed to compare data from two groups.

3. Results

3.1. Quantitative detection of hemin-induced NETs

Feasibility of quantitative analysis was checked by using lambda DNA in RPMI 1640, and linearity between 0.0625 ng/mL and 5.0 ng/mL was confirmed ($r^2 = 0.994$) (Supplemental Fig. 1).

NET formation induced by PMA and hemin is shown in Fig. 1-a and b, respectively. The NET induced by PMA (0.1–10 nM) and hemin (1.5–15.3 μM) was dose-dependent. Although we previously demonstrated that PP IX had a more potent ROS-producing capability than hemin [11], PP IX did not induce NETs in this case (Fig. 1-b). The probability of LPS contamination in reagents was unlikely because LPS alone did not induce NETs under these conditions, and treatment with polymyxin B did not affect the fluorescence intensity (Fig. 1-c).

3.2. Hemin-induced NETs depend on NADPH oxidase and ROS but not on TLR-4

NET formation is mediated by several integrated mechanisms comprising autophagy, ROS production through NADPH oxidase, neutrophil elastase, and histone citrullination by peptidylarginine deiminases 4 (PAD4) [24]. For this study, we investigated whether hemin induced-NETs are ROS- dependent. We found that although DPI inhibited NET formation induced by PMA and hemin, Mito-TEMPO inhibited only PMA-induced NET formation (Fig. 2-a). Thus, the effect of hemin on NET formation was shown to be NADPH oxidase-derived ROS-dependent.

Neutrophils have been found to be activated through TLR-4 by high-mobility group box-1 (HMGB1) [25], and heme reportedly activates endothelial cells through TLR-4 [26]. For this study, we investigated the effects of TLR-4 signaling blockade on hemin-induced NET generation. The addition of 10 and 30 μM of TAK-242 was found to significantly inhibit the expression of LPS-induced monocytes CD11b ($p < 0.01$), indicating the efficacy of TAK-242 action on TLR-4 inhibition (Supplemental Fig. 2). However, since TAK-242 did not inhibit hemin-induced NET generation (Fig. 2-b), the NET-producing effects of hemin were not considered to have been generated through TLR-4 signaling.

