Introduction

Neutrophils or polymorphonuclear neutrophils (PMNs) are the most abundant and short-lived white blood cells. They represent the essential component of the innate immune system and are also involved in inflammatory disorders. PMNs are the first cells to reach the site of infections and their migration is crucial for the resolution of infections. In consequence defects in PMN chemotaxis result in severe infections [1,2]. PMNs kill ingested/phagocytosed pathogens by generating reactive oxygen (ROS) [3] and nitrogen species (RNS) [4] as well as by nonoxidative mechanisms [5].

Neutrophil adhesion, chemotaxis, bacterial killing, and apoptosis are modulated by nitric oxide (NO) [6] which along with hydrogen peroxide may act as intracellular signal transducers [7]. The cellular redox status is maintained by a delicate balance between ROS and RNS (RONS) production and their effective scavenging by cellular enzymatic and nonenzymatic antioxidants [8,9]. A perturbation in this balance is thus detrimental for cellular homeostasis. Activated human PMNs are a major source of ROS generation [10] and also synthesize NO [11]. Several research groups, including ours, have shown the involvement of NADPH oxidase (NOX-2) in NO-mediated augmentation of PMN free radical generation [12–14].

Increased oxidative stress is also of critical importance in many pathological conditions and is often associated with defective neutrophil chemotaxis and enhanced propensity to infection [15,16]. Impaired PMN chemotaxis, bactericidal activity, and oxidative stress are a hallmark of diabetes mellitus (DM), a devastating metabolic disorder [17,18] and therefore it is important to understand the molecular mechanisms underlying defective PMN functions. S-Glutathionylation (S-thiolation), a redox-mediated posttranslational modification of cysteine residues described in a substantial number of proteins, plays an important role in the cellular adaptation to oxidative stress [19,20]. Cysteines surrounded by basic residues have

Original Contribution

L-Plastin S-glutathionylation promotes reduced binding to β-actin and affects neutrophil functions

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ABSTRACT

Posttranslational modifications (PTMs) of cytoskeleton proteins due to oxidative stress associated with several pathological conditions often lead to alterations in cell function. The current study evaluates the effect of nitric oxide (DETA-NO)-induced oxidative stress-related S-glutathionylation of cytoskeleton proteins in human PMNs. By using in vitro and genetic approaches, we showed that S-glutathionylation of L-plastin (LPL) and β-actin promotes reduced chemotaxis, polarization, bactericidal activity, and phagocytosis. We identified Cys-206, Cys-283, and Cys-460 as S-thiolated residues in the β-actin-binding domain of LPL where cys-460 had the maximum score. Site-directed mutagenesis of LPL Cys-460 further confirmed the role in the redox regulation of LPL. S-Thiolation diminished binding as well as the bundling activity of LPL. The presence of S-thiolated LPL was detected in neutrophils from both diabetic patients and db/db mice with impaired PMN functions. Thus, enhanced nitroxidative stress may result in LPL S-glutathionylation leading to impaired chemotaxis, polarization, and bactericidal activity of human PMNs, providing a mechanistic basis for their impaired functions in diabetes mellitus.

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Abbreviations: GMP, guanosine monophosphate; IAM, iodoacetamide; NCF, neutrophil cytosolic factor; NOS, nitric oxide synthase; ROS, reactive oxygen species
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the highest susceptibility for S-thiolation \[21,22\]. S-Glutathionylation takes place via thiol-disulfide exchange with oxidized glutathione, by reaction of oxidant-induced protein thyl radicals with reduced glutathione, or by reaction of a nitrosothiol with another thiol \[23,24\]. NO-induced oxidative stress also causes glutathionylation of several proteins. Although NO induces protein S-nitrosylation, several studies suggest that NO/GSH may cause conversion of S-nitrosylation into the more stable modification, S-glutathionylation \[25,26\]. Proteins, which are normally not glutathionylated, also undergo this modification, leading to altered protein function in pathological conditions \[27,28\] such as atherosclerosis, cancer, neurodegenerative diseases, and diabetes \[29–31\].

L-Plastin (LPL), an important β-actin-bundling protein, has many cysteine residues and is exclusively present in leukocytes \[32\]. LPL phosphorylation results in its enhanced affinity for β-actin \[33\] and modulates PMN migration, maintenance of lamellipodia, filopodia, and integrin function \[34\]. On the contrary LPL-deficient PMNs are unable to kill bacterial pathogens and even lose their capability of generating an adhesion-dependent respiratory burst \[35\]. Recently, Sakai et al. reported that FMLP-mediated ROS production induced β-actin S-glutathionylation in human PMNs \[36\]; of note, S-glutathionylation of the actin interacting proteins is yet to be demonstrated.

The current study was undertaken to assess the molecular mechanisms involved in impaired PMN functions following high oxidative stress. Human PMN chemotaxis, polarization, phagocytosis, and bactericidal activity were investigated under conditions of high oxidative stress both in vitro (treatment with NO donor, DETA-NO) and ex vivo (PMNs from diabetic subjects and db/db mice). Our results highlight the role of S-glutathionylation of L-plastin and β-actin in impaired neutrophil migration, polarization, and bacterial killing.

