Effects of sulphurous water on human neutrophil elastase release

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***Abstract*:**
Background: Molecules bearing a sulphide (HS) group, such as glutathione, play a fundamental role in the defensive system of human airways, as shown by the fact that the lining fluid covering the epithelia of the respiratory tract contains very high concentrations of glutathione: the lungs and nose, respectively, contain about 140 and 40 times the concentrations found in plasma. Consequently, various low-weight soluble molecules bearing an HS group (including N-acetylcysteine, mesna and thiopronine, and prodrugs such as stepronine and erdosteine) have been used for therapeutic purposes. HS groups can also be therapeutically administered by means of sulphurous thermal water containing HS groups. The aim of this study was to investigate the direct activity of such water on the release of elastase by activated human neutrophils.
Method: After the neutrophils were incubated with increasing amounts of sulphurous water or the HS/hydrogen sulphide donor sodium hydrosulphide (NaHS), elastase release was initiated by N-formyl-methionyl-leucyl-phenylalanine and measured by means of spectrofluorimetry using methylsuccinylalanylprolylvalyl-methylcoumarin amide as the fluorogenic substrate. To verify the presence of direct action on elastase we determined the diameter of the area of elastinolysis on elastine–agarose gel plates.
Results: The sulphurous water significantly inhibited elastase release at HS concentrations ranging from 4.5 to 18 μg/ml, as assayed using the iodometric method; in the case of NaHS, the inhibition was significant at HS concentrations ranging from 2.2 to 18 μg/ml. The concentration-effect regression lines of both were parallel and neither showed any direct elastolytic activity.
Conclusions: Previous claims concerning the activity of sulphurous water have been based on the patients' subjective sense of wellbeing and on symptomatic (or general) clinical improvements that are not easy to define or quantify exactly. Our findings indicate that, in addition to its known mucolytic and antioxidant activity, sulphurous water also has an anti-elastase activity that may help to control the inflammatory processes of upper and lower airway diseases.

***Keywords*:** human neutrophil elastase, hydrogen sulphide/sulphide, sodium hydrosulphide, sulphurous water

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Introduction

Many lung disorders, including chronic obstructive pulmonary disease, asthma, acute respiratory distress syndrome, cystic fibrosis, acute idiopathic pulmonary fibrosis, fibrosis due to xenobiotics (e.g. paraquat, nitrofurantoin or bleomycin) and silicosis/asbestosis, are characterized by the 'common denominator' of acute or chronic airway inflammation at the alveolar or bronchial level. The inflammatory responses elicited by exogenous or endogenous stimuli are protective insofar as they destroy or remove the damaging agent and injured tissues, and thus promote tissue repair [Lengas *et al.* 1994]; however, uncontrolled responses lead to excessive cell/tissue damage, chronic inflammation, and the destruction or remodelling of normal tissues [Rahman and MacNee, 2000; Brown and Jones, 1996].

The inflammatory or immune response of the airways is characterized by the activation of epithelial cells and resident macrophages, which leads to the production of chemotactic signals, and the subsequent recruitment and activation

of eosinophils, monocytes, lymphocytes but mainly neutrophils attracted to the lungs and airway lumen by these stress signalling pathways [Kim and Nadel, 2004; Thompson *et al.* 1989]. Polymorphonuclear leucocytes (PMNs) are the central components of inflammatory processes and, when stimulated, show a variety of cell responses, such as chemotactic migration, phagocytosis and the release of reactive oxygen species (ROS) and reactive nitrogen species (respiratory burst) [Robbins *et al.* 2000; Badwey and Karnovsky, 1980], and various degrading enzymes contained in different types of cytoplasmic granules [Fujie *et al.* 1999; Wrigt, 1988].

Serine proteinase elastase is one of the most abundant lysosomal proteinases contained in PMNs [Junger *et al.* 1992], and it plays an important role in respiratory tract inflammation [Janoff, 1985], because of the abundance of elastin at pulmonary level. The destructive capacity of the released elastase is usually offset by protease inhibitors, mainly the α-1-proteinase inhibitor. However, the ROS released by PMNs at the same time as elastase and other proteases can inactivate these inhibitors, thus allowing free proteases to cause tissue damage [Iwamura *et al.* 1993]. The therapeutic use of low molecular weight antioxidants and/or elastase inhibitors can therefore help to prevent PMN-mediated tissue damage [Iwamura *et al.* 1993].