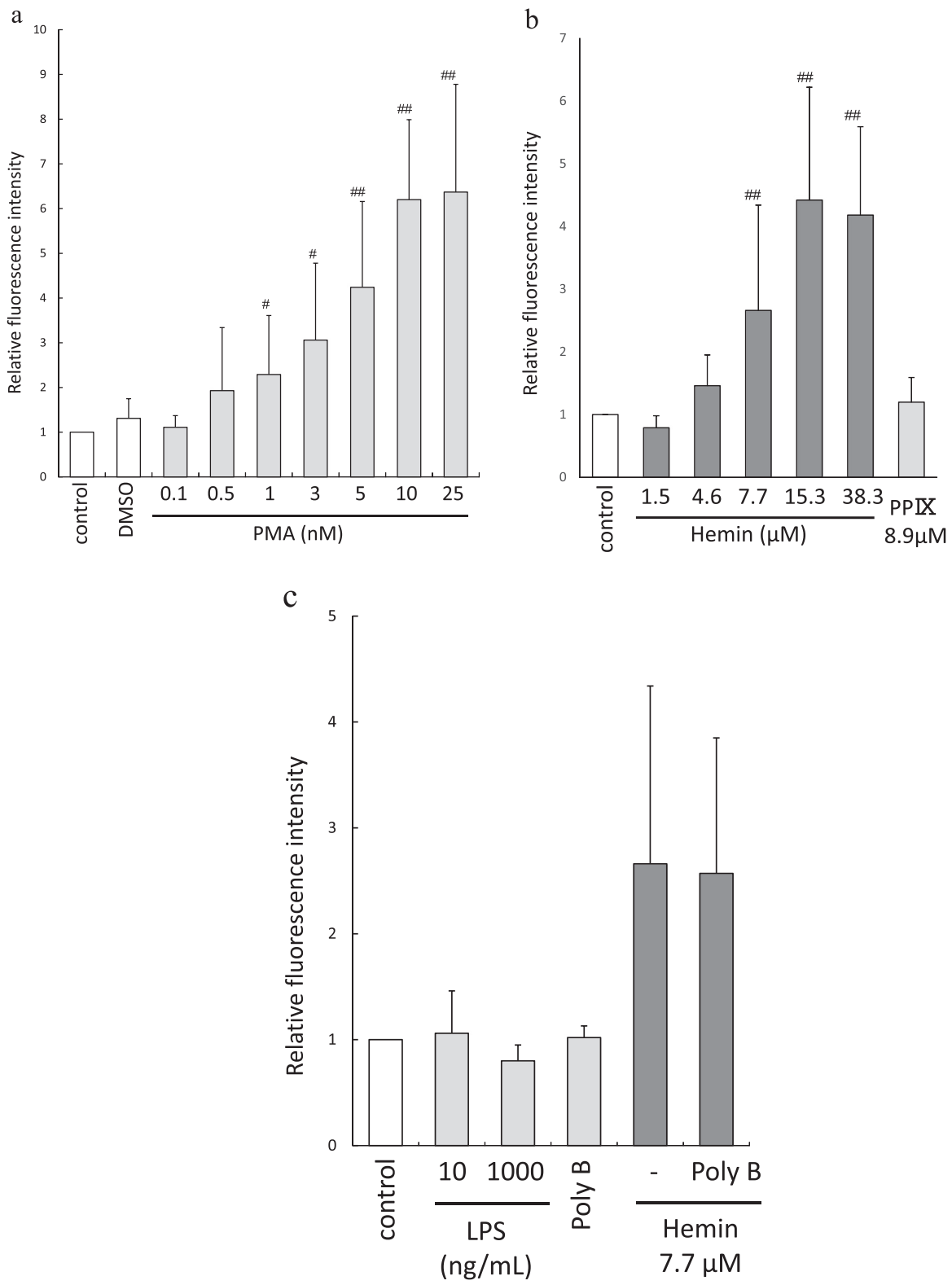

Fig. 1. Quantitative analyses of PMA- or hemin-induced NET generation. (a) PMA-induced NET formation. NETs were formed dose-dependently (0.1 and 10 nM of PMA; n = 10–20). #, $p < 0.05$, ##, $p < 0.01$ against control. (b) Hemin-induced NET formation. NETs were generated dose-dependently (1.5 μM–38.3 μM of hemin; n = 10–20). PP IX did not induce NET production. ##, $p < 0.01$ against control. (c) The effects of Poly B on hemin-induced NETs. LPS or Poly B alone did not induce NETs. Hemin-induced NETs were not inhibited by the presence of Poly B (n = 4–10).

3.3. Hydrogen peroxide enhanced hemin-induced NET production

As oxidative stress is induced by several acute illnesses including sepsis, stroke, myocardial infarction, or multiple trauma [27,28], we studied the effects of hemin under oxidative stress generated by hydrogen peroxide (H_2O_2). High concentrations of H_2O_2 (10 mM) tended to induce NETs as reported previously [29,30], but concentrations lower than 1 mM did not induce NETs individually (Fig. 3). Changes brought about by the

