Thiotaurine Modulates Human Neutrophil Activation

Elisabetta Capuozzo, Alessia Baseggio Conrado, and Mario Fontana

Abbreviations

fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
HTAU	Hypotaurine
PMA	Phorbol 12-myristate 13-acetate
PKC	Protein kinase C
ROS	Reactive oxygen species
TAU	Taurine
TTAU	Thiotaurine

1 Introduction

Neutrophils are well recognized as one of the major players during acute inflammation. They are typically the first leukocytes to be recruited to an inflammatory site and can eliminate pathogens by multiple means. Two different microbicidal mechanisms occur within the neutrophils: the oxidative and the non oxidative systems. The oxygen-dependent mechanism acts through generation of reactive oxygen species (ROS), and the oxygen-independent mechanism acts through production of antimicrobial peptides and proteolytic enzymes. During inflammation, neutrophils are activated in response to several agonists generating superoxide anion and other ROS by NADPH oxidase-dependent mechanisms. This functional response, termed

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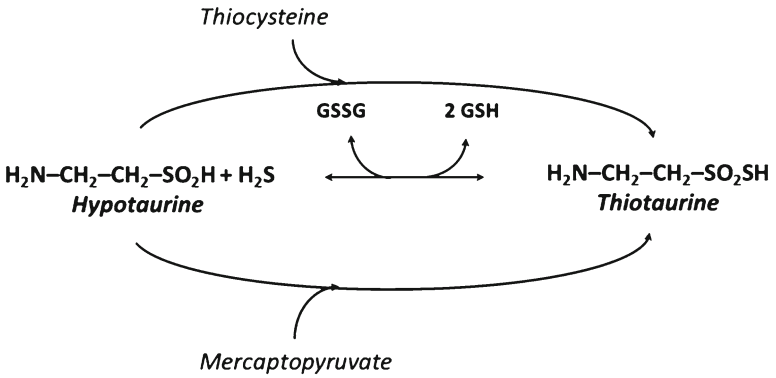
oxidative burst, contributes to host defense, but it can also result in collateral damage of host tissues. NADPH oxidase is a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to superoxide anion. NADPH oxidase is activated by a variety of agents including *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA). These stimuli trigger biochemical cascades leading to the phosphorylation of several proteins of the NADPH oxidase system (Morel et al. 1991). In addition to the well-documented PKC pathway, one of these cascades involves activation of members of the mitogen-activated protein kinase (MAPK) family. Several studies have demonstrated that MAPK pathways such as extracellular signal-regulated kinases (ERK) 1/2 and p38 MAPK are activated in human neutrophils (El Benna et al. 1996; Nick et al. 1997; Dewas et al. 2000).

Taurine is the most abundant free amino acid in most animal tissues and plays an important role in several essential biological processes (Huxtable 1992). A large number of reports have demonstrated the key role of taurine and its derivatives in the innate immune response (Schuller-Levis and Park 2004). It is widely recognized that taurine and related compounds such as hypotaurine and taurine chloramine exert a regulatory role in acute inflammation (Green et al. 1991; Marcinkiewicz and Kontny 2014; Kim and Cha 2014). The protection by taurine and its derivatives on inflammatory injury may be due to modulation of NADPH oxidase activity. It is noteworthy that taurine chloramines decrease PMA-stimulated superoxide production in human neutrophils by inhibiting phosphorylation of subunits of NADPH oxidase, eventually blocking the assembly of NADPH oxidase complex (Choi et al. 2006). Recently, it has been shown that thiotaurine (2-aminoethane thiosulfonate), a biomolecule structurally related to hypotaurine and taurine, prevents spontaneous apoptosis of human neutrophils (Capuozzo et al. 2013) and counteracts the damaging effect of oxidants in diabetic rat (Budhram et al. 2013). Interestingly, thiotaurine contains a sulfane sulfur that can be released as hydrogen sulfide (H_2S) (Westley and Heyse 1971; Capuozzo et al. 2013). It has been shown that H_2S plays relevant roles, modulating several pathophysiological processes, including inflammation (Zanardo et al. 2006; Predmore et al. 2012). Taken together, these observations raise the possibility that thiotaurine, analogously to taurine and its derivatives, could modulate neutrophil activation.

