

ORIGINAL ARTICLE

Taurine chloramine inhibits functional responses of human eosinophils *in vitro*

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Summary

Background Eosinophils are prominent effectors of allergic inflammation. Taurine-chloramine (TauCl), a derivative of the amino acid taurine, shows antioxidant properties in different cell systems but its effects on eosinophils have not been reported.

Objective To study the effects of TauCl and taurine on functional responses of isolated human eosinophils activated by different stimuli.

Methods Human eosinophils were purified from the blood of healthy donors by a magnetic bead separation system. The effects of TauCl and taurine (0.1–1 mM) were investigated on the generation of superoxide anion (ferricytochrome-c reduction microassay), calcium signal (fluorimetry), p47^{phox}–p67^{phox} translocation (Western blot), leukotriene C₄ (LTC₄) production (enzymeimmunoassay), eosinophil peroxidase (EPO) release (spectrophotometry), eosinophil cationic protein (ECP) release (radioimmunoassay), apoptosis (flow cytometry with annexin V-propidium iodide), and nuclear factor-κB (NF-κB) activation (Western blot).

Results TauCl inhibited superoxide anion generation triggered by *N*-formyl-Met-Leu-Phe (fMLP; 30 nM), phorbol myristate acetate (1 nM) and serum opsonized zymosan (0.5 mg/mL) with similar potency (IC₅₀~200 μM) for the three stimuli, while taurine (0.1–1 mM) was scarcely effective. TauCl but not taurine inhibited p47^{phox}–p67^{phox} translocation. TauCl (200 μM) and taurine (1 mM) did not modify the [Ca²⁺]_i responses to fMLP. TauCl inhibited the release of EPO (IC₅₀~200 μM) and reduced ECP and LTC₄ production from fMLP-activated eosinophils while taurine was without significant effects. TauCl (1 mM) did not change constitutive apoptosis but significantly attenuated the ability of granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-5 to prevent apoptosis. The activation of eosinophil NF-κB induced by GM-CSF and IL-5 was suppressed by TauCl.

Conclusion Taurine is without significant *in vitro* effects on human eosinophil functions but its derivative TauCl inhibits oxidative burst and generation of inflammatory mediators, and reverses the survival effect produced by inflammatory cytokines. Therefore, endogenous TauCl may help to suppress excessive inflammatory response in eosinophils at inflammatory sites.

Keywords apoptosis, eosinophil cationic protein, eosinophil peroxidase, human eosinophils, leukotriene C₄, superoxide anion, taurine, taurine-chloramine

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Introduction

Eosinophils are key effector cells for the pathogenesis of allergic inflammation. Eosinophils release oxidant [superoxide anion, eosinophil peroxidase (EPO)] and non-

oxidant [leukotriene C₄ (LTC₄), eosinophil cationic protein (ECP)] proinflammatory mediators [1]. Enhanced levels of reactive oxygen species (ROS) and/or defective endogenous antioxidant defences appear involved in allergic inflammation [2, 3]. Eosinophils are very sensitive to oxidant stress [4, 5] and therefore, antioxidants could modulate eosinophil function and have a potential role in the treatment of allergic inflammation.

¹These two authors have contributed equally to this work.

Taurine is a ubiquitous β -amino acid found at high concentrations in the cytosol of human neutrophils, and intracellular concentrations of taurine in human eosinophils are close to that in neutrophils and higher than that found in other blood cells [6]. Taurine, either intracellularly or released to the extracellular medium, has been proposed to protect cells against attack by oxidants and as a modulator of inflammation [7, 8] including that in allergic asthma [9, 10]. Among other activities, taurine is a scavenger of hypochlorous acid (HOCl), a damaging oxidant produced by the myeloperoxidase–hydrogen peroxide–chloride system of activated neutrophils and monocytes. The reaction of taurine with HOCl forms taurine chloramine (TauCl) which is much less reactive and toxic than HOCl [11]. TauCl acts extracellularly but also within cells since it is transported into phagocytes, and it has been reported to down-regulate the inflammatory response in macrophages [12–15] and neutrophils [16, 17]. EPO may also react with hydrogen peroxide and chloride to generate HOCl, and its subsequent reaction with taurine also produces TauCl [18] that may contribute to killing of parasites by eosinophils [19]. Therefore, eosinophils may be exposed to TauCl formed by phagocytes at their vicinity in the inflammatory site but also to their own generated TauCl. However, neither the effects of taurine nor those of its derivative TauCl have been studied on the functional responses of eosinophils.

Therefore, the present work addresses the question of whether eosinophil functional responses related to the inflammatory role of these cells in allergic diseases may be inhibited by taurine and TauCl. Different stimuli were selected for eosinophil generation of superoxide anion (O_2^-). These included *N*-formyl-Met-Leu-Phe (fMLP), a soluble activator that binds to a specific membrane receptor, serum opsonized zymosan (SOZ), a particulate agonist that binds through the C3b receptor, and phorbol 12-myristate 13-acetate (PMA), that causes translocation of protein kinase C. Because fMLP has proven to be a valuable stimulus relevant to the activation of human eosinophils by allergens [20], we also examined the influence of TauCl and taurine on fMLP-induced calcium signal, and release of LTC_4 , ECP and EPO. Furthermore, since survival of eosinophils is related to oxidant stress and may be relevant to allergic inflammation [5, 21], the effects of TauCl and taurine on the constitutive and granulocyte-monocyte colony-stimulating factor (GM-CSF)- and IL-5-delayed apoptosis of eosinophils was also examined.

