BBA - Molecular Cell Research 1865 (2018) 444–454

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

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

S-Glutathionylation of p47phox sustains superoxide generation in activated neutrophils

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ARTICLE INFO

Keywords:
Neutrophils
Oxidative burst
NADPH oxidase (NOX-2)
Reactive oxygen species (ROS)
Nitric oxide (NO)
Phorbol 12-myristate 13-acetate (PMA)
N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)
p47phox (NCF-1)
Phosphorylation
S-Glutathionylation
Sustain ROS

ABSTRACT

Post-translational modifications (PTMs) induced conformational changes of proteins can cause their activation or inactivation. Neutrophils clear pathogen through phagocytosis and oxidative burst generation, while participate in inflammation through sustained and uncontrolled generation of ROS. In activated PMNs, cytosolic NOX-2 subunit p47phox following phosphorylation interacts with p67phox, p40phox and along with Rac2 translocate to the membrane. Phosphorylation of p47phox subunit occurs in both short spurts as well as sustained ROS generation, suggesting towards the unidentified molecular mechanism(s) driving these two diverse outcomes by various stimuli. The present study demonstrates that in PMA or NO treated neutrophils a subunit of NOX2, p47phox gets glutathionylated to sustain ROS generation along with a decrease in catalase, Grx-1 activity and change in GSH/GSSG ratio. Surprisingly, fMLP treated cells neither showed sustained ROS production nor glutathionylation of p47phox. S-Glutathionylation was always secondary to phosphorylation of p47phox and inhibition of glutathionylation did not alter phosphorylation but specifically impaired sustained ROS production. Interestingly, forced S-glutathionylation of p47phox converted the fMLP induced ROS generation into sustained release of ROS. We then identified the glutathionylation susceptible cysteine residues of p47phox by LC-MS/MS with IAM switch mapping. Site-directed mutagenesis of cysteine residues further mitigated p47phox S-glutathionylation. Thus, we demonstrate that p47phox S-glutathionylation plays an essential key role in the sustained ROS generation by human neutrophils.

1. Introduction

Neutrophils are an important and indispensable component of innate immunity in host defence [1,2]. NADPH oxidase (NOX) mediated ROS generation by human neutrophils plays an important role in microbial killing following phagocytosis as well as during NETosis [3,4]. Moreover, in the inflammatory milieu neutrophils are major contributors of ROS and cytotoxic proteases [5,6]. Thus efforts to identify and characterize the regulatory mechanisms of NOX activity are highly desirable to mitigate uncontrolled and sustained ROS mediated inflammatory conditions.

NADPH oxidase 2 (NOX-2), is the major source of ROS in neutrophils, which catalyse the generation of superoxide from oxygen and NADPH [7,8]. This phagocyte oxidase consists of different protein components that are spatially regulated in resting/un-stimulated neutrophils. On activation, cytosolic components like p47phox, Rac2 and p67phox undergoes different post translation modifications to form complex and migrate to associate with the membrane protein, flavocytochrome *b*558 [9,10]. Neutrophils can produce a sharp spurt of respiratory burst or sustained ROS production that most likely depend on diverse signals like stimulator type, concentration and regulatory signaling involved [11,12]. Under normal conditions, neutrophils maintain reduced conditions by maintaining low and essential amounts of ROS and RNS due to adequate levels of antioxidants and scavenging enzymes. Diverse stimuli cause differential activation of NOX system. For example, PMA, a potent NOX activator, releases excessive superoxide radicals, while nitric oxide (NO), an extremely reactive, freely diffusible moiety [13,14] causes thiol oxidation and also involved in generation of RNS such as $\cdot NO_2$, N_2O_3, $ONOO^-$ that further depends on the redox environment and NO concentration. fMLP a bacterial component is known to induce short respiratory burst in neutrophils. In general, excessive RNS/ROS production modulates important neutrophil functions as well as survival and commonly associated with inflammatory diseases, thus warrants investigations to better

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https://doi.org/10.1016/j.bbamcr.2017.11.014
Received 3 August 2017; Received in revised form 8 November 2017; Accepted 26 November 2017
Available online 02 December 2017

understand the differential ROS release mechanisms in detail.