The usefulness of soluble nonprotein thiol molecules in reducing oxidant-induced respiratory airway injury and inflammation has been mainly attributed to the presence of reducing sulphide (HS) groups (sulphydryl or thiol) belonging to the cysteine amino acid in glutathione. However, HS groups can also be administered therapeutically by means of sulphurous waters. The efficacy of inhalations of sulphurous waters in reducing the clinical signs of inflammation in various respiratory diseases has been described in clinical reports, but there is still a lack of data concerning their direct effects on elastase release by activated human neutrophils.

The aim of this study was to investigate this particularly important aspect of the generation and progression of inflammation of the airway.

Materials and methods

Collection of human PMNs

Peripheral venous blood was obtained from healthy adults by the Blood Donors Department of Niguarda Hospital (Associazione Volontari Italiani Sangue Comunale, Milan, Italy). The Ethics Committee of the hospital approved the study and informed consent was obtained from each donor, none of whom had received any medication for at least 2 weeks. The blood (5 ml) was stratified in 3 ml of a polymorphprep cell separation medium (Axis-Shield, Oslo, Norway), and the PMNs were separated by means of density gradient centrifugation. After centrifugation, the upper mononuclear cell band was removed and discarded, and the lower PMN band was removed and washed in RPMI 1640 medium containing glutamine (Sigma Chemical Co., St Louis, MO, USA). When necessary, any residual erythrocytes in the granulocyte preparation were lysed using a 0.15 mol/L NH_4Cl solution (pH 7.4). After the aggregates were disrupted by being passed through a needle with an internal diameter of 150 μm, the PMNs were collected, washed in RPMI 1640, and tested for viability by means of trypan blue exclusion. The number of cells in the final cell suspension used for each test was adjusted following counting in a Bürker chamber (Nomarski interference contrast microscopy).

Elastase release assay

PMN elastase release was spectrofluorometrically measured as described by Sklar *et al.* [1982] and De Vries *et al.* [1990]. The cells (5×10^6/ml) were suspended in a buffer (composition in mM: NaCl 147, KCl 5, $MgSO_4$ 0.3, $MgCl_2$ 1, $KHPO_4$ 1.9, Na_2HPO_4 1.1 and glucose 5.5) containing 20 μM of a fluorogenic substrate that is highly specific for neutrophil elastase, that is, methylsuccinylalanyl-prolylvalyl-methylcoumarin amide (MeO-Suc-Ala-Ala-Pro-Val-MCA) (Sigma), 3 μg/ml cytochalasin B (Sigma), and either sulphurous water or sodium hydrosulphide (NaHS), which is commonly used as a hydrogen sulphide (H_2S)/HS donor because it dissociates to Na^+ and HS^- and, depending on the ionic environment, HS^- can partially bind H^+ to form undissociated H_2S [Lowicka and Beltowski, 2007]. When stimulated by an inducer such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma), cytochalasin B converts phagocytic neutrophils to secretory cells; it also promotes the disaggregation of the intracellular actin network, which facilitates the fusion of secretory granules with cell membranes and thus enhances the degranulation process and the phagocyte secretion of lysosomal enzymes [Lowicka and Beltowski, 2007; Southwick and Stossel, 1983]. The cell suspension was

equilibrated for 15 min at 37°C before the addition of the stimulus. The nonfluorescent substrate was hydrolysed by the enzyme to liberate intensely fluorescent 4-methyl-7-coumarin amide (MCA) (Sigma), and the fluorescence was recorded using a Perkin Elmer LS-5 fluorescence spectrophotometer with excitation and emission wavelengths of 380 and 460 nm, respectively. After the incubation period, fMLP was added and the increase in the signal was determined. The enzyme rate was calculated using MCA as the standard and was considered a reflection of the amount of elastase released [De Vries *et al.* 1990]. The total elastase content of the PMNs was determined by cell lysis in 0.1% Triton X-100 (Sigma) as described by Sklar *et al.* [1982]. Enzyme release was quantified as a percentage using the formula:

$$\%\ \text{inhibition} = \left\{1 - \left[\left(\text{ABS}_{(S+1)} - \text{ABS}_{(S+B)}\right)/\left(\text{ABS}_{(1)} - \text{ABS}_{(B)}\right)\right]\right\} \times 100$$

where ABS = absorbance, S = sample, I = inducer and B = buffer [Johansson *et al.* 2002].

