



## Research paper

## Pleiotropic effects of 4-hydroxynonenal on oxidative burst and phagocytosis in neutrophils



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## ABSTRACT

Metabolic control of cellular function is significant in the context of inflammation-induced metabolic dysregulation in immune cells. Generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide are one of the critical events that modulate the immune response in neutrophils. When activated, neutrophil NADPH oxidases consume large quantities of oxygen to rapidly generate ROS, a process that is referred to as the oxidative burst. These ROS are required for the efficient removal of phagocytized cellular debris and pathogens. In chronic inflammatory diseases, neutrophils are exposed to increased levels of oxidants and pro-inflammatory cytokines that can further prime oxidative burst responses and generate lipid oxidation products such as 4-hydroxynonenal (4-HNE). In this study we hypothesized that since 4-HNE can target glycolysis then this could modify the oxidative burst. To address this the oxidative burst was determined in freshly isolated healthy subject neutrophils using 13-phorbol myristate acetate (PMA) and the extracellular flux analyzer. Neutrophils pretreated with 4-HNE exhibited a significant decrease in the oxidative burst response and phagocytosis. Mass spectrometric analysis of alkyne-HNE treated neutrophils followed by click chemistry detected modification of a number of cytoskeletal, metabolic, redox and signaling proteins that are critical for the NADPH oxidase mediated oxidative burst. These modifications were confirmed using a candidate immunoblot approach for critical proteins of the active NADPH oxidase enzyme complex (Nox2 gp91phox subunit and Rac1 of the NADPH oxidase) and glyceraldehyde phosphate dehydrogenase, a critical enzyme in the metabolic regulation of oxidative burst. Taken together, these data suggest that 4-HNE-induces a pleiotropic mechanism to inhibit neutrophil function. These mechanisms may contribute to the immune dysregulation associated with chronic pathological conditions where 4-HNE is generated.

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## 1. Introduction

Phagocytosis is an innate mechanism for the clearance of pathogens and apoptotic and senescent cells by the immune system [1,2]. Neutrophils are one of the major cell types actively involved

in this process to maintain tissue homeostasis and to defend against invading pathogens [2,3]. Environmental, metabolic, and genetic factors have all been shown to impact the efficiency and the dynamics of phagocytosis in humans and in animal models [4]. The inability of neutrophils to execute phagocytosis can lead to the development of severe and chronic pathological conditions and dysregulation of the immune response. In particular, this inability has been observed in pathological conditions such as chronic granulomatous disease, severe glucose-6-phosphate dehydrogenase (G6PD) deficiency and glycogen storage disease type 1b [5,6]. Inadequate clearance of cellular debris and pathogens through phagocytosis also leads to enhanced recruitment of phagocytic cells to the inflammatory foci and amplification of the inflammatory response and oxidative stress [6,7].

Phagocytosis is a complex process that involves pattern

**Abbreviations:** HNE, 4-hydroxynonenal; ROS, reactive oxygen species; PMA, 3-phorbol myristate acetate; XF assay, extracellular flux assay; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear granulocytes; *S. aureus*, *Staphylococcus aureus*; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH Oxidase

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recognition, cellular signaling, and cytoskeletal reorganization which is critical for phagosome formation, engulfment and degranulation [4,8]. The high energetic requirement of phagocytosis is satisfied to a great extent by glycolysis [9]. In addition, glucose metabolism through the pentose phosphate pathway generates NADPH, a required reducing substrate for the oxidative burst in activated neutrophils [10,11]. We have previously shown that the neutrophil oxidative burst can significantly inhibit the oxidative and glycolytic metabolism of lymphocytes, preventing them from undergoing clonal expansion and producing cytokines [10]. These findings highlight the need for active metabolic machinery to execute neutrophil phagocytosis and the oxidative burst. Clinically, inefficient phagocytosis can increase the incidence of bacterial and fungal infections, skin abscess, inflammatory bowel disease, oral ulcers and organ damage [2,5].

NADPH oxidase plays a critical role in controlling the process of neutrophil activation and phagocytosis [12]. Generation of large amounts of reactive oxygen species (ROS; superoxide and hydrogen peroxide) by NADPH oxidase through the oxidative (respiratory) burst is a key event in the process of phagocytosis and recycling of macromolecules [4]. A sustained oxidative burst is associated with chronic pathological conditions and generates reactive metabolites of macromolecules in tissues and vascular compartments [13]. This can result in modification of cellular proteins, lipids and DNA and can cause cellular and tissue dysfunction. Membrane lipids are one of the major targets of oxidative damage and the generation of secondary reactive molecules [14]. Non-enzymatic lipid peroxidation induced by oxidants such as superoxide, hydrogen peroxide, hypochlorous acid and peroxynitrite generates reactive lipid species such as 4-hydroxynonenal (4-HNE) from polyunsaturated fatty acids (PUFA) of membrane lipid bilayers [15–17]. These reactive species are capable of modifying cellular enzymes and cytoskeletal proteins and amplifying the oxidative reactions in cells and tissues [15,18]. Extensive modification of cellular proteins and DNA can impact the cellular homeostasis by altering cell signaling, metabolic pathway dynamics, metabolite flux, and ATP synthesis which in turn can affect critical neutrophil functions such as cellular motility, phagocytosis and microbial killing [19,20].

Experimental models of inflammatory diseases demonstrate that 4-HNE is produced by activated neutrophils which can serve as a potent chemoattractant for further leukocyte recruitment to the inflammatory foci [16,17,21]. 4-HNE is a reactive and diffusible aldehyde which can form adducts with several nucleophilic amino acid residues such as arginine, lysine and cysteine [19,22]. It is then possible that 4-HNE treatment modifies several neutrophil proteins that are involved in the oxidative burst response, glycolysis and phagocytosis. Acute and chronic inflammatory responses such as those associated with inflammatory disease are suggested to induce generation of 4-HNE in tissue and vascular compartments [23]. Rapid recruitment of neutrophils to the site of injury and increased oxidative stress support this hypothesis. Accumulation of 4-HNE and 4-HNE-modified proteins has been detected in aging and in various diseases such as cancer, atherosclerosis, neurodegenerative disorders, metabolic syndrome, diabetes and autoimmune diseases [20,24]. Recent studies have suggested that 4-HNE can range from 0.05 to 0.15  $\mu\text{M}$  in healthy human blood and serum [25–27]. Under pathological conditions, the tissue and plasma membrane concentration of 4-HNE increases significantly and can reach  $> 100 \mu\text{M}$  in locations close to the core of the lipid peroxidation sites [26,28].

In chronic inflammatory conditions, the phagocytic cells exist in a highly oxidative and reactive environment. Interestingly, several lines of evidence suggest that exposure of neutrophils to oxidative stress can modify cellular function [29]. Previous studies have shown that 4-HNE can modify neutrophil and macrophage

oxidative burst responses in human and mouse samples [29,30]. Compromised oxidative burst responses to the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (fMLP) and PMA by 4-HNE treated macrophages have also been shown in vitro studies, suggesting oxidative damage to these cell types [31]. However, the mechanisms of these responses are not well established. Although neutrophils are designed to survive the reactivity of the inflammatory foci, prolonged inflammation combined with inadequate antioxidant defenses can make these cells susceptible to oxidative modifications by reactive lipids. It is proposed that oxidative modification of the cellular proteins alter the critical processes involved in oxidative burst and phagocytosis. Exposure of 4-HNE to isolated cells can modify key glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase [32]. The critical subunits of NADPH oxidase enzyme and Rac1 are also found to be modified by reactive lipids and electrophiles in inflammatory conditions similar to that observed in pathological situations [33]. These processes signify a possible regulatory role for 4-HNE and related bioactive reactive lipids in controlling NADPH oxidase activity during conditions of increased oxidative stress. Due to the high energy and metabolic demand of phagocytosis, it is proposed that the metabolic pathways of glucose utilization can also serve as check points of oxidative burst and phagocytosis. Since several of the metabolic enzymes can be regulated by redox mechanisms, understanding the role of secondary metabolites of oxidative stress on neutrophil function has the potential to uncover viable targets of intervention in chronic inflammatory diseases.

