Chapter 19
Thiotaurine Prevents Apoptosis of Human Neutrophils: A Putative Role in Inflammation

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Abstract Thiotaurine, a metabolic product of cystine, contains a sulfane sulfur atom that can be released as H_2S, a gaseous molecule with a regulatory activity on inflammatory responses. The influence of thiotaurine on human leukocyte spontaneous apoptosis has been evaluated by measuring caspase-3 activity in human neutrophils. Addition of 100 μM thiotaurine induced a 55% inhibition of caspase-3 activity similar to that exerted by 100 μM H_2S. Interestingly, in the presence of 1 mM GSH, an increase of the inhibition of apoptosis by thiotaurine has been observed. These results indicate that the bioactivity of thiotaurine can be modulated by GSH, which promotes the reductive breakdown of the thiosulfonate generating H_2S and hypotaurine. As thiotaurine is able to incorporate reversibly reduced sulfur, it is suggested that the biosynthesis of this thiosulfonate could be a means to transport and store H_2S.

Abbreviations

GSH	Glutathione
HTAU	Hypotaurine
TTAU	Thiotaurine

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19.1 Introduction

Thiotaurine (2-aminoethane thiosulfonate) is a biomolecule structurally related to hypotaurine and taurine. Thiosulfonates (RSO_2SH), including thiotaurine, have been occasionally detected among the products of biochemical reactions involving sulfur compounds. Thiotaurine is a metabolic product of cystine in vivo (Cavallini et al. 1959, 1960) and is produced by a spontaneous transulfuration reaction involving thiocysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961). Moreover, an enzyme capable of oxidizing thiols to sulfinates and thiosulfonates, in the presence of inorganic forms of sulfur has been detected in a number of animal tissues (Cavallini et al. 1961). 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).

Recently, it has been shown that hydrogen sulfide (H_2S), an endogenously generated gaseous molecule, plays relevant signal roles, modulating several pathophysiological functions (Predmore et al. 2012). Though desulfuration of cysteine constitutes the main source of H_2S in mammals, thiotaurine contains a sulfane sulfur atom that can be released as H_2S (Westley and Heyse 1971). It is widely recognized that hypotaurine, taurine, and H_2S exert a regulatory activity on inflammatory responses (Green et al. 1991; Whiteman and Winyard 2011). However, thiotaurine has never been investigated for a bioactivity in inflammation.

In the present study, the influence of thiotaurine on human leukocyte spontaneous apoptosis has been evaluated. Neutrophil apoptosis is an important process because it provides a signal for neutrophil removal promoting resolution of inflammation, and because it results in the loss of functional neutrophil responsiveness (Savill and Fadok 2000; Simon 2003). On the other hand, increased survival in the inflamed tissue permits neutrophils to fulfill their effector functions most efficiently (Lee et al. 1993; Savill et al. 2002). Thus, modulation of apoptosis may have a major effect on the inflammatory process.

As several studies suggest a critical role of caspase-3 in both spontaneous and Fas receptor-mediated apoptosis in neutrophils (Weinmann et al. 1999; Ottonello et al. 2002), we tested the effect of thiotaurine on neutrophil apoptosis by measuring the caspase-3 activity in cell lysates of human neutrophils.

19.2 Materials and Methods

19.2.1 Chemicals

Thiotaurine was prepared from hypotaurine and elemental sulfur (Cavallini et al. 1959). L-Glutathione reduced, hypotaurine, sodium hydrosulfide (NaHS), sulfur, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate, acetyl-Asp-Glu-Val-Asp-7-amido-4-

methylcoumarin (Ac-DEVD-AMC, caspase-3 substrate) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). All other chemicals were analytical grade.

19.2.2 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 (PBS), 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.

19.2.3 Measurement of H_2S

Aliquots of the sample were mixed with distilled water to a final volume of 0.5 mL. Then 0.25 mL zinc acetate (1% w/v), 0.25 mL *N,N*-dimethyl-*p*-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 0.2 mL $FeCl_3$ (30 mM in 1.2 M HCl) were added. After 15 min at room temperature, the absorbance of the resulting solution was measured at 670 nm (Siegel 1965). All samples were assayed in duplicate and H_2S was calculated against a calibration curve of sodium hydrosulfide (NaHS, 2–100 μM).