The effect of sulphurous water on elastase release was investigated by incubating the PMNs for 15 min at 37°C with increasing amounts of sulphurous water collected from a spring called 'Fontanino dell'Acqua Marcia', Acqui Terme (Alessandria, Italy), in order to obtain HS group concentrations ranging from 1.1 to 18 μg/ml (log scale) as assayed using the iodometric method [American Public Health Association, 2000]. The same procedure was used to investigate the effects of NaHS at the same HS group concentrations.

Measurement of the direct effect of sulphurous water on elastase

To investigate whether sulphurous water and NaHS have direct hydrolytic activity on elastase (elastinolysis), we used a slightly modified version of the method of Carp and Janoff [1978], which allows the results to be directly visualized. Briefly, 1% agarose gel plates buffered to a pH of 8.6 with 0.4 M tris-HCl and containing 0.8% (w/v) powdered bovine elastine (neck ligament) (Sigma) were prepared in petri plates. Wells (3 mm in diameter) were then punched out of the hardened agarose and the small gel discs removed by suction. As required by the method, a 10 μl sample of standard elastase solution (porcine pancreatic elastase: activity = 4 units/mg of protein) (Sigma) was placed into the central well of the agarose plate as a reference. The diameters of the areas of elastinolysis were measured after 24 h at 37°C. The effect of sulphurous water and NaHS was investigated by mixing increasing amounts (from 1.1 to 18 μg/ml of HS) with the elastase, and the diameters of the areas of elastinolysis were measured using the same procedure.

Statistical analysis

Four assays were made of each concentration for each test, and the statistical significance of the differences was calculated by means of one-way analysis of variance, followed by multiple paired comparisons using Dunnett's test. The differences were considered statistically significant when the p-value was ≤ 0.05. The concentration-effect regression lines of the sulphurous water and NaHS data were analysed (GrapfPad Prism), and their slopes were compared to verify the presence of parallelism.

Results

As shown in Figure 1, the incubation of human PMNs in the presence of 30 nM fMLP led to a linear increase in fluorescence after an initial lag period of approximately 10 s. This behaviour is in line with the findings of De Vries *et al.* [1990]. The enzyme content in the cell lysate was measured and the amount of elastase released after 30 nM fMLP was 65.50 ± 10.11% of the total cell enzyme content. In order to determine whether sulphurous water inhibited elastase

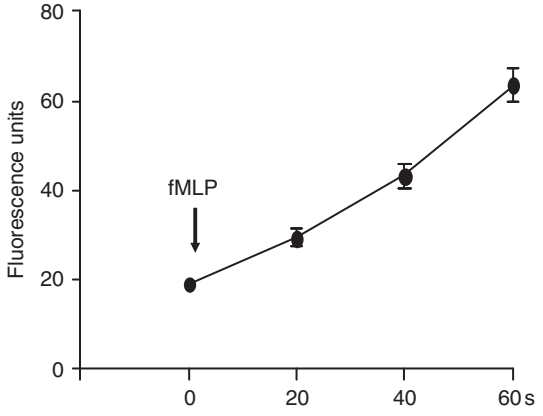

Figure 1. Time course of elastase release by human neutrophils in response to 30 nmol/L of fMLP. The increase in fluorescence (release of 4-methyl-7-coumarin amide) indicates the rate of hydrolysis (cleavage) of methylsuccinylalanylprolylvalyl-methyl-coumarin amide. fMLP, N-formyl-methionyl-leucyl-phenylalanine.