**Materials and methods**

**Materials**

Percoll was from Amersham Biosciences Corp. (Uppsala, Sweden), antibodies against L-plastin, β-actin, GFP, and LPL siRNA were from Santa Cruz Biotechnology (Santa Cruz, USA), and anti-glutathione monoclonal antibody was purchased from Virogen (Wattertown, MA). The phosho-Ser Ab against L-plastin was a kind gift from Dr. Eric Brown, University of California (Genentech, San Francisco, USA), Biotinylated GSH monooethyl ester (BioGEE) was from Molecular Probes, β-G-actin was from Cytoskeleton (Bocchout, Belgium), and purified human L-plastin protein was obtained from Origene Technologies Inc. (Rockville), mEmerald-Plastin-N-10 was a gift from Michael Davidson (Addgene plasmid No. 54233). Lipofectamine LTX and Plus reagent were obtained from Invitrogen (Carlsbad, CA). Recombinant human GM-CSF was purchased from Peprotech (Rocky Hill, USA), VAS-2870 was from EMD Millipore (Billerica, USA), and all the primers used in the study were obtained from Integrated DNA Technology (India). DETA NONOate (DETA-NO), N-ethylmaleimide (NEM), N-Formyl-Met-Leu-Phe (FMLP), dithiothreitol (DTT), and all other chemicals used in the study were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

**Isolation of PMNs from human blood and mice bone marrow**

Neutrophils were isolated from the blood of the healthy volunteers and diabetic patients as described previously \[4\]. Diabetic patients were recruited based on the criteria of the American Diabetes Association. A total of 17 patients were included in the study (10 females, 7 men, age 21–55), fasting glucose was 156 mg/dl and HbA1c ≥ 9%. Sex-matched and age-matched healthy volunteers were selected for the comparison. C57BL/6 control and the age-matched db/db mice (leptin receptor knockout), iNOS knockout mice (iNOS\(^{-/-}\)), and p47NOS knockout mice (p47\(^{NOS-/-}\)/neutrophil cytosolic factor 1 null mice. NCF1\(^{-/-}\)) were procured from The Jackson Laboratory (Bar Harbor, ME, USA). 12 to 16-week-old mice were used in all the experiments. Purity of the isolated human neutrophils and of the mouse bone marrow neutrophils was assessed by using CD15 and Ly6G labeling, respectively, using flow cytometry (FACS Calibur, BD, USA). Purity of the isolated neutrophils was always more than 90% with > 95% viability as confirmed by trypan blue. The study protocol for human blood samples was approved by ethical committees of KGMU and CSIR-CDRI, Lucknow, India. Studies with C57BL/6 wild-type, diabetic mice (db/db), iNOS knockout mice (iNOS\(^{-/-}\)), and p47NOS knockout mice (NCF1\(^{-/-}\)) were performed according to the Institutional Animal Ethics Committee of CSIR-CDRI, India.

**ROS generation**

PMNs (1 × 10\(^6\) cells/ml) loaded with 2,7-dichlorofluorescein diacetate (DCF-DA, 10 μM) for 10 min were subsequently treated with DETA-NO (300 μM) for 15 and 30 min at 37 °C. Ten thousand events were acquired using FACS Calibur (Becton Dickinson, USA) and subsequently ROS generation was assessed using the Cell Quest program.

In the current study, cells were treated with the NO donor for 15 or 30 min (as noted in the subsequent sections), centrifuged, and resuspended to assess the effects on FMLP-induced chemotaxis and polarization, or incubated with bacteria to evaluate the effect on phagocytosis and ROS generation.

**F-actin content**

PMNs (1 × 10\(^6\) cells/ml) were stimulated with DETA-NO (300 μM) at 37 °C for 15 and 30 min. After stimulation, cells were fixed with 4% PFA for 30 min at room temperature and then permeabilized with 0.1% Triton X-100. The cells were incubated with phalloidin-TRITC (0.25 μM) for 45 min at room temperature and were subsequently stained with DAPI (1 μg/ml). Specimens were mounted in Antifade and images were acquired using a Carl Zeiss confocal microscope (Oberkochen, Germany) equipped with DPSS-561 laser source using a 63x oil objective lens. Images were further processed using the Adobe Photoshop software. Quantitative assessment was made after the extraction of Phalloidin-TRITC with methanol overnight, and fluorescence was measured using a BMG LABTECH POLARstar omega microplate reader (Ortenberg, Germany) \[36\]. F-actin content was also measured after labeling the cells with phalloidin–FITC, and was analyzed by a flow cytometer (FACS Calibur, BD USA).

**Immunofluorescence**

Human PMNs after stimulation with DETA-NO (300 μM) for 15 min at room temperature were assessed for S-glutathionylation by following the method of Sakai et al. \[36\]. Cells were incubated with GSH (1:500) and L-plastin (1:500) antibodies overnight at 4 °C, followed by staining with secondary antibodies (1:500) for 1 h at room temperature, and nuclei were stained with DAPI (1 μg/ml). After mounting, images were acquired using a Carl Zeiss confocal microscope (Oberkochen, Germany) equipped with DPSS-561 laser source using a 63x oil objective lens.

**Phagocytosis assay**

The phagocytosis assay was performed as described previously \[4\]. FITC-coated bacteria were added to the PMN (1:50) suspension containing 1 mM calcium and 1 mM magnesium chloride to assess phagocytosis at 37 °C for 15 min. Samples were acquired using
FACS Calibur at 488-nm excitation. Trypan blue (200 μg/ml) was added to quench the fluorescence of adherent bacteria.

Neutrophil polarization and migration assay

For the neutrophil polarization assay, control and DETA-NO-treated (300 μM for 15 min) human PMNs (40,000 cells in HBSS [containing 10 mM glucose, 1 mM calcium, and 1 mM magnesium chloride]) were seeded on poly-L-lysine-coated coverslips. For DTT treatment, cells were treated with DETA-NO followed by incubation with DTT (1 mM) for 10 min at 37 °C and then DTT was removed by washing the cells with HBSS. PMNs were stimulated with 10 μM fMLP for 10 min in a CO2 incubator. After stimulation, the cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100. The cells were then stained with phalloidin–TRITC (0.25 μM) for 1 h at room temperature. Nuclei were stained with DAPI (1 μg/ml). After mounting, images were acquired using a TCS SP8 Leica confocal microscope (Oberkochen, Germany) equipped with a DPSS-561 laser source using a 63x oil objective lens.