In this study we investigated the impact of 4-HNE on phagocytosis in human neutrophils and how oxidative burst influences phagocytosis. In addition, we identified 4-HNE protein targets using mass spectrometry techniques. The findings from these studies present potential mechanisms of 4-HNE induced metabolic alterations in neutrophils that may lead to impairment of phagocytosis in pathological conditions.

## 2. Methods

### 2.1. Neutrophil isolation from human blood

All study protocols for collection and handling of human samples were reviewed and approved by the Institutional Review Board, University of Alabama at Birmingham. Blood samples (2 tubes, 8.5 ml/tube) were collected from 12 different healthy volunteers (25–55 years of age) in vacutainers (BD Biosciences) containing 1.5 ml ACD solution (trisodium citrate, 22.0 g/l; citric acid, 8.0 g/l; and dextrose 24.5 g/l) and processed within 15 min of collection. Neutrophils were isolated from freshly drawn blood from healthy donors as described [34,35]. Briefly, the blood tubes were centrifuged at 500g for 10 min at room temperature to collect the buffy coat and separate the platelet-rich plasma. The buffy coat containing the peripheral blood mononuclear cells (PBMC) and the polymorphonuclear granulocytes (PMN) was diluted 1:4 using RPMI cell culture media without serum and antibiotics and carefully applied onto the Histopaque density gradient (Histopaque 1.077/1.119, 3 ml each) in a 15 ml conical Falcon centrifuge tube and centrifuged for 20 min at 700g at room temperature. Following centrifugation, the PBMC and PMN layers were clearly visible and separated. The PBMC layer formed on top of the Histopaque 1.077 layer and the PMN layer formed at the interface between Histopaque 1.077 and 1.119 were collected separately, diluted 1:4 using RPMI media and pelleted by centrifuging at  $700 \times g$  for 10 min at room temperature. CD15<sup>+</sup> neutrophils were purified from the PMN fraction using the MACS protocol (Milteneyi Biotec). The PMN pellet resuspended in 80  $\mu\text{l}$  of RPMI

containing 0.05% bovine serum albumin (BSA) was incubated with magnetic bead labeled anti-CD15 (for neutrophils) antibodies for 15 min at 4 °C. The antibody-labeled cells were collected by positive selection by passing through the columns placed in the magnetic field. The cells adhered to the magnetic field were eluted, resuspended in the assay medium (XF-DMEM without serum and antibiotics) and counted.

## 2.2. Determination of oxidative burst in human neutrophils

To determine the oxidative burst in neutrophils, were seeded on Cell-Tak coated XF assay plates (75,000 cells/well) and attached by low speed centrifugation as previously described [34]. Briefly, the seeded plates were centrifuged at 201g on a swinging bucket rotor for 1 s without brake. The centrifugation was repeated at 296g for 1 s after turning the plates 180°. The oxidative burst was assessed using the XF96 analyzer from Seahorse Biosciences which measures real-time O<sub>2</sub> consumption in cells non-invasively by measuring the oxygen consumption rate following PMA (100 ng/ml) activation. The attached cells were pretreated with 4-HNE for 2 h prior to PMA activation. The area under the non-mitochondrial oxygen consumption rate curve was calculated after normalizing to the number of cells per well. Glycolytic changes following PMA treatment were also calculated by analyzing the area under the curve of the extracellular acidification rate (ECAR).

## 2.3. Assessment of phagocytosis by human neutrophils

Freshly isolated neutrophils were resuspended in XF-DMEM medium (pH 7.4, 37 °C) and seeded at 75,000 cells per well (in 75 µl) on Cell-Tak-coated 96-well microtiter assay plates (Corning). The seeded neutrophils were attached to the plates by centrifugation as described above. The neutrophils were treated with 4-hydroxynoneneal (4-HNE) at 30 µM concentration at 37 °C for 1 h. The ability of 4-HNE-treated neutrophils to phagocytose bacteria was performed by modified phagocytosis assay using FITC-labeled opsonized *Staphylococcus aureus* (*S. aureus*, S285, Life technologies, NY, USA). The labeled bacteria ( $2 \times 10^8$  bacteria/ml) were added to the cells seeded in the assay plate and incubated at 37 °C for 15 min [36,37]. Phagocytosis was stopped by washing the cells with ice-cold PBS. Fluorescence of the internalized bacteria was determined by quenching the extracellular fluorescence using 0.04% trypan blue (Sigma-Aldrich, USA) and then normalized to the total fluorescence intensity per well. Increased intracellular fluorescence corresponds to increased phagocytosis.

## 2.4. Determination of 4-HNE-protein adducts in human neutrophils

Ethanol stocks of 4-HNE or alkyne-HNE (aHNE) were diluted in XF media and added to cells within 5 min of making the lipid dilution in media. Neutrophils ( $2 \times 10^6$  cells per sample) were treated for 1 h at room temperature by vehicle (ethanol), 30 µM 4-HNE or 50 µM aHNE in a total volume of 600 µl of XF media. The concentrations of 4-HNE and aHNE for the determination of HNE-protein adducts were chosen based so that the level of inhibition of the PMA-induced oxidative burst was approximately the same. After the reaction, cells were pelleted and washed once with 200 µl PBS. Pelleted cells were then lysed on ice in 10 mM Tris (pH 7.4) with 1% Triton X-100, containing protease inhibitor and 5 mM PMSF. Lysates were cleared by centrifugation at 16,860g for 10 min and supernatants were collected. Protein concentrations were measured by Bradford assay (Bio-Rad) per manufacturer's protocol. Lysates were then either processed for SDS-PAGE western analysis or click chemistry.

## 2.5. Determination of cellular targets of 4-HNE in human neutrophils

Pre-cleared neutrophil cell lysates were treated with 10 mM sodium borohydride and incubated for 30 min. The Cu(I)-catalyzed click reaction [38] was initiated by adding 2 mM ascorbate (protected from light), 1 mM cupric sulfate, and 0.5 mM Azide-PEG3-biotin to each sample and incubated on a shaker for 1.5 h at room temperature. Samples were either stored at -20 °C or immediately processed. Protein was precipitated using 2 times the volume of ice cold methanol for 30 min on ice and then centrifuged at 20,817g for 10 min. The pellet was washed once with 100 µl of ice cold methanol and centrifuged at 20,000g for 5 min. Methanol was removed and the pellets were re-suspended in 50 µl RIPA (50 mM Tris pH 7.4, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 150 mM sodium chloride, 1 mM EDTA, and 1% v/v NP-40) buffer containing PIC and 5 mM PMSF. Protein concentrations were estimated assuming no protein loss occurred during the click and precipitation procedure. Clicked lysates were either loaded onto SDS-PAGE gel for processing or biotin affinity precipitation.