Thiotaurine is a thiosulfonates (RSO_2SH) which has been occasionally detected among the products of biochemical reactions involving sulfur compounds. Thiotaurine is a metabolic product of cysteine in vivo (Cavallini et al. 1959; Cavallini et al. 1960) and is produced by a spontaneous transsulfuration reaction involving thiocysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961) (Scheme 1). Moreover, a sulfurtransferase which catalyzes the transfer of sulfur from mercaptopyruvate to hypotaurine with production of thiotaurine has been also reported (Sörbo 1957; Chauncey and Westley 1983).

In the present study, thiotaurine has been assessed for an activity on functional response of human neutrophils. The results reveal that thiotaurine modulates fMLP- and PMA-mediated activation of human neutrophils, by inhibiting total ROS generation and superoxide anion production. Compared with fMLP-activated



Scheme 1 Biochemical pathway of thiotaurine

neutrophils, PMA-activated neutrophils were more susceptible to thiotaurine inhibition, suggesting that thiotaurine may interfere with the PKC-dependent pathway of neutrophil activation.

2 Materials and Methods

2.1 Chemicals

Thiotaurine (2-aminoethane thiosulfonate) was prepared from hypotaurine and elemental sulfur (Cavallini et al. 1959). Taurine, hypotaurine, sodium hydrosulfide (NaHS), sulfur, luminol, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). Cytochrome *c* from horse heart and cytochalasin B were from Fluka Chemie GmbH (Buchs, CH). MeOSuc-Ala-Ala-Pro-Val-AMC, elastase substrate, was from Enzo Life Sciences (Lausen, CH). All other chemicals were analytical grade.

2.2 Isolation of Neutrophils

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90–98 % neutrophils were obtained by one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) (Ferrante and Thong 1980). The cells were suspended in isotonic phosphate-buffered saline, pH 7.4, with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90 % up to 6 h after purification. The incubations were carried out at 37 °C.

2.3 Respiratory Burst of Neutrophils: Luminol Enhanced Chemiluminescence

Human neutrophils were activated by 1 μM fMLP or by 1 μg/mL PMA. Total ROS production was evaluated by luminol enhanced chemiluminescence method (Klink et al. 2003). Neutrophils were distributed into 96-well black plate (1×10^6 cells/well) and incubated with test compound at various concentrations for 5 min at 37 °C. Then, PMA or fMLP was added to the cells to initiate respiratory burst and luminol (10 μM) to enhance chemiluminescence. Cytochalasin B (1 μg/mL) was added to fMLP-stimulated neutrophils. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Chemiluminescence intensity was given in relative luminescence units (RLU). Data were expressed as the area under the curve of chemiluminescence (total RLU).

2.4 Respiratory Burst of Neutrophils: Superoxide Anion Production

Human neutrophils were activated by 1 μM fMLP or by 1 μg/mL PMA. Superoxide production by NADPH oxidase was estimated by measuring the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm ($\varepsilon = 21{,}100\ M^{-1}cm^{-1}$ for ferrocytochrome *c*) by a modification of the method described by Lehmeyer et al. (1979). The incubation mixture contained 2×10^6 cells/mL, 80 μM cytochrome *c* in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose. After 3 min of preincubation at 37 °C, the reaction was started by adding PMA or fMLP. Cytochalasin B (1 μg/mL) was added to fMLP-stimulated neutrophils. The controls contained, in addition, 20 μg/mL superoxide dismutase. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve.

2.5 Determination of Neutrophil Degranulation by Elastase Release

Degranulation of azurophilic granules was determined by elastase release (Sklar et al. 1982). Elastase release was measured by hydrolysis of the elastase substrate (MeOSuc-Ala-Ala-Pro-Val-AMC). Briefly, isolated neutrophils (2×10^6 cells/mL) were resuspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 1 μg/mL cytochalasin B at 37 °C. The elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) was added at a final concentration of 40 μM. After 3 min of preincubation at 37 °C, the reaction was started by adding 1 μM fMLP, and elastase activity was monitored fluorometrically (excitation wavelength 380 nm, emission wavelength 460 nm).

2.6 Statistical Analysis

Results are expressed as mean ± SEM for at least three separate experiments. Graphics and data analysis were performed using GraphPAD prism 4 software. Statistical analyses were performed using the Student's *t*-test or the ANOVA and Bonferroni post hoc test. $p \leq 0.05$ was deemed significant.