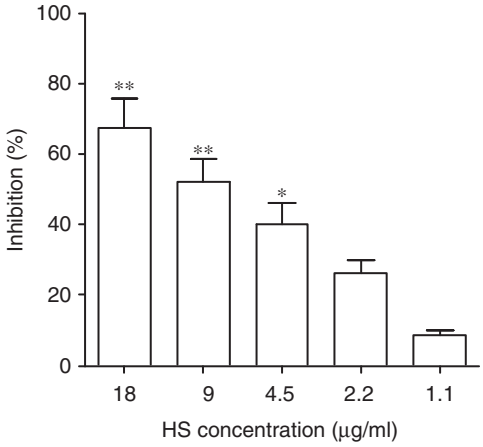

Figure 2. Effects of sulphurous water on the release of elastase by N-formyl-methionyl-leucyl-phenylalanine-activated neutrophils. The results (mean values ± standard error of the mean) are expressed as the percentage of elastase production inhibited by various sulphurous water concentrations. $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. HS, sulphide.

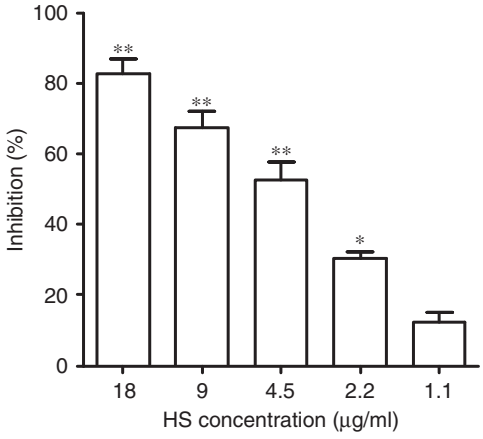

Figure 3. Effects of NaHS on the release of elastase by N-formyl-methionyl-leucyl-phenylalanine-activated neutrophils. The results (mean values ± standard error of the mean) are expressed as the percentage of elastase production inhibited by various NaHS concentrations. $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. HS, sulphide; NaHS, sodium hydrosulphide.

release from human neutrophils, the PMNs were pre-incubated with progressively increasing amounts of water, which showed that the inhibition was dependent on time and HS concentration. The effect of 1.1 and 2.2 μg/ml HS was not significant, but elastase release was significantly inhibited at concentrations ranging from 4.5 to 18 μg/ml (Figure 2). When NaHS was tested, the inhibition of elastase release was statistically significant at concentrations ranging from 2.2 to 18 μg/ml (Figure 3).

Figure 4 shows the sulphurous water concentration-effect regression line with confidence intervals ($r^2 = 0.683$; $p \leq 0.001$), and Figure 5 that of NaHS ($r^2 = 0.764$; $p \leq 0.001$). The slopes of the two regression lines were not significantly different and can be considered parallel.

Figures 6 and 7 show the results of the elastase-induced radial diffusion of elastolysis in elastine–agarose gel. The increasing amounts of water or NaHS did not lead to any significant changes from the baseline, thus suggesting that they had no direct effect on elastase activity.

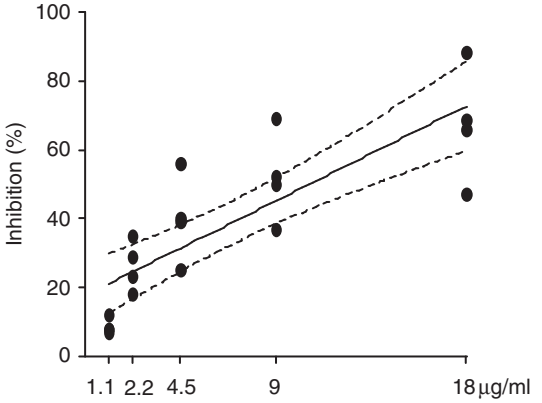

Figure 4. Concentration-effect regression line of elastase inhibition by sulphurous water (dotted lines = confidence intervals).

