Comparison of the Neutrophil Proteome in Trauma Patients and Normal Controls

Liz M.B. Teles, Elaine N. Aquino, Anne C.D. Neves, Carlos H.S. Garcia, Peter Roepstorff, Belchor Fontes, Mariana S. Castro and Wagner Fontes

Abstract: Background: Neutrophils have an impressive array of microbicidal weapons, and in the presence of a pathogen, progress from a quiescent state in the bloodstream to a completely activated state. Failure to regulate this activation, for example, when the blood is flooded with cytokines after severe trauma, causes inappropriate neutrophil activation that paradoxically, is associated with tissue and organ damage. Acidic proteomic maps of quiescent human neutrophils were analyzed and compared to those of activated neutrophils from severe trauma patients. The analysis revealed 114 spots whose measured volumes differed between activated and quiescent neutrophils, with 27 upregulated and 87 downregulated in trauma conditions. Among the identified proteins, grancalcin, S100-A9 and CACNB2 reinforce observed correlations between motility and ion flux, ANXA3, SNAP, FGD1 and Zfyve19 are involved in vesicular transport and exocytosis, and GSTP1, HSPA1, MAOB, UCH-L5, and PPA1 presented evidence that activated neutrophils may have diminished protection against oxidative damage and are prone to apoptosis. These are discussed, along with proteins involved in cytoskeleton reorganization, reactive oxygen species production, and ion flux. Proteins such as Zfyve19, MAOB and albumin-like protein were described for the first time in the neutrophil. In this work we achieved the identification of several proteins potentially involved in inflammatory signaling after trauma, as well as proteins described for the first time in neutrophils.

Keywords: Neutrophils, trauma, proteome, systemic inflammatory response syndrome, inflammation.

INTRODUCTION

Trauma is major cause of mortality in the Western world, for people under the age of 45 [1-2]. The mechanical energy of severe trauma may injure tissues and organs, and usually results in intense bleeding and hemorrhagic shock, leading to tissue ischemia. Other insults, such as surgical interventions or infections, may aggravate the patient's condition [3-4]. Fluid resuscitation after prolonged shock involves reperfusion that carries inflammatory mediators from ischemic tissue to multiple organs, in which may lead to the development of the complex phenomenon of systemic inflammatory response syndrome (SIRS) and acute respiratory distress syndrome (ARDS). This trauma-induced immune response involves the activation of diverse cell types, mainly polymorphonuclear neutrophils which, besides controlling infection by phagocytosing bacteria, are well known for their ap
and function, including adhesion, migration, phagocytosis, generation of reactive oxygen species (ROS), and degranulation of stored inflammatory proteins. These processes are performed and regulated by proteins in a complex interactive network. Activation mechanisms identified to date largely involve post-transcriptional checkpoints [8]. In addition to post-translational modifications, splicing and allelic variation can contribute to the tight control of the synthesis of cytokines, ion channels and metabolism-related proteins.

Currently, laboratory evaluation of patients with suspected sepsis or inflammatory disorders such as SIRS and ARDS uses tests with poor sensitivity and specificity. The complexity of the clinical presentation of SIRS, ARDS and sepsis requires new assays with early diagnostic and prognostic value. Possible candidates for these assays maybe found in neutrophil proteins, which are investigated in this work by comparative analysis of the proteomes of quiescent and trauma-activated cells.

**MATERIALS AND METHODS**

**Sample Preparation**

Neutrophils for the control group were obtained from volunteer donors with no history of chronic diseases, no recent inflammatory diseases, and no medication use within the last 60 days. Polymorphonuclear leukocytes were obtained from major torso trauma patients at the HC-FMUSP, 24-48 h after the traumatic event. Trauma patients had an anatomical injury severity score (ISS) of 29, received no viscera injury and no signs of infection. Donors and patients were 25-45 years old. The project was approved by the ethical committee of HC-FMUSP (process # 2001045) before donors and patients were contacted.

Neutrophils were isolated as described previously [10] with minor modifications. Briefly, blood was collected in heparinized solution and centrifuged through 60% and 70% density layers of Percoll (Amersham Biosciences, Uppsala, Sweden). Residual erythrocytes were removed by osmotic lysis. An aliquot was taken for cell counting in Neubauer chambers and viability analysis by nigrosine test. Purification lysis. An aliquot was taken for cell counting in Neubauer chambers and viability analysis by nigrosine test. Purification of blood products transfusion up to the moment of sample collection [9], and presented no hollow viscer injury and no signs of infection. Donors and patients were 25-45 years old. The project was approved by the ethical committee of HC-FMUSP (process # 2001045) before donors and patients were contacted.