3 Results

3.1 Effect of Thiotaurine and Related Compounds on Respiratory Burst of Human Neutrophils

The stimuli which trigger in vitro neutrophil response include substances, such as the chemotactic peptide fMLP, that bind to specific receptors, or substitutes of diacylglycerol, like PMA, that activate protein kinase C (PKC) directly. To investigate if thiotaurine could influence neutrophil response, thiotaurine was explored for its effect on respiratory burst. Preincubation of human neutrophil with 100 μM thiotaurine strongly reduced PMA-induced respiratory burst, as evaluated by luminol enhanced chemiluminescence (Fig. 1). Thiotaurine caused a decrease of respiratory burst of PMA-stimulated neutrophils in a dose-dependent manner (Fig. 1, inset). Addition of 100 μM thiotaurine induced 64.4 % inhibition of total ROS production by PMA-activated cells (Fig. 2). Conversely, fMLP-stimulated cells were slightly

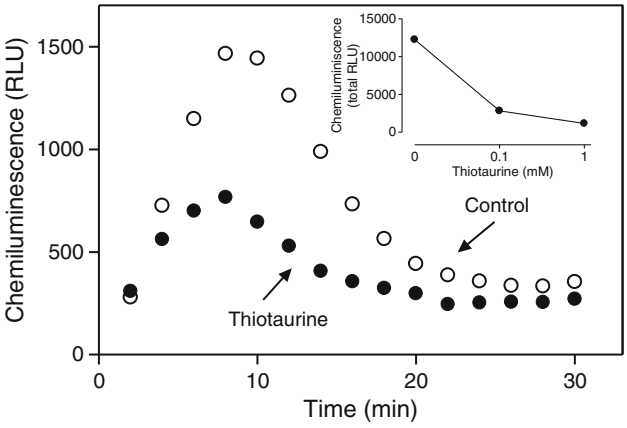


Fig. 1 Inhibition of human neutrophil respiratory burst in response to PMA by thiotaurine. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated with 100 μM thiotaurine for 5 min at 37 °C. Then, 1 μg/mL PMA was added to initiate respiratory burst. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Chemiluminescence intensity was given in relative luminescence units (RLU). Inset, dose-dependent inhibition of PMA-stimulated neutrophil respiratory burst by thiotaurine. Data were expressed as the area under the curve of chemiluminescence (total RLU)

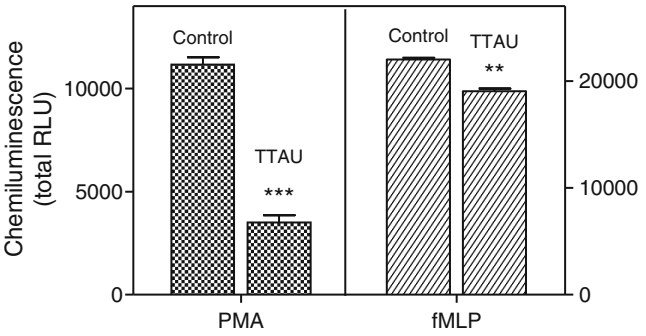


Fig. 2 Effect of thiotaurine on PMA- and fMLP-stimulated neutrophil respiratory burst. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated with 100 μM thiotaurine for 5 min at 37 °C. Then, 1 μg/mL PMA or 1 μM fMLP was added to initiate respiratory burst. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Data were expressed as the area under the curve of chemiluminescence (total RLU). **$p<0.01$ and ***$p<0.001$, compared with the control value (PMA- or fMLP-stimulated neutrophil luminol enhanced chemiluminescence in the absence of thiotaurine)

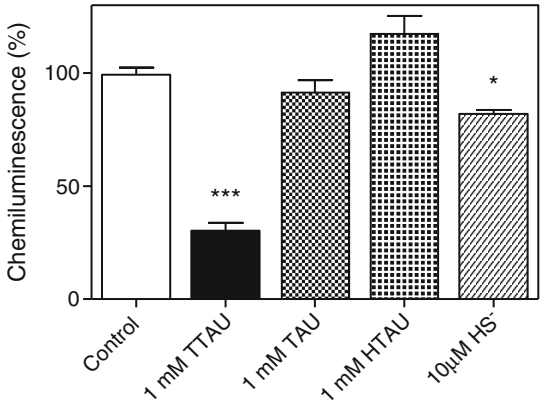


Fig. 3 Comparison of the effect of thiotaurine-related compounds and hydrogen sulfide on PMA-stimulated neutrophil respiratory burst. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated at 37 °C. Thiotaurine and other test compounds at the indicated concentrations were added 5 min before activation by 1 μg/mL PMA. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Data were estimated from the area under the curve of chemiluminescence and expressed as percentage of the control. *$p<0.05$ and ***$p<0.001$, compared with the control value (PMA-stimulated neutrophil luminol enhanced chemiluminescence in the absence of thiotaurine)

affected by thiotaurine. A 13.4 % inhibition of respiratory burst was observed in fMLP-stimulated cells (Fig. 2).