Migration of control and DETA-NO-treated (300 μM for 15 min) cells was measured in the Neuro Probe 48-well microchemotaxis chamber. The lower wells were filled with the chemoattractant (100 nM fMLP) and kept at 37 °C for 10 min. The filter membrane (5 μm pores) was placed over the bottom wells. After the silicone gasket was applied, the upper wells were filled with 18,000 cells/well (in HBSS containing 10 mM glucose, 1 mM calcium, and 1 mM magnesium chloride). The filled chamber was incubated at 37 °C in humidified air with 5% CO2 for 2 h. The transwell membrane was then removed, and the number of cells migrated was quantitated by staining the membrane with DAPI (1 μg/ml) visualized under a Leica fluorescence microscope.

Bactericidal assay

Control and DETA-NO-treated (300 μM for 15 and 30 min) PMNs were incubated with Escherichia coli (containing pCMV6-entry vector) for 30 min. After incubation, the cells were washed twice with HBSS to remove nonphagocytosed bacteria. The cells were lysed with sterilized water and the lysates (100 μl) were seeded on LB agar plates containing kanamycin (25 μg/ml) and incubated overnight at 37 °C. Live bacteria were counted and expressed as the number of colony-forming units (CFU). The bactericidal assay was also performed in PMNs following silencing with LPL siRNA.

LPL silencing in human PMNs

LPL silencing was performed using the Nucleofector II electroporation device (Amaxa Biosystems, Cologne, Germany) and the Nucleofector program T-019. After transfection, the cells were cultured in RPMI containing 10% FBS and 100 ng/ml GM-CSF to cultured in RPMI containing 10% FBS and 100 ng/ml GM-CSF to

Treatment of LPL siRNA.

Detection of S-glutathionylation with BioGEE

One vial of BioGEE (Stock 1.78 mM, final concn: 296 μM) was suspended in 100 μl of HBSS (containing 10 mM glucose, 1 mM calcium, and 1 mM magnesium chloride), kept for 30 min at 37 °C, and then added to 1 × 10^7 cells/ml. The cells were incubated at 37 °C and after 30 min cells were pelleted and washed once with HBSS followed by stimulation with DETA NO (300 μM for 30 min) at 37 °C. Cells were lysed with buffer containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, protease inhibitor cocktail, 5 mM diisopropylfluorophosphate (DFP), 0.5% NP-40, and 100 mM NEM. Proteins were pulled down with neurtavdin beads and samples were run on nonreducing SDS-PAGE and subsequently probed with Strep-HRP.

Immunoprecipitation (IP) and Western blotting

Human PMNs (total protein, 500 μg) were lysed with ice-cold lysis buffer at 4 °C for 30 min. The supernatant was preclarified with protein A/G agarose (Amersham Biosciences, Upsala, Sweden) and incubated with 1 μg of β-actin or L-plastin antibody overnight at 4 °C. Subsequently, 20 μl of protein A/G agarose was added and incubated for 3 h at room temperature. The beads were washed, resuspended in gel loading buffer, denatured at 95 °C for 10 min, and subsequently analyzed by Western blotting.

Proteomic identification

The BioGEE-labeled and NO-stimulated PMNs were suspended in ice-cold lysis buffer and subjected to the pulldown assay using neurtavdin agarose beads. After heat denaturation, an equal amount of protein was loaded and subjected to 10% SDS-PAGE followed by Coomassie staining. The Coomassie-stained bands of different molecular weights were excised from the gel and subjected to mass spectrometry by MALDI-TOF through a commercial source (Sandal Proteomics, Hyderabad).

Iodoacetamide (IAM) switch to label cysteine residues

Human PMNs (1 × 10^6 cells/ml) were treated with DETA-NO (300 μM) for 30 min. After treatment, the cells were lysed with lysis buffer (containing 150 mM NaCl, 50 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM DFP, protease inhibitor cocktail, and 100 mM NEM). Excess NEM was removed by passing the cell lysates through a Sephadex desalting column. Cell lysates were reduced with 1 mM TCEP at 56 °C for half an hour. After reduction, the lysates were incubated with 50 mM IAM for 45 min in the dark at room temperature. The proteins were separated on SDS-PAGE and stained with Coomassie. The Coomassie-stained protein band corresponding to LPL was excised and processed for in-gel digestion at the NCBS C-CAMP proteomics facility using the protocol of Shevchenko et al. [37]. Full mass spectrometry in a mass range between m/z 300 and m/z 200 was performed in an Orbitrap mass analyzer with a resolution of 30,000 at m/z 400 and an AGC target of 2 × 10^5. The strongest signals were selected for collision-induced dissociation (CID)–MS/MS in the LTQ ion trap.

For LC-LTQ Orbitrap MS analysis, samples were resolubilized in 2% [v/v] acetonitrile, 0.1% [v/v] formic acid in water and injected onto an Agilent 1200 (Agilent, Santa Clara, CA, USA) nanoflow LC system that was in-line coupled to the nansoelectrospray source of an LTQ-Orbitrap discovery hybrid mass spectrometer (Thermo Scientific, San Jose, CA, USA). Peptides were separated on Zorbax 300SB-C18 (Agilent, Santa Clara, CA, USA) by a gradient developed from 2% [v/v] acetonitrile, 0.1% [v/v] formic acid to 80% [v/v] acetonitrile, 0.1% [v/v] formic acid in water over 70 min at a flow rate of 300 nL/min. Full MS in a mass range between m/z 300 and m/z 2000 was performed in an Orbitrap mass analyzer with a resolution of 30,000 at m/z 400 and an AGC target of 2 × 10^5. The strongest signals were selected for CID–MS/MS in the LTQ ion trap at a normalized collision energy of 35% using an AGC target of 1 × 10^6 and two microscans. Dynamic exclusion was enabled with one repeat counts during 45 s and an exclusion period of 180 s. Peptide identification was performed by CID-based MS/MS of the selected precursors, which also revealed the site of cysteine modification. For protein/peptide identification, MS/MS data were searched against the Uniprot/Swissprot amino acid sequence database (downloaded in August 2013) using an in-house Mascot server (version 2.4) through the Proteome Discoverer 1.4 software. The
search was set up for full tryptic peptides with a maximum of three missed cleavage sites. NEM, carbamidomethyl on cysteine, and oxidized methionine were included as variable modifications. The precursor mass tolerance threshold was 10 ppm, and the maximum fragment mass error was 0.8 Da. The significance threshold of the ion score was calculated based on a false discovery rate of < 1%, estimated by the peptide validator node of the Proteome Discoverer software.