Methods

Isolation of human eosinophils and cytotoxicity assessment

Human blood from healthy donors was obtained in heparin, and PMNs were separated by standard labo-

ratory procedures [22]. Eosinophils were then separated by depletion of neutrophils with anti-CD16-coated magnetic microbeads using the magnetic cell-separation system (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) as outlined [23, 24]. Eosinophils of >95% purity (determined by May-Grünwald-Giemsa) and viability (trypan blue exclusion) were used. For cytotoxicity assessment, the percentage of lactate dehydrogenase (LDH) release compared with values in cell lysates was taken as marker for cell damage by using a commercially available colorimetric assay as outlined [24]. This investigation was approved by the institutional ethics committee and informed consent was obtained from all donors.

Superoxide anion generation

Release of superoxide anion from eosinophils was studied as described [25]. In brief, generation of superoxide anion was measured as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* with a modified microassay. With 96-well microtitre plates and a 200 μ L reaction volume, 10^5 cells were added to 100 μ mol/L of cytochrome *c* in Hanks balanced salt solution (HBSS) with 0.1% gelatin and 5 μ g/mL of cytochalasin B. To initiate the reaction, the cells were incubated with fMLP (30 nM), PMA (1 nM) or SOZ (SOZ, 0.5 mg/mL). Immediately after the addition of activator, the reaction wells were measured for absorbance at 550 nm in a Microplate Autoreader (EL309, Bio-Tek Instruments Inc., Winooski, VT, USA) followed by repeated readings for 60 min. Each reaction was performed in duplicate and against an identical control reaction which contained 20 μ g/mL of SOD (approximately 2000 U/mg protein). Results were adjusted to represent a 1 mL reaction volume, and superoxide anion generation was calculated with an extinction coefficient of 21.1×10^3 mol/L/cm as nanomols of cytochrome *c* reduced per 5×10^5 cells per time (min) minus SOD control. Treated cells were pre-incubated with taurine or TauCl (0.1, 0.5 or 1 mM) for 30 min at 37 °C before cell activation and the exposure was continued until the end of experiment. Control experiments were carried out in parallel with drug vehicle. Drug effects are expressed as percent inhibition from control values.

In order to determine the ability of taurine/TauCl to react directly with the oxygen metabolites generated by activated cells, formation of superoxide anion of a comparable magnitude was triggered in a cell-free assay system but otherwise similar conditions by a xanthine/xanthine oxidase system as outlined [25]. Possible interaction of the substances tested with the assay system (ferricytochrome *c*, xanthine–xanthine oxidase) was excluded (not shown). Neither taurine nor TauCl affected the uptake of opsonized zymosan [16].

Intracellular Ca^{2+} -levels

Measurement of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) was performed as described [26]. Cells (10^6 mL) were suspended in HBSS containing Ca^{2+} 1 mM, glucose 1 mM, bovine serum albumin 0.5%, and Fura-2/AM 5 μM , and incubated for 45 min at 37 °C. After loading, eosinophils were washed in pre-warmed HBSS and resuspended (5×10^5 cells/mL). Then, cells were incubated at 37 °C for 60 min with taurine (1 mM), TauCl (200 μM) or its vehicle before addition of fMLP (30 nM). The fluorescence intensity was monitored (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) using a spectrofluorometer (Perking Elmer LS50, Waltham, MA, USA) with thermally controlled cuvette holder and a magnetic stirrer. The peak values of $[\text{Ca}^{2+}]_i$ and the area under the curve over 60 s after activation were determined as outlined [26].

Analysis of the translocation of p47^{phox} and p67^{phox} to eosinophil membrane

Eosinophils (5×10^5 mL⁻¹) were pre-incubated in HBSS containing 0.5 mM CaCl_2 , 5.6 mM glucose and 20 mM HEPES (pH 7.4) in the presence of taurine (1 mM), TauCl (1 mM) or its vehicle for 30 min at 37 °C, and then stimulated with fMLP (100 nM) for 10 min. The translocation to the cell membrane of p47^{phox} and p67^{phox} was performed as outlined [24]. After standing on ice for 10 min, eosinophils were spun at 1500 g for 5 min at 4 °C and resuspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2 , 1.25 mM EGTA 10 mM PIPES, pH 7.4) supplemented with 0.8 mM PMSF, 10 $\mu\text{g}/\text{mL}$ pepstatin A, and 50 $\mu\text{g}/\text{mL}$ leupeptin. Cells were then disrupted by sonication on ice and spun at 500 g for 5 min at 4 °C. Resulting postnuclear supernatants were further centrifuged at 200 000 g for 20 min at 4 °C. The pellet, membrane fraction, was resuspended in 35 μL of 1 $\times$ sample buffer and kept at -80 °C until analysis. The protein content was measured using the Pierce bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL, USA). For immunoblot analysis, equal amounts (30 $\mu\text{g}/\text{lane}$) of protein from the membrane fraction were subjected to SDS-PAGE on 12% polyacrylamide gel, and the separated proteins were electrophoretically transferred onto nitrocellulose filters, blocked and probed with mouse anti- p47^{phox} and anti p67^{phox} monoclonal antibodies (BD Biosciences, San Jose, NJ, USA). After washing, blots were further probed with horseradish peroxidase-conjugated rabbit anti mouse IgG (DakoCytomation A/S, Glostrup, Denmark), and detected with the ECL Western Blotting Detection System (Amersham International PLC, Amersham Place Little Chalfon, Buckinghamshire, UK).