As thiotaurine can release sulfane sulfur producing hydrogen sulfide and hypotaurine, the effect of these compounds on neutrophil respiratory burst has been evaluated. Thiotaurine (TTAU, 1 mM) was more effective than taurine (TAU, 1 mM), hypotaurine (HTAU, 1 mM) and hydrogen sulfide (HS^-, 10 μM) (Fig. 3).

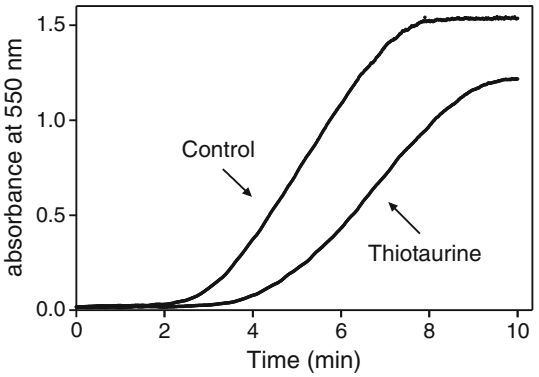


Fig. 4 Time course of superoxide anion generation by PMA-activated neutrophils with/without 1 mM thiotaurine. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in Sect. 2. The absorbance at 550 nm was monitored. Thiotaurine was added 3 min before activation

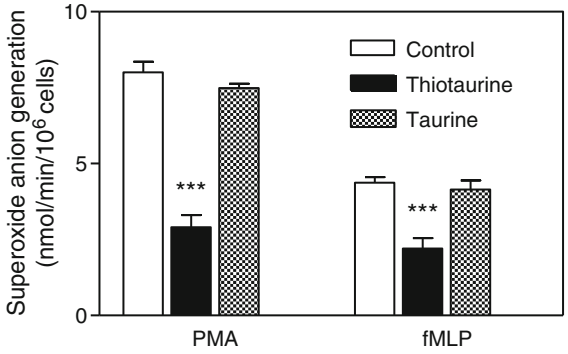


Fig. 5 Effect of thiotaurine and taurine on PMA- and fMLP-stimulated superoxide anion generation. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in Sect. 2. Neutrophils were activated by 1 μg/mL PMA or 1 μM fMLP at 37 °C and the absorbance at 550 nm was monitored. Thiotaurine or taurine (1 mM) was added 3 min before activation. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. ***$p < 0.001$, compared with the control value (PMA- or fMLP-stimulated neutrophil superoxide anion generation in the absence of thiotaurine)

3.2 Thiotaurine Inhibition of Superoxide Anion Generation by Human Neutrophils

Superoxide anion is produced by neutrophils as a result of NADPH oxidase activation. NADPH oxidase has been activated by chemotactic peptide, fMLP or by PKC activator, PMA. Addition of 1 mM thiotaurine to human neutrophils in suspension did not stimulate superoxide anion generation. However, 1 mM thiotaurine added to neutrophils 3 min before activation by 1 μg/mL PMA led to an inhibition of superoxide anion generation, as estimated by measuring cytochrome *c* reduction (Fig. 4). Thiotaurine (1 mM) exhibited 58.6 and 49.4 % inhibition of superoxide anion generation by PMA- and fMLP-stimulated human neutrophils, respectively (Fig. 5).