Discussion

The first point of impact of airborne xenobiotic environmental pollutants in the airways is the lining fluid covering the epithelia of the respiratory tract. This contains an array of defensive molecules, including soluble low molecular weight antioxidants, metal-binding proteins, enzymes, sacrificial reactive proteins and unsaturated lipids [Chabot *et al.* 1998; Cross *et al.* 1994]. It also contains very high concentrations of reduced glutathione: the lungs and nose, respectively, contain about 140 and 40 times the amount found in plasma [Meister, 1994; Heffner and Repine, 1989; Patterson and Rhoades, 1988; Cantin *et al.* 1987]. This is an important indication of the natural defensive strategy adopted by the airways. They make use of glutathione, a molecule bearing an HS group, as a fundamental part of their defensive system against the various types of aggression leading to

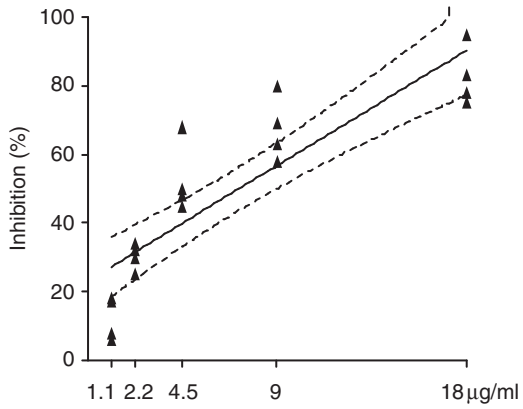

Figure 5. Concentration-effect regression line of elastase inhibition by sodium hydrosulphide (dotted lines = confidence intervals).

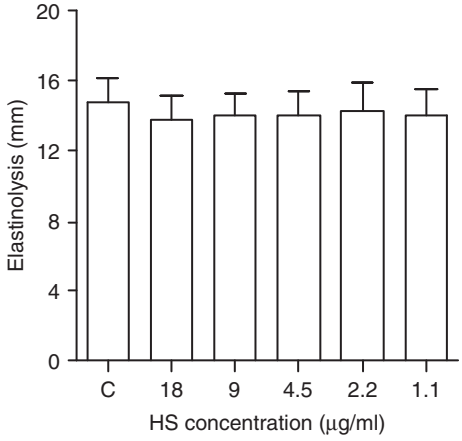

Figure 6. Elastase hydrolysis as measured by means of radial diffusion in elastin–agarose gel plates. The results (mean values ± standard error of the mean) are expressed as the millimetric diameters of the elastinolysis zones of the different sulphurous water concentrations. C, control; HS, sulphide.

respiratory tract injury and inflammation [Rahman, 2005] and, on the basis of this observation, a number of low-weight soluble molecules bearing an HS group (such as N-acetylcysteine, mesna, thiopronine and dithiothreitol, or the prodrugs stepronine and erdosteine) have been used for therapeutic purposes [Rahman, 2005; Allegra *et al.* 2002; Braga *et al.* 2000, 1989]. It can be observed that, following this line of defence, HS groups can also be therapeutically administered not only by means of such thiol molecules, but also by sulphurous waters, which contain different concentrations of HS groups. The activity of these groups has been generally described on the basis of clinical responses, such as the patients'

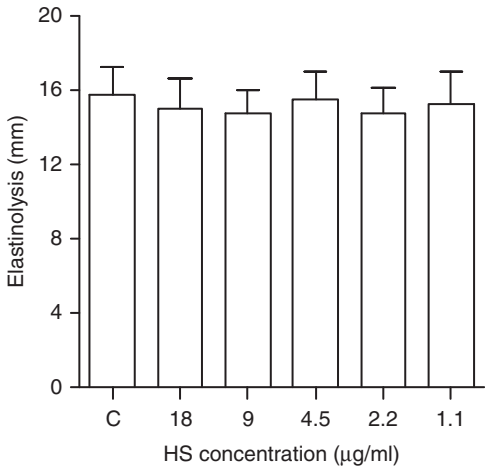

Figure 7. Elastase hydrolysis as measured by means of radial diffusion in elastin–agarose gel plates. The results (mean values ± standard error of the mean) are expressed as the millimetric diameters of elastinolysis zones of the different concentrations of sodium hydrosulphide tested. C, control; HS, sulphide.

subjective sense of wellbeing, and symptomatic (or general) clinical improvements whose parameters are not easy to define or quantify. However, a more recent direct approach has shown that the sulphurous water of the Fontanino dell'Acqua Marcia interacts with human neutrophils and has interesting antioxidant activity *in vitro* against the oxidants released during neutrophil bursts [Braga *et al.* 2008]. Following this line of research, subsequent findings showed that the water also reduces the release of elastase from activated neutrophils, thus extending the range of its effects on the degranulation of cytoplasmic neutrophil granules.

