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Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation

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ABSTRACT

High availability of NO, oxidative stress and neutrophil extracellular trap (NETs) contents are often noticed at the site of inflammation/infection. Studies from this lab and others have reported NO mediated free radical generation from neutrophils; role of NO in NETs formation however remains undefined so far. The present study was therefore undertaken to explore the effect of NO donors on NET release from human neutrophils (PMNs), using confocal/scanning microscopy, measuring the extracellular DNA content and NET-bound elastase activity. Addition of NO donors (SNAP and SNP) to adhered PMNs led to a time and concentration dependent NETs release, which was blocked by *N*-acetyl cysteine, suggesting involvement of free radicals in NETs formation. Free radical formation by NO donors was assessed by using DCF-DA, DMPO-nitrone antibody and by p47 phox migration to the neutrophils membrane. NO mediated formation of free radicals and NETs was significantly reduced by the pretreatment of neutrophils with diphenyleneiodonium (DPI), a NADPH-oxidase inhibitor and 4-aminobenzoic acid hydrazide (ABAH), a myeloperoxidase inhibitor, suggesting role of enzymatic free radical generation by NO donors. We thus demonstrate that NO by augmenting free radical formation in human neutrophils mediates NETs release.

Introduction

Neutrophils (PMNs), the first line of defense, are recruited first to the site of infection, inflammation or injury [1]. PMNs internalize and destroy infectious agents by the sequential formation of phagosomes, recruitment of lysosomes and various types of granules to release proteolytic enzymes/microbicidal peptides, and free radical formation, to mediate killing of endocytosed pathogens [1–3]. PMNs an active player in inflammatory pathologies, thus pose a challenge of being a classical friend or foe.

Formation of NETs, a novel mechanism, to eliminate invading pathogens has been demonstrated [4]. NETs formation could also be a way of PMNs death, which is distinct from apoptosis and necrosis. High circulating levels of DNA have been assigned to NETs in malaria and sepsis patients [5,6]. NETs formation by the addition of PMA or IL8, indicated that in addition to bacteria, cytokines or PKC activation also induce NETs release [4,7]. Platelet TLR4 mediated neutrophil activation and NETs formation has been reported in severe sepsis [7]. *In vivo* NETs contents are expectedly abundant at the site of infection and acute inflammation [4,8,9]. Neutrophils from chronic granulomatous disease (CGD) patients could not form NETs, it was therefore delineated that NADPH-oxidase dependent generation of reactive oxygen species (ROS) is needed for NETs release [10]. Identification of new mechanisms and mediators of NETs release is thus an area of intense research and have immense implications so as to identify new therapeutic targets.

Augmented inducible nitric oxide synthase (iNOS) activity at the inflammatory site is often observed even before any obvious inflammatory changes. ROS, NO, nitrated proteins, DNA or neutrophil proteolytic enzymes at the site or in circulation, are well documented in inflammatory bowel disease [11,12], cholera or shigellosis dysentery [13], chronic inflammatory airway diseases [14,15], malaria [5], pelvic inflammatory disease [16,17] and sepsis [6,7]. Previous studies from this lab as well as others have identified NO as an important modulator of free radical generation in PMNs [18–22]. Since NO and PMNs are

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well established mediators of inflammation [1,11], we thought it logical to investigate effect of NO donors on human PMNs in NETs release and also explored the possible role of free radicals [23].

Methods

Isolation of human neutrophils

Blood from healthy volunteers was collected after their consent from the anti-cubital vein (Heparin, 10 U/ml), was layered on Histopaque 1119 and centrifuged for 20 min at 800*g*. The lower interphase having granulocytes was washed with RPMI 1640 medium and was loaded on the discontinuous Percoll gradients as described earlier [24]. Isolated PMNs were suspended in RPMI 1640 medium containing 0.5% FBS. The purity and viability of the isolated PMNs was ascertained by CD15-FITC and PI staining using Flow cytometer (Becton Dickinson, USA), which was never less than 95%. The study was approved by the institutional committee and was conducted according to the Declaration of Helsinki.

NETs formation

Confocal microscopy

Neutrophils (1×10^6), plated on glass bottomed cover-slips (precoated with 0.001% poly-L-lysine) were treated with NO donors, [sodium nitroprusside (SNP, 100 μM), *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 100–500 μM)], PMA (10–50 nM) or vehicle for 30–180 min in a CO_2 incubator (RS Biotech, UK) at 37 °C. Effect of various interventions such as *N*-acetyl-L-cysteine (NAC, 5 mM), diphenyleneiodonium chloride (DPI, 10 μM), 4-aminobenzoic acid hydrazide (ABAH, 100 μM), or 7-nitroindazole (7-NI, 1 mM) was also monitored on NETs release by incubating PMNs for 15 min at 37 °C and then treated with SNP or SNAP for 3 h. After fixation samples were stained overnight with 20 μg/ml of rabbit polyclonal elastase antibody (Calbiochem, USA) and were visualized after treatment with the secondary antibody (1:200, chicken anti-rabbit AF 488 antibody, Molecular Probes, USA) by confocal microscope [25] and assessed for the incidence of NETs formation [9]. DNA was stained with propidium iodide (PI, 20 μg/ml), Hoechst 33342 (3 μg/ml) or Sytox green (5 μM).

