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Mass spectrometric phosphoproteome analysis of small-sized samples of human neutrophils

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ABSTRACT

Background: Global analysis of stimulus-dependent changes in the neutrophil phosphoproteome will improve the understanding of neutrophil signal transduction and function in diverse disease settings. However, gel-free phosphoproteomics of neutrophils in clinical studies is hampered by limited sample amounts and requires protein extract stability, efficient tryptic digestion and sensitive phosphopeptide enrichment in a protease-rich environment. For development of an appropriate workflow, we assessed neutrophil protein stability in urea-based lysis buffers and determined feasibility of gel-free phosphoproteomic analyses using polymer-based metal ion affinity capture (PolyMAC).
Methods: Western blotting, phosphopeptide enrichment and mass spectrometric analyses of samples of neutrophils were performed.
Results: Degradation of proteins in neutrophil extracts was observed after preparation with a urea-containing lysis buffer and could be prevented by addition of highly concentrated protease inhibitors. Subsequent tryptic digestion and PolyMAC-based phosphopeptide enrichment proved efficient with accordingly prepared neutrophil samples. Applying the new workflow, formyl–methionyl–leucyl–phenylalanine-induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) was detected after gel-free and gel-based phosphoproteomic analyses as proof of principle from 20 ml of whole blood. Furthermore, phosphorylation of other ERK1/2 pathway-associated proteins was monitored.
Conclusion: We provide a workflow for efficient, gel-free phosphoproteome analyses with small-sized neutrophil samples, suitable for application in clinical studies.

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1. Introduction

Neutrophils represent the first line of defense against invading pathogens and also play crucial roles in the pathogenesis of non-infectious diseases like acute lung injury (ALI)[1] and transfusion-related acute lung injury (TRALI) [1–4]. While induction of the neutrophil antimicrobial arsenal is a physiological response to infection, its aberrant mobilization is thought to be causative for lung tissue damage occurring during ALI and TRALI [2,5]. The elucidation of the underlying mechanisms might thus help to develop strategies for appropriate modulation of neutrophil effector functions. Proteomic techniques are powerful tools for the global analysis of cellular responses and were already used in several studies characterizing the neutrophil proteome [6].

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[1] Abbreviations: 1D-PAGE: 1-dimensional polyacrylamide gel electrophoresis; ALI: acute lung injury; IPA: ingenuity pathway analysis; LC–MS/MS: liquid chromatography–tandem mass spectrometry; MOAC: metal oxide affinity chromatography; PIC: protease inhibitor cocktail; PolyMAC: polymer-based metal ion affinity capture; PolyMAC-Ti: polymer-based metal ion affinity capture with titanium dioxide-functionalized soluble nanopolymers; TRALI: transfusion-related acute lung injury.

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Neutrophils show both, rapid responses by molecular reorganization processes and long-term effector functions determined by induced gene expression and protein synthesis (e.g. cytokine production) [7]. Both response types are mediated by activation of particular signaling pathways. Such cascades involve alterations of the phosphorylation pattern of certain signaling molecules, which may render them active or inactive. Thus, the analysis of the phosphoproteome is another potential approach for the characterization of physiological and pathological mechanisms of neutrophils and has been applied so far e.g. to investigate regulatory mechanisms in neutrophil exocytosis [8].

Proteome analyses of neutrophils are, however, challenging, as neutrophils contain a large pool of endogenous proteases as part of their microbicidal arsenal [9]. Usage of non-denaturing Tris-buffers for lysis leads to rapid protein degradation after cell disruption and hampers proteome analyses. Thus, such buffer systems were described to require the addition of protease inhibitors [6]. In contrast, buffers containing high concentrations of urea possess strong denaturing properties themselves [10]. Such buffers are therefore preferred when native protein conformation is negligible i.e. when lysates are intended to be used only for comparative proteomic approaches. However, it has not been shown so far whether the denaturing properties of urea buffers are sufficient to stabilize neutrophil extracts, and according proteomic studies conducted to date were varying regarding addition of protease inhibitors [11–14].

Application of mass spectrometry for phosphoproteomic approaches so far required the enrichment of phosphorylated peptides from relatively large sample sizes. However, the amount of neutrophil protein available from blood donations, particularly from patients, is limited. Recently, advances have been made in developing phosphopeptide enrichment strategies using metal oxide affinity chromatography (MOAC) [15]. A new technique designated polymer-based metal ion affinity capture with titanium dioxide-functionalized soluble nanopolymers (PolyMAC-Ti) was developed and proved highly selective for enrichment of phosphorylated peptides [15,16].

In this study, we aimed to develop a preparation protocol for the stable extraction of neutrophil protein using an urea-based lysis buffer. We assessed whether efficient phosphopeptide enrichment is feasible with small-sized neutrophil extracts using PolyMAC-Ti and whether such samples would be suitable for mass spectrometry-based phosphoproteome analyses.