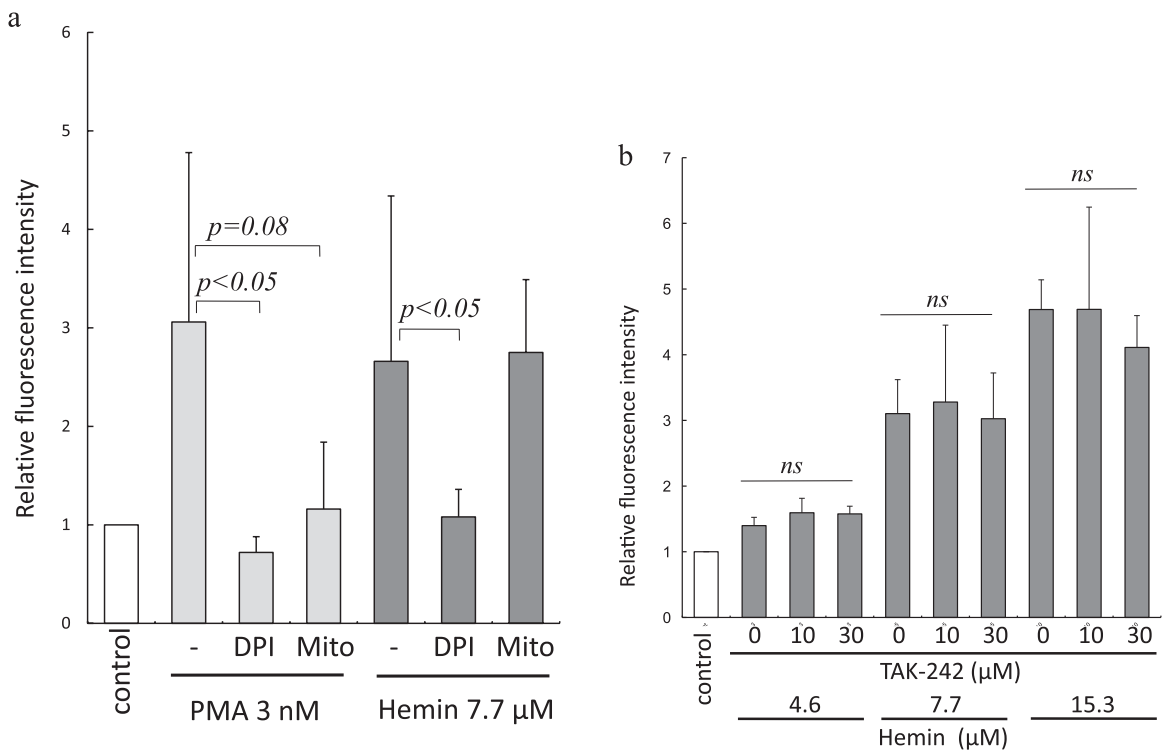

Fig. 2. ROS-dependent and TLR-4 independent characteristics of hemin-induced NETs. (a) Inhibition of PMA- and hemin-induced NETs by DPI. Mito-TEMPO inhibited only PMA-induced NETs (n = 7–12). (b) Effects of TAK-242 on hemin-induced NETs. Addition of TAK-242 did not suppress hemin-induced NETs (n = 4). ns = not significant.

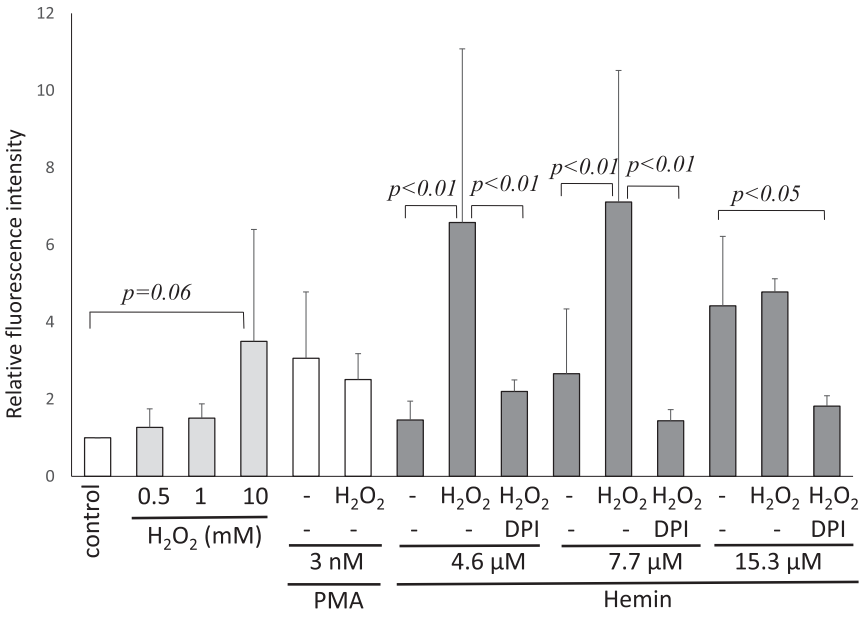

Fig. 3. Effects of hydrogen peroxide on NET formation. H_2O_2 (10 mM) induced NET formation, while concentrations lower than 1 mM failed to do so. Addition of H_2O_2 did not affect PMA-induced NETs, but, enhanced hemin-induced NET production. H_2O_2 -enhanced NETs were sensitive to DPI (n = 10–20).

combination of PMA and H_2O_2 were insignificant alteration in comparison with those produced by PMA alone, although 0.5 mM H_2O_2 significantly increased NET formation induced by 4.6 or 7.7 μM hemin (Fig. 3). This augmented NET formation was inhibited by DPI (Fig. 3), similarly to the result shown in Fig. 2-a. NET formation from 15.3 μM was not enhanced by H_2O_2 probably due to the toxic effects of hemin [31].

