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Original Contribution

Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species

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ABSTRACT

Aim: Neutrophil extracellular traps (NETs) are mesh-like DNA fibers clad with intracellular proteins that are cast out from neutrophils in response to certain stimuli. The process is thought to depend on reactive oxygen species (ROS) generated by the phagocyte NADPH-oxidase and the ROS-modulating granule enzyme myeloperoxidase (MPO), but when, how, and where these factors contribute is so far uncertain. The neutrophil NADPH-oxidase can be activated at different cellular sites and ROS may be produced and processed by MPO within intracellular granules, even in situations where a phagosome is not formed, e.g., upon stimulation with phorbol myristate acetate (PMA).
Objectives: We investigated the subcellular location of ROS production and processing by MPO in the context of PMA-induced NET formation.
Results: Complete neutralization of extracellular ROS was not sufficient to block NET formation triggered by PMA, indicating that intragranular ROS are critical for NETosis. Employing a set of novel MPO-inhibitors, inhibition of NET formation correlated with inhibition of intragranular MPO activity. Also, extracellular addition of MPO was not sufficient to rescue NET formation in completely MPO-deficient neutrophils and specific neutralization by luminol of MPO-processed ROS within intracellular granules led to a complete block of PMA-triggered NET formation.
Conclusion: We show for the first time that inhibition of intragranular MPO activity, or neutralization of intragranular MPO-processed ROS by luminol effectively block NET formation. Our data demonstrate that ROS must be formed and processed by MPO in order to trigger NET formation, and that these events have to occur within intracellular granules.

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1. Introduction

Neutrophils belong to the first line innate immune defense against microbes and have the capacity to eliminate invading pathogens by different mechanisms. The most prominent defense mechanism, phagocytosis, is a well-characterized process that involves uptake of the prey into an intracellular compartment, the phagosome. Subsequently, the phagosome matures by fusion with a variety of different preformed neutrophil granules, creating an inhospitable compartment filled with toxic levels of proteases, antimicrobial peptides, and other microbicidal agents, most notably reactive oxygen species (ROS). These ROS are produced by an electron transporting enzyme system, the NOX2-containing NADPH-oxidase, which generates superoxide at the expense of NADPH. The combined effects of different ROS and the protein/peptide antimicrobials most often kill the engulfed prey [1].

An alternative defense mechanism by which neutrophils trap, and possibly kill, microbes is through the formation of so-called neutrophil extracellular traps (NETs) [2]. The NETs are extracellular cobweb-like fibrils made up by the neutrophil's nuclear DNA,

Abbreviations: CGD, chronic granulomatous disease; CL, chemiluminescence; DAG, sn-1,2-dioctanoylglycerol; DPI, diphenyleneiodonium; HRP, horseradish peroxidase; KRG, Krebs-Ringer phosphate buffer; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PEG, polyethylene-glycol; PHPA, p-hydroxyphenylaceticacid; PMA, phorbol myristate acetate; PKC, protein kinase C; ROS, reactive oxygen species; RT, room temperature; SOD, superoxide dismutase

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which are clad with histones, myeloperoxidase (MPO), antimicrobial peptides, and proteases, all cast out from the cells by an active process called NETosis. This rather spectacular mode of cell death is implicated not only in the context of infectious diseases, but has also been shown to be of importance under non-infectious conditions, *e.g.*, in autoimmune diseases by exposing nuclear and granule material to the immune system [2–5], and in cardiovascular disease by promoting the generation of thrombin [6].

In vitro, NET formation occurs after exposure of neutrophils to a wide range of stimuli, including synthetic chemicals, microbes, microbial products, and cytokines (reviewed in [7]). The by far most potent and commonly used trigger of NET formation in experimental systems is the protein kinase C (PKC) activator phorbol myristate acetate (PMA). This stimulus has been used to explore the subcellular signaling pathways preceding NETosis [8–10], NET composition, and the immunological defense (microbial killing) properties of these structures [11]. PMA is a very potent neutrophil stimulus that activates the superoxide anion producing NADPH-oxidase, and it is well established that PMA-triggered NETosis is strictly dependent on the ROS formed through this activation. This is illustrated most clearly by the fact that neutrophils from chronic granulomatous disease (CGD) patients (or respective animal models), that are unable to form ROS due to a genetically defective NADPH-oxidase, fail to form NETs in response to PMA [12–15]. Additionally, PMA-driven NET formation is completely blocked by the potent NADPH-oxidase inhibitor diphenyleneiodonium (DPI) [12,16]. Exactly how, and where in the cell, the ROS contribute to launching NET expulsion is still unknown.