## 2.6. Biotin affinity precipitation using neutravidin resin

Biotin affinity precipitation was performed using Neutravidin Plus UltraLink Resin (ThermoScientific, USA). Micro Bio-Spin columns (Bio-Rad, #732-6204) were loaded with 20 µl of 50% neutravidin slurry using a large orifice pipet tip. The resin was equilibrated to RIPA buffer by three 100 µl washes. Thirty micrograms of "clicked" lysates were loaded onto the equilibrated resin. The volume was brought up to 100 µl with RIPA lysis buffer and lysates were incubated on resin for 1 h at room temperature on a shaker. After incubation, the flow through was collected into a fresh tube for further analysis. The resin was washed six times with 100 µl volumes of RIPA lysis buffer and then resuspended in 300 µl of RIPA buffer in a fresh 0.6 ml tube using a 1 ml pipet tip. The resin was allowed to settle for 10 min and the supernatant was removed, leaving a meniscus of RIPA solution over the resin. The bound proteins were eluted using 15 µl of 2X Laemmli sample buffer containing β-mercaptoethanol, by vortexing and heating at 80 °C for 10 min. Within 5 min of heating, the supernatant was collected into a clean 0.6 ml tube for analysis by SDS-PAGE or stored at -20 °C for later analysis.

## 2.7. Determination of protein targets of aHNE in human neutrophils by tandem mass spectrometry

Affinity enriched fractions were separated by gel electrophoresis and stained with Coomassie Blue. The stained bands were excised and the staining was removed by an overnight wash in 50% 100 mM ammonium bicarbonate/50% acetonitrile. Disulfide bonds were reduced using dithiothreitol (25 mM) at 50 °C for 30 min followed by alkylation of free thiols groups with iodoacetamide (55 mM) for 30 min in the dark. After removal of excess alkylating agent, the gel pieces were evaporated to dryness prior to reswelling in 100 mM ammonium bicarbonate buffer and overnight digestion using mass spectrometry grade trypsin (12.5 ng/ml). Tryptic peptides were extracted using solution of 1% formic acid in water and acetonitrile (50/50) and then evaporated to dryness in a Speedvac. Samples were resuspended in 30 µl of ddH<sub>2</sub>O with 0.1% formic acid for mass spectrometry evaluation.

An aliquot (5 µl) of each digest was loaded onto a Nano cHiPLC 200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å reverse-phase trap cartridge (Eksigent, Dublin, CA) at 2 µl/min using an Eksigent autosampler. After washing the cartridge for 4 min with 0.1% formic acid in double-distilled water (ddH<sub>2</sub>O), the bound peptides were flushed onto a Nano cHiPLC column (200 µm ID × 15 cm ChromXP C18-CL 3 µm 120 Å, Eksigent, Dublin, CA) with a 45 min linear

(5–50%) acetonitrile gradient in 0.1% formic acid at 1000 nl/min using an Eksigent Nano1D+LC (Dublin, CA). The column was washed with 90% acetonitrile–0.1% formic acid for 10 min and then re-equilibrated with 5% acetonitrile–0.1% formic acid for 10 min. The SCIEX 5600 Triple-ToF mass spectrometer (Sciex, Toronto, Canada) was used to analyze the protein digest. The IonSpray voltage was 2300 V and the declustering potential was 80 V. Ion spray and curtain gases were set at 10 psi and 25 psi, respectively. The interface heater temperature was 120 °C.

Eluted peptides were subjected to a time-of-flight survey scan from  $m/z$  400–1250 to determine the top twenty most intense ions for MS/MS analysis. Product ion time-of-flight scans at 50 ms were carried out to obtain the tandem mass spectra of the selected parent ions over the range from  $m/z$  400–1500. Spectra are centroided and de-isotoped by Analyst software, version 1.6 TF (Sciex). A  $\beta$ -galactosidase trypsin digest was used to establish and confirm the mass accuracy of the mass spectrometer.

### 2.8. Protein pilot 4.5 search queries

The tandem mass spectrometry data were processed to provide protein identifications using an in-house Protein Pilot 4.5 search engine (Sciex) using the Homo sapiens Spot protein database and using a trypsin digestion parameter. Proteins of significance were accepted on the criteria of having at least two peptides detected with a confidence score of 95% or greater.

## 3. Results

### 3.1. 4-HNE inhibits neutrophil phagocytosis

Freshly isolated human neutrophils from healthy donors were used to determine the impact of 4-HNE on phagocytosis. As shown in Fig. 1A, pretreatment with 4-HNE (0–30  $\mu$ M) for 2 h inhibited the ability of neutrophils to phagocytize fluorescently labeled heat inactivated bacteria (*S. aureus*) in a concentration dependent manner.

Previous studies have shown that the oxidative burst plays a critical role in the bactericidal process of phagocytosis [14,39]. One of the mechanisms that control the oxidative burst is to regulate

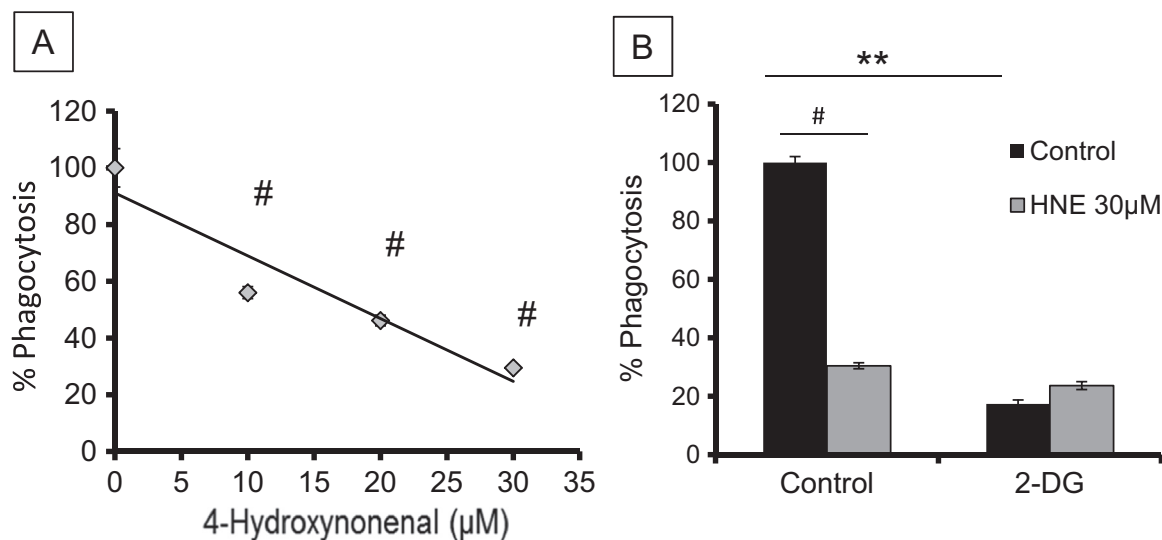
the supply of NADPH, the substrate for NADPH oxidase activity. Inhibiting hexokinase with 2-deoxyglucose treatment prevented the ability of neutrophils to undergo phagocytosis in control cells to a similar extent as 30  $\mu$ M 4-HNE (Fig. 1B). These data support a critical role for glucose in regulating neutrophil phagocytosis. 4-HNE did not have any significant additive effect on 2-DG mediated inhibition of phagocytosis consistent with them acting at similar targets (Fig. 1B).

