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A Proteomic Approach to Study the Effect of Thiotaurine on Human Neutrophil Activation

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Abstract Thiotaurine, a thiosulfonate related to taurine and hypotaurine, is formed by a metabolic process from cystine and generated by a transulfuration reaction between hypotaurine and thiocysteine. Thiotaurine can produce hydrogen sulfide (H_2S) from its sulfane sulfur moiety. H_2S is a gaseous signaling molecule which can have regulatory roles in inflammatory process. In addition, sulfane sulfur displays the capacity to reversibly bind to other sulfur atoms. Thiotaurine inhibits PMA-induced activation of human neutrophils, and hinders neutrophil spontaneous apoptosis. Here, we present the results of a proteomic approach to study the possible effects of thiotaurine at protein expression level. Proteome analysis of human neutrophils has been performed comparing protein extracts of resting or PMA-activated neutrophils in presence or in absence of thiotaurine. In particular, PMA-stimulated neutrophils showed high level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression compared to the level of the same glycolytic enzyme in the resting neutrophils. Conversely, decreased expression of GAPDH has been observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. This result, confirmed by Western blot analysis, suggests again that thiotaurine shows a bioactive role in the mechanisms underlying the inflammatory process, influencing the energy metabolism of activated leukocytes and raises the possibility that

thiotaurine, acting as a sulfur donor, could modulate neutrophil activation via persulfidation of target proteins, such as GAPDH.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TTAU, thiotaurine; PMA, phorbol 12-myristate 13-acetate, PMNs, human neutrophils.

1 INTRODUCTION

Thiotaurine (2-aminoethane thiosulfonate) is produced *in vivo* from cystine (Cavallini et al. 1959 and 1960) and is generated spontaneously by transulfuration between the persulfide analogue of cysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961). In several animal tissue thiol oxidation to sulfinates and thiosulfonates can enzymatically occur when inorganic sulfur is present (De Marco and Tentori 1961; Cavallini et al. 1961). Furthermore, thiotaurine is formed by a sulfurtransferase catalyzing sulfur transfer from mercaptopyruvate to hypotaurine (Sörbo 1957; Chauncey and Westley 1983). Structurally, thiotaurine is a thiosulfonate (RSO_2SH) related to hypotaurine (RSO_2H) and taurine (RSO_3H). However, it exhibits peculiar biological properties distinct from those exerted by the structurally related sulfur compounds (Westley and Heyse 1971; Luo and Horowitz 1994; Capuozzo et al. 2015). It has been previously reported that thiotaurine can also produce hydrogen sulfide (H_2S) by its sulfane sulfur moiety (Capuozzo et al. 2013). Sulfane sulfur exhibits the singular capacity to reversibly bind to other atoms of sulfur (Toohey 1989). Related to this chemical behaviour, sulfane sulfur moiety has been reported to have regulatory effects in various biological processes (Beinert 2000; Mueller 2006). In addition, the gaseous signaling molecule H_2S promotes several physiological effects from cardioprotection to being an anti-inflammatory mediator and a neuromodulator (Zanardo et al. 2006; Whiteman and Winyard 2011; Whiteman et al. 2011). A dominant way for transmission of sulfide-based signals includes activation or inactivation of enzymes via post-translational modification of reactive cysteine thiols (RSH) to persulfide (RSSH) (Toohey 2011; Yadav et al 2016). However, which are the chemical intermediates associated to H_2S signaling remain difficult to identify in spite of the various pathophysiological effects displayed by this gasotransmitter (Mishanina and al. 2015).
Previously, it has been shown that thiotaurine inhibits neutrophil activation in response to PMA, a diacylglycerol substitute that activates protein kinase C, or to fMLP, a ligand which binds to specific leukocyte receptors.

3 Thiotaurine and Human Neutrophil Activation

Thiotaurine can attenuate leukocyte functions by the inhibition of PMA-induced ROS generation, and of superoxide anion production in human neutrophils activated by PMA or fMLP (Capuozzo et al. 2015). Moreover, thiotaurine reduced apoptosis of human neutrophils (Capuozzo et al. 2013). The protection of mouse cerebellar granule neurons from potassium deprivation-induced apoptosis by interfering with the activation of caspase-3 has been also observed (Dragotto et al. 2015). Here, we suggest that key enzymes of neutrophil activation cascade can be modulated by sulfane sulfur of thiotaurine. Relatively to this hypothesis, proteomic profiling of human neutrophils can be applied to understand possible effects of thiotaurine treatments at protein expression level. The aim of this approach is to identify and to analyze proteins that change their expression level or undergo post-translational modifications, such as phosphorylation, nitrosylation/nitration, persulfidation etc. In particular, we performed proteome analysis of human neutrophils comparing protein extracts of resting neutrophils, PMA-activated neutrophils, and PMA-activated neutrophils treated with thiotaurine.