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Samples yielding 2 x 10^7 cells/mL (98% neutrophils, 99% viable) were immediately subjected to cell lysis in protein solubilization buffer (7 M urea, 2 M thiourea, 1% v/v Triton X-100, 0.5% v/v Pharmalyte 4-7, 65 mmol/L dithiothreitol - DTT) containing protease inhibitors. Cell lysis was in a shaker for 2 h at 25 °C. After protein solubilization, the supernatant following centrifugation of the cell lysate was frozen at -80 °C until electrophoretic separation.

**Two-Dimensional (2D) Polyacrylamide Gel Electrophoresis**

Neutrophil protein extracts were submitted to protein quantification [11] and diluted in protein solubilization buffer, pH 4-7, to a final protein concentration of 0.14 μg/μL. A sample of 350 μL in lysis buffer was applied to 18-cm immobilized pH gradient (IPG) gel strips with a linear pH range of 4 to 7 (Amersham Biosciences, Uppsala, Sweden) and submitted to isoelectric focusing using an IPGphor (Amersham Biosciences, Uppsala, Sweden) [12]. After focusing, IPG gel strips were submitted to protein reduction in 125 mM DTT and alkylation in 135 mM iodoacetamide. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% T gels run on a Protean II system (Bio-Rad, Hercules, CA, USA) at 25 mA per gel until the dye front reached the end. Proteins were visualized by ammoniacal silver staining, chosen for the improved sensitivity and compatibility to mass spectrometry [6, 13].

**Images and Statistical Analysis**

Images from the total of 18 gels (nine gels of each group, comprising biological and technical replicates) were analyzed with ImageMaster Platinum software (Amersham Biosciences, Uppsala, Sweden). The experimental design was validated according to Chich et al. [14] Volumes for each spot were normalized against the total volume of the detected spots. For both the normal donors and the severe trauma patients, one gel was assigned as a standard for the remaining eight gels. After pairing within each group, the two master gels were matched. Paired spots were tested for normality of volumes using the Kolmogorov-Smirnov test. Significance of differences in spot volumes was assessed using an Independent-Samples T-test (for parametric samples) or Mann-Whitney test (for non-parametric samples). As noted by Birron and Magalhães et al. [15-16], two additional stringency criteria were applied. To avoid false positive results in statistical parameters, the difference in spot volume ratio was required to be at least twelve between the groups of gels, and the spot was required to be present in at least five gels in each group. Spots were considered specifically present if they appeared in at least five gels in a group, and were not detected in any gels of the other group.

**Peptide Mass Fingerprinting (PMF) and Tandem Mass Spectrometry Fragmentation (MS/MS)**

Silver-stained spots were excised, destained, in-gel digested with trypsin (Promega, Madison, USA), and extracted as previously described [17]. Before application on matrix-assisted laser desorption/ionization (MALDI) targets, digests were desalted using reversed-phase chromatography micro-columns and eluted on the surface of AnchorChip 384/600 targets (Bruker Daltonics, Leipzig, Germany) with a matrix mix of α-cyano-4-hydroxycinnamic acid and 2,5 dihydroxybenzoic acid, 1:1 (v/v), 5 μg/μL final concentration [18].

PMF used a MALDI-TOF/TOF mass spectrometer (Autoflex II; Bruker Daltonics, Leipzig, Germany), in positive and reflector mode, after external calibration using a standard peptide mixture ranging from 757 to 2465 mass units. Spectra were internally calibrated with peaks from keratin contaminant digestion and trypsin autolysis. Selected peaks were used in PMF searches for protein identification, and those that did not fulfill the validation criteria described below were subjected to MS/MS fragmentation. MS/MS fragmentation was carried out using the LIFT technique, which, combined with peptide mass fingerprinting, increased the identification rate [19]. Mass spectra were analyzed using FlexAnalysis and Biotools software (Bruker Daltonics). Pro-
tein identification used Mascot software. Searches were performed against the NCBInr database [20] with 50-100 ppm mass tolerance and a fragmentation mass tolerance of 90 ppm. Search parameters were restricted to *Homo sapiens* and one missed cleavage was allowed. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine and N-terminal acetylation as variable modifications.