Site-directed mutagenesis of LPL

Site-directed mutagenesis of LPL Cys460 → Ser460 was performed using a QuickChange site-directed mutagenesis kit (Strategene, Santa Clara, USA). The mutated plasmid was generated following the manufacturer’s instruction. Mutation was confirmed by DNA sequencing. HEK293T cells were cultured in DMEM medium with 10% FBS and 100 μg/ml penicillin/streptomycin. Cells were transfected with 2.5 μg of wild-type and mutated LPL clone using LTX-plus liposomal transfection reagent. After 24 h of transfection, cells were treated with dexamethasone (0.5 mM) for 30 min followed by lysis with lysis buffer containing 100 mM NEM. For invasion assay, cells were trypsinized and seeded on polycarbonate membranes. LPL expression was monitored by Western blotting with anti-GFP antibody and flow cytometry.

Cell invasion assay

Cell invasion assay was performed using Boyden chamber. Polycarbonate filters (8 μm pores) were coated with Matrigel on the upper surface and fibronectin (10 mg/ml) on the lower surface. HEK293T (20,000) cells were seeded and incubated for 24 h. Then the filters were fixed with 4% PFA and stained with DAPI (1 μg/ml). Invaded cells on the lower surface were counted under a fluorescent microscope.

Real-time PCR

Total RNA was isolated from human PMNs with the help of TRI reagent, and cDNA was synthesized using the RevertAid H Minus first-strand cDNA synthesis kit (Fermentas Life Science, Vilnius, Lithuania) using the oligo(dT) primer. Grl 1 mRNA was quantified by real-time PCR using the primer sequence GxlF 5′-AGCCACACACCACTACTAAG-3′ and GrlXR 5′-CTGACCACACTCTGTGGTTAC-3′ using the Light Cycler instrument (Roche Applied Science, Lewes, UK) with the 2− Maxima SYBR Green RT-PCR Master Mix (Roche Applied Science, Lewes, UK). β-Actin was used as the normalization control.

In vitro S-glutathionylation of LPL

To induce S-glutathionylation, LPL (1 μM) was incubated with oxidized glutathione (GSSG 2 mM) for 30 min at room temperature. After incubation, the samples were boiled in Laemmli sample buffer (without DTT) for 5 min, separated on SDS-PAGE, and probed with the anti-GSH antibody.

In vitro actin binding and bundling assay

The effect of LPL S-glutathionylation on β-actin binding and bundling was investigated as described by Janji et al. with some modifications [33]. Recombinant human LPL (1 μM) was S-glutathionylated in vitro with GSSG (2 mM) and excess of GSSG was removed using a desalting column. Human G-actin (6 μM) was polymerized overnight at 4°C in the presence of LPL or LPL-SSG in actin polymerization buffer containing 100 mM KCl, 1 mM MgCl₂, 1 mM ATP, 0.5 mM EGTA, 50 mM sodium phosphate buffer, pH 7.0. Actin and LPL were separated by ultracentrifugation at 200,000 g for 30 min. For the bundling assay, G-actin was polymerized overnight and centrifuged at 12,000 g for 15 min for separation of spontaneously formed β-actin bundles, and then incubated with LPL or LPL-SSG for 1 h. LPL-induced β-actin bundles were sedimented by centrifugation at 12,000 g for 15 min.

Statistical analysis

Data in the manuscript have been reported as mean ± SEM from at least 3–5 independent experiments. The data were analyzed by one-way ANOVA followed by Newman-Keul’s post hoc analysis. Comparisons between the control and the treated samples were performed using Student’s t test. A P value of < 0.05 was considered to indicate statistical significance.

Results

Effect of NO on PMN chemotaxis and polarization

Nitric oxide is elevated in various inflammatory disorders and diseases, so we first investigated the possible role of human neutrophil migration, a crucial event common to these conditions. To that end we used fMLP (100 nM) as a chemoattractant to induce human neutrophil migration, as previously described [38]. Neutrophil chemotaxis in NO-pretreated cells was significantly decreased in the experimental cells as compared to vehicle-treated cells in a concentration-dependent manner with the most significant diminution observed at 100 and 300 μM concentration of DET-A-NO (Fig. S1A). Thus, for the subsequent experiments only one concentration of DETA-NO (300 μM) was used. NO-induced inhibition of human PMN migration was reversed by treatment with DTT (Fig. 1A, B), suggesting redox regulation of the process. Neutrophil polarization is critical for the chemoattractant-induced PMN migration and is characterized by a change in the shape of the neutrophils, from round to polarized. The NO-treated human PMNs exhibited reduced formation of pseudopods as compared to the control cells when stimulated with fMLP (Fig. 1C, D), confirming the previous reports of defective polarity and migration in NO-pretreated cells [39]. Moreover, enhanced migration of bone marrow neutrophils from iNOS−/− mice confirmed the previously defined role of intracellular NO in neutrophil migration (Fig. 1E, F).

NO acts in a cGMP-dependent and independent manner; therefore, cGMP involvement was assessed by using ODQ, a guanylate cyclase inhibitor. Pretreatment of human PMNs with ODQ (10 μM) did not affect NO-induced decrease in chemotaxis, indicating that a cGMP-independent pathway was responsible for the effect (Fig. S1B).