2 MATERIALS AND METHODS

2.1 Chemicals

Thiotaurine (2-aminoethane thiosulfonate) was synthetized reacting elemental sulfur and hypotaurine according to Cavallini et al. (1959). All chemicals were analytical grade.

2.2 Isolation of Human Neutrophils

Human leukocytes were purified from freshly drawn heparinized blood of healthy donors and isolated by one-step procedure with the Ficoll-Hypaque medium purchased from Axis-Shield, Oslo, Norway (Ferrante and Thong 1980). Ice-cold isotonic phosphate-saline buffer, pH 7.4, containing 5 mM glucose was used to suspend the cells. To check cell viability trypan blue exclusion test was used obtaining a survival value higher than 90 % up to 6 h after purification.

2.3 Activation of Human Neutrophils

Human neutrophils were activated by 1 μg/mL PMA. The incubation mixture contained 15×10^6 cells/mL in phosphate-saline buffer with 5 mM glucose, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$. When present, thiotaurine was 1 mM. After 5 min at 37°C, the reaction was started by adding PMA to cell suspension to trigger the oxidative burst. Neutrophils were collected by centrifugation at 2,750 x g after 15 min incubation at 37°C and lysed in 0.5 mL ice-cold lysis buffer, pH 7.4, containing 10 mM Tris, 0.5% NP40, 60 mM KCl, and 1 mM EDTA. Protease inhibitors were added to a final concentration of 10 μM APMSF (4-amidinophenylmethanesulfonyl fluoride), 10 μg/mL aprotinin, and 10 μg/mL pepstatin.

2.4 Proteomic Analysis of Human Neutrophils

Cell lysates were centrifuged at 14,000 x g for 20 min and protein concentration in the supernatant was determined by Bradford assay. A volume corresponding to 300 μg protein of control and treated cell samples were precipitated with cold ethanol (overnight at -20°C). The protein separation by pI and molecular weight was performed according to Cattaneo et al. (2015).
The interesting spots were processed via tryptic proteolysis, the peptide mixtures were analysed by MALDI-ToF mass spectrometry (AutoFlex II, Bruker Daltonics, Bremen, Germany) and the resulting peptide mass fingerprints used to identify proteins by Mascot search engine (Di Domenico et al. 2016).

2.5 Western blot analysis

Equal amount (15 μg) of total protein/lane were separated by electrophoresis on a 4-12% gradient SDS-polyacrylamide gel (Bolt® Bis-Tris Plus gels, Life Technologies, Carlsbad, CA, USA), as previously described (Canterini et al. 2013; Palladino et al. 2016). Membranes were incubated overnight at 4°C with anti-GAPDH (Sigma-Aldrich, 1:8000 dilution) and anti-β actin (AbCam, 1:1000 dilution) primary antibodies, then washed and incubated with the appropriate secondary antibody. Blots were evaluated by using a Gel Doc 2000 videodensitometer (Biorad,

Hercules, CA, USA). After normalization of the band densities against the β-actin, the percentage was averaged for three replicates of three different biological samples and compared between groups to find out statistically significant (two-way ANOVAs, $p \leq 0.05$) differences.

3 RESULTS

3.1 Human neutrophil proteome profiles

Two-dimensional gel electrophoresis (2-DE) method was applied to evaluate protein expression profiles in human neutrophils. Colloidal Coomassie staining of a 2-DE gel showed at least 300 protein spots in the cell lysates. A representative proteome profile of resting human neutrophils is shown in figure 1. Gels obtained from lysates of resting neutrophils, resting neutrophils treated with 1 mM thiotaurine, PMA-activated neutrophils, and PMA-activated neutrophils treated with 1 mM thiotaurine were compared to identify differences in protein patterns. The protein pattern analysis focused on the protein spots with different expression levels. Colloidal Coomassie-stained gels were comparatively evaluated by dedicated software for image analysis in order to define quantitative changes of protein density spots from treated and untreated cells. Only spots with a statistically significant variation in abundance were excised from the gel, undergone proteolysis and MS analysis. The identification of protein spots was allowed by the database search considering experimental results from Peptide Mass Fingerprinting MALDI-ToF. Among the recognized proteins, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed a significantly different expression level in neutrophils activated by PMA and neutrophils treated with 1 mM thiotaurine before PMA activation as compared to resting neutrophils. As highlighted in the boxes of figure 1, the PMA-stimulated neutrophils showed high level of expression of glycolytic GAPDH enzyme compared to the level of the same enzyme in the resting neutrophils. Conversely, a decreased amount of GAPDH was observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. Tryptic peptide mass fingerprint of GAPDH spot is shown in figure 2.