To further validate Mascot results, we applied stringency criteria that the peptide mass error distribution should fit a distribution curve, and coherence between theoretical and experimental mass and pl values should be within 15%. Peptides containing missed cleavages and methionine oxidation were manually verified. To reduce redundancy and validate the sequence data, significant results (p < 0.05) were analyzed in GPMAW software (Lighthouse data, Odense, Denmark) for multiple peptide location.

**RESULTS**

An average of 850 spots were detected on each gel. After comparison and statistical analysis using the stringency criteria described above, 114 spots showed differential volumes, with 357 spots undetectable in activated neutrophils, and 244 spots exclusively found in activated neutrophils from severe trauma patients. Fig. (1) shows the 2D map for the quiescent neutrophil state highlighting the identified and the differentially expressed proteins. Table 1 summarizes information about each identified protein. In the section below, after each protein, the number in parenthesis indicates where the protein can be found in Table 1 and on Fig. (1).

Image of a 2D gel obtained from quiescent neutrophils. Spots whose measured volumes differed between the control and trauma conditions are in white. Boxes show examples of spot volume comparisons between the conditions. In the 3D view of the spot, the peak height represents staining intensity and the corresponding gel image is below. Numeric identification corresponds to the proteins in Table 1.

The unique identified protein upregulated in activated neutrophils was PPA1 (1398) or pyrophosphatase enzyme. Proteins downregulated in trauma included ubiquitin carboxyl-terminal hydrolase L5 (UCH-L5) (1179), L-plastin (354), vimentin (521), β-actin (729), grancalcin (1282), S100-A9 (1543), α-soluble N-ethylmaleimide-sensitive factor (NSF)-attachment protein (SNAP) (1024), annexin A3 (ANXA3) (1052), heat shock 70 kDa protein 1 (HSPA1) (358), glutathione S-transferase P (GSTP1) (1336), laminin receptor 1 (RASSA) (811), and Rho GDP dissociation inhibitor (RhoGD1) (1262) complex.

In the set of proteins detected only in quiescent cells, we identified KIAA0564 (71), histidyl-tRNA synthetase (HisRS) (526), interferon-induced protein with tetraticopeptide repeat 2 (IFIT2) (595), modulator of retrovirus infection (MRI) (1501), postmeiotic segregation increased 2-like (PMS2L3) (1360), pseudouridylate synthase 7 homolog (Saccharomyces cerevisiae)-like (PUS7L) (1479), NCK-associated protein 1-like (NCKAP1L) (102), heat shock 70 kDa protein 1-like (HSP90B1) (508), desmin (603), albumin-like (679), zinc finger FYVE domain 19 (ZFyve19) (680), faciogenital dysplasia 1 protein (FGD1) - a protein reported as containing the domains FYVE, RhoGEF and PH (546), antibody-dependent cellular cytotoxicity (ADCC)-enhanced Fcγ fragment (629), monoamine oxidase type B (MAOB) (695), calcium channel voltage-dependent beta-2 subunit (CACNB2) (904) and chloride intracellular channel (CLIC4) (982).

**DISCUSSION**

Adams and Botha *et al.* demonstrated that trauma modulates neutrophil function [21-22]. Our results showing differences in proteins in neutrophils from trauma patients and normal controls corroborate these findings. Of the 114 spots showing different volumes, 27 were upregulated and 87 were downregulated after activation by systemic inflammation caused by trauma. Previously, we identified 22 proteins [6] by 2D-acidic mapping of the quiescent neutrophil. In this article, 29 additional proteins were identified, enhancing the identification sensitivity of our previous study. The number of successful identifications was rather low because of contamination with keratin and low abundance in the gels. Among proteins differentially expressed between quiescent and activated neutrophils, we identified 1 upregulated and 12 downregulated proteins, and 16 that were below the detection level in polymorphonuclear leukocytes from trauma patients.

The identification of pyrophosphatase (PPA1) (1398) as upregulated in trauma is in accordance with the findings of Borregaard and Herlin, that activated neutrophils enhance the glucose consumption and consequently the production of ATP and NADPH which will be used in ROS production [23]. After trauma, the augmented level of glucagon hormone maintains blood glucose levels until depauqueration. To generate energy the cells potentiate some biochemical processes as oxidation of fatty acids, which requires the pyrophosphatase enzyme (PPA1). Also, PPA1 is associated with apoptosis induction [24], consistent with the functions of other proteins described below.