Phagocytosis and bacterial killing in NO-treated PMNs

The effect of exogenous NO on phagocytosis and bactericidal activity was examined in human PMNs. Phagocytosis was significantly decreased at 30 min in NO-pretreated cells as compared to control PMNs and this inhibition was also reversed by DTT (Fig. 2A). NO treatment also reduced bactericidal activity. In untreated PMNs, the number of bacteria was significantly reduced, but a greater number of CFU were detected in the NO-pretreated cells, an effect reversed by DTT (Fig. 2B, C).

Effect of NO on protein S-glutathionylation

Previous studies from our group have shown that NO augmented ROS generation in human PMNs [13,40]. ROS generation was measured using DCF-DA. PMNs treated with DETA-NO showed significant enhancement of ROS generation at 15 and 30 min (Fig. 3A). Involvement of NADPH oxidase in NO-induced free radical generation has been documented by this lab [13,41]. Consistent with our previous observations, involvement of NOX-2 was further
confirmed by using PMNs from NCF1−/− mice where NO-induced free radical generation was significantly reduced (Fig. 3B). During phagocytosis, PMNs generate ROS/RNS to kill the internalized pathogens. Therefore we next examined phagocytosis-induced ROS generation in NO-pretreated PMNs. There was significant inhibition in ROS generation after bacterial phagocytosis in NO-pretreated cells as compared to untreated cells (Fig. 3C).

Since oxidative stress may cause reversible or irreversible S-glutathionylation of proteins, we measured it in neutrophils pretreated with DETA-NO for 15 min. Protein S-glutathionylation was detected using a monoclonal anti-GSH antibody and NEM treatment, which alkylates thiol groups and reduced the signal, confirming the specificity of the anti-GSH antibody binding (Fig. 3D). NO-induced S-glutathionylation was also significantly decreased in PMNs from NCF1−/− mice, supporting the involvement of NADPH oxidase in ROS formation in NO-induced S-glutathionylation (Fig. 3E).

To further confirm the presence of NO-induced S-glutathionylation, we used another experimental approach where PMNs were loaded with BioGEE and then treated with NO. As shown in Fig. 3F, an increase in the amount of proteins undergoing glutathionylation was observed. Treatment with DPI (a NOX and NOS inhibitor), VAS-2870 (a NOX inhibitor), and cPTIO (an NO scavenger) reduced the amount of BioGEE-labeled proteins (Fig. S1C). To identify the specific proteins modified by S-glutathionylation, immunoprecipitates from NO-pretreated PMNs were separated by SDS-PAGE. After Coomassie staining, S-glutathionylated proteins were identified by MALDI-TOF. Among others two important cytoskeleton proteins L-Plastin (LPL) or lymphocyte cytosolic protein (LCP1) and β-actin were identified. Since LPL glutathionylation occurred prior to β-actin and little is known about it, we further explored in detail the potential significance of this phenomenon.

Lysates of BioGEE-labeled and DETA-NO-stimulated cells were precipitated with neutravidin beads and probed with the anti-LPL
Fig. 2. Effect of NO on phagocytosis and bactericidal activity of human PMNs. (A) Phagocytosis of FITC-labeled bacteria in human PMNs following treatment with DETA-NO (300 μM). Human PMNs (1 × 10⁶ cells/ml) were treated with DETA-NO (300 μM) for 15 and 30 min followed by incubation with FITC-labeled bacteria (1:50). (B) Bar diagram represents CFU of E. coli in control and NO-treated (300 μM) PMNs (1 × 10⁶ cells/ml). Treatment with DTT (1 mM) leads to reversal of NO-induced inhibition. Data represent mean ± SEM of five independent experiments. ***P < 0.001 vs control neutrophils, $P < 0.05$ vs control neutrophils with bacteria, #P < 0.05 vs NO-treated (30 min) cells with bacteria, @@@P < 0.001 vs neutrophils treated with NO for 15 min.

Fig. 3. NO induced oxidative stress. (A) Nitric oxide induced ROS generation in PMNs from healthy control neutrophils. PMNs (1 × 10⁶ cells/ml) were incubated with DCF-DA for 10 min followed by stimulation with DETA-NO (300 μM) for 15 and 30 min. (B) Comparison of NO-induced ROS generation in wild-type and NCF1⁻/⁻ mice. (C) ROS generation in control and NO-treated cells after phagocytosis of bacteria. (D) Detection of S-glutathionylation using the anti-GSH antibody. PMNs were stimulated with DETA-NO (300 μM) followed by lysis with NEM (100 mM)-containing lysis buffer. S-Glutathionylated proteins were detected by Western blotting using the anti-GSH monoclonal antibody. Pretreatment with NEM (100 mM) for 15 min reduced the S-glutathionylation signal. (E) NO-induced S-glutathionylation in wild-type and NCF1⁻/⁻ mice. (F) Human PMNs (1 × 10⁷ cells/ml) were incubated with BioGEE (300 μM) for 30 min and then treated with DETA-NO (300 μM) for 30 min. BioGEE-labeled proteins were probed with Strep-HRP. Data represent mean ± SEM of five independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control neutrophils, @P < 0.05 (Student’s t test) vs control neutrophils treated with NO for 15 min, @@P < 0.001 vs control neutrophils with bacteria.
and anti-β-actin antibodies. As shown in Fig. 4A and B, NO-induced glutathionylation of LPL and β-actin was clearly detected. S-Thiolation of LPL and β-actin was further confirmed by immunoprecipitation using LPL and β-actin antibodies and by probing with the anti-GSH antibody (Fig. 4C, D). We also confirmed LPL S-glutathionylation by immunostaining of PMNs (Fig. 4E). Pulldown with the anti-LPL antibodies from BioGEE-labeled lysates and probing with streptavidin–HRP also demonstrated that in NO-treated PMNs, LPL undergoes S-glutathionylation (Fig. S1D). Since NO or related species can lead to S-nitrosylation and tyrosine nitration, we checked these possibilities by using anti-SNO and anti-nitrotyrosine antibodies in Western blotting. However, no such posttranslational modifications were detected (Fig. S1E). Our next objective was to identify the susceptible cysteine residues of LPL undergoing glutathionylation. We performed redox switch using IAM to label oxidized cysteine residues. LC–MS/MS data from IAM switch mapped potential LPL S-glutathionylation sites: Cys-206, Cys-283, and Cys-460. Control sample was used to check

