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High oxidative stress adversely affects NFκB mediated induction of inducible nitric oxide synthase in human neutrophils: Implications in chronic myeloid leukemia

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ABSTRACT

Increasing evidence support bimodal action of nitric oxide (NO) both as a promoter and as an impeder of oxygen free radicals in neutrophils (PMNs), however impact of high oxidative stress on NO generation is less explored. In the present study, we comprehensively investigated the effect of high oxidative stress on inducible nitric oxide synthase (iNOS) expression and NO generation in human PMNs. Our findings suggest that PMA or diamide induced oxidative stress in PMNs from healthy volunteers, and high endogenous ROS in PMNs of chronic myeloid leukemia (CML) patients attenuate basal as well as LPS/cytokines induced NO generation and iNOS expression in human PMNs. Mechanistically, we found that under high oxidative stress condition, S-glutathionylation of NFκB (p50 and p65 subunits) severely limits iNOS expression due to its reduced binding to iNOS promoter, which was reversed in presence of DTT. Furthermore, by using pharmacological inhibitors, scavengers and molecular approaches, we identified that enhanced ROS generation via NOX2 and mitochondria, reduced Grx1/2 expression and GSH level associated with NFκB S-glutathionylation in PMNs from CML patients. Altogether data obtained suggest that oxidative status act as an important regulator of NO generation/iNOS expression, and under enhanced oxidative stress condition, NOX2-mtROS-NFκB S-glutathionylation is a feed forward loop, which attenuate NO generation and iNOS expression in human PMNs.

1. Introduction

Neutrophils (PMNs), the most abundant leukocyte and the first line of cellular defense against invading microorganisms play a key role in the innate immunity [1]. Activated PMNs generate reactive oxygen (ROS) and nitrogen species (RNS) to execute the ingested/phagocytosed pathogens, on the other hand enhanced ROS/RNS production lead to tissue damage and inflammation [2,3]. Augmented inducible nitric oxide synthase (iNOS) activity and NO production following PMNs activation also modulate ROS production [4,5].

NO affects chemotaxis, adhesion, aggregation, microbial killing and apoptosis of neutrophils under diverse pathophysiological conditions [6,7]. Moreover, NO mediated modulation of free radicals generation in PMNs by multiple mechanisms have been reported [8]. NO in lower concentrations augmented ROS generation, while higher concentrations attenuated ROS formation through inhibition of NADPH oxidase activity or ROS scavenging [9–12]. Previous study from this lab also demonstrated the biphasic role of NO on the reactive species formation in PMNs following treatment with ascorbate, arachidonic acid and fMLP [6,13–17]. In tumors low flux NO fosters cell survival, while high levels of NO exerts cytotoxic effects [5,18,19]. Alternatively, increasing evidence suggests that ROS itself can influence the intracellular NO signalling, however the precise molecular mechanism is not identified [20,21]. Acute or

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sub-acute oxidative stress in endothelial cells reduces NOS activity through caveolin-1 inhibition, ETB dissociation, and eNOS phosphorylation [22].

Enhanced ROS generation in several pathological conditions such as rheumatoid arthritis, atherosclerosis, diabetes and myeloid leukemias has been associated with defective neutrophil functions [23–26]. An intricate balance between ROS/NO generation and cellular enzymatic or non-enzymatic antioxidants maintain the cellular redox, while perturbation in these parameters adversely affect homeostasis [27,28]. Redox imbalance often oxidises reactive thiols on specific cysteine residues of proteins, leading to their S-nitrosylation, S-glutathionylation or sulfenic acid modification [29]. In a recent study we have shown that high nitroxidative stress impaired neutrophil migration, polarization and bacterial killing by S-glutathionylation of L-plastin and β-actin [30]. Oxidative or nitrosative stress regulates activation of several transcription factors and proteins such as NFκB, HIF1α, Nrf2 and STAT by reversible or irreversible modifications of cysteine thiols affecting iNOS expression [21,31].

The present study was undertaken to assess the regulation of iNOS expression and NO generation in human PMNs under high oxidative stress conditions. Herein, we demonstrate that enhanced oxidative stress attenuated NFκB transcriptional activity as evident from prevention of LPS/cytokines induced NO generation and iNOS expression in PMNs of healthy control and chronic myeloid leukemia (CML) patients. Present study illustrates that under high ROS, S-glutathionylation of NFκB (p50 and p65 subunits) severely limits iNOS expression due to its reduced binding to iNOS promoter. Furthermore, using PMNs from CML patients we attributed NADPH oxidase (NOX) specifically NOX2, and mitochondrial ROS (mtROS) mediated NFκB S-glutathionylation. Results obtained thus established that under high oxidative stress conditions NOX2-mtROS mediated S-glutathionylation of NFκB (p50 and p65 subunits) attenuated NO generation and iNOS expression in PMNs of CML patients.