Quantitation of leukotriene C_4 production

LTC_4 assay was carried out as outlined [27]. After isolation, eosinophils were resuspended in HBSS with HEPES

(10 mM), calcium (1 mM), and magnesium (1 mM), pH 7.4, supplemented with 0.1% gelatine and 20 mM L-serine to a final concentration of 10^6 in 200 μL incubation volume. Eosinophils were pre-incubated with taurine (1 mM), TauCl (1 mM) or their vehicle for 30 min at 37 °C with cytochalasin B (5 $\mu\text{g}/\text{mL}$) added after the first 5 min of pre-incubation. Thereafter, the cells were stimulated with fMLP (1 μM) for 30 min at 37 °C. LTC_4 synthesis was terminated by immersion of the tubes in ice and the addition of 3 vol of ice-cold methanol. Cells were pelleted by centrifugation at 1500 g for 20 min at 4 °C. The methanolic supernatants (containing LTs released by cells) and extracts of cell pellets treated with 100% methanol for 18 h at 4 °C (containing LTs retained intracellularly) were evaporated to dryness in a speed vacuum concentrator, and stored at -80 °C before enzymeimmunoassay (EIA). Samples were reconstituted to original volume with ice-cold EIA buffer and LTC_4 was quantified as described by the manufacturer (Biotrak, RPN 224, Amersham Int.). Absorbances were measured at 450 nm with a microtitre plate photometer (Multiscan MKII, Labsystems, Haverhill, MA, USA). The assay uses horseradish peroxidase labelled LTC_4 and a rat anti- LTC_4 . The sensitivity of the assay is 0.5 pg/well. The assay has 100% and 30% cross-reactivity for LTD_4 and LTE_4 , respectively; cross-reactivity for LTB_4 was 0.3%, and cross-reactivity for other related compounds is negligible (<0.006%).

Determination of eosinophil peroxidase release

Release of EPO was measured as outlined [25]. Eosinophils were resuspended in HBSS-FBS, and aliquots of 10^5 cells in 100 μL were loaded onto microplate wells. Taurine (0.1–1 mM), TauCl (1 μM –1 mM) or their vehicle was added to each well and the plate was incubated for 30 min at 37 °C. Then, cells were activated with fMLP (1 μM plus 5 $\mu\text{g}/\text{mL}$ of cytochalasin B). The substrate solution (0.1 mM *o*-phenylenediamine dihydrochloride in 0.05 M Tris-HCl containing 0.1% Triton X-100 and 1 mM H_2O_2) was added to wells and the plate incubated (30 min, 37 °C) before stopping the reaction (4 M sulphuric acid), followed by centrifugation at 350 g for 5 min. Duplicate aliquots of supernatant (100 μL) were transferred onto a new plate. Kinetic assay for EPO was performed for supernatant of treated and untreated cells. The absorbance was determined at 492 nm using a Microplate Autoreader (EL309, Bio-Tek Instruments). The EPO release is expressed in peroxidase units/ 10^6 cells as determined from comparison with a standard curve. Drug effects are also expressed as per cent inhibition from control values.

Eosinophil cationic protein production

All assays were performed in duplicate at a cell concentration of 2.5×10^5 cells/mL in supplemented Dulbecco's

phosphate-buffered saline (PBS) [28]. Cell suspensions were pre-incubated with taurine (1 mM), TauCl (1 mM) or its vehicle for 30 min at 37 °C before stimulation; thereafter, the cells were activated with fMLP (100 nM) for 30 min. Experiments were terminated by centrifugation and supernatants were prepared for storage (−20 °C). Aliquots of the samples were taken for ECP measurements by a RIA method according to the manufacturer instructions (anti-ECP-¹²⁵I; Pharmacia, Uppsala, Sweden).

Eosinophil apoptosis and survival

Human eosinophils were analysed for apoptosis after 24 h of culture by flow cytometry using annexin V-fluorescein isothiocyanate and PI following the protocol indicated by the manufacturer (Annexin-V-Fluos; Roche Applied Science, Barcelona, Spain) in a flow cytometry analyzer (CyAn TM ADP; DakoCytomation A/S DK-2600), and as previously outlined [29]. Cells (1×10^4) were distinguished as early apoptotic (annexin V⁺, PI[−]), late apoptotic and/or necrotic (annexin V⁺ and PI⁺), and viable non-apoptotic (annexin V[−] and PI[−]) cells. Apoptosis was measured in the absence and presence of rhGM-CSF (1 ng/mL) or IL-5 (20 ng/mL) in cells treated with taurine and TauCl (each at 1 mM) or their vehicle. The concentration of GM-CSF and IL-5 were selected to produce a significant eosinophil survival effect as previously reported [21, 30].

In additional experiments, the effect of taurine and TauCl on eosinophil survival after 4 days of culture was studied as previously outlined [30]. Freshly isolated eosinophils were resuspended at a concentration of 2×10^6 cells/mL in supplemented RPMI. Then, 25 µL (~50 000 cells) of the cell suspension were cultured in a 96-well plate containing 75 µL supplemented RPMI with rhGM-CSF (1 ng/mL), and taurine and TauCl (each at 1 mM) were added simultaneously with the GM-CSF. The cells were cultured for a period of 4 days after which viability was assessed in duplicate by trypan blue exclusion.

Nuclear factor-κB activity assay

Eosinophils were incubated with or without rhGM-CSF (1 ng/mL) or IL-5 (20 ng/mL) for 24 h in the presence of taurine (1 mM), TauCl (1 mM) or vehicle. Cells were lysed in ice (10 min) in 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, and 15 µg/mL aprotinin and centrifuged at 18 000 g for 15 min. Protein concentrations were determined by using a BCA protein assay kit (Pierce). Equal amounts (30 µg/lane) of protein were subjected to SDS-PAGE [10% (w/v)] gel and separated proteins were electroblotted on polyvinylidene difluoride membranes (Bio-Rad Laboratories Ltd, Hertfordshire, UK) Western

analysis was performed with antibodies against nuclear factor-κB (NF-κB) p65 (c-20) (Santa Cruz Biotechnology INC, Santa Cruz, CA, USA) and against phosphorylated NF-κB p65 Ser536 (Cell Signalling Technology, Beverly, MA, USA).