### 3.2. 4-HNE inhibits oxidative burst and glycolysis in neutrophils

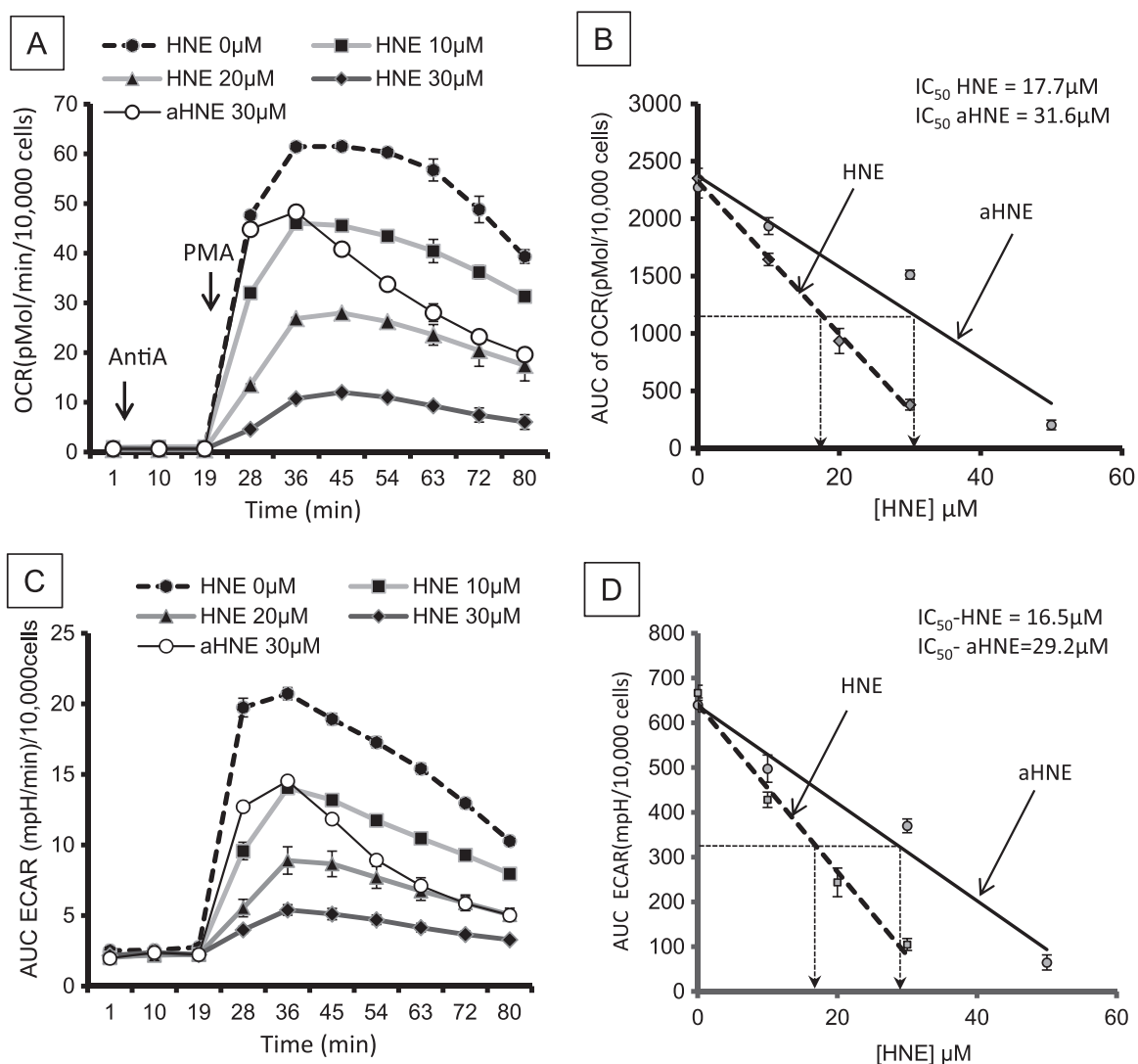
The ability of neutrophils to undergo oxidative burst and the amount of ROS generated can be determined using the extracellular flux analyzer by measuring PMA-stimulated oxygen consumption rate under conditions where mitochondrial respiration is inhibited using antimycin A [34,40]. To assess the effects of 4-HNE on neutrophil oxidative burst and glycolysis the effects of 4-HNE were compared with alkyne HNE (aHNE), which will be used in the study as a probe to detect HNE-protein adducts. As shown in Fig. 2A, the addition of PMA causes a rapid increase in OCR that declines slowly over time in control cells. Pretreatment (2 h) of neutrophils with 4-HNE or aHNE concentration dependently inhibited the PMA-induced oxidative burst response with an  $IC_{50}$  of  $17.7 \pm 2.9 \mu$ M for 4-HNE and  $31.6 \pm 3.3 \mu$ M for aHNE (Fig. 2A and B). aHNE is approximately 1.8 times less potent than 4-HNE in inhibiting the oxidative burst.

We next assessed whether a 2 h pre-treatment of 4-HNE or aHNE inhibits PMA-stimulated glycolysis in neutrophils under identical conditions described above. As shown in Fig. 2C and D, 4-HNE (0–30  $\mu$ M) or aHNE (0–50  $\mu$ M) pretreatment caused a concentration-dependent inhibition of glycolysis with an  $IC_{50}$  of  $22.4 \pm 1.4 \mu$ M for 4-HNE and  $29.2 \pm 1.84$  (Fig. 2D) which are not significantly different than the concentrations which inhibit the oxidative burst.

The inhibitory effects of 4-HNE on oxidative burst and glycolysis could potentially be dependent on the extent of neutrophil activation. In the next set of experiments, we determined the impact of 4-HNE on the concentration-dependent effects of PMA on both the oxidative burst and glycolysis. Neutrophils were pre-treated with 4-HNE (30  $\mu$ M) for 2 h before measuring the oxidative burst and glycolysis with different doses of PMA (0–100 ng/ml). As



**Fig. 1.** 4-HNE inhibits phagocytosis in human neutrophils. (A) Pretreatment of neutrophils with 4-HNE for 2 h inhibits the ability of neutrophils to phagocytize fluorescently labeled *S. aureus* in a dose-dependent manner. (B) The effect of inhibition of glucose metabolism for 1 h prior to the assay using 2-deoxyglucose (2-DG) on phagocytosis. Percent phagocytosis relative to the total bacterial fluorescence in the control groups not treated with 4-HNE was calculated. Mean  $\pm$  sem from 3 independent donors. #,  $p \leq 0.0001$ , \*\*,  $p \leq 0.001$  compared to the untreated control neutrophils.



**Fig. 2.** 4-HNE inhibits oxidative burst and glycolysis in human neutrophils. Representative profiles of PMA-stimulated (A) oxygen consumption rate and (C) extracellular acidification rate of neutrophils pre-treated with 4-HNE at the indicated concentration for 2 h using the extracellular flux analyzer are shown. The area under the curve of OCR (B) and ECAR (D) following PMA stimulation in 4-HNE or alkyne HNE (aHNE) treated neutrophils. The  $IC_{50}$  of 4-HNE and aHNE for OCR and ECAR was calculated from 3 independent samples (shown in Figs. 2B & 2D). Mean  $\pm$  sem from  $n=5-6$  replicates with an individual representative donor. #,  $p \leq 0.05$  compared to the respective untreated control.