Composing the trauma downregulated proteins class, ubiquitin carboxyl-terminal hydrolase L5 (UCH-L5) (1179) is a deubiquitinating enzyme and degradation of proteins by the ubiquitin-proteasome pathway is critical in regulating the levels of most cellular proteins and in the rapid elimination of misfolded proteins [25]. UCH-L5 was also described as associated with a chromatin-remodeling complex and can participate in the regulation of transcription or DNA repair [26]. The finding of this protein as downregulated after activation suggests a decrease in repair mechanisms, protein processing, ubiquitin turnover and in the regulation of transcription, as proposed by Hossain *et al.* [27].

L-plastin (354) is an actin-bundling protein which collaborates to the modifications on leukocyte cytoskeleton at membrane ruffles during activation [28]. Vimentin (521) is an intermediate filament cytoskeletal protein which participates in neutrophil adhesion and transmigration, and can be secreted to the extracellular space collaborating in cytotoxic processes, such as bacteria killing and oxidative metabolite production [29]. Beta-actin (729) is the major protein component of the cytoskeleton, can assemble into filaments and generate a motile machine capable of efficient and rapid
Figure 1. Proteomic map of neutrophils.

Table 1. Neutrophil proteins identified by MALDI-TOF PMF and MS/MS fragmentation. Spot numbers coincide with Fig. (1)