Drugs and solutions; statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. SOZ was prepared immediately before use by boiling in saline (10 mg/mL) zymosan A particles for 10 min, washing twice in HBSS, and then resuspending in HBSS at 5 mg/mL; zymosan (0.5 mg/mL) was then incubated with 10% autologous serum. Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout. TauCl was prepared in our laboratory as outlined [31]. Briefly, 25 µL of 200 mM NaOCl was added to ice-cold 1 mL of 10 mM taurine in PBS with mixing. TauCl was used immediately after preparation. Each preparation of TauCl was monitored by measuring its UV absorption spectra (190–350 nm) in a UV spectrophotometer to assure that taurine formed a monochloramine without dichloramine formation. The maximum wavelength of TauCl was 252 nm whereas that of OCl[−] was 292 nm. The concentration of TauCl was determined spectrophotometrically assuming $\epsilon_{252 \text{ nm}} = 415 \text{ M/cm}$ [12, 31].

Data are presented as mean ± SE mean of *n* experiments. The IC₅₀ values were calculated from the concentration-inhibition curves by non-linear regression analysis. Statistical analysis of data was carried out by ANOVA followed by Bonferroni's test or by Student's *t* test as appropriate (GraphPad Software Inc., San Diego, CA, USA). Significance was accepted when *P* < 0.05.

Results

Taurine-chloramine produced no cytotoxicity in human eosinophils

The cytotoxicity of TauCl and taurine on human eosinophils has not been reported previously. Therefore, to exclude that any inhibition of eosinophil functions observed with these compounds was due to cell damage, we first examined the effect of TauCl and taurine on LDH release as a marker of cell injury. In this respect, the maximal concentrations used in the present study for TauCl and taurine showed no cytotoxicity (values are expressed as % of total LDH) as shown by $3.08 \pm 0.77\%$ for TauCl 1 mM (*n* = 5) and $2.86 \pm 0.69\%$ for taurine 1 mM (*n* = 5) which were not significantly different from $2.66 \pm 0.57\%$ in control experiments (*n* = 5).

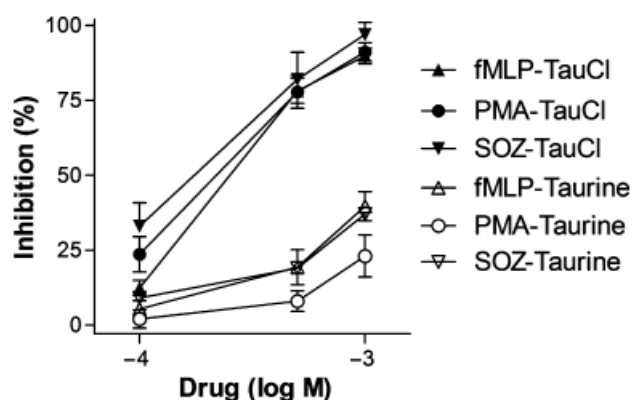


Fig. 1. Inhibitory effects of taurine and taurine-chloramine (TauCl) on the superoxide anion generation evoked by *N*-formyl-Met-Leu-Phe (fMLP; 30 nM), phorbol myristate acetate (PMA; 1 nM) and serum opsonized zymosan (SOZ; 0.5 mg/mL) in isolated human eosinophils as indicated. Data are mean $\pm$ SEM of five independent experiments.

Taurine-chloramine inhibited N-formyl-Met-Leu-Phe, phorbol 12-myristate 13-acetate- and serum opsonized zymosan-induced superoxide anion release

Oxidant generation by eosinophils is relevant to inflammatory damage. Since TauCl suppressed oxidative burst from activated human neutrophils [32], we tested first the ability of this taurine derivative to inhibit the generation of superoxide anion from human eosinophils activated by stimuli acting through different mechanisms such as fMLP (30 nM), PMA (1 nM) and SOZ (0.5 mg/mL). The concentrations of these stimuli were selected to produce similar levels of oxidant generation (7.7 ± 1.4 , 11.2 ± 3.1 and 11.7 ± 1.6 nmol superoxide anion generated by 5×10^5 cells for fMLP, $n = 5$; PMA, $n = 5$; and SOZ, $n = 5$; respectively). TauCl decreased, in a concentration-dependent way, the superoxide anion generation triggered by fMLP, PMA and SOZ, with similar inhibitory potencies for the three stimuli ($-\log IC_{50} = 3.63 \pm 0.08$, 3.66 ± 0.07 and 3.78 ± 0.04 , respectively) as shown in Fig. 1. Taurine, up to 1 mM, produced only moderate inhibitions of oxidative burst.