2. Materials and methods

2.1. Reagents and antibodies

RevertAid™ H minus first strand cDNA synthesis kit was obtained from Fermentas Life Sciences (Vilnius, Lithuania). 2X maxima SYBR green PCR master mix was purchased from Roche Applied Science (Lewes, UK). Antibodies against iNOS, p50, p65, CD15, NOX2/gp91^{phox}, and control, NOX2 or NOX4 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-GSH, Prx-SO_3 and NOX4 antibodies were obtained from Abcam (Cambridge, USA). NFκB activation inhibitor, SN-50 and VAS-2870 was obtained from Calbiochem (San Diego, USA). Immunoprecipitation starter pack was procured from GE Healthcare (Piscataway, USA). All primers used in the present study were from Integrated DNA technology (India). MitoTEMPO was procured from Enzo Life Sciences (New York, USA). Biotinylated GSH monoethyl ester (BioGEE), Amplex red assay kit and MitoSOX red were obtained from Molecular probes (Eugene, USA). Glutathione fluorometric assay kit was from BioVision (California, USA). All other chemicals used in the study were purchased from Sigma Aldrich Co. (St. Louis, USA).

2.2. Neutrophils isolation from control and CML patients

Buffy coat from healthy blood donors was obtained from blood centre, King George Medical University (KGMU), Lucknow. The donors were free from any major disease and any prior medication for last 72 h. CML patients enrolled in this study were 18–70 years old (mean age-39 years) with a confirmed diagnosis of Ph^+ chronic phase CML. Peripheral blood samples from CML patients were collected in sodium citrate (0.129 M, pH 6.5, 9:1v/v) after written consent from the patients. KGMU institutional ethics committee approved the consent procedure and the procedure involved in blood collection. PMNs from healthy volunteers and CML patients were isolated as described previously [2], and purity was ascertained by Flow cytometer (FACS Calibur, Becton Dickinson, NJ, USA) using CD15 antibody and it was always more than 95%. Viability of the isolated PMNs was more than 95%, as assessed by Trypan blue exclusion test. Neutrophil precursors (myeloblast/promyelocytes, myelocytes/metamyelocytes and band cells/segmented neutrophils represented as bands 3, 2 and 1 respectively) from CML patients peripheral blood were isolated according to method described by Kumar et al. [32]. The purity of band 3, 2 and 1 was assessed by CD11b, CD15 and CD16, and also by Giemsa staining [33]. The study protocol was approved by the ethical committees of KGMU and CSIR-Central Drug Research Institute, Lucknow and was conducted in accordance with the declaration of Helsinki.

2.3. Treatment of PMNs with LPS and cytokines mixture

PMNs isolated from healthy volunteers were pre-treated with vehicle, NFκB activation inhibitor (NFI, 2 μM) or SN-50 (25 μM) for 30 min followed by treatment with LPS (1 μg/ml) + mixture of cytokines (IFNγ, TNFα and IL1β-20 ng/ml each) for 4h. NFκB activation inhibitor, a quinazoline compound act as a potent inhibitor of NFκB transcriptional activation and LPS-induced TNF-α production [34], while SN50, a synthetic peptide contains cell membrane permeable hydrophobic region and nuclear localization sequence, and inhibited the translocation of the NFκB active complex into the nucleus [35]. In other set of experiments PMNs isolated from healthy volunteers or CML patients were exposed to thiol oxidizing agent diamide at the indicated time and concentrations after being primed with vehicle, thiol alkylating agent N-ethylmaleimide (NEM, 50 μM) or sulfhydryl reducing agent dithiothreitol (DTT, 500 μM) for 30 min. These cells were further stimulated with LPS (1 μg/ml) + mixture of cytokines (IFNγ, TNFα and IL1β-20 ng/ml each) for 4h. PMNs from healthy volunteers were also treated with PMA (30 nM) at the indicated time followed by stimulation with LPS (1 μg/ml) + mixture of cytokines (IFNγ, TNFα and IL1β-20 ng/ml each) for 4h.

2.4. NOX2 silencing in human PMNs

Control or NOX2 siRNA (100 pM) were transfected in PMNs of healthy control and CML patients using the Nucleofactor II electroporation device (Amaxa Biosystems, Cologne, Germany) and the Nucleofector™ program T-019 [2,30]. After transfection, the cells were cultured in RPMI containing 10% FBS and 100 ng/ml GM-CSF. Viability of the PMNs was assessed 24 h after transfection by PI staining and found to be ~75%.

2.5. Treatment of K562 cells

Human CML cell line K562 obtained from ATCC was cultured in RPMI-1640 with 10% heat inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, at 37 °C in 5% CO_2 incubator. To assess the importance of NADPH oxidase and mtROS, K562 cells were treated with NADPH oxidase inhibitor, VAS-2870 (25 μM); mitochondrial superoxide scavenger, mitoTEMPO (50 nM) or complex I inhibitor, rotenone (10 μM) and analysed after 24 h.