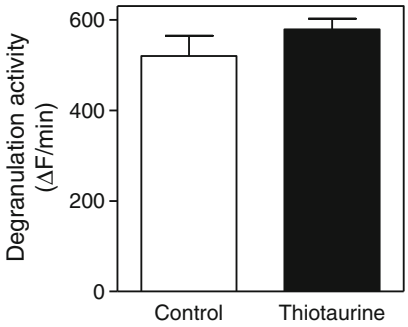


Fig. 6 Effect of thiotaurine on fMLP-stimulated neutrophil degranulation. Degranulation activity was determined by elastase release. Elastase activity was measured fluorometrically by hydrolysis of the elastase substrate (MeOSuc-Ala-Ala-Pro-Val-AMC) as described in Sect. 2. Neutrophils were incubated with 1 mM thiotaurine at 37 °C for 3 min before activation by 1 μM fMLP

3.3 Effect of Thiotaurine on Human Neutrophil Degranulation

Pretreatment of neutrophils with thiotaurine up to 5 mM concentration did not affect fMLP-induced azurophilic degranulation as evaluated by measuring the activity of released elastase (Fig. 6). Addition of thiotaurine to unstimulated neutrophils had no effect on their degranulation activity.

4 Discussion

Thiotaurine, a biomolecule structurally related to hypotaurine and taurine, has recently been found to prevent spontaneous apoptosis of human neutrophils (Capuozzo et al. 2013). In the present study, the influence of thiotaurine on human neutrophil functional responses was examined to further characterize its bioactivity toward proinflammatory leukocytes, which play a pivotal role in acute inflammation. Thiotaurine (0.1–1 mM) inhibited neutrophil activation in response to PMA or fMLP, as indicated by the inhibition of total ROS production, evaluated by luminol enhanced chemiluminescence, and of superoxide anion generation, estimated by measuring cytochrome *c* reduction. These results reveal that this thiosulfonate can attenuate leukocyte functions.

Important functional responses such as activation of NADPH oxidase leading directly to superoxide anion production likely occur through more than one signal transduction pathway, depending on the type of stimuli tested. In this respect, it has been reported that fMLP triggers both ERK 1/2-MAPK and PKC pathways to phosphorylate $p47^{phox}$ leading to NADPH oxidase activation, whereas PMA-induced phosphorylation of $p47^{phox}$ is mainly dependent on conventional PKC activation

(Dewas et al. 2000). Interestingly, PMA-activated neutrophils were considerably more susceptible to the inhibitory effects of thiotaurine than fMLP-activated cells. Thiotaurine strongly inhibits PMA-induced total ROS production in human neutrophils. Conversely, fMLP-induced response is influenced by this biomolecule at minor extent. Moreover, fMLP-induced neutrophil azurophilic degranulation is completely unaffected by thiotaurine. Taken together, these data suggest that thiotaurine modulates the activation pathways which trigger neutrophil response by interfering mainly with a PKC-dependent pathway.

The molecular mechanism by which thiotaurine modulates human leukocyte activation is difficult to explain taking into account only these results. Thiotaurine exhibits a peculiar activity on the neutrophil functional response compared to structurally related compounds. Under our experimental conditions, hypotaurine and taurine are not effective in modulating the activation of neutrophils. Although thiotaurine and related compounds are well-known free radical scavengers, the inhibition of respiratory burst of human neutrophils by thiotaurine could not be ascribed only to its antioxidant action (Aruoma et al. 1998; Fontana et al. 2004; Acharya and Lau-Cam 2013). However, thiotaurine contains a sulfane sulfur that can be released as hydrogen sulfide (H_2S), which plays regulatory roles in inflammation (Zanardo et al. 2006). In agreement, we found that H_2S inhibits respiratory burst as evaluated by luminol enhanced chemiluminescence. Moreover, sulfane sulfur moiety displays the unique ability to attach reversibly to other sulfur atoms (Toohey 1989). On the basis of this property, sulfane sulfur has been found to have regulatory effects in diverse biological systems (Beinert 2000; Mueller 2006). These functions include activation or inactivation of enzymes (Toohey 2011). It is possible that sulfane sulfur of thiotaurine can modulate by this mechanism enzymes involved in the signalling cascades leading to human neutrophil activation. In this regard, we suggested a role of thiotaurine as a biochemical intermediate in the transport, storage and release of sulfide in mammalian cells, such as neutrophils (Capuozzo et al. 2013). This hypothesis is further supported by the fact that hypotaurine, present in leukocytes at millimolar concentration (Learn et al. 1990), can readily incorporate H_2S formed during inflammation with production of thiotaurine (De Marco and Tentori 1961).

The cellular mechanism by which thiotaurine exerts a regulatory effect on neutrophil functional responses requires further study. However, these results indicate that thiotaurine displays a notable bioactive role that may provide new insights into the molecular inflammatory mechanisms and lead to the development of new therapeutic approaches for inflammation.

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