| Spot | Expression Parameter | p-value | Accession Number | Name       | Activity                                      | pI (theor/exp) | Mr (theor/exp) (kDa) | PMF Score | PMF C(%) | MS/MS Score | MS/MS peptides                  |
|------|----------------------|---------|------------------|------------|-----------------------------------------------|----------------|----------------------|------------|----------|-------------|---------------------------------|
| 71   | Q                    |         | gi|3043652   | KIAA0564  | Translation                                    | 6.37 / 6.56    | 163 / 176             | 354        | 39       | -           |                                 |
| 102  | Q                    |         | gi|34485727  | NCKAP1L   | Structural / Motility                          | 6.39 / 5.53    | 130 / 120            | 97         | 19       | -           |                                 |
| 354  | ↓T (4.59)            | 0.003   | gi|346733    | L-plastin | Structural / Cytoskeleton remodelling           | 5.20 / 5.20    | 71 / 70              | 259        | 64       | 138         | R.NWXMIGMVNGP.R V K.MSLIPDSQTR.T K.FSLVGIGQV[DQSSG.R |
| 358  | ↓T (3.92)            | 0.024   | gi|94248072  | HSPA1A    | Protein metabolism / Oxidative protection      | 5.48 / 5.56    | 70 / 75              | 361        | 60       | 67          | R.TTFSYVAFDTR.L R.ARFEEGCSDFLR.S K.NQVAINPQNTVFAKR.L |
| 508  | Q                    |         | gi|24256496  | HSPA1L    | Protein metabolism / Oxidative protection      | 5.76 / 5.21    | 71 / 66              | 88         | 20       | -           |                                 |
| 521  | ↓T (5.28)            | 0.003   | gi|62414289  | vimentin  | Structural / Motility                          | 5.06 / 5.03    | 54 / 62              | 201        | 49       | 71          | R.SLYASSPGVYATR.S R.TYSLSALRPSTSR.S R.ISLPLPNSLSILR.E K.FADLSLEANN4RNL.R Q |
| Spot | Expression Parameter | p-value | Accession Number | Name | Activity | pI (theor/exp) | Mr (theor/exp) (kDa) | PMF Scoreb | PMF C(%)c | MS/MS Scored | MS/MS peptides |
|------|----------------------|---------|------------------|------|----------|---------------|------------------|-------------|-----------|--------------|----------------|
| 526  | Q                    | gi|62766469 | HisRS      | Translation | 5.72 / 5.51 | 58 / 62 | 181 | 46 | - | |
| 546  | Q                    | gi|94389088 P98174.2 | FGD1      | Granule transport | 5.57 / 5.32 | 60 / 58 | 91 | 22 | - | |
| 595  | Q                    | gi|53082755 | IFIT2     | Translation/apoptosis | 6.32 / 6.59 | 55 / 59 | 82 | 23 | - | |
| 603  | Q                    | gi|408188 | desmin    | Structural/motility | 5.21 / 5.70 | 53 / 60 | 238 | 60 | - | |
| 629  | Q                    | gi|171848866 | ADCC-Eah. Fc Fragment | Cell stimulation | 6.37 / 5.35 | 26 / 54 | 78 | 60 | 39 | R.VVSLTVLHGWLSQGK.E |
| 679  | Q                    | gi|763431 | albumin-like | Structural | 5.69 / 5.66 | 53 / 56 | 162 | 46 | 96 | K.KVFQVSTPTLVEVSR.N R.BHFDFYSVUWLRL.K DVFGLMFYFAR.R |
| 680  | Q                    | gi|19612854 | Zfyve19   | Granule transport | 5.21 / 6.13 | 48 / 53 | 109 | 27 | - | |
| 695  | Q                    | gi|38202207 | MAOB      | Amine metabolism/oxidative protection | 7.20 / 6.79 | 59 / 52 | 103 | 34 | - | |
| 729  | ▼T (2.74) 0.027     | gi|4250401 | β-actin   | Structural/motility | 5.56 / 5.27 | 41 / 49 | 80 | 31 | 63 | R.AVPSIVGRFR.H K.SYELPDQVITIONER.F |
| 811  | ▼T (2.67) 0.040     | gi|125969 | RSSA      | Adhesion receptor | 4.79 / 4.69 | 33 / 46 | 192 | 53 | 212 | K.FAAATGATPIAGR.F R.AIVAINPADVSVISSR.N R.FTPGFTNQIQAFFPR.L |
| 904  | Q                    | gi|50039531 | CACNB2    | Voltage-gated calcium channel | 6.41 / 6.16 | 46 / 43 | 84 | 31 | - | |
| 982  | Q                    | gi|7330335 | CLIC4     | Voltage-gated chloride channel | 5.45 / 5.60 | 29 / 38 | 76 | 41 | - | |
| 1024 | ▼T (5.76) 0.003     | gi|16242794 | SNAP      | Granule transport | 5.23 / 5.23 | 34 / 33 | 197 | 75 | - | |
| 1052 | ▼T (6.34) 0.001     | gi|4826643 | ANXA3     | Structural/Granule transport | 5.63 / 5.63 | 37 / 36 | 268 | 62 | 499 | K.ALLTLADGR.R K.LTDFEYR.N M.ASWIVGRHR.G + Acetyl (Protein N-term) R.NTPAFLAER.L K.GIGTFDLTINR.I K.GAGTNDELIELITR.T K.GELGSHFEDLLIADVCR.N |
| 1179 | ▼T (2.47) 0.006     | gi|55859537 | UCH-L5    | Protein metabolism/DNA repair | 4.95 / 4.88 | 29 / 32 | 78 | 52 | - | |
| 1262 | ▼T (10.66) 0.011    | gi|56676393 | Rho GDI  | Motility/NADPH oxidase assembly | 5.10 / 5.12 | 23 / 29 | 167 | 81 | 38 | K.YVQHTYR.T |
| 1282 | ▼T (8.29) 0.001     | gi|7943195 | Grancalcin | Structural/Motility | 5.77 / 4.87 | 19 / 28 | 69 | 28 | 33 | R.ALTDFR.K R.CLTQSGINGYTSPFSLETCR.I |
chemotaxis in polymorphonuclear leukocytes [30]. The downregulation of these three cytoskeleton related proteins is probably related to their post-translational processing, either by secretion, proteolysis or by uptake in complexes, during the remodeling process, instead of a decreased gene expression. This confirms the findings of Suchard and Boxer [31] and Mambole et al. [32] who mention the cleavage of cytoskeletal proteins upon activation. The findings of Fessler et al. [8, 33], showing a low correlation between the transcriptome and the proteome of neutrophils are also supportive for this downregulation.

Laminin receptor 1 or RSSA (811) can function as a leukocyte surface receptor for laminin - a glycoprotein of the endothelium [34]. After exposure to cytokines in inflammatory events, the expression of laminin in endothelial cells is upregulated [35]. A study using rabbit circulating polymorphonuclear leukocytes shows laminin on their surfaces and it is presumably bound to the receptor [36], suggesting a strong binding. Our finding of fewer amounts of laminin receptor 1 after cell activation can represent an increased link between the receptor and neutrophil laminin, before the development of chemotaxis, adhesion and transmigration.