The intracellular signalling pathways and mechanisms that regulate the function of human PMNs responses to pro-inflammatory stimuli are only partially understood, but it is known that divalent cations (especially calcium ions) play an important regulatory role in a variety of cell types and that increased intracellular calcium levels is a key element of stimulus–response coupling [Suda *et al.* 2005], particularly in PMN activation [Gandry *et al.* 1990; Lew *et al.* 1985, 1984]. The intracellular release of calcium depletes the level of stored calcium, and this simultaneously triggers calcium influx by increasing membrane permeability, thus restoring the intracellular calcium level [Khalfi *et al.* 1996; Truett *et al.* 1988].

This fMLP-induced calcium influx is mediated by the activation of a calcium channel localized at the plasma membrane [Suda *et al.* 2005], a mechanism that is known as 'capacitative calcium entry' [Jan *et al.* 1999], and is considered to be an important route of calcium influx in neutrophils [Suda *et al.* 2005; Chen and Jan, 2001]. Calcium mobilization also triggers the release of elastase by PMNs in response to fMLP: that is, the inhibition of calcium mobilization inhibits elastase release [Khalfi *et al.* 1996; De Vries *et al.* 1990].

It has recently been reported that H_2S inhibits the activity of L-type calcium channels, thus inhibiting calcium mobilization [Sun *et al.* 2008]. H_2S is present in sulphurous water because 30–40% of the thiol species in aqueous solutions at pH values of about 7 (the pH of most thermal waters) are in the form of undissociated H_2S and 60–70% in the form of HS^- (the S^{2-} form is present in negligible concentrations) [World Health Organization, 2003; National Research Council, 1979; Hunisch, 1977]. As the sulphurous water of the Fontanino dell'Acqua Marcia (pH 7.6) does not have a direct elastinolytic effect, and as H_2S is free to enter PMNs (it can diffuse through biological membranes and tissues because it is not dissociated and with a solubility in a lipophilic environment of approximately five times that in water [Wang, 2002]), the observed final reduction in elastase release is probably attributable to H_2S entering the cells and its activity in reducing calcium mobilization.

This mechanism is in line with data relating to sodium sulphide (Na_2S) (another H_2S/HS donor), which depresses calcium-dependent cytoskeleton activities, and inhibits both the degranulation of PMNs by fMLP and the release of β-glucuronidase [Meriggio *et al.* 1997]. Another molecule that is used to investigate the effects of thiols is NaHS, which is similar to Na_2S insofar as both are used as H_2S/HS producers. Our findings show that NaHS reduces neutrophil elastase release in a way that is similar to that of sulphurous water. Knowledge of the role of H_2S in human physiology and pathology is still fragmentary and controversial because it has been reported to have both protective and deleterious effects [Lowicka and Beltowski, 2007], and so its interactions with the cell membrane and cytoplasm metabolic pathways of PMNs are still unclear.

Our findings add sulphurous water to mesna, nacystelyn, captopril [Vanderbist *et al.* 1996; Stolk *et al.* 1986], N-acetylcysteine and the metabolite I of erdosteine, all of which are thiolic compounds that reduce elastase release [Braga *et al.* 2006; Eklund *et al.* 1988]. At present, to characterize the H_2S/HS interchanges in water, air, the air–water interface and the interactions in cell biochemical pathways is very complex as many parameters (including pH variations and the ox/redox potential of the environment) can influence the distribution of the two chemical species, and further studies are needed to clarify the biological role of H_2S/HS. However, our observations can help to characterize the action of sulphurous waters and may also be clinically relevant in improving the control over airway inflammation because, although sulphurous water only contains about 30–40% of H_2S, the preferred way of administering sulphurous water is through repeated sessions of inhalation of nebulized or aerosolized water lasting several minutes (i.e. inhalation lasts about 15–20 min) and thus several millilitres of water are released.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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