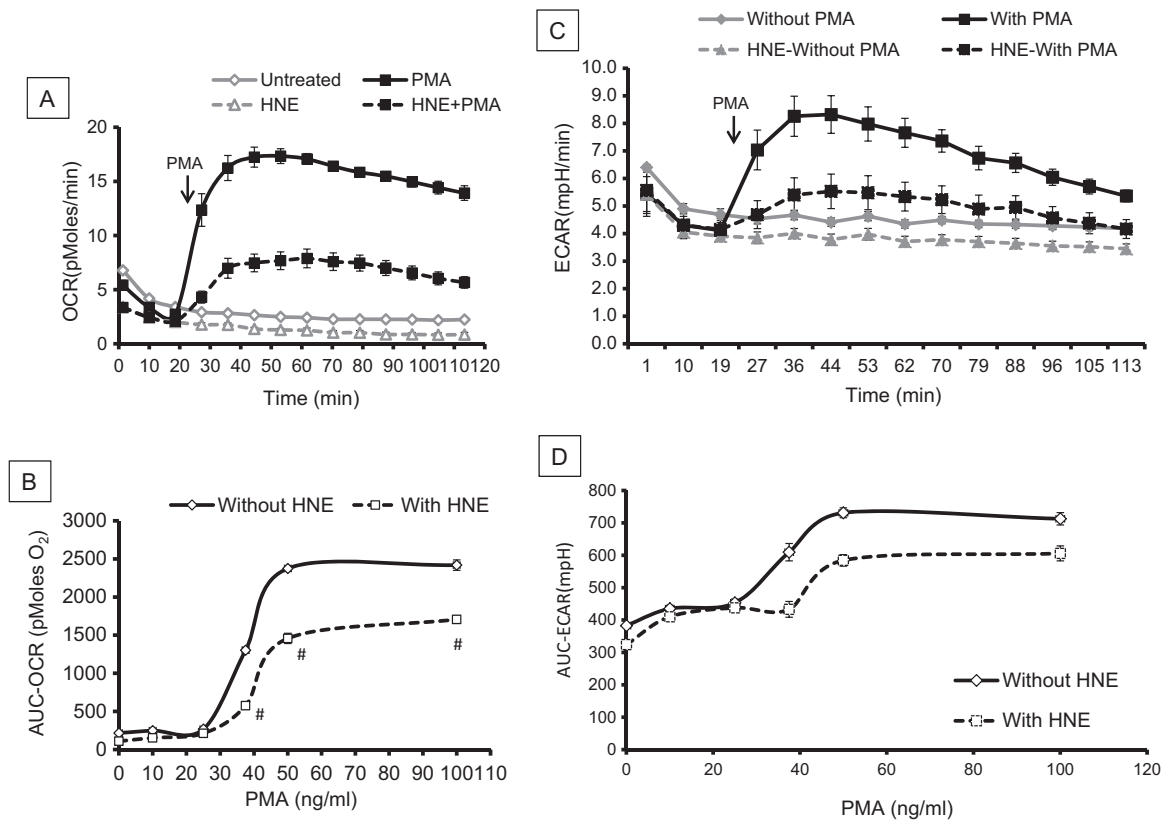
shown in Fig. 3A and B, 4-HNE treatment inhibited both the maximal OCR and the rate at which the maximal OCR was achieved. Pretreatment of 4-HNE also inhibited maximal glycolysis (Fig. 3C and D).

### 3.3. aHNE formation of protein adducts in neutrophils

4-HNE reacts with cellular protein targets to form covalent adduct which can result in alterations in protein function [15–17,22,38]. 4-HNE-modified proteins were identified in the next series of experiments using alkyne HNE (aHNE) and the azido Click-Chemistry technique [22]. As shown in Fig. 2, aHNE is less potent compared to the unmodified 4-HNE in inhibiting the oxidative burst and for this reason the concentration of aHNE was increased to 50  $\mu$ M using the same conditions for the treatment with 4-HNE (Fig. 2). The total proteins after reaction were collected and separated on SDS-PAGE gels. No significant differences in the total protein pattern on the blot were evident using Ponceau S staining for total protein (Fig. 4A). Affinity detection of the biotin tagged proteins showed no positive signal in the control or 4-HNE sample but multiple bands in the samples treated with aHNE

indicating that the tagging protocol was effective (Fig. 4B). The functional data suggested that 4-HNE was capable of targeting both proteins in glycolysis and/or the NADPH oxidase or associated signaling pathways. To test this, Biotin tagged proteins were affinity purified and blotted against NOX2 (gp91phox), GAPDH or Rac1. As shown in Fig. 4C, the total cell lysate had detectable levels of NOX2, GAPDH and Rac 1 which were not altered by aHNE treatment. After biotin pull down the levels of all three proteins were substantially enhanced compared to the total cell lysate in the aHNE treated sample and compared to the control (Fig. 4D–F). Western blots of NOX2 (Fig. 4G) and GAPDH (Fig. 4H) show that there is no difference in the expression levels of NOX2 or GAPDH in neutrophils between each control donor lysate samples.

It is clear from Fig. 4B that a significant number of proteins have been modified by aHNE under these conditions. To determine members of the aHNE proteome in neutrophils the affinity enriched samples shown in Fig. 4C were used and subjected to mass spectrometry. The analysis of the control neutrophil cell lysate subjected to the affinity enrichment protocol revealed 45 proteins which were assigned as false positives. After subtraction of the false positives a total of 93 proteins, represented by at least two



**Fig. 3.** 4-HNE treatment inhibits the maximal oxidative burst response and shifts the neutrophil activation curve to the right. Neutrophils were pretreated with 4-HNE and activated with different doses of PMA as shown. Figs. A and C show the representative profiles of PMA (37.5 ng/ml)-induced oxidative burst and glycolysis  $\pm$  4-HNE (30  $\mu$ M) treatment respectively. Figures B and D, demonstrate the comparison of the dose dependent PMA activation and glycolysis respectively of neutrophils  $\pm$  4-HNE treatment. Mean  $\pm$  sem from  $n=5-6$  replicates. #,  $p \leq 0.005$  compared to neutrophils not treated with 4-HNE.