Post-translational modifications (PTMs) regulate protein functions by regulating conformational changes. Phosphorylation a well explored PTM regulates various important cellular functions [15,16]. Another key and physiologically relevant PTM, S-glutathionylation modifies several proteins involved in basic cellular functions including actin, L-Plastin, tyrosine kinases, phosphatases, and transcription factors like NFkB [17,18] and these modified proteins can further cross talk with other post-translational mechanisms. We have recently established the significance of L-Plastin S-glutationylation in human neutrophil chemotaxis and polarization [19]. S-glutathionylation is a reversible PTM at cysteine residues and modulated by intracellular GSH [20]. De-regulated protein S-glutathionylation has been associated with several diseases like diabetes mellitus, myocardial ischemia-reperfusion and cancer [21,22].

NOX-2, cytosolic and activation unit, p47phox (also known as neutrophil cytosolic factor 1, NCF-1) presents at maximum concentration in neutrophils and consists of 390 amino acids [23]. p47phox plays a substantial role in the assembly and activation of NOX2, via post-translational modification. The C-terminal fragment consist of multiple phosphorylated serines (303–379) [24] and the primary role of phosphorylation at serine residue in NADPH oxidase activation in different types of cells including neutrophils is well investigated [25]. In addition, primary amino acid sequence of human p47phox consists four cysteine residues including Cys-98, Cys-111, Cys-196 and Cys-378 and the side chain of cysteine profounds variety of protein modification for protein regulation [26,27]. However the cysteine residues present in p47phox remains unexplored for regulatory role in NOX activation.

Although phosphorylation signaling and role of different protein kinases is known in ROS generation, occurrence of p47phox phosphorylation in short spurts as well as sustained ROS generation, suggesting unidentified mechanism(s) driving these varied outcomes. Nevertheless molecular mechanism responsible for sustained activation of NOX system remains ill defined. By using diverse stimuli including fMLP, NO and PMA mediated human neutrophil ROS stimulation; here we report for the first time the mechanistic insights of sustained NOX activation, via S-glutathionylation of p47phox. Moreover, our results further support the essential role of phosphorylation for ROS generation and that is pre-requisite for p47phox glutathionylation in neutrophils. We thus identified a novel and the key role of S-glutathionylation of p47phox in a sustained ROS generation. This would be important for many diseases that are linked with sustained ROS derived inflammatory conditions.

2. Methods and materials

Biotinylated glutathione monoethyl ester (BioGEE, was purchased from Molecular probes (Eugene). DETA NONOate (DETA-NO), dextranT-500, 2′,7′-dichlorofluorescein diacetate (DCF-DA), dihydroethidium (DHE), diisopropyl fluorophosphate (DFP), diphenyleneiodoniumchloride (DPI), dithiothreitol (DTT), N-formyl-Met-Leu-Phe (fMLP), luminol, HRP, 2-Hydroxyethyl disulphide, phorbol 12-myristate 13-acetate (PMA), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4),and sodium arsenite, TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) were from Sigma Aldrich Co. (St. Louis, MO, USA). VAS-2870 was procured from EMD Millipore (Billerica, USA). Neutravidin agarose Resin and Zeba Spin Desalting Columns were obtained from Thermo Scientific (San Jose, CA, USA). Lipofectamine LTX and Plus reagent were obtained from Invitrogen (Carlsbad, CA). Anti-glutathione monoclonal antibody, Anti-S-nitrocysteine antibody (HY8E12); ab94930, Anti-NCF1 (phospho S304) antibody; (ab63554); Anti-Peroxiredoxin-SO3 antibody (ab16830) was purchased from abcam (Cambridge, MA). Antibody against p47phox (D-10) sc17845 was obtained from Santa Cruz Biotechnology (Santa Cruz, USA).β Actin (A2066), anti-rabbit IgG-HRP, anti-mouse IgG-HRP, antibodies were procured from Sigma Aldrich Co. (MO, USA). NCF1 (NM_000265) Purified Human Protein was obtained from Origene Inc.