High magnification and low magnification images were captured by Carl Zeiss (Germany) or Olympus (Japan) confocal microscopes by using appropriate lenses and filters. The mean fluorescence intensities were quantified using LSM Software Version 4.2 (Carl Zeiss, Germany).

Scanning electron microscopy (SEM)

Neutrophils (5×10^5) suspended in RPMI medium containing 0.5% FBS were allowed to adhere on poly-L-lysine coated sterile cover-slips and then incubated with various interventions, PMA (20 nM) or SNAP (100 μM) for various time intervals. Cells were fixed overnight with 2% paraformaldehyde + 2.5% glutaraldehyde, post fixed in cacodylate buffer containing 1% osmium tetra-oxide and 1% tannic acid for 1 h, dehydrated by ethanol (30–100%), dried, mounted on stub and sputter coated with gold palladium alloy, and analyzed under FEI-XL30 scanning electron microscope [4].

Elastase activity and DNA estimation

Neutrophils (1×10^6) suspended in RPMI 1640 medium (Phenol red free) containing 0.5% FBS were seeded on poly-L-lysine precoated tissue culture plates and subsequently treated with vehicle, SNAP or PMA for 3 h. Elastase activity in supernatant following DNAase treatment was assayed by using substrate *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide (100 μM) [10], while extracellular DNA was determined by Sytox green (Invitrogen, USA), as reported earlier to assess DNA/NETs release [4,10].

Free radical generation

Confocal microscopy

2,7-Dichlorodihydro-fluorescein diacetate (DCF-DA) loaded neutrophils (1×10^6 cells) were dispensed on poly-L-lysine coated cover-slips and treated with SNP (100 μM) and subsequently free radical generation was monitored for 30 min using a BioRad Confocal Microscope with a Plan Apo 60X/1.4 NA oil immersion objective. Data was captured with Bio Rad Laser Sharp 2000 software 5.1 and photographs were processed using Adobe Photoshop software [26].

Flow cytometry

PMNs (2×10^6cells) were incubated with vehicle or various interventions [DPI (10 μM), 7-NI (1 mM), ABAH (100 μM) or *N*-acetyl-L-cysteine (NAC, 5 mM)] for 15 min at 37 °C, loaded with DCF-DA, (10 μM) for 5 min, and finally SNP (1 μM–1 mM) or SNAP (1 μM–1 mM) was added. Ten thousand events were acquired and free radical generation was determined by using FACS Calibur (Becton Dickinson, USA) [21].

Assessment of ROS and RNS formed in the PMNs lysate

Fluorescence of the reaction mixture containing PMNs lysate (1×10^6 cells), Tris buffer (100 mM, pH 6.0) or phosphate buffer (50 mM, pH 6.0), DCF-DA (10 μM), sodium nitrite ($NaNO_2$-1 mM) and or H_2O_2 (4.4 mM), was continuously monitored for 10 min at 488 nm_{ex} and 520 nm_{em} in the presence and absence of ABAH (100 μM) or 7-NI using a flourimeter (Varian, Cary Eclipse, Netherlands). Suitable controls were used for each set.

Assessment of 7-NI on MPO enzyme activity MPO activity was measured in the presence of vehicle, 7-NI or ABAH by using purified protein (Sigma, St. Louis, MO) as described by Sethi et al. [20]. MPO protein aliquot was suspended in 50 mM phosphate buffer (pH 6.0), incubated with vehicle, 7-NI or ABAH for 5 min, subsequently *o*-dianisidine (7.9 mM), and hydrogen peroxide (4.4 mM) were added to the incubation mixture. The enzyme kinetics was monitored for 3 min at 15 s intervals at 460 nm to determine the MPO enzyme activity.

Western blot analysis

NO mediated free radical formation was also determined by DMPO. PMNs (5×10^7 cells) were incubated at 37 °C for 30–180 min with SNAP (100 μM) or PMA (20 nM) and DMPO (50 mM) in the presence and absence of NAC (5 mM). Samples (30 μg protein) were loaded on 10% SDS–PAGE and transferred to PVDF membrane, which was blocked with 3% skimmed milk in TBST and then incubated with rabbit DMPO-nitrone antibody (1:3000 in 1.5% skimmed milk TBST) for 2 h at room temperature [27].

In other set of experiment to assess the translocation of p47 phox from the cytosol to neutrophil membranes, membrane fraction was prepared from cells (1×10^7cells) pre-treated with PMA (20 nM) or SNAP (100 μM) at 37 °C for 60–180 min [28]. Membrane fraction (20 μg protein) was run on 10% SDS–PAGE and subsequently transferred to PVDF membrane. The membrane was blocked overnight, incubated with p47 phox antibody (1:500 in 1.5% skimmed milk TBST) for 2 h at room temperature. Both proteins were detected after incubation with HRP-conjugated anti-rabbit IgG (1:20,000) for 2 h by enhanced chemiluminescence detection reagents (Millipore, USA).

Statistical analysis

Results have been represented as mean ± SEM of at least 3–5 independent experiments. Student's *t*-test or analysis of variance for repeated measurements was used. When the *F* value was significant further pair wise comparisons were done by using