![Fig. 4.](image)
effective blocking by NEM, where TCEP was not used. In this sample only NEM-modified peptides were observed. NEM-modified spectra for Cys 206, 283, and 460 are shown in Figs. S2A–C. Representative MS/MS spectra of IAM-modified Cys-206, Cys-283, and Cys-460 are shown in Fig. 5A, C, and D. All these residues were localized in the β-actin-binding domain of LPL (Fig. 5B). Among these residues Cys-460 was found in most of the samples and with the maximum protein score.

In vitro S-glutathionylation of LPL and interaction with β-actin

To further assess LPL glutathionylation, purified recombinant human LPL protein was treated in vitro with oxidized glutathione (GSSG). As shown in Fig. 5E GSSG (2 mM) induced S-glutathionylation of human LPL, which was reversed by treatment with DTT (10 mM for 10 min). To explore the potential relevance of this observation, the effects of LPL S-thiolation on β-actin bundling and on the interaction of LPL with β-actin were investigated. LPL S-glutathionylation reduced β-actin binding as well as bundling activity of LPL in vitro (Fig. 5F, G).

Mutagenesis of LPL cysteine460 to serine460 residue

To further confirm the role of the LPL cysteine residues in the redox regulation, we replaced a serine residue at highly glutathionylated site cysteine 460 using site-directed mutagenesis. Among the identified cysteine residues Cys-460, Cys-283, and Cys-206, Cys-460 had a maximum score; therefore we mutated this cysteine residue. Mutation was confirmed by DNA sequencing (Fig. S2D). Control and mutant LPL clones were overexpressed in HEK293T cells, because LPL is not expressed in these cells. Expression of wild-type and mutated LPL was similar as confirmed by Western blotting and flow cytometry (Fig. 6A, B). S-Glutathionylation was induced in HEK293T cells by treatment with diamide (0.5 mM) for 30 min. Wild-type LPL was S-glutathionylated by treatment with diamide while mutated LPL was resistant to diamide-induced glutathionylation (Fig. 6C). Since LPL expression induced invasive properties in HEK293T cells [33], an invasion assay was performed. Whereas expression of wild-type as well as mutated LPL conferred invasive properties in HEK293T cells, diamide pretreatment augmented invasion in wild-type transfectected
cells but not in mutated LPL overexpressed cells (Fig. 6D), which further confirmed the significance of Cys-460 glutathionylation in the regulation of LPL functions. Cell–cell contact formation was also reduced in wild-type and mutated LPL overexpressed cells as confirmed by their culture in suspension (data not shown).

Effect of NO-induced S-glutathionylation on the F-actin content

Since β-actin S-glutathionylation inhibits its polymerization and F-actin formation, we next investigated whether NO-induced oxidative stress also affects β-actin polymerization. Neutrophils treated with DETA-NO showed a significantly less amount of F-actin as compared to the control resting cells (Fig. 7A, B). Fluorescence of phalloidin–TRITC was also measured after methanol extraction, confirming the previous results (Fig. 7C). Changes in phalloidin–FITC/TRITC fluorescence were also consistent with a decline in NO-treated PMNs (Fig. 7D, E).

Effect of LPL silencing in human PMNs

To check the functional involvement of LPL in human PMNs a loss of function strategy by means of siRNA silencing was employed. We observed significant reduction of neutrophil migration in LPL-deficient PMNs (Fig. 8A, B). Consistently, when infected with E. coli, bacterial killing was markedly reduced in LPL-deficient PMNs as compared to control PMNs (Fig. 8C). To assess the role of LPL in NO-induced actin S-glutathionylation, actin glutathionylation and F-actin formation were assessed after NO treatment in control and LPL-silenced PMNs. NO-induced decrement in F-actin formation (Fig. 8D) and enhancement of actin glutathionylation (Fig. 8E) were not significantly affected in LPL-silenced PMNs, which suggest that NO-induced actin glutathionylation is independent of LPL. Phagocytosis was also not significantly affected in LPL-silenced PMNs (Fig. 8F). LPL expression in LPL siRNA-treated cells was significantly reduced (Fig. 8G).

Functional response of PMNs and LPL S-glutathionylation in diabetic subjects

Next we assessed LPL S-glutathionylation in pathological conditions. Since diabetes mellitus is associated with enhanced oxidative stress and impaired PMN responses, we hypothesized that defective PMN function in diabetes might be associated with enhanced S-glutathionylation of LPL in diabetic subjects. To test this hypothesis, LPL S-glutathionylation and various functional responses in PMNs from diabetic patients were compared with those of neutrophils from control subjects. Enhanced LPL S-glutathionylation was observed in PMNs of diabetic patients, which was reduced after treatment with DTT (Fig. 9A). Basal ROS generation was also enhanced in diabetic patients’ neutrophils (Fig. 9B). Glutaredoxin is a thiol disulfide oxidoreductase, which catalyzes both the formation and the reduction of mixed disulfides between protein thiols. Grx-1 mRNA expression in neutrophils was also significantly reduced in PMNs from diabetic patients as compared to controls (Fig. 9C), this potentially favoring enhanced S-glutathionylation in diabetes. To validate these findings, migration of PMNs from diabetic patients was compared with that of control neutrophils and found to be significantly reduced in the diabetic patients (Fig. 9D). PMN treatment with DTT (1 mM for 10 min) partially reversed this inhibition. Bactericidal activity of neutrophils was also significantly reduced in PMNs from diabetic patients as compared to controls (Fig. 9E). Bacterial phagocytosis was also measured and marginally affected in diabetic patients (Fig. S2F).