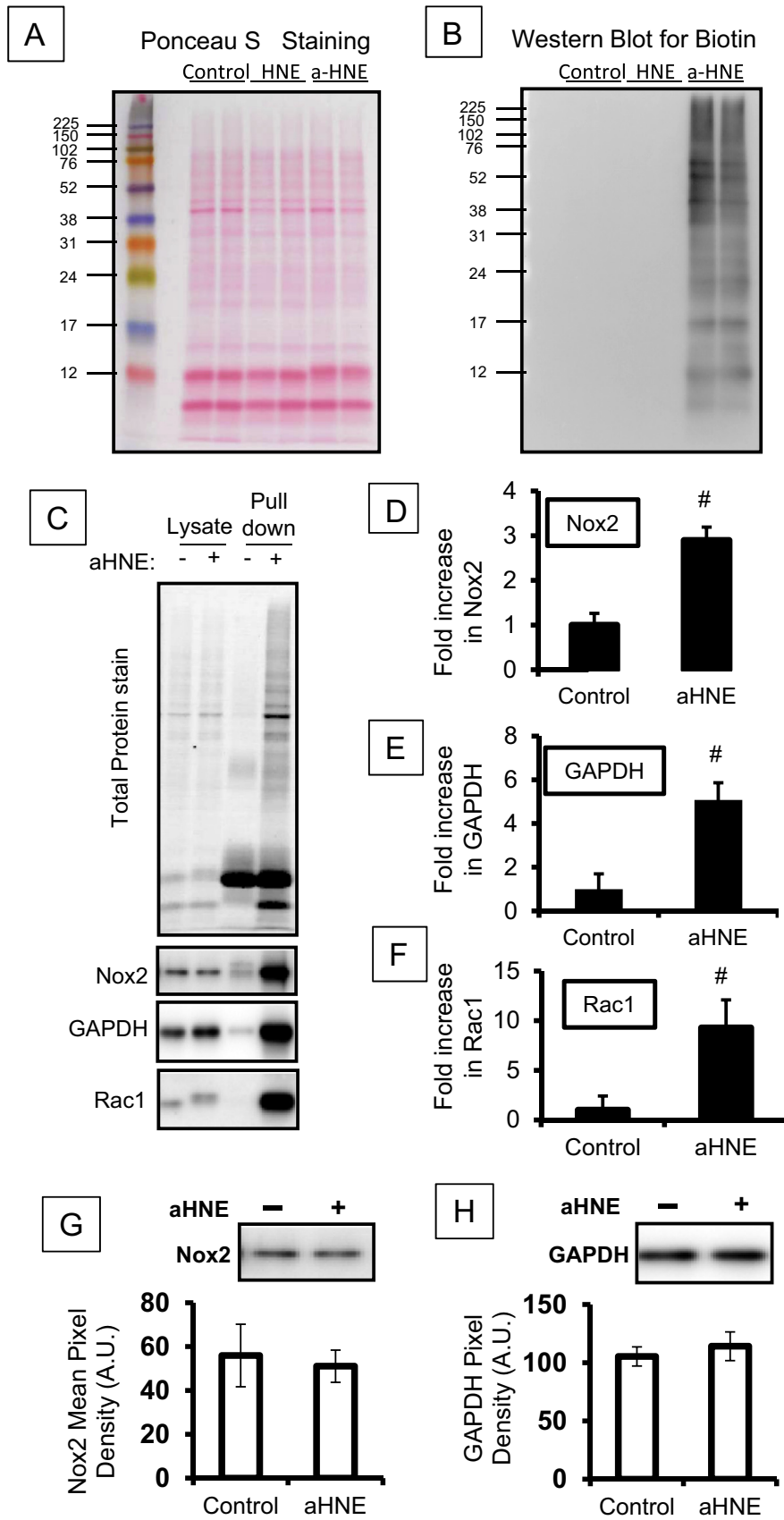
peptide fragments and exhibiting more than 95% homology with corresponding regions of the native protein in the aHNE treated sample were identified and grouped according to their major cellular functions. Using this approach, proteins involved in the cytoskeleton, cellular metabolism, redox homeostasis, inflammatory response, NADPH oxidase activity and small GTPases were identified as potential targets of aHNE in neutrophils (Tables 1–6).

#### 4. Discussion

Neutrophils mediate key components of the cellular immune response which involves cellular adhesion, migration to the site injury, phagocytosis of opsonized molecules and degradation and turnover of phagocytic metabolites [14,29,37,41]. Cytokines and chemokines enable these processes by generating a concentration gradient of the chemotaxins which are recognized by the cell receptors of the phagocytic cells [4,13]. In response to the chemotaxins, neutrophils and macrophages undergo activation, which changes the metabolic responses of the cell and lead to the generation of reactive oxygen and nitrogen species [13,14]. In order to meet the rapidly changing cellular energy and metabolite demands of activation, phagocytic cells increase the metabolic rate through glycolysis and/or switch the metabolic phenotype from oxidative to glycolytic. The interdependent operation of several cellular processes, the irreversible nature of phagocytosis and the involvement of large quantities of reactive metabolites necessitate an extremely high level of cellular regulation in the phenotypic adaptation [1,4,10,29,41,42].

Oxidative burst plays critical roles in the cellular function of

neutrophils. Clinical conditions such as chronic granulomatous disease have chronic inflammation and inefficient clearance of cellular debris due to defective NADPH oxidase activity [5,43]. When neutrophils undergo an oxidative burst, large quantities of ROS are generated within a short duration and at a high concentration close to the cellular compartment [1,2,14,42]. In addition, activated neutrophils undergo rapid apoptosis [8,44] and NETosis [45,46], releasing DNA, myeloperoxidase and proteases creating a highly reactive and oxidative environment capable of inducing lipid peroxidation and generating reactive lipid species such as 4-HNE. The uncontrolled generation of reactive metabolites in chronic inflammatory diseases has been shown to induce cellular and organelle dysfunction in a variety of cell types [47,48]. In vivo and in vitro experimental models support this hypothesis and suggest that increased oxidative stress has profound effects on leukocytes. However, the impact of reactive lipid peroxidation products generated secondary to uncontrolled oxidative burst, on neutrophil function and the mechanisms involved, are not well characterized. In this study we demonstrate that the non-enzymatic lipid peroxidation product, 4-HNE can inhibit phagocytosis, oxidative burst and cellular metabolism, the key functions that regulate the immune response of human neutrophils and monocytes. Mass spectrometry analyses demonstrate that 4-HNE forms adducts with proteins involved in key pathways of neutrophil oxidative burst, phagocytosis, redox homeostasis and glucose metabolism (Tables 1–6). This study also confirms the formation of neutrophil protein-4-HNE adducts using candidate proteins found to be modified by mass spectrometry [49–51]. The PMA stimulated oxidative burst is a well characterized experimental system to determine neutrophil immune response, which directly stimulates the protein kinase C pathway to trigger the assembly of active



**Fig. 4.** 4-HNE modifies NADPH Oxidase (Nox-2), glyceraldehyde-3-phosphate dehydrogenase and Rac1 in human neutrophils. Total cell lysates of vehicle, 4-HNE or alkyne-HNE treated neutrophils stained with (A) Ponceau S or (B) streptavidin-HRP biotin staining. Immunoprecipitation of the aHNE modified proteins were probed for NOX2 (gp91phox subunit), GAPDH and Rac1 (Figure C, representative blots) with densitometric quantitation of the bands (D, E and F) from three independent donors. Nox2 (G) and GAPDH (H) total protein levels in primary human neutrophils prior to 4-HNE/aHNE treatment in samples used for the detection of adducts. Mean  $\pm$  sem from n=3. #,  $p \leq 0.05$  compared to the control.