19.2.4 Detection of Neutrophil Apoptosis by Caspase-3 Activity Assay

Caspase-3 activity was tested in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (Ac-DEVD-AMC) (Nicholson et al. 1995). Neutrophils (5×10^6 cells), preincubated in PBS with 5 mM glucose at 37°C for 3.5 h, were collected by centrifugation and lysed in 0.5 mL of 50 mM HEPES buffer, pH 7.4, containing 5 mM 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 10 μM 4-amidinophenylmethanesulfonyl fluoride (APMSF), 10 μg/mL pepstatin, and 10 μg/mL aprotinin. The reaction was started by adding 100 μL aliquots of the lysates in 2 mL solutions containing 16 μM AcDEVD-AMC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT, and 2 mM EDTA, pH 7.4. The assay mixture was incubated at 20°C in the dark for 1 h. The fluorescence (excitation

wavelength 360 nm, emission wavelength 460 nm) increase was compared with an appropriate blank control containing 10 μM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor (Nicholson et al. 1995) or standard preparations of recombinant caspase-3 (Sigma). A calibration curve obtained with standard AMC solutions was employed for quantitative analysis.

19.2.5 HPLC Analysis

Hypotaurine and thiotaurine were determined by HPLC using the *o*-phthaldialdehyde reagent (Hirschberger et al. 1985). Analyses were performed as previously described (Fontana et al. 2005), using a Waters 474 scanning fluorescence detector ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 450$ nm). The elution times of hypotaurine and thiotaurine were 22 min and 27 min, respectively.

19.2.6 Statistics

Results are expressed as means ± SEM for at least three separate experiments performed in duplicate. Graphics and data analysis were performed using GraphPad Prism 4 software.

19.3 Results

19.3.1 Influence of Thiotaurine on Human Neutrophil Spontaneous Apoptosis

Spontaneous apoptosis was evaluated by measuring caspase-3 activity in lysates of neutrophils (5×10^6 cells/mL) that were preincubated at 37°C for 3.5 h. When the preincubation step was performed in the presence of thiotaurine (TTAU), a concentration-dependent decrease of caspase-3 activity was observed (Fig. 19.1). As thiotaurine contains a sulfane sulfur atom that can be released as H_2S, the influence of NaHS on caspase-3 activity has been also evaluated. With 100 μM thiotaurine the reduction of caspase-3 activity was 55 ± 3%, similar to that exhibited by 100 μM NaHS (57 ± 3%). Control experiments (not shown) indicated that neither TTAU, nor NaHS, at concentrations ranging from 0.01 to 0.2 mM, affected the activity of recombinant caspase-3.

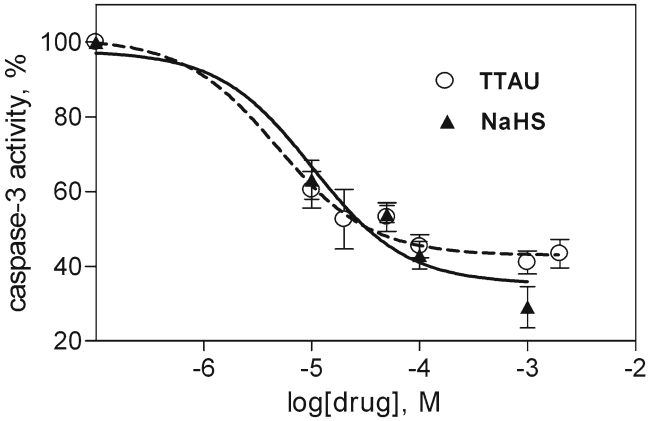

Fig. 19.1 Effect of thiotaurine and NaHS on caspase-3 activity. Neutrophils (5×10^6 cells/mL) were incubated at 37°C for 3.5 h with different concentrations of thiotaurine (TTAU) or NaHS. Caspase-3 activity was determined as described in Sect. 2

19.3.2 Effect of Glutathione on Thiotaurine-Induced Inhibition of Caspase-3 Activity

It is reported that glutathione (GSH) regulates neutrophil apoptosis by affecting caspase-3 activity (O'Neill et al. 2000). This effect has been attributed to its antioxidant activity (Wedi et al. 1999). To gain insights into the mechanism of inhibition by TTAU, the inhibitory effect of this thiosulfonate on caspase-3 activity has been compared with that of GSH (Fig. 19.2).

Under our experimental conditions, the inhibitory effect of 1 mM GSH (58±3%) on caspase-3 activity is similar to that of 0.1 mM TTAU. Interestingly, the inhibition of spontaneous apoptosis by 0.1 mM TTAU increases to 76±4% when GSH is present in the preincubation step.

19.3.3 Reductive Breakdown of Thiotaurine by Glutathione: Generation of H_2S and Hypotaurine

It is well known that thiol compounds such as GSH promote reductive breakdown of thiosulfonates generating H_2S and sulfinates (Chauncey and Westley 1983). Spontaneous and GSH-catalyzed H_2S release has been analyzed in the presence or in the absence of human neutrophils. Figure 19.3 shows that the release of H_2S is stimulated by GSH and it increases with the incubation time. Furthermore, it can be seen that the amount of H_2S results lower in the presence of cells. This result may depend on different factors, such as H_2S binding to proteins (Cavallini et al. 1970) or H_2S uptake by cells (Mathai et al. 2009).