3.4. Morphological confirmation of enhancement of NET production by hemin with hydrogen peroxide

Morphological observation under a fluorescence microscope was carried out after staining with Sytox Green. Hemin alone, at a concentration of 7.7 μM or 15.3 μM, induced nuclear swelling with little NET-like structure formation (Fig. 4-b or d, respectively) in comparison with control (Fig. 4-a). However, co-stimulation with 0.5 mM H_2O_2 induced accelerated formation of NET-like structures. Representative results of stimulation with 7.7 μM hemin with H_2O_2 are shown in Fig. 4-c, and those of 15.3 μM hemin with H_2O_2 are shown in Fig. 4-e. Immunostaining studies revealed that both PMNs stimulated with 15.3 μM hemin alone (Fig. 4-f, g, h) and those stimulated with hemin combined with 0.5 mM H_2O_2 (Fig. 4-i, j, k) displayed positive staining with citrullinated histone H3 (Fig. 4-f, i, green) as well as with MPO (Fig. 4-g, j, red), indicating that both hemin alone and hemin in conjunction with H_2O_2 induced NET formation.

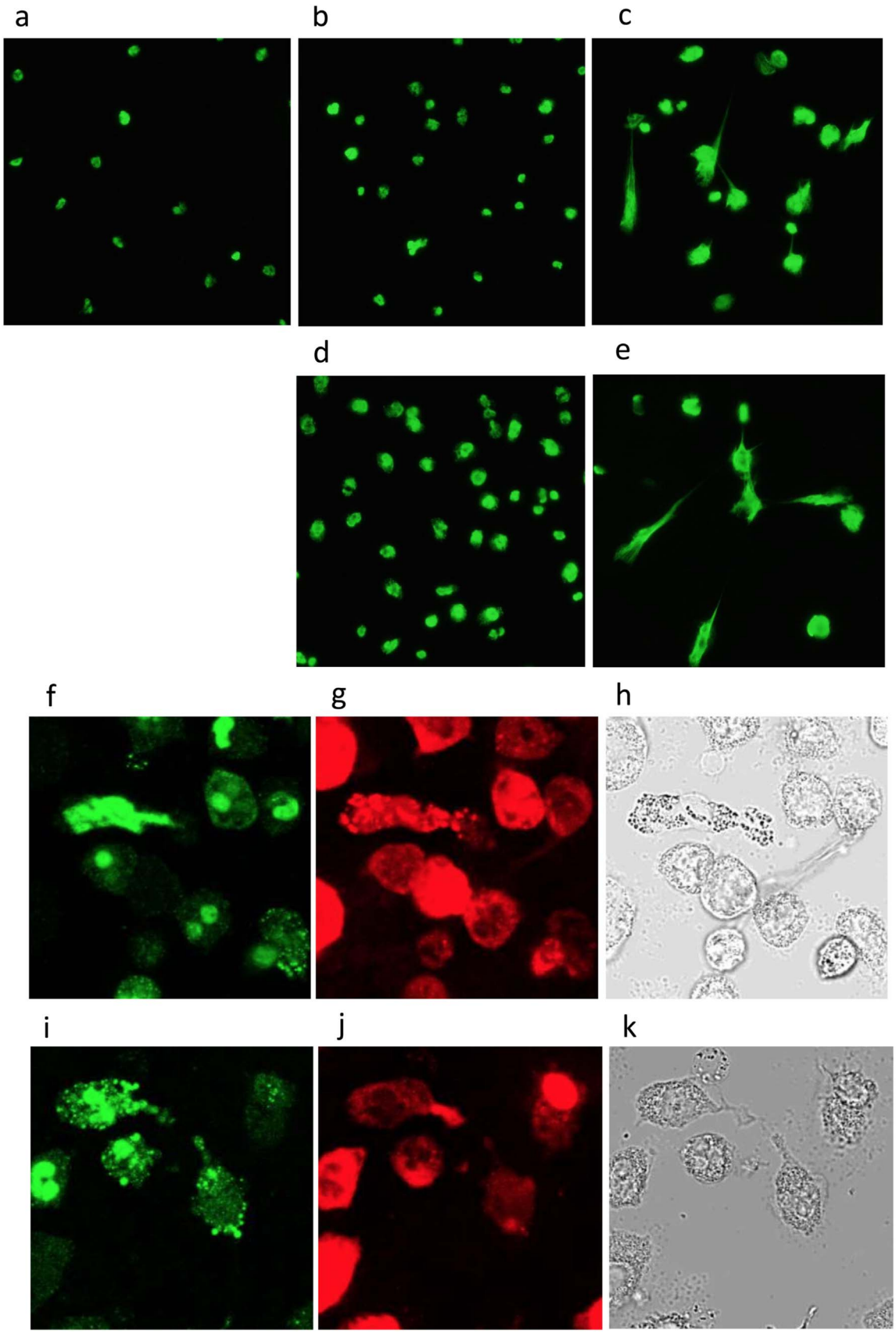

Fig. 4. Morphological observation of hemin-induced NETs. Representative fluorescence microscopy images after Sytox Green staining (a~e), and immunostaining with anti-citrullinated histone H3 (f, i) and MPO (g, j) are shown as phase contrast microscopy images (h, k). (a) control, (b) 7.7 μM hemin, (c) 7.7 μM hemin with 0.5 mM H_2O_2, (d) 15.3 μM hemin, (e) 15.3 μM hemin with 0.5 mM H_2O_2, (f,g,h) 15.3 μM hemin, (i,j,k) 15.3 μM hemin with 0.5 mM H_2O_2.

4. Discussion

NETs are though to exert protective effects because of their antimicrobial activity. However, NETs also contribute to destructive events, such as thrombus formation and organ damage. Although morphological observation using fluorescent microscopy or confocal laser scanning microscopy is most frequently used in in vitro studies of NETs, a quantitative analysis of morphological changes is neither simple nor easy. For the study reported here, a quantitative method described by Palmer [14] was used to detect NET formation by using Sytox Green, which is frequently employed for morphological observation. As linearity between 0.0625 and 5.0 ng/mL lambda DNA had been confirmed, quantitative analysis was feasible and carried out.

It confirmed the capability of hemin to induce NET production. As PP IX did not induce NET formation, hemin-induced NETs were considered to be iron dependent, a finding consistent with that from a previous report [13].

Although NADPH oxidase-derived ROS is required to generate NETs, another ROS-independent pathway has also been reported [32–34]. As shown in our study, hemin- and PMA-induced NETs were NADPH oxidase dependent, but the contribution of mitochondria-derived ROS might be different with these two inducers. Formation of NETs by hemin combined with tumor necrosis factor (TNF) was also reported to be dependent on ROS [13].