Taurine-chloramine inhibited membrane translocation of p47^{phox} and p67^{phox} without alteration of intracellular Ca²⁺ response to N-formyl-Met-Leu-Phe

Since activation of formyl-peptide receptors is relevant in mediating the response of human eosinophils to allergens [20], we further studied the influence of TauCl in the eosinophil response to fMLP. The oxidative burst in eosinophils follows the assembly of NADPH oxidase on the plasma membrane [4]. Eosinophils activated with fMLP (100 nM) showed membrane translocation of p47^{phox}

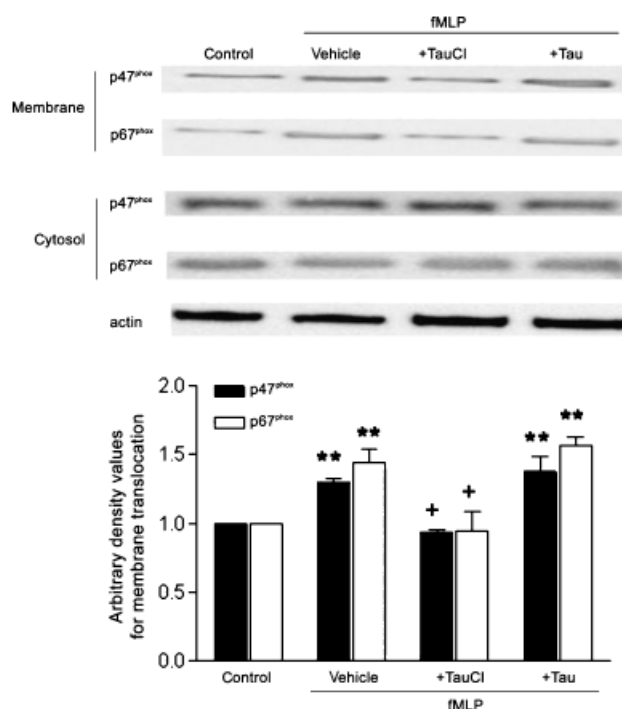


Fig. 2. Inhibitory effects of taurine chloramine (TauCl; 1 mM) on translocation to the membrane of cytosolic components of NADPH oxidase, p47^{phox} and p67^{phox}, in -stimulated human eosinophils. Isolated human eosinophils were stimulated with *N*-formyl-Met-Leu-Phe (fMLP; 100 nM) and the membrane and cytosolic fractions obtained. Both fractions were loaded and separated by 12% SDS-PAGE, and immunoblotting was performed with antibodies against p47^{phox} and p67^{phox}. A representative immunoblot of three different experiments is shown in the upper panel. Bar graphs (lower panel) shows the relative levels of p47^{phox} and p67^{phox} translocation to plasma membrane based on densitometry of normalized immunoblot signals. Data are mean $\pm$ SEM of three independent experiments; ** $P < 0.01$ from control; + $P < 0.05$ from fMLP alone.

Table 1. Effect of taurine and taurine-chloramine (TauCl) on the intracellular calcium ([Ca²⁺]_i) response elicited by fMLP (30 nM) in human eosinophils

	<i>n</i>	Baseline value (nM)	Δ (nM)	AUC _{0–60 s} (nM s)
Control	5	147 $\pm$ 14	227 $\pm$ 85	7646 $\pm$ 742
Taurine (1 mM)	5	165 $\pm$ 33	191 $\pm$ 27	9252 $\pm$ 611
TauCl (200 μ M)	5	160 $\pm$ 13	341 $\pm$ 43	6716 $\pm$ 914

Data are mean $\pm$ SEM of *n* independent experiments. Differences among groups by ANOVA were not significant ($P > 0.05$).

The responses are assessed as the increase of [Ca²⁺]_i (Δ ; peak value minus baseline), and the area under the curve in the interval 0–60 s (AUC_{0–60 s}). fMLP, *N*-formyl-Met-Leu-Phe.

and p67^{phox}, and this translocation to membrane was markedly inhibited by TauCl but was not affected by taurine (Fig. 2).

The oxidant production by fMLP in human eosinophils requires an intracellular calcium signal [33]. Thus,

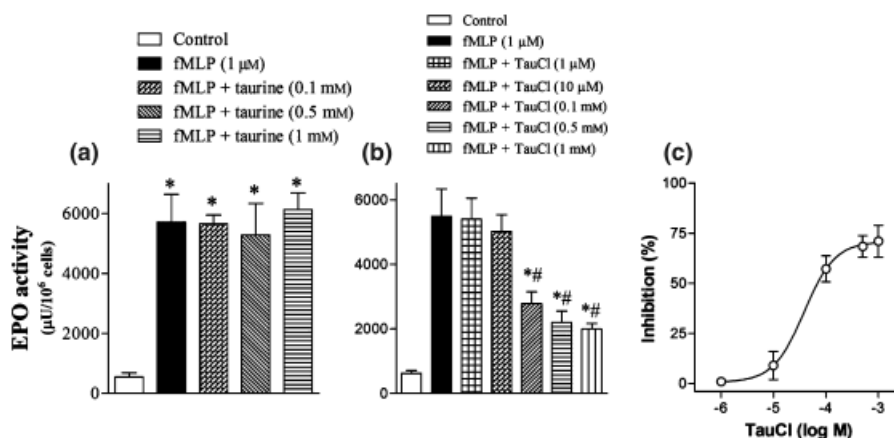


Fig. 3. The effects of taurine (a) and taurine-chloramine (TauCl) (b) on eosinophil peroxidase (EPO) release triggered by *N*-formyl-Met-Leu-Phe (fMLP) in isolated human eosinophils. The concentration–response curve for the inhibitory effects of TauCl is shown in (c). Data are mean±SEM of three to six independent experiments for each drug concentration. * $P < 0.05$ compared with control values; # $P < 0.05$ from fMLP alone.