2.1. Isolation and purification of neutrophils

Human neutrophils were isolated from peripheral blood, obtained from healthy donors. After Dextran sedimentation and Percoll density gradient, layer enriched in neutrophils was isolated, washed and re-suspended in HBSS containing glucose (10 mM), Ca^{2+}(1 mM), Mg^{2+}(1 mM). Purity of the isolated human neutrophils was assessed by using CD15 labelling using flow cytometry (FACS Calibur, BD, USA). Microscopic evaluation of isolated cells indicated purity of isolated PMNs was ~95% and viability as tested by Trypan blue exclusion was > 95%. Experimental studies with human blood samples were approved by ethical committees of KGMU and CSIR-CDRI, Lucknow, India [28].

2.2. ROS/RNS and superoxide measurement

Human neutrophils (1×10^6 cells/ml) were loaded with DHE (10 μM) or DCF (10 μM) for 10 min and then stimulated with DETA NO (300 μM), PMA (30 nM) or fMLP (10 μM) for 30 min to induce generation of superoxide and other reactive oxygen species. Fluorescence of 10,000 cells was acquired on FACS Calibur (Becton Dickinson, NJ, USA) and subsequently analyzed using Cell Quest programme. ROS generation was also checked by using luminometry it was peroxidase-dependent and catalase-sensitive technique, which detect extracellular hydrogen peroxide. 2×10^5 cells were plated in 96 well plate and were incubated with 10 μM of Luminol, 10 mU horseradish peroxidase in HBSS buffer for 10 min on a Labtech white plate. Reactions were performed by addition of various interventions like fMLP (10 μM) or PMA (30 nM) or DETA NO (300 μM). Luminescence was measured at every 40 s for 60 min using Labtech-96 Microplate Luminometer. To test effect of interventions, cells were treated with vehicle, VAS (25 μM), Ro-31-8220 (10 μM), Sodium orthovanadate (PI) (20 μM), NEM (100 mM), Sodium arsenite (50 μM) or GSH (1 mM) for 15 min at 37 °C in dark, before treatment with stimulator [29,30].

2.3. Measurement of superoxide by DHE using HPLC

The specificity of DHE for superoxide production was further assessed using HPLC (Agilent technologies, USA) to avoid non-specific signals due to fluorescent probes interaction with macromolecules. Cells (1×10^6) were pre-incubated with DHE (5 μM, 30 min), prior to the treatment with VAS (25 μM, 15 min) followed by stimulation with DETA NO (300 μM, 30 min) and PMA (30 nM, 30 min), cells were then lysed in 1 × PBS containing 0.1% Triton X-100. The lysate were vortexed, mixed with 0.5 ml of 1-butanol, and centrifuged for 5 min at 5000 rpm. The butanol phase was separated and dried under vaccum at its boiling temperature (40 °C) (Lab concocentri-vap micro IR, MO, USA). Dried samples were resuspended in 100 μl of HPLC grade water and 20 μl of sample was injected into HPLC (Agilent technologies, USA) equipped with a fluorescence detector. Oxidised product of DHE was then separated using C18 (Zorbax Eclipse Plus 100 × 4.6 mm, 3.5-μm particles) column at a flow rate of 0.5 ml/min. The amount of DHE oxidised products hydroxyethidium and ethidium were measured at excitation wavelength 480 nm and emission at 580 nm. HPLC peak area in individual sample was normalized with the protein concentration of each sample [31].

2.4. Determination of intracellular Glutathione (GSH)

GSH content in human neutrophils was determined by HPLC (Agilent technologies, USA) equipped with a fluorescence detector. Cells (10^7) were sonicated in 3.5% perchloric acid, centrifuged at 10,000 rpm for 5 min, and were then divided into two aliquots. For total glutathione determination, one aliquot was diluted with 20