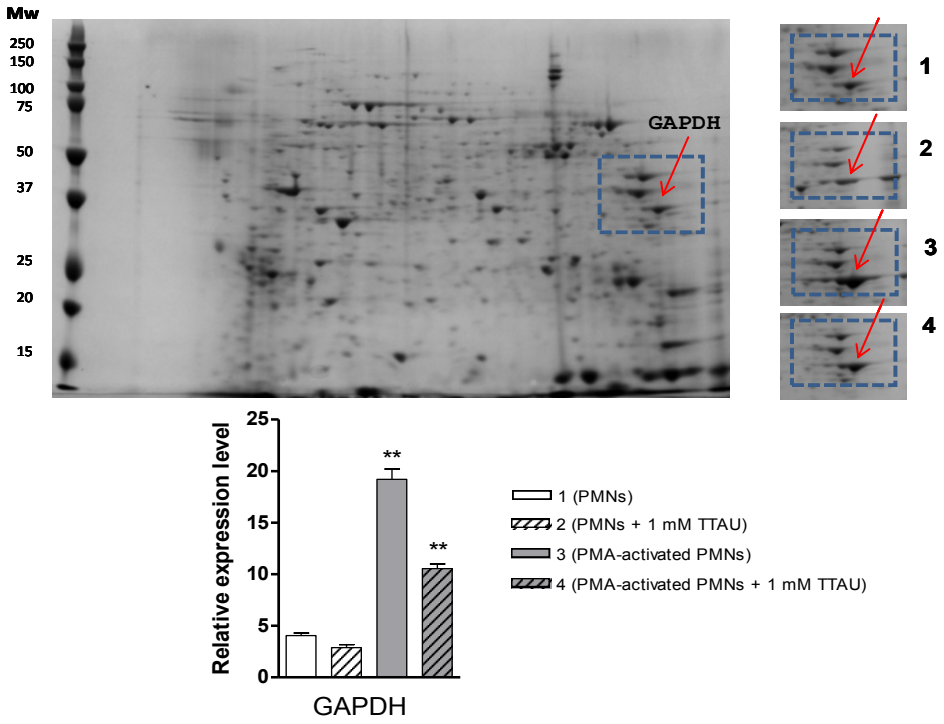

Fig. 1. 2-DE human neutrophil proteome map: effect of thiotaurine on expression level of GAPDH. Proteins were separated in the pH 3–10 NL range and the 200–15 kDa molecular mass range and visualized by colloidal Coomassie staining. In the boxes, protein spot corresponding to GAPDH is indicated by a red arrow. The relative intensities of GAPDH expression in resting and PMA-activated neutrophils with/without 1 mM thiotaurine are shown in the bar graph. **p $\leq$ 0.05 was evaluated by Student's t-test.

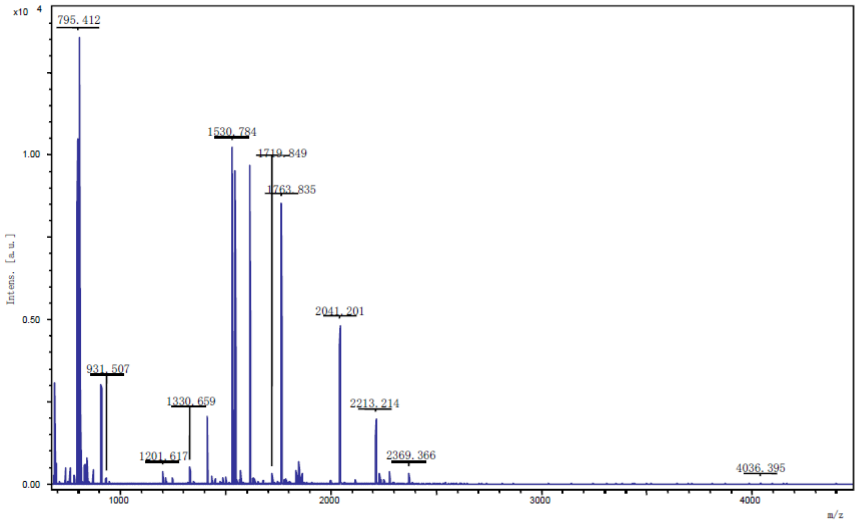

Fig. 2. MALDI-ToF mass spectrum of tryptic peptide fingerprint of GAPDH spot

3.2 Western blot analysis of GAPDH

To verify proteome data, Western blot analysis with antibodies directed against the GAPDH protein was performed. Results, reported in figure 3, show that 1 mM thiotaurine decreases the expression level of the glycolytic enzyme GAPDH in PMA-activated neutrophils (lane 4), thereby confirming the data obtained by proteomic analysis. Thiotaurine does not affect the expression level of GAPDH in resting human neutrophils.