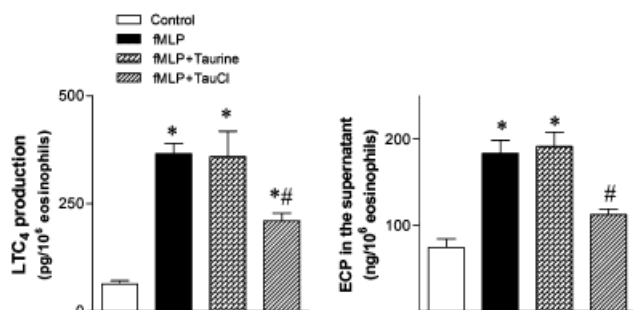


Fig. 4. Effects of taurine and taurine-chloramine (TauCl) (each at 1 mM) on the generation of leukotriene C₄ (LTC₄; left panel) and eosinophil cationic protein (ECP; right panel) by isolated human eosinophils stimulated with *N*-formyl-Met-Leu-Phe (fMLP). Data are mean±SEM of three independent experiments. * $P < 0.05$ compared with control values; # $P < 0.05$ from fMLP alone.

addition of fMLP (30 nM) resulted in a rapid initial increase in $[Ca^{2+}]_i$ followed by a decline towards baseline levels (not shown). However, neither TauCl nor taurine significantly altered the peak and AUC_{0–60s} responses to fMLP in human eosinophils (Table 1).

*Taurine-chloramine inhibited eosinophil peroxidase release and leukotriene C₄ and eosinophil cationic protein production in *N*-formyl-Met-Leu-Phe-activated eosinophils*

Activation of eosinophils with fMLP caused also the release of EPO and other non-oxidant inflammatory mediators such as LTC₄ and ECP. TauCl produced a concentration-related inhibition of EPO release ($-\log IC_{50} = 3.62 \pm 0.13$; Fig. 3) and reduced the increased production of LTC₄ and ECP while taurine was without significant inhibitory effects (Fig. 4).

Taurine-chloramine attenuated the ability of granulocyte-monocyte colony-stimulating factor and interleukin-5 to prevent eosinophil apoptosis

Since oxidant stress has been related to apoptosis [5], we investigated the effects of TauCl on spontaneous apoptosis as well as in the presence of GM-CSF and IL-5, inflammatory cytokines which enhance eosinophil survival. Apoptosis was measured after 24 h of culture by using annexin V and PI staining of eosinophils. Taurine (1 mM) produced no change in apoptosis in the absence and presence of cytokines. TauCl did not alter constitutive apoptosis but attenuated the GM-CSF- and IL-5-induced inhibition of apoptosis (Fig. 5a). A representative experiment is shown in Fig. 6.

To confirm whether this effect of TauCl observed at 24 h was also present at later stages, we determined survival of eosinophils after 4 days of culture in the absence and presence of GM-CSF. In these conditions, taurine was without effect but TauCl decreased survival of eosinophils cultured with GM-CSF without alteration of spontaneous death rate (Fig. 5b).

Taurine-chloramine blocked the activation of eosinophil nuclear factor- κ B by granulocyte-monocyte colony-stimulating factor and interleukin-5

A key mechanism in the cytokine-mediated survival of eosinophils is the activation of NF- κ B [34]; therefore, we next investigated the effect of TauCl on the activation of NF- κ B promoted by GM-CSF and IL-5, relevant cytokines to enhance survival of eosinophils in inflammatory sites [35]. Exposure of human eosinophils to GM-CSF (1 ng/mL) or IL-5 (20 ng/mL) resulted in an increase in the levels of p65 phosphorylation at the Ser⁵³⁶ that was decreased in TauCl (1 mM)-treated eosinophils but remained unaffected by taurine (1 mM) (Fig. 7).

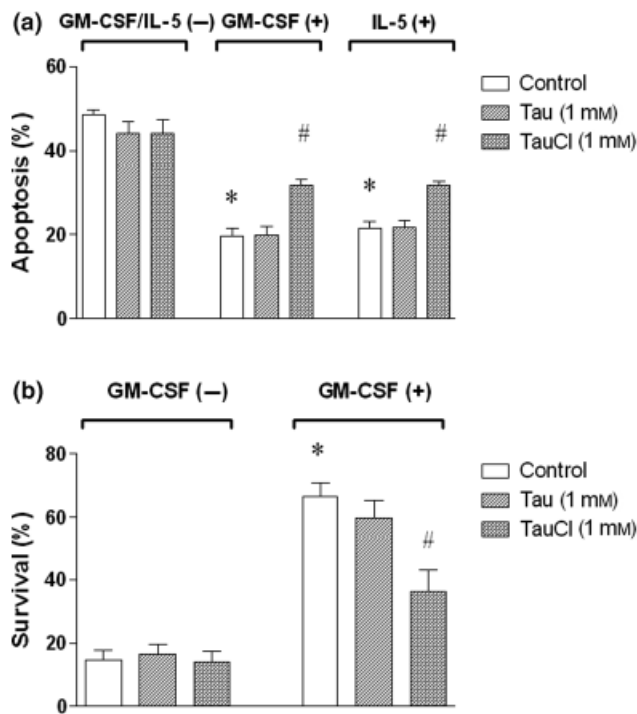


Fig. 5. (a) Effects of taurine and taurine-chloramine (TauCl) (each at 1 mM) on apoptosis assessed as annexin V positive at 24 h of culture of isolated human eosinophils. Eosinophil apoptosis was examined in the absence and in the presence of granulocyte-monocyte colony-stimulating factor (GM-CSF) (1 ng/mL) or IL-5 (20 ng/mL) as indicated. Columns are mean $\pm$ SEM of five independent experiments; * P < 0.05 compared with absence of cytokine; # P < 0.05 compared with the corresponding control. (b) Effect of taurine and taurine-chloramine (TauCl) on the survival of isolated human eosinophils, measured by trypan blue exclusion, at 4 days of culture in the absence and presence of GM-CSF (1 ng/mL) as indicated. Columns are mean $\pm$ SEM of four independent experiments. * P < 0.05 compared with absence of GM-CSF; # P < 0.05 compared with the corresponding control.