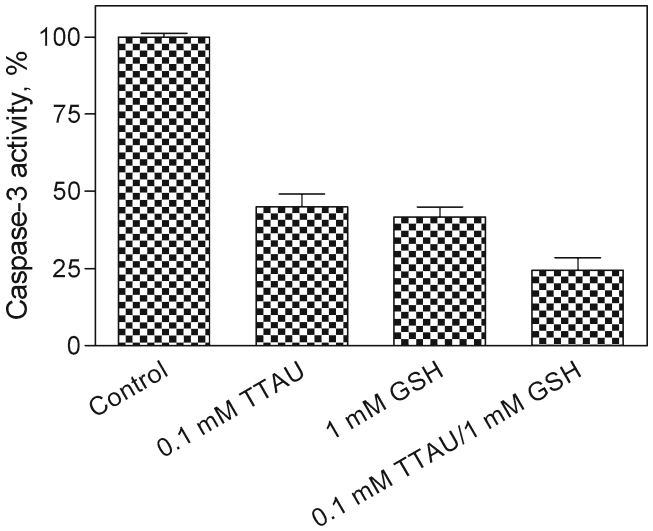

Fig. 19.2 Influence of glutathione on thiotaurine inhibition of caspase-3 activity. Neutrophils (5×10^6 cells/mL) were incubated at 37°C for 3.5 h in the absence (control) and in the presence of 0.1 mM thiotaurine (TTAU) or 1 mM glutathione (GSH) or both compounds. Caspase-3 activity was determined as described in Sect. 2

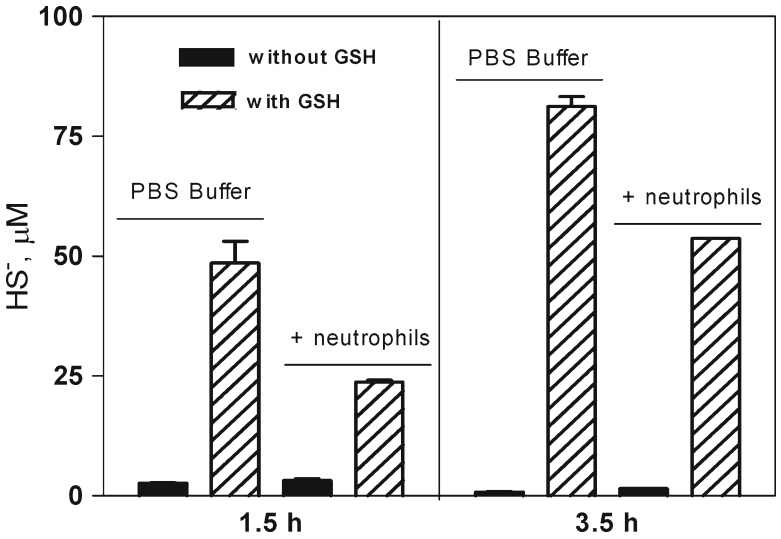

Fig. 19.3 Generation of H_2S by thiotaurine: effect of glutathione. 1 mM thiotaurine (TTAU) was added to neutrophils (5×10^6 cells/mL) and incubated at 37°C for 1.5 and 3.5 h. When present, glutathione (GSH) was 1 mM. The controls (PBS Buffer) were performed in the same conditions without neutrophils. H_2S was determined spectrophotometrically as described in Sect. 2