Heme binds to TLR-4 on endothelial cells at sites other than the LPS-binding site, and produces TNF and von Willebrand factor [26,35]. However, our experiments with TAK-242 showed that the binding sites of hemin on neutrophils were different from TLR-4. Although hemin can penetrate the cellular membrane because of its hydrophobic character [36], a protoporphyrin ring transporter, heme carrier protein 1 (HCP1), is reportedly present and functions in duodenal cells [37], hematopoietic stem cells [38], and astrocytes [39]. Therefore, studies of the function of HCP1 in neutrophils are also necessary.

It is important for biological responses to occur that low concentrations of hemin-induced NETs are enhanced by low concentrations of hydrogen peroxide. Hemin and hydrogen peroxide have also been reported by Larsen et al. to have this additive effect on the cell death of hepatocytes in vitro, who emphasized that hemin might be a major factor in the pathophysiology of sepsis [7]. The results reported here seem to suggest that oxidative stress generated by oxidizing agents such as hydrogen peroxide [29,30], superoxide [16] or hypochlorous acid [40] amplifies the NET producing activity induced by hemin. This means that hemin can be expected to become a therapeutic target for hemolysis-related events.

As hemin can be easily released from oxidized hemoglobin (methemoglobin) under conditions of oxidative stress [36], the pathological implications of hemin-induced NETs should be considered. Hemin combined with TNF induced NETs in vivo, and these NETs were life-threatening to mice [13]. Moreover, hemin can also induce expression of HO-1, an anti-inflammatory protein [41,42], pointing to the need for in vivo studies under various conditions.

In summary, in the quantitative assay system, hemin alone induced NETs in a dose-dependent manner, which in turn was iron- and NADPH oxidase derived ROS-dependent. In addition, NET-forming capacity was enhanced in the presence of low concentrations of hydrogen peroxide. These results indicate that even a small amount of hemin produced by hemolysis might have pathophysiological implications that warrant further investigation.

Conflict of interest disclosure

The authors declare no conflict of interest.

Acknowledgement

This work was supported by JSPS Grant-in-Aid 15K08660 and Hyogo Medical Association Grant MRF-H-07-14 to K.S.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.07.009.

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