Discussion

The main finding of the present work is that TauCl, a derivative of the amino acid taurine, is able to inhibit a wide array of functional responses of human eosinophils *in vitro* while taurine produced only weak or no effects. Cytotoxicity was not involved in the inhibitory effects of TauCl as no significant release of LDH, a gross marker of cell damage, was found for TauCl (1 mM), the highest concentration explored in the present study, which is consistent with reports in other leukocytes [13].

TauCl inhibited the oxidative burst of eosinophils activated with soluble, receptor dependent (fMLP) or independent (PMA), and particulate (opsonized zymosan) stimuli with similar potency that was $\sim 200 \mu\text{M}$. We are not aware of other studies examining the effect of TauCl or taurine on the release of oxygen radicals from eosinophils. This inhibitory effect of TauCl in human eosinophils is in keeping with findings from other studies showing that this

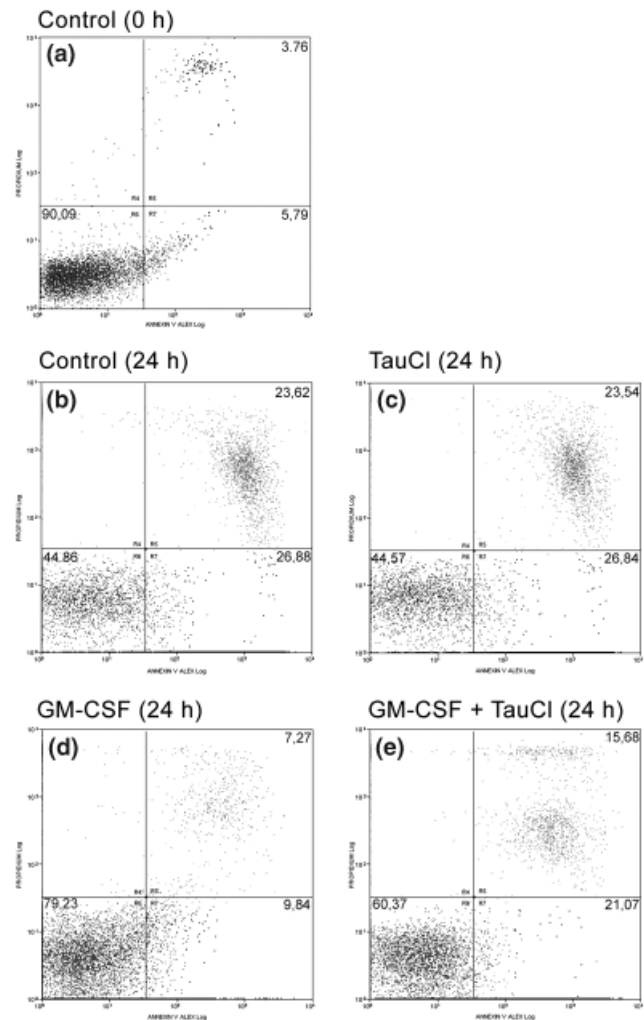


Fig. 6. A representative flow cytometry showing annexin V staining (x-axis) and propidium iodide (PI) staining (y-axis) to assess apoptosis of human eosinophils at 0 h (a) and at 24 h of culture (b–e) in the absence (b, c) and presence (d, e) of granulocyte-monocyte colony-stimulating factor (GM-CSF) (1 ng/mL), and in the absence (b, d) and presence (c, e) of taurine-chloramine (TauCl; 1 mM). Viable non-apoptotic eosinophils were quantified as the percentage of total population of cells that were negative for both annexin V and PI staining. Early apoptotic cells were annexin V positive and PI negative. Late apoptotic cells were annexin V and PI positive. The numbers represent the percentage of cells in each quadrant. Note that TauCl did not change spontaneous apoptosis (i.e. absence of GM-CSF) (cf. c vs. b) but increased apoptotic percentages obtained in the presence of GM-CSF (cf. e vs. d).

compound, in a similar concentration range, was able to reduce the generation of ROS, nitric oxide, inflammatory cytokines (TNF- α and IL-6) and prostaglandin E_2 in murine neutrophils and macrophages [12–17].

Additional studies were carried out to see whether TauCl interferes with the intracellular calcium signal produced after cell activation by different stimuli and considered relevant to the oxidative burst in eosinophils [33]. In these

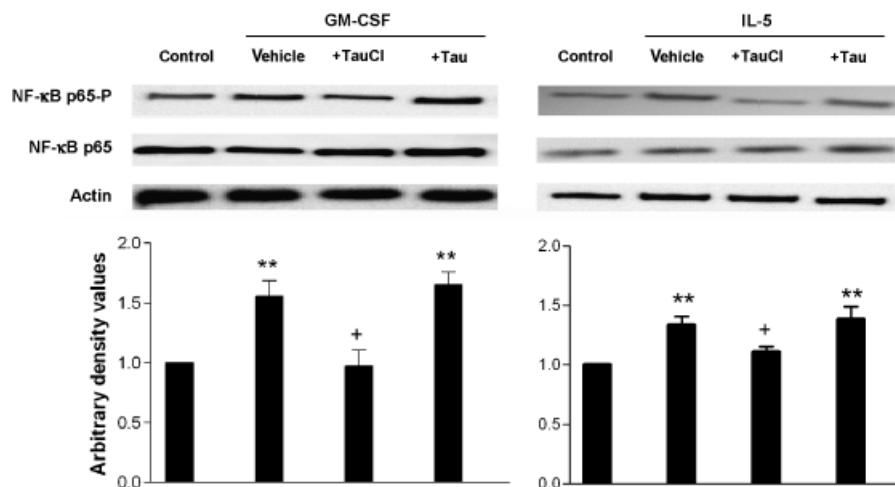


Fig. 7. Eosinophil lysates were prepared from 10^6 cells per condition after activation by granulocyte-monocyte colony-stimulating factor (GM-CSF) (1 ng/mL) or IL-5 (20 ng/mL) in the presence of taurine chloramine (TauCl; 1 mM), taurine (Tau; 1 mM) or vehicle. The whole cell lysates were separated by 10% SDS-PAGE, gels were transferred to nitrocellulose and immunoblotted with antibodies for NF- κ B p65-P and NF- κ B p65. A representative blot of three different experiments is shown in the upper panel. Bar graphs (lower panel) shows the relative levels of immunoblot signals based on densitometry analysis. Data are mean $\pm$ SEM of three independent experiments; ** P < 0.01 from control; + P < 0.05 from GM-CSF alone.