**Table 1**

Cytoskeletal/cytoskeleton interacting proteins are targets of HNE modification in human neutrophils. The list of cytoskeletal/cytoskeleton interacting proteins modified by aHNE in neutrophils as determined by mass spectrometry are reported. The protein accession ID and the number of peptides identified with 95% confidence are shown.

| No | Protein name                                 | Accession I.D. | Peptides | % Coverage |
|----|--|----------------|----------|------------|
| 1  | Beta-actin-like protein 2                    | P61160         | 3        | 20.81      |
| 2  | Plastin-2                                    | O15143         | 2        | 23.12      |
| 3  | Myosin-9                                     | O15144         | 3        | 20.67      |
| 4  | Alpha-actinin-1                              | O15145         | 2        | 17.42      |
| 5  | Tubulin alpha-1B chain                       | P59998         | 4        | 26.19      |
| 6  | Tubulin alpha-1C chain                       | P61158         | 4        | 22.97      |
| 7  | Moesin                                       | P12814         | 11       | 26.23      |
| 8  | Actin-related protein 2/3 complex subunit 4  | Q562R1         | 36       | 55.32      |
| 9  | Actin-related protein 3                      | Q14019         | 2        | 33.80      |
| 10 | Actin-related protein 2/3 complex subunit 1B | P52907         | 2        | 18.88      |
| 11 | Actin-related protein 2/3 complex subunit 3  | P47756         | 2        | 24.91      |
| 12 | Vinculin                                     | P21333         | 2        | 7.18       |
| 13 | Myosin light chain 6B                        | P40121         | 4        | 31.32      |
| 14 | Myosin light polypeptide 6                   | P26038         | 10       | 41.42      |
| 15 | Myosin-I $\beta$                             | P14649         | 2        | 11.54      |
| 16 | F-actin-capping protein subunit alpha-1      | P60660         | 2        | 26.49      |
| 17 | F-actin-capping protein subunit beta         | P35579         | 12       | 20.10      |
| 18 | Actin-related protein 2                      | O00160         | 2        | 8.56       |
| 19 | Actin-related protein 2/3 complex subunit 2  | P13796         | 42       | 78.47      |
| 20 | Macrophage-capping protein                   | Q9Y490         | 3        | 8.70       |
| 21 | Talin-1                                      | P68363         | 4        | 16.85      |
| 22 | Coactosin-like protein                       | Q9BQE3         | 4        | 14.03      |
| 23 | Filamin-A                                    | P50552         | 2        | 23.68      |
| 24 | Vasodilator-stimulated phosphoprotein        | P18206         | 6        | 23.72      |

**Table 2**

Cellular metabolism-related proteins are targets of aHNE in human neutrophils. Metabolic proteins that are modified by aHNE were identified by mass spectrometry. Table demonstrates the list of proteins with protein accession ID, number of peptides identified with 95% confidence, and the percent coverage of the peptides identified to the sequence of the protein with 95% confidence are shown.

| No | Protein name                                      | Accession | Peptides | % Coverage |
|----|---|-----------|----------|------------|
| 1  | Alpha-enolase                                     | P52209    | 18       | 48.24      |
| 2  | Pyruvate kinase isozymes M1/M2                    | P06733    | 33       | 72.12      |
| 3  | 6-phosphogluconate dehydrogenase, decarboxylating | P04075    | 7        | 42.58      |
| 4  | L-lactate dehydrogenase A chain                   | P11413    | 11       | 54.17      |
| 5  | Transketolase                                     | P06744    | 12       | 39.07      |
| 6  | Phosphoglycerate kinase 1                         | P06737    | 11       | 26.09      |
| 7  | Glucose-6-phosphate isomerase                     | P52790    | 9        | 25.35      |
| 8  | Glucose-6-phosphate 1-dehydrogenase               | P00338    | 16       | 44.58      |
| 9  | Glycogen phosphorylase, liver form                | P07195    | 6        | 23.05      |
| 10 | Transaldolase                                     | P40925    | 2        | 12.87      |
| 11 | Hexokinase-3                                      | Q96G03    | 2        | 18.95      |
| 12 | Triosephosphate isomerase                         | P00558    | 14       | 36.21      |
| 13 | Fructose-bisphosphate aldolase A                  | P00491    | 4        | 28.72      |
| 14 | L-lactate dehydrogenase B chain                   | P14618    | 30       | 68.36      |
| 15 | UTP-glucose-1-phosphate uridylyltransferase       | P37837    | 10       | 43.62      |
| 16 | Purine nucleoside phosphorylase                   | P29401    | 15       | 45.75      |
| 17 | Malate dehydrogenase, cytoplasmic                 | P60174    | 8        | 41.37      |
| 18 | Phosphoglucomutase-2                              | Q16851    | 5        | 29.72      |

NADPH oxidase complex on the plasma membrane [1,4,52]. Several methods have been employed to determine the oxidative burst in neutrophils, which either lack sensitivity or are limited throughput [52–54]. In addition these methods determine the products of oxidative burst which are reactive and have

**Table 3**

aHNE modifies redox homeostasis related proteins in human neutrophils. The redox homeostasis-related proteins modified by aHNE in neutrophils as determined by mass spectrometry are reported. The protein accession ID, number of peptides identified with 95% confidence, and the percent coverage of the peptides identified to the sequence of the protein with 95% confidence are shown.

| No | Protein name  | Accession | Peptides | % Coverage |
|----|---|-----------|----------|------------|
| 1  | ATP synthase subunit alpha, mitochondrial               | P25705    | 2        | 8.68       |
| 2  | Catalase  | P04040    | 5        | 22.39      |
| 3  | Glutathione reductase, mitochondrial                    | P00390    | 2        | 8.81       |
| 4  | Glutathione S-transferase omega-1                       | P78417    | 2        | 11.2       |
| 5  | Glutathione S-transferase P                             | P09211    | 4        | 36.67      |
| 6  | Heat shock protein HSP 90-alpha                         | P07900    | 2        | 14.89      |
| 7  | Myeloperoxidase   | P05164    | 4        | 13.29      |
| 8  | Protein disulfide-isomerase                             | P07237    | 7        | 34.06      |
| 9  | Protein DJ-1  | Q99497    | 2        | 16.4       |
| 10 | Ras-related C3 botulinum toxin substrate 2              | P15153    | 10       | 43.23      |
| 11 | Synaptic vesicle membrane protein VAT-1 homolog         | Q99536    | 3        | 22.9       |
| 12 | Thioredoxin-dependent peroxide reductase, mitochondrial | P30048    | 2        | 10.16      |

**Table 4**

Proteins associated with inflammatory response are modified by aHNE in human neutrophils. aHNE modified proteins related to inflammatory pathways in neutrophils as determined by mass spectrometry are reported. The protein accession ID, number of peptides identified with 95% confidence, and the percent coverage of the peptides identified to the sequence of the protein with 95% confidence are shown.

| No | Protein name   | Accession | Peptides | % Coverage |
|----|--|-----------|----------|------------|
| 1  | Annexin A3   | P12429    | 18       | 70.28      |
| 2  | Annexin A4   | P09525    | 4        | 24.45      |
| 3  | Annexin A5   | P08758    | 3        | 20.94      |
| 4  | Annexin A6   | P08133    | 12       | 36.11      |
| 5  | Arachidonate 5-lipoxygenase                                      | P09917    | 3        | 5.19       |
| 6  | Arachidonate 5-lipoxygenase-activating protein                   | P20292    | 2        | 25.47      |
| 7  | Coronin-1A   | P31146    | 10       | 52.93      |
| 8  | Guanine nucleotide-binding protein G(i) subunit alpha-2          | P04899    | 7        | 47.04      |
| 9  | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 | P62873    | 5        | 31.18      |
| 10 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 | P62879    | 4        | 22.65      |
| 11 | Heat shock 70 kDa protein 1 A/1B                                 | P08107    | 12       | 39.00      |
| 12 | Heat shock cognate 71 kDa protein                                | P11142    | 6        | 20.59      |
| 13 | Integrin alpha-M   | P11215    | 4        | 7.29       |
| 14 | Leukotriene-B(4) omega-hydroxylase 1                             | P78329    | 3        | 10.00      |
| 15 | Leukotriene-B(4) omega-hydroxylase 2                             | Q08477    | 4        | 14.42      |
| 16 | Peptidyl-prolyl cis-trans isomerase A                            | P62937    | 6        | 55.76      |

competing targets within the cells and in the assay medium [55,56]. In this method for the determination of neutrophil oxidative burst using the extracellular flux analyzer, the amount of oxygen consumed by activated neutrophils is accurately determined [35,57]. Neutrophil mitochondria do not consume significant amount of oxygen and hence almost all of the oxygen consumed following PMA activation is dedicated for the oxidative burst, which can be quantified by calculating the area under the oxygen consumption rate time course [10,58]. In addition, the assay is performed in the presence of antimycin A, an inhibitor of mitochondrial complex III to exclude any oxygen consumption from the mitochondria [57].