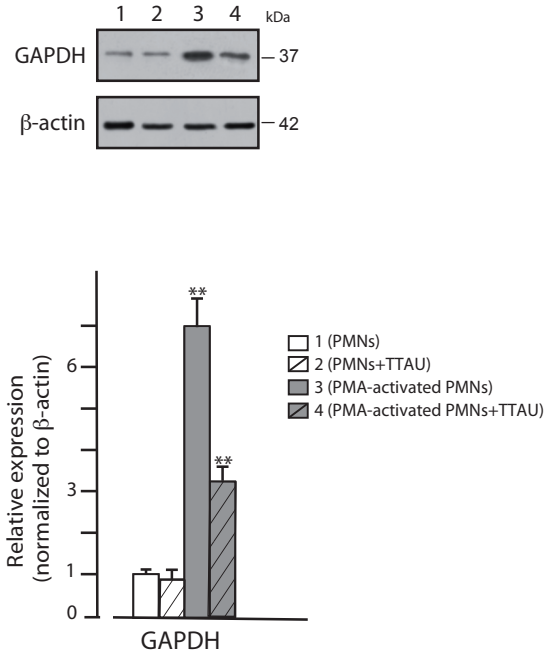

Fig. 3. Western blot analysis of human neutrophil proteins: effect of thiotaurine on expression level of GAPDH. Western blot analysis of protein extracts from human neutrophils and probed with primary antibodies direct against GAPDH. Histograms represent the GAPDH abundance (mean ± SEM) determined by protein band densitometry of three separate experiments, β-actin was used as internal standard. ** $p \leq 0.01$

4 DISCUSSION

Proteome analysis of human neutrophils comparing protein extracts of resting neutrophils, PMA-activated neutrophils, and PMA-activated neutrophils treated with thiotaurine highlighted a different expression level of some proteins. The expression level of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as identified by mass spectrometry and Western blot analysis caught our attention. The PMA-stimulated neutrophils showed high level of expression of GAPDH

compared to the level of the same enzyme in the resting neutrophils. Conversely, decreased amount of GAPDH was observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. This result suggests that thiotaurine exhibits a bioactive role in the mechanisms underlying the inflammatory process, influencing the energy metabolism of activated leukocytes, but especially it gives insights in the cell mechanism involved in the modulation of human leukocyte activation by thiotaurine. Interestingly, one of the first proteins shown to undergo persulfidation, alternatively called sulfhydration, was GAPDH. It has been described a sevenfold increase of GAPDH activity after sulfhydration of cysteine 150 (Mustafa et al. 2009). Conversely, cysteine residue nitrosylation abolishes GAPDH activity (Hara et al. 2005). Protein persulfidation is considered a major pathway in sulfide signaling (Mustafa et al. 2009; Paul and Snyder 2012). Despite the various examples reported in literature, it is still unclear whether protein sulfhydration by H_2S is important in cell signalling and which are the mechanisms underlying this modification (Mishanina et al. 2015). The low intrinsinc reactivity of H_2S towards oxidized thiols like disulfides has also raised questions regarding the direct involvement of H_2S in signaling and led to the consideration of alternative sulfur donors in protein persulfidation reactions (Kabil and Banerjee 2014; Cuevasanta et al. 2015; Yadav et al. 2016). Moreover, biological H_2S donors and how this gasotransmitter is mobilized from cell stores remain to be identified (Paul and Snyder 2015). Up to now, the main source of H_2S *in vivo* is the desulfuration of cysteine by enzymes of the transulfuration pathway and 3-mercaptopyruvate sulfurtransferase (Singh et al. 2009; Kabil and Banerjee 2014). It has been suggested that sulfane sulfur pool can represent physiological stores able to release H_2S. (Kimura 2011). At this regard, thiotaurine with its sulfane sulfur moiety can be part of the sulfur store pool and represent a biologically relevant sulfur donor in protein persulfidation reactions. It has been demonstrated that under reducing condition, such as in the presence of GSH, H_2S is released from thiotaurine (Chauncey and Westley 1983). Accordingly, in human neutrophils GSH induced the release of H_2S from thiotaurine (Capuozzo et al. 2013). In conclusion, our result raises the possibility that thiotaurine, acting as a sulfur donor, could modulate neutrophil activation via persulfidation of target proteins, such as GAPDH. Furthermore, this result would confirm that thiotaurine takes part to mammalian biochemical pathways involved in sulfide transport, release and storage. This role is further supported by the ability of hypotaurine to readily incorporate H_2S with thiotaurine generation (De Marco and Tentori 1961). This reaction between H_2S and hypotaurine, the latter present at millimolar

concentration in leukocytes, very likely occurs during inflammation (Learn et al. 1990).

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