additional experiments, the chemoattractant peptide fMLP was selected as the activator stimulus since activation of formyl-peptide receptors is a relevant mechanism involved in the activation of human eosinophils by allergens [20]. We found that neither TauCl (200 μ M) nor taurine (1 mM) alter basal values of $[Ca^{2+}]_i$ or the fMLP-induced calcium signal. This finding is consistent with the observation that the agonist-induced activation of NADPH-oxidase and the subsequent ROS generation may proceed decoupled from the transient $[Ca^{2+}]_i$ rise [36]. Therefore, the inhibitory effect of TauCl on ROS generation by fMLP-activated eosinophils has to be exerted on a different target.

A critical step in the activation of the superoxide-generating NADPH oxidase is the assembly of membrane-bound cytochrome b_{558} with cytosolic $p47^{phox}$ and $p67^{phox}$ which have then to translocate to the plasma membrane [37]. Eosinophils display a higher superoxide production upon stimulation as compared with neutrophils [38], and the components of the NADPH oxidase are expressed more abundantly in eosinophils than in neutrophils [4]. TauCl has been recently shown to inhibit phorbol-stimulated superoxide production in human neutrophils by inhibiting the phosphorylation and translocation of $p47^{phox}$ [39]. In the present study we show that TauCl elicited a marked inhibition of $p47^{phox}$ translocation in human eosinophils while taurine was without effect.

To find whether the inhibitory effects of TauCl on ROS generation extend to other functional responses of human eosinophils, we examined its effect on the fMLP-induced release of the cytotoxic products EPO and ECP which are markers of granular secretion [28, 40]. Incubation with TauCl produced a dose-dependent decrease of the release of EPO with a potency of $\sim 200 \mu$ M. The release of ECP

from fMLP-activated eosinophils was virtually suppressed by TauCl (1 mM). The fMLP-induced production of LTC_4 , a lipid mediator which is a marker of non-oxidant inflammatory activity of the eosinophils, was also inhibited by TauCl. A possible mechanism of action of how TauCl can inhibit early fMLP-induced events such as LTC_4 production and ECP release may be related to the inhibition by TauCl of the mitogen-activated protein kinase cascade triggered by the activation of formyl peptide receptors, a G-protein coupled receptor, in eosinophils [41, 42]. Thus, TauCl has been shown to inhibit Ras activation and ERK1/2 phosphorylation in murine macrophages and p38 phosphorylation in a human myeloid cell line [43]. However, the clinical relevance of these inhibitory effects is uncertain since the concentration levels of TauCl locally attainable are not known although taurine concentrations in bronchoalveolar lavage fluid is augmented in asthmatics [10].

Apoptosis is now considered an important mechanism in the resolution of inflammation, and many *in vitro* studies have shown that an array of inflammatory mediators and pro-inflammatory cytokines present in inflamed sites augment the survival of human granulocytes [44, 45]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism, whereas many inhibitors of apoptosis show antioxidant properties [5]. Taurine, at concentrations higher than those used in the present study, was reported to inhibit spontaneous as well as Fas- and sodium arsenite-triggered apoptosis in human eosinophils [5]. In the present study, we show that TauCl (up to 1 mM) was not affecting the spontaneous apoptosis and survival of cultured eosinophils.

Interestingly, when the same concentration of TauCl was added to eosinophils incubated with GM-CSF to

extend their life span in culture, we found an augmented apoptosis at 24 h and decreased survival at 4-days of culture. Similar results were found for another relevant cytokine, IL-5. A possible explanation for this finding is related to the importance of NF- κ B activation to prolong survival of eosinophils [46, 47]. Thus, GM-CSF and IL-5 activate NF- κ B, and incubation of human eosinophils with inhibitors of NF- κ B activation resulted in abrogation of the pro-survival effects of GM-CSF and IL-5 [35, 48]. The finding that NF- κ B activation is inhibited by TauCl in human eosinophils is in keeping with a similar finding in Jurkat cells [49] and suggests that this mechanism may explain the pro-apoptotic effects of TauCl in eosinophils exposed to GM-CSF and IL-5. Interestingly, a recent report indicated that taurine bromamine, also generated at inflammatory sites, exerted an anti-inflammatory effect by a similar process to that of TauCl since inhibited cytokine-induced NF- κ B activation in a myeloid-committed eosinophil cell line [50]; therefore, both taurine derivatives generated by activated eosinophils may concurrently have a potential anti-inflammatory role in allergic disease.

In conclusion, the amino acid taurine is without significant effects on human eosinophil functions but its derivative TauCl inhibits oxidative burst and generation of inflammatory mediators, and reverses the survival effect produced by inflammatory cytokines such as GM-CSF. Therefore, endogenous TauCl may help to suppress excessive inflammatory response in eosinophils at inflammatory sites although it is currently difficult to envisage an anti-inflammatory therapy of benefit to allergic disease based on this molecule.

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