Chemotaxis, bactericidal activity, and LPL glutathionylation in db/db mice

Enhanced LPL S-glutathionylation was also noted in the neutrophils obtained from db/db mice as compared to the wild-type mice (Fig. 10A). ROS generation was also enhanced in the PMNs of
db/db mice (Fig. 10B), while bacterial killing and migration in response to fMLP were significantly reduced (Fig. 10C, D, and E).

Discussion

Neutrophils are the most abundant and short-lived WBCs in human blood and constitute the first line of defense against invading pathogens [42]. These cells generate the highest amount of ROS and also play a vital role in inflammatory pathologies [43]. It is now well accepted that resting as well as activated human PMNs generate NO, though in lesser amounts as compared to mice PMNs [4]. Under infectious or inflammatory conditions expression of iNOS is further upregulated by LPS and/or inflammatory cytokines [44,45].

The migration of PMNs to the site of infection is crucial for the effective resolution of infection [46]. Successful chemotaxis requires not only increased motility but also sustained directionality. This study demonstrates that exogenous treatment with NO reduced fMLP-induced neutrophil chemotaxis independently of cGMP. Previously reported increased chemotaxis of PMNs in iNOS−/− mice [47] was also observed by us (Fig. 1E) and was further augmented by fMLP, thereby underscoring the regulatory role of NO in PMN migration. The concentration of NO during sepsis at inflammatory sites is believed to be in the micromolar range. DETA-NO at the concentrations employed in our study (100–300 μM) releases approximately 0.5–1.5 μM NO and hence likely mimicking pathophysiological levels [48]. Treatment with NO donor led to impaired chemotaxis, polarization, phagocytosis, and ROS generation following phagocytosis. Even though NO-mediated defective neutrophil migration has been reported in sepsis and other pathologies, the molecular mechanisms involved have not been investigated in depth [49,50]. Cell polarization, an initial step for directional migration, is partly accomplished through cell elongation. PDGF and Rac-1-driven fibroblast migration were reported as attenuated by NO due to reduced filopodia formation [39]. In agreement with this we also observed that NO affected PMN polarization. In the current study phagocytosis and bactericidal activity in NO-pretreated human PMNs were also attenuated (Fig. 2) but were prevented by DTT, suggesting the role of redox regulation in NO-mediated responses. PMNs migrate to the site of infection, phagocytose the invading pathogens, and subsequently kill them through oxidative and nonoxidative mechanisms [3,5]. All these crucial steps required for the resolution of the infection were found to be reduced to a variable extent in NO-pretreated PMNs. On the other hand intracellular iNOS is critical for the bacterial killing and NO production is enhanced during phagocytosis [4]. Enhanced ROS production under basal conditions in neutrophils and reduced bactericidal activity were observed in various pathological conditions [17]. Surprisingly we found that prior exposure to NO negatively regulated chemotaxis, phagocytosis, and bacterial killing due to increased oxidative stress and were prevented by DPI, VAS-2870, NAC, and DTT as well as in PMNs from NCF-1−/− mice. NO-induced free radical generation in PMNs is well established [40,51] and enhanced ROS leading to oxidative modifications of proteins has also been reported in several other cell types [52,53]. Peroxynitrite generated from NO and O2 cause oxidation and nitration of susceptible molecules [54,55]. S-Glutathionylation is a functionally relevant redox-sensitive reversible protein posttranslational modification [56] and has also been
proposed as a signaling mechanism under both physiological and pathological conditions [56]. Several proteins are S-nitrosylated during exposure to NO [57,58]; however, such modifications are labile and their stability is often affected by the presence of thiols, predominantly by GSH (glutathione), which finally leads to protein S-glutathionylation [26,59]. The current study demonstrated that in human PMNs, NO-induced RONS generation led to enhanced protein S-glutathionylation. Involvement of NADPH oxidase in S-glutathionylation was confirmed by reduced signal of the glutathionylation in PMNs from NCF1−/− mice and by the NOX inhibitors VAS-2870 (Fig. 3E, S1C). A large number of cytoskeleton proteins are in fact modified by S-glutathionylation, which includes β-actin, myosin, profilin, vimentin, spectrin, and tubulin [60]. We have now identified and established S-thiolation of two neutrophil proteins β-actin and L-plastin, which are crucial for cytoskeleton arrangements. In the case of LPL, we believe this is the first description of S-glutathionylation.

LPL, a member of the fimbrin family of actin-binding proteins, is a leukocyte-specific β-actin binding protein and is characterized by two actin-binding domains and a headpiece region containing two EF hand-type calcium-binding domains [61]. Plastin-mediated bundling of actin filaments enables the maintenance of structures such as lamellipodia and filopodia [34]. LPL controls polarization and migration of chemokine-stimulated T lymphocytes [62]. Modulation of PMN functions by LPL was confirmed by knocking down LPL. LPL silencing reduced PMN migration and bacterial killing in human PMNs, indicating the significance of LPL in PMN functions (Fig. 8). LPL undergoes phosphorylation at the serine residue in the headpiece region [61,62], which regulates LPL function in leukocytes. LPL phosphorylation was, however, not affected in NO-pretreated cells as observed using the anti-pSer LPL antibody (Fig. S1F).