2. Material and methods

2.1. Volunteers

All experiments were conducted according to the regulations of the ethical committee of the University Medicine Greifswald. All donors gave informed consent.

2.2. Lysis and wash buffer for preparation of neutrophils

The standard lysis buffer (UT) contained 8 mol/l urea/2 mol/l thiourea (Sigma-Aldrich, Steinheim, Germany) and was supplemented with 25 mmol/l sodium pyrophosphate (Sigma), 100 mmol/l sodium orthovanadate (pH 10; Sigma), 500 mmol/l β-glycerophosphate (Merck, Darmstadt, Germany), 1 mol/l sodium fluoride (Sigma), 500 mmol/l ethylenediaminetetraacetic acid (EDTA, pH 8; Merck) and 100 mmol/l Tris(2-carboxyethyl)phosphine (Sigma) to inhibit activity of phosphatases.

For lysis optimization EDTA-free complete ULTRA mini tablets (Roche Diagnostics, Mannheim, Germany) were added to UT to yield an 8×, 4×, 3×, 2× or 1× protease inhibitor concentration (compared to the manufacturer's suggestion).

For preparation of phosphatase inhibitors-containing wash buffer (PI-WB), phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS; Biochrom AG, Berlin, Germany) was supplemented with phosphatase inhibitors as described for UT.

2.3. Isolation of neutrophils

Neutrophils were isolated from EDTA-anticoagulated whole blood (20 ml) as described [17]. The cell suspensions obtained after isolation were analyzed using a Sysmex pocH-100i hematology analyzer (Sysmex Deutschland GmbH, Norderstedt, Germany) and a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA). A detailed method and result description is provided in Supplementary File 1. Directly after hematology analyzer-based isolate characterization, cell concentration was adjusted to 2×10^8 cells/ml with PBS. For samples used for optimization of preparation, 1×10^7 neutrophils (50 μl) were immediately transferred into 4 ml of chilled PI-WB (Section 2.2) and were washed and centrifuged twice for 3 min at 550 ×*g* and 4 °C. Pellets were resuspended in respective lysis buffers (Section 2.2) and immediately snap-frozen in liquid nitrogen.

2.4. Treatment of neutrophils

To allow reversal of isolation-induced activation (priming), cells were incubated under rotation for 1 hour (h) at 37 °C in PBS. Subsequently, 1×10^7 neutrophils (50 μl) were adjusted to a concentration of 2.5×10^6 cells/ml with PBS and treated with 10 μmol/l formyl–methionyl–leucyl–phenylalanine (fMLP; Sigma) for 5 min at 37 °C. Untreated neutrophils (1×10^7) served as control. Suspensions were washed and lysed as described in Section 2.3.

2.5. Cell lysis

In experiments utilizing the in-house standard lysis protocol, suspensions were subjected to six freeze and thaw cycles prior to ultrasonication. Otherwise, this step was omitted and cells were sonicated on ice in three cycles of 3 s at 60% power using a Sonoplus ultrasonic probe (Bandelin, Berlin, Germany). Cell debris was pelleted for 1 h at 4 °C and 17000 ×g. Supernatants were collected and protein concentrations were measured in triplicate using a Bradford assay [18]. Extract aliquots were stored at −80 °C.

2.6. In-solution protein digestion

Samples containing 15 μg of protein were diluted with 20 mmol/l ammonium bicarbonate (Sigma) and reduced with 2.5 mmol/l dithiothreitol (final concentration; Amersham Biosciences, Uppsala, Sweden) for 1 h at 60 °C. Subsequently, proteins were alkylated for 30 min at 37 °C using 10 mmol/l iodoacetamide (final concentration; Sigma). Samples were digested with porcine trypsin (Promega, Madison, WI, USA) at a ratio of 1:10 for 3 h at 37 °C. For tryptic digestion, the urea concentration was consistently adjusted to 1 mol/l (1:8 dilution). Digestion was stopped by adding acetic acid (final concentration 1%; v/v). Digests (5 μg) were subsequently subjected to 1-dimensional polyacrylamide gel electrophoresis (1D-PAGE) as described in Section 2.7.

2.7. One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

Protein (30 or 50 μg) or peptide samples (5 μg) were mixed with 4× lithium dodecyl sulfate (LDS) sample buffer, 10× reducing agent (both Life Technologies, Carlsbad, CA, USA) and HPLC grade water (Avantor, Deventer, Netherlands) according to the manufacturer's instructions. Reduction was performed either at room temperature (RT) for 15 min or by boiling at 95 °C for 5 min. Samples were loaded onto midi-sized pre-cast gradient gels (NuPAGE 4–12% Bis–Tris, Life Technologies) and separated according to the manufacturer's instructions using 2-(*N*-morpholino)ethanesulfonic acid sodium dodecyl sulfate (MES SDS)

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