Neutrophils are capable of producing vast amounts of ROS (not least when stimulated with PMA) and these radicals can be produced at different cellular sites. The major part of the membrane-bound component of the NADPH-oxidase, the b-cytochrome, is located in the membranes of specific and gelatinase granules of resting cells, while a minor part resides in the secretory vesicles and plasma membrane [17]. Upon stimulation, the cytosolic components of the NADPH-oxidase translocate to the membrane-bound b-cytochrome to build an active electron-transporting enzyme. The orientation of the NADPH-oxidase is such that these ROS are never released into the cytosol of the cells, but are separated from cytoplasmic constituents by the membrane in which the b-cytochrome is anchored. Many stimuli, *e.g.*, chemoattractants, will exclusively activate the plasma membrane localized oxidase, leading to ROS release to the extracellular surroundings [18]. In contrast, during phagocytosis the oxidase is assembled in granule membranes that fuse with the phagosome [19], resulting in ROS production within the phagolysosome. Additionally, ROS can be formed within neutrophils in the complete absence of phagocytosis [20]; these ROS are generated in granules or organelles derived thereof [21–23] and are thus also separated from the cytosol by membranes [22]. Such non-phagosomal intracellular ROS production, from here on referred to as intragranular ROS, can be induced by natural agonists [24], but PKC-activation triggered by PMA is one of the most prominent routes leading to such activation [25–27].

Intracellular oxidants are well known to participate in various cell signaling events by altering the intracellular redox state, affecting cytosolic targets such as the cysteines of protein tyrosine phosphatases [28,29]. This knowledge mostly stems from non-phagocytic cells, where the majority of radicals are generated within the cytoplasm, e.g., by the xanthine oxidase [30]. In phagocytic cells, the scenario is different; large amounts of NADPH-oxidase-derived ROS are produced within membrane-enclosed organelles, e.g. neutrophil granules. The possibility that intragranular ROS are important for PMA-induced NET formation has so far been overlooked and not tested directly.

In addition to the NADPH-oxidase and the ROS that stem from its activation, the granule enzyme MPO has also been shown indispensable for PMA-induced NET formation [31–33]; neutrophils from rare MPO-deficient individuals cannot make NETs upon PMA stimulation [32]. The enzymatic activity of MPO is normally involved in the processing of primary ROS (superoxide anion and hydrogen peroxide) to secondary ROS (*e.g.*, hypohalous acid) [34]. Although MPO is clearly critical for NET formation, its enzymatic activity may not be crucial as the mere presence of the protein has been shown to suffice in driving the chromatin decondensation that precedes NETosis [31]. However, pharmacological MPO inhibitors are in fact capable of decreasing NET formation, although the process is not fully blocked [16,35,36].

In this study, we aimed at elucidating the role of intragranular ROS and MPO in PMA-induced NET formation. Using a novel approach employing membrane-permeable MPO inhibitors and selective ROS scavengers, we show that ROS generated and processed by MPO within intracellular granules regulate NETosis. This is to our knowledge the first cellular function that has been ascribed to these intragranular oxidants and indicates that such ROS production may be of importance in health and disease.

2. Materials and methods

2.1. Isolation of neutrophils

Human neutrophils were isolated from buffy coats from healthy donors, or from peripheral blood, as first described by Boyum [37]. Cells were washed in Krebs-Ringer phosphate buffer (KRG) containing glucose (10 mM) and Mg^{2+} (1.5 mM) and resuspended in KRG supplemented with Ca^{2+} (1 mM). Buffy coats were obtained from the hospital blood bank after de-identification and according to the Swedish legislation section code 4§ 3 p SFS 2003:460, no informed consent is needed. Peripheral blood from one MPO-deficient individual and healthy controls were obtained after written informed consent and the study was approved by the Regional Ethical Review Board in Gothenburg, Sweden.

2.2. Quantification of NET formation

Neutrophils (5×10^4 cells/well) in RPMI (without phenol red) were added to black 96-well plates with Sytox Green (1.25 μM; Molecular Probes (Life Technologies Europe BV), Stockholm, Sweden) and stimulated with PMA (50 nM; Sigma-Aldrich, St.Louis, Missouri) or DAG (25 μM; Avanti Polar Lipids, Alabaster, Alabama) with or without the following inhibitors or scavengers; DPI (10 μM; Sigma-Aldrich), MPO inhibitors (0.01–10 μM;), luminol (560 μM; Sigma-Aldrich), or isoluminol (560 μM; Sigma-Aldrich), SOD (50 U/ml; Worthington, Lakewood, New Jersey) and catalase (2000 U/ml; Worthington). Exogenous MPO isolated from human blood [38] (a kind gift from Professor Inge Olsson, Lund University) was added to some samples. Fluorescence was measured (excitation 485 nm, emission 535 nm) in a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany) after incubation at 37 °C with 5% CO_2 for 3 h or indicated time points. The MPO-inhibitors were synthesized by AstraZeneca R&D Mölndal, Sweden. Tx1 and Tx4 have been previously described [39] and for intellectual property reasons, the chemical structure of AZM198 is not disclosed. For others to reproduce the data presented herein, AstraZeneca may provide the compound under a standard material transfer agreement (contact: Erik.Michaelsson@astrazeneca.com).

2.3. Visualization of NET formation

Visualization of NETosis was performed as described by Brinkmann et al. [40]. Briefly, neutrophils in RPMI with 2% fetal

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