The current study thus demonstrates regulation of LPL by S-glutathionylation leading to inhibiting the interaction with actin. Interestingly, our observation is supported by the previously reported inhibition of PMN function by LPL alkylation with bromophenacyl bromide [63]. Even though in the current study, the role of S-glutathionylation in the regulation of LPL activity has been confirmed by several approaches, we cannot completely rule out the presence of S-nitrosylation.

Experiments using pure and nonphosphorylated LPL protein following S-glutathionylation in vitro did not yield pronounced effects on interaction and β-actin bundling. However, LPL S-glutathionylation marginally reduced the interaction as well as the bundling of β-actin (Fig. S5F, G). LPL contains a total of 10 cysteine residues, among which three were identified by the IAM switch technique to be susceptible to oxidative posttranslational modifications [64]. These residues are Cys-206, Cys-283, and Cys-460, present in the actin-binding domain of LPL. Cys residues surrounded by basic amino acids are more susceptible targets for oxidation [21]. Although the crystal structure of human LPL protein is not fully resolved, from the molecular modeling simulations using a Swiss model via ExPASy web server (http://swissmodel.expasy.org/), we found that Cys-206, Cys-283, and Cys-460 were present at the surface of the β-actin binding domains and were surrounded by basic amino acids (histidine, lysine, and arginine). Mutation of the LPL Cys-460 to serine had no effect on LPL functions in HEK293T cells but was resistant to diamide-induced LPL glutathionylation and inhibition of cell invasion, thus highlighting the importance of LPL Cys-460 in the redox regulation of LPL functions.

Chen et al. showed that PMNs deficient in L-plastin failed to kill Staphylococcus aureus both in vitro and in vivo, and that this defect

Fig. 8. Effect of LPL silencing on PMN chemotaxis and bactericidal activity. (A) Representative images of fMLP (100 nM)-induced neutrophil migration in control and LPL-silenced human PMNs. Scale bars: 100 μm in the images. (B) The same data are represented as bar diagram. (C) Silencing of LPL in human PMNs leads to reduced bactericidal activity. (D) Phalloidin–FITC fluorescence in control and LPL-silenced human PMNs after NO treatment. (E) NO induced actin S-glutathionylation in control and LPL-silenced PMNs. (F) LPL expression after silencing with LPL siRNA. Data represent mean ± SEM of three to five independent experiments. *P < 0.05 (Student’s t test). ***P < 0.001 vs scrambled siRNA-transfected cells.
arises from failure to mount an efficient adhesion-dependent respiratory burst [35]. In the current study PMNs treated with DETA-NO for 15 and 30 min showed reduced actin interaction and impaired bactericidal activity as compared to the control untreated PMNs, thus corroborating the importance of LPL-actin interactions. Phagocytosis-induced respiratory burst was also reduced in PMNs pretreated with NO (Fig. 3C). Yan et al. had shown reduced bactericidal activity and oxidative burst in Gsr (glutathione reductase)-deficient mice [65]. Gsr is involved in the reduction of glutathione disulfide to glutathione.

S-Glutathionylation has been encountered in association with a wide range of diseases, including cancer, lung disease, cardiovascular diseases, neurodegenerative diseases, and diabetes [56]. Diabetes mellitus is a disease characterized by increased superoxide generation and enhanced oxidative stress, and neutrophil dysfunction is one of the major causes of diabetic complications. Diabetic condition is also associated with delayed wound healing and susceptibility to infections. Park et al. observed more susceptibility to Staphylococcus aureus infection in db/db mice due to defects in innate immunity [66]. Early reports described that glutathione metabolism is impaired in erythrocytes from patients with diabetes mellitus [67] and reduced GSH as well as augmented GSSG levels were also found [68,69]. Our study also corroborated the higher oxidative stress in diabetic patients and db/db mice as evidenced by enhanced DCF fluorescence in resting neutrophils. Reduced chemotaxis and enhanced LPL S-glutathionylation were also observed in the PMNs from diabetic subjects. A decrease in neutrophil migration in db/db mice was previously reported by Spiller et al. [70] and is in agreement with our observation. This effect was attributed to the reduced expression of CXCR2 and high levels of AGP (α1-acid glycoprotein) [70,71]. The fact that DTT in our study partially reverted the inhibition of migration suggests a potential role for S-glutathionylation in this phenomenon. Glutaredoxins are thiol–disulphide oxidoreductases that reduce protein disulphides and a number of studies indicate that human Grx1 catalyze the reduction of mixed disulphides [72]. The reduced level of Grx-1 in the PMNs from diabetic patients further supports the presence of enhanced S-glutathionylation in diabetes (Fig. 9C). Increased levels of LPL S-glutathionylation are consistent with this scenario and emphasize the importance of LPL in the regulation of PMN functions.

Altogether our findings support that enhanced LPL S-glutathionylation and associated changes in the function of PMNs in db/db mice and diabetic patients represent an important molecular and regulatory mechanism to control PMN functions and may contribute to explain defective PMN functions in various pathological conditions.
Fig. 10. L-plastin S-glutathionylation in control and db/db mice. (A) LPL S-glutathionylation in control and db/db mice. Cells (5 × 10⁶ cells/ml) were lysed with NEM (100 mM) containing lysis buffer. LPL was resolved by 8% SDS-PAGE and blotted with anti-GSH antibody. The blot is representative from three different experiments. (B) ROS generation was measured by DCF-DA (10 μM). (C) Comparison of bactericidal activity in neutrophils from control and db/db mice. (D) fMLP (100 nM)-induced neutrophil migration was assessed in control and db/db mice. Scale bars: 100 μm in the images. (E) The same data are represented as bar diagram. Data represent mean ± SEM of three to five independent experiments. *P < 0.05 (Student’s t test), **P < 0.01 vs control neutrophils, $P < 0.05 vs db/db mice neutrophils.

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Appendix A. Supporting information

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References

contain mitochondrial as well as nuclear DNA and exhibit inflammatory potential. Cytometry A 81:238–247; 2011.