Grancalcin (1282) is a cytosolic Ca\(^{2+}\)-binding protein expressed at high levels in neutrophils which translocates to the primary and secondary granules membranes upon leukocyte activation [37]. In activated neutrophils, granalcin is related with L-plastin through Ca\(^{2+}\) dependence in adhesion and spreading processes [38] and the downregulation of both indicates a cell that is more likely to adhere and migrate, since the molecules are probably complexed (by interaction with L-plastin) in the activated state [39]. Also, S100-A9 (1543) is one of the major calcium-binding molecules expressed in neutrophils [40]. It is targeted to the cell surface upon calcium influx and is released from the leukocyte during the activation processes [41] inducing chemotaxis to other neutrophils and degranulation [42], and this is reasonable with our finding of S100-A9 downregulation. Annexin A3 (ANXA3) (1052) is a calcium-dependent phospholipid-binding protein which participates in intracellular membrane fusion and granule aggregation in neutrophils, as observed when these cells engulfed yeast particles and ANXA3 accumulated at periphagosomal area [43]. The α-soluble NSF attachment protein (SNAP) (1024) has a widespread involvement in vesicular transport pathways leading to membrane fusion among endoplasmic reticulum, Golgi apparatus and secretory vesicles, conducting to exocytosis [44]. Our findings of SNAP and ANXA3 downregulation, associated to the fact that this class of proteins is secreted from diverse cell types in exosomes [45], suggest that the activated neutrophils of those trauma patients have released these proteins.

Heat shock 70 kDa protein 1 or HSPA1A (358) can stabilize existing proteins against aggregation, protecting against oxidative damage and preventing apoptosis [46]. This protein is also considered an autoantigen chemotactic for leukocytes, released by exocytosis during leukocyte activation [47]. Such facts are coherent with the fewer amounts of this protein. Glutathione S-transferase P or GSTP1 (1336) is a cytosolic enzyme that plays an important role in detoxification processes [48]. When these cells engulfed yeast particles and ANXA3 accumulated at periphagosomal area [43]. The α-soluble NSF attachment protein (SNAP) (1024) has a widespread involvement in vesicular transport pathways leading to membrane fusion among endoplasmic reticulum, Golgi apparatus and secretory vesicles, conducting to exocytosis [44]. Our findings of SNAP and ANXA3 downregulation, associated to the fact that this class of proteins is secreted from diverse cell types in exosomes [45], suggest that the activated neutrophils of those trauma patients have released these proteins.

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the GTPases required for Fcγ receptor-mediated phagocytosis [51]. These processes are consistent with the diminished quantities of RhoGDI found in activated neutrophils.

In the set of proteins detected only in quiescent cells, we identified a subset that participates in translation processes: KIAA0564 (71), HisRS (526), IFIT2 (595), MRI (1501), PMS2L3 (1360) and PUSTL (1479). The activity of such proteins involves nucleotide binding, gene expression modulation, synthesis and processing of tRNA, DNA repair, regulation of proteasomal processing, of alternative splicing and of apoptosis. The unique presence of these six proteins in quiescent leukocytes added to the downregulation of UCH-L5 described above might be alluded to the fact that many neutrophil activation responses use constitutive biochemical pathways and enzymes performing post-translational modifications in a higher rate than new protein synthesis [52] and reinforces the hypothesis of suppressed reparative pathways in a cell inclined to programmed death. In addition, Fessler et al. described poor concordances between mRNA transcriptions and protein quantitation in polymorphonuclear leukocytes [8, 33].

NCK-associated protein 1-like (NCKAPIL) (102) collaborates in Rac activation, actin polymerization and myosin regulation in neutrophil chemotaxis [53]. Soon after receiving the activating stimulus, neutrophils initiate the process of shape change and movement, when complex interactions among cytoskeleton proteins, such as beta-actin and NCKAPIL, are occurring [54] and may explain its absence in the activated cell state. Heat shock 70 kDa protein 1-like (HSPA1L) (508), an allelic variant of the HSPA1A, already described as downregulated above, confirms the reduced level of this protein family, reinforcing the aspects related to oxidative damage discussed above.

Yet, desmin (603) is a filament found in neutrophils, like vimentin. It is enriched on activated neutrophils phagosomes [55] and connects to F-actin, promoting formation of pseudopods [56] and preventing its detection as unmodified.