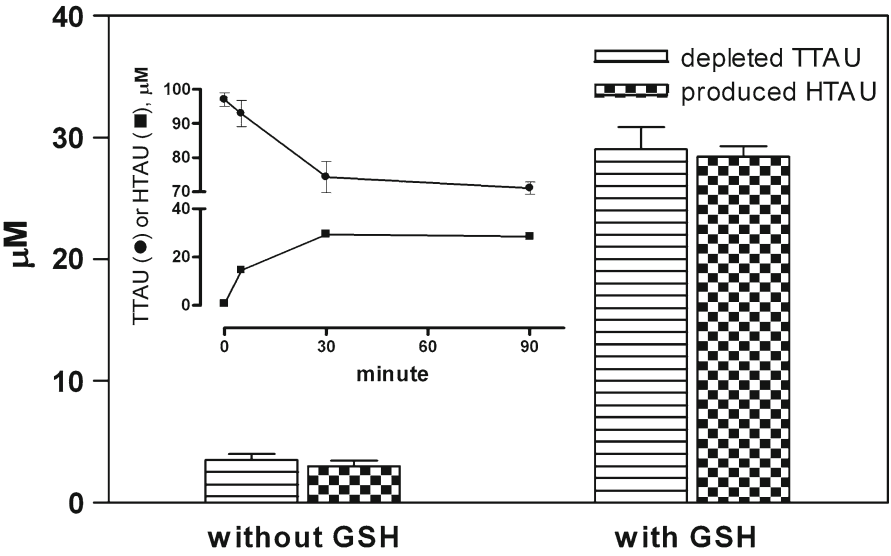

Fig. 19.4 Generation of hypotaurine by thiotaurine: effect of glutathione. 0.1 mM thiotaurine (TTAU) was added to neutrophils (5×10^6 cells/mL) and incubated at 37°C. When present, glutathione (GSH) was 1 mM. Hypotaurine (HTAU) and TTAU concentrations were determined by HPLC as described in Sect. 2. The amounts of depleted TTAU and produced HTAU, after 90 min incubation in the absence or in the presence of GSH, are compared. *Inset* time-course of the reaction of TTAU with GSH

Figure 19.4 shows that, in human leukocytes, GSH promotes the generation of hypotaurine (HTAU) as the main metabolite of TTAU. The production of HTAU increases with time (up to 30 min) and a stoichiometry of approximately 1 mol of HTAU produced/mol of TTAU depleted is observed (inset). The fate of TTAU has been evaluated also in the absence of cells; the results were similar to those observed with human leukocytes. Furthermore, GSH-mediated breakdown of TTAU in human neutrophils activated by phorbol 12-myristate 13-acetate (PMA) produces also taurine, the oxidative product of HTAU (not shown).

19.4 Discussion

These results indicate that the thiosulfonate, thiotaurine, may exert regulatory effects on inflammation influencing lifespan of human neutrophils. Mature circulating neutrophils are constitutively committed to apoptosis. During inflammatory response, survival of neutrophils recruited into the inflamed area is significantly prolonged. Increased survival in the inflamed tissue permits neutrophils to fulfill their effector functions most efficiently. On the other hand, macrophage-mediated elimination of apoptotic neutrophils from the inflamed area has been recognized as a crucial mechanism for promoting resolution of inflammation (Savill and Fadok 2000; Simon 2003).

It is recognized that the production of reactive oxygen species by activated cells accelerate the apoptosis and that superoxide release is required for spontaneous apoptosis (Ottonello et al. 2002; Scheel-Toellner et al. 2004). Moreover, the spontaneous and FAS-mediated apoptosis are prevented by antioxidants, such as GSH (Wedi et al. 1999). This effect has been ascribed to the ability of GSH to scavenge reactive oxygen species (Watson et al. 1997). It has been also shown that thiotaurine is highly effective in counteracting the damaging effect of oxidants (Acharya and Lau-Cam 2012). Thus, it is possible that the delay of spontaneous apoptosis of human neutrophils by thiotaurine may be related to its antioxidant activity. On the other hand, our results show that the inhibitory effect of thiotaurine on caspase-3 activity was higher than that of GSH. Moreover thiotaurine, in the presence of GSH, is more effective in influencing neutrophil apoptosis. These findings suggest that alternative or additional mechanisms of inhibition can be involved. It is well-known that GSH can act as a catalyst of the reductive breakdown of thiotaurine with generation of hypotaurine and H_2S (Chauncey and Westley 1983). Accordingly, we found that human neutrophils generate H_2S from thiotaurine with GSH as a necessary reductant in the reaction. It has been previously reported that H_2S promotes the short-term survival of neutrophils by inhibition of caspase-3 cleavage (Rinaldi et al. 2006). Our results confirm the effect of H_2S on prolonging the survival of neutrophils. Hence, it is likely that the sulfane sulfur of thiotaurine released as H_2S in the presence of GSH, may contribute to the observed effect on neutrophil survival.

The biological relevance of thiotaurine in mammalian is still a challenge to biochemical research. Biological roles have been sporadically reported (Costa et al. 1990; Baskin et al. 2000). On the contrary, in some marine organisms a key role for thiotaurine in the transport of sulfur has been strongly demonstrated (Pruski et al. 2001; Pruski and Fiala-Médioni 2003). Morevover, the metabolic origin of thiotaurine in mammalians is subject to debate, as is its fate. One pathway for thiotaurine metabolism is via transulfuration reactions with hypotaurine being the main intermediate (Cavallini et al. 1961; De Marco and Tentori 1961). These reactions can be spontaneous or catalyzed by sulfur transferases (De Marco et al. 1961; Chauncey and Westley 1983). Our experiments show that hypotaurine is the main metabolite of thiotaurine with a 1:1 stoichiometry, suggesting a role of thiotaurine as a biochemical intermediate in the transport, storage, and release of sulfide also in mammalians. 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).

Since thiotaurine as well as hypotaurine, taurine, and H_2S can modulate leukocyte functional responses, it would be worthy to investigate the metabolic and functional interplay between these sulfur compounds at inflammatory sites.

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