Neutrophil activation causes rapid assembly of the NADPH oxidase subunits and triggers oxygen consumption, which is consistent with the rapid rise in OCR curve following the addition



**Table 5**

NADPH Oxidase-related proteins are modified by aHNE in human neutrophils. NADPH Oxidase related proteins modified by aHNE in neutrophils as determined by mass spectrometry are reported. The protein accession ID, number of peptides identified with 95% confidence, and the percent coverage of the peptides identified to the sequence of the protein with 95% confidence are shown.

| No | Protein name                               | Accession | Peptides | % Coverage |
|----|--|-----------|----------|------------|
| 1  | Adenylosuccinate synthetase isozyme 2      | P30520    | 2        | 20.18      |
| 2  | Cytochrome b-245 heavy chain               | P04839    | 2        | 18.60      |
| 3  | Nicotinamide phosphoribosyltransferase     | P43490    | 2        | 29.53      |
| 4  | Nicotinate phosphoribosyltransferase       | Q6XQN6    | 3        | 16.73      |
| 5  | Rab GDP dissociation inhibitor beta        | P50395    | 9        | 41.80      |
| 6  | Rho GDP-dissociation inhibitor 2           | P52566    | 9        | 55.22      |
| 7  | Ras-related C3 botulinum toxin substrate 2 | P15153    | 10       | 43.23      |

**Table 6**

Small GTPase mediated signal transduction proteins modified by aHNE in human neutrophils. Small GTPase mediated signal transduction proteins modified by aHNE in neutrophils as determined by mass spectrometry are reported. The protein accession ID, number of peptides identified with 95% confidence, and the percent coverage of the peptides identified to the sequence of the protein with 95% confidence are shown.

| No | Protein name                              | Accession | Peptides | % Coverage |
|----|---|-----------|----------|------------|
| 1  | 14-3-3 protein beta/alpha                 | P31946    | 2        | 26.42      |
| 2  | ADP-ribosylation factor 1                 | P84077    | 4        | 33.70      |
| 3  | ADP-ribosylation factor 3                 | P61204    | 4        | 33.70      |
| 4  | ADP-ribosylation factor 4                 | P18085    | 3        | 31.67      |
| 5  | ADP-ribosylation factor 5                 | P84085    | 3        | 31.11      |
| 6  | ADP-ribosylation factor-like protein 8B   | Q9NVJ2    | 2        | 16.13      |
| 7  | Ras GTPase-activating-like protein IQGAP1 | P46940    | 3        | 6.46       |
| 8  | Ras-related protein Rab-10                | P61026    | 3        | 26.50      |
| 9  | Ras-related protein Rab-1A                | P62820    | 2        | 24.88      |
| 10 | Ras-related protein Rab-1B                | Q9H0U4    | 2        | 18.91      |
| 11 | Ras-related protein Rab-27A               | P51159    | 2        | 23.98      |
| 12 | Ras-related protein Rab-3D                | O95716    | 2        | 24.20      |
| 13 | Ras-related protein Rab-7a                | P51149    | 3        | 38.65      |
| 14 | Ras-related protein Rap-1A                | P62834    | 3        | 26.63      |
| 15 | Ras-related protein Rap-1b                | P61224    | 3        | 26.63      |
| 16 | Ras-related protein Rap-1b-like protein   | A6NIZ1    | 2        | 14.67      |

of PMA (Figs. 2A & 3A). After reaching a peak, oxygen consumption declines over 30–60 min initially and then slowly to reach the basal values. The profile of the curve demonstrates the dynamics of oxidative burst that includes rapid assembly of the enzyme complex, disassembly and metabolic switching to provide substrates essential for NADPH oxidase activity. 4-HNE is a highly reactive aldehyde that generated at sites of inflammation at high concentrations (~100  $\mu$ M) during pathological conditions and is known to have multiple targets inside the cell [10,26–28]. Previous studies have shown that 4-HNE has the potential to act as chemotactic agent to attract neutrophils to the site of inflammation [59]. It is likely that neutrophils at sites of inflammation or tissue injury get exposed to very high concentrations of reactive aldehydes and other intermediates of oxidative stress that exceed the concentrations used in this study. 4-HNE treatment inhibits the initial rate, peak and total amount of respiratory burst suggesting inhibition of multiple pathways associated with oxidative burst (Figs. 2A & 2B). Inhibition of ECAR (Figs. 2C & 2D) confirms the inhibition of the glucose metabolism with 4-HNE treatment. This response of neutrophil is different from other immune cells which tend to activate the glycolytic pathway when they are encountered with pro-inflammatory stimuli and oxidative stress.

In this study we utilized alkyne HNE to identify protein

adducts. Although the click chemistry approach is highly specific to determine aHNE-protein adducts, the alkyne tag decreases the reactivity of 4-HNE by approximately 40%. The lower reactivity necessitated increasing the aHNE concentration appropriately to match the reactivity of 4-HNE in the determination of protein adducts.

Approximately 100 protein targets were identified (Tables 1–6) including members associated with both metabolic and signaling pathways critical for neutrophil function. Phagocytosis is a complex but highly regulated process that involves metabolic, signaling and structural components of the cell [4,8,60]. For example, modification of the cytoskeletal proteins such as myosin, tubulin and actin-related peptides by 4-HNE could affect the ability of neutrophils to phagocytize *S. aureus* (Fig. 1). This is consistent with loss of function of cytoskeletal proteins by 4-HNE modification in oligodendrocytes and malaria infected erythrocytes [51,61]. Inhibition of the cytoskeletal proteins could affect cellular motility and phagosome formation. Interestingly, actin, one of most abundant cellular target of 4-HNE was found to be heavily modified in neutrophils but not included among the modified proteins by mass spectrometry as actin was also present in the control protein sample. Using a candidate protein approach direct evidence for modification of Rac1, GAPDH and Nox2 was found (Fig. 4). Interestingly, modification of Rac1 by other lipid electrophiles has previously been reported [62].

In summary, 4-HNE forms protein adducts with a broad range of proteins essential for the normal functioning of the oxidative burst and phagocytosis and its overall effects in inhibiting neutrophil function can be ascribed to the pleiotropic effects on both cell structure, metabolism and signaling. Since this reactive lipid intermediate is produced at sites of inflammation the partial suppression of this essential mechanism of innate immunity may promote a chronic inflammatory response and the failure to kill pathogens.

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