Presenting folding similarity with albumin and vitamin D-binding protein, the albumin-like protein (679), found for the first time in neutrophils, binds to actin within the cytoskeleton [57] and to C5a, enhancing chemotaxis. After binding, the protein goes through proteolytical processing [58], what could explain its undetectable amounts in neutrophils after trauma. Zinc finger FYVE domain 19 (Zfyve19) (680), also seen for the first time in neutrophils, is a zinc binding finger protein probably involved in vesicle trafficking and phosphoinositide metabolism. The absence of Zfyve19 in activated cells could be due to its proteolytical processing after interaction with phosphatidylinositol 3-phosphate followed by recruitment to endo- and exocytical membranes [59]. This finding is reinforced by the FGD1 protein, that presents a FYVE domain [60], also detected only in quiescent cells. Again, this work presents for the first time the presence of a third protein, monoamine oxidase type B (MAOB) (695), in the neutrophil. This protein localizes at the outer membrane of mitochondria and the byproducts of the reaction catalyzed by MAOB are reactive nitrogen species (RNS) and ROS, known inducers of mitochondrial damage and apoptosis [61]. MAOB disappearance after neutrophil activation could be associated to the increased free radicals generated in the granules during this leukocyte state.

In antibodies, the Fab region opsonizes the antigen, and the Fcγ region is a ligand for the Fcγ receptor, presented as FcγRIII on the quiescent polymorphonuclear leukocyte surface [7]. Upon activation of neutrophils, or during apoptosis, FcγRIII is released from the cell surface by proteolytic cleavage, and the enzyme responsible for this process is probably a membrane-bound metalloprotease released from granules [62]. The finding of antibody-dependent cellular cytotoxicity (ADCC)-enhanced Fcγ fragment (629) only on the quiescent set is coherent with neutrophil activation by trauma and consequent release of FcγRIII bound to its ligand.

In a subset of ion channel proteins, we have found CACNB2 and CLIC4. Calcium channel voltage-dependent beta-2 subunit (CACNB2) (904) allows calcium influx, participating on the activation of NADPH oxidase, adhesion, degranulation and synthesis of IL-8 [63]. The filling of intracellular Ca<sup>2+</sup> stores involves physical coupling at plasma membranes of the calcium channel proteins with IP<sub>3</sub> (inositol triphosphate) receptors and this fact could be the explanation for the absence of CACNB2 in activated neutrophils [64]. Chloride intracellular channel 4 (CLIC4) (982) is an anion exchanger channel for a proper cytoplasmic or phagosome acidification, through a Cl⁻/H<sup>+</sup> antiporter activity [65]. Variations in intracellular pH allowed by CLIC4 modulate adhesion, chemotaxis, phagocytosis, secretion of enzymes and ROS production in neutrophils. Since chloride channels operate in neutrophils after β₂-integrins cross linking [66], we hypothesize that CLIC4 could be undetectable in stimulated cells due to its interaction with integrins. The finding for CLIC4 is supported by the observed downregulation of GSTP1, its structural homolog.

In summary, although recent intensive study has clarified many events involving the role of neutrophils in the inflammatory response, many questions remain to be answered. Our results show differences between the proteomic patterns of quiescent and the trauma-induced activated neutrophils, with some proteins described here for the first time (albumin-like, Zfyve19 and MAOB) as associated with either quiescent or trauma-activated neutrophils. As illustrated by Fig. (2), among the identified proteins, a considerable subset is related to cytoskeletal reorganization, or cell motility and adhesion (L-plastin, vimentin, beta-actin, RhoGDI, NCKAPIL and desmin). Some, such as granulin C1, S100-A9, CACNB2 and CLIC4 reinforce observed correlations between motility and ion flux. Another group of identified proteins contains SNAP, ANXA3 and Zfyve19, which are involved in vesicular transport and exocytosis.

We have presented evidence that activated neutrophils may have diminished protection against oxidative damage and are prone to apoptosis, through downregulation of GSTP1, UCH-L5 and HSPA1A, upregulation of PPA1, and absence of HSPA1L and MAOB. Quantitative changes in translational and repair proteins also support this hypothesis.
CONCLUSION

In conclusion, the results of this study allowed the identification of a set of differences between the proteomic pattern of quiescent and trauma-activated neutrophils. Some of the identified proteins are involved in the neutrophil cytoskeleton reorganization, others suggest that the neutrophil is a cell with diminished protection against oxidative damage and prone to apoptosis at the initial hours after trauma. Some proteins were described for the first time in neutrophils. Our findings may contribute to the development of laboratory and clinical strategies for controlling deleterious inflammatory responses, as seen after severe trauma and sepsis.

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