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Antioxidant Effect of Sulphurous Thermal Water on Human Neutrophil Bursts: Chemiluminescence Evaluation

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Key Words

Sulphurous thermal water · HS group · Antioxidant activity · Polymorphonuclear neutrophils · Luminol-amplified chemiluminescence · Cell-free system

Abstract

Background: The activities of the HS (sulfhydryl or thiolic) group in the cysteine of glutathione or various low-weight soluble molecules (thiolic drugs), such as N-acethylcysteine, mesna, thiopronine and dithiotreitol or stepronine and erdosteine (prodrugs), include its antioxidant activity in the airways during the release of reactive oxygen or nitrogen species (ROS, RNS) by polymorphonuclear neutrophils (PMNs) activated in response to exogenous or endogenous stimuli. ***Objective:*** In addition to being administered by means of thiolic molecules, the HS group can also be given by means of the inhalation of sulphurous thermal water. The aim of this study was to investigate the effect of sulphurous thermal water on the release of ROS and RNS during the bursts of human PMNs. ***Methods:*** The luminol-amplified chemiluminescence methodology was used to investigate the ROS and RNS released by PMNs stimulated with N-formyl-methionyl-leucyl-phenylalanine and phorbol-12-myristate-13-acetate, before and after incubation with sulphurous water. Effects on cell-free systems were also investigated. ***Results:*** The water significantly reduced the luminol-amplified chemiluminescence of N-formyl-methionyl-leucyl-phenylalanine- andphorbol-12-myristate-13-acetate-activated PMNs on average from 0.94 to 15.5 μg/ml of HS, even after the addition of L-arginine, a nitric oxide (NO) donor. Similar findings have also been obtained in a cell-free system, thus confirming the importance of the presence of the HS group (reductive activity). ***Conclusions:*** The positive effects of the activity of sulphurous thermal waters has been partially based on the patients' subjective sense of wellbeing and partially on not always easy to quantify symptomatic (or general) clinical improvements. Our findings indicate that, in addition to their known mucolytic activity and trophic effects on respiratory mucosa, the HS groups present in the sulphurous thermal water of this spring also have antioxidant activity that contributes to the therapeutic effects of the water in upper and lower airway inflammatory diseases.

Introduction

The upper and lower airway epithelia are in contact with inhaled air, and hence the interface where impact all airborne xenobiotic substances such as environmental pollutants, cigarette smoke, allergens, micro-organisms, hydrocarbons, ion acid aerosol, reactive gases and combustion-product particles, etc. [1]. The response of

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the airway epithelia to such exposure is to activate cellular and biohumoral defense mechanisms, including epithelial cells and resident macrophages, thus leading to the recruitment and activation of neutrophils, eosinophils, monocytes and lymphocytes which are attracted towards the airways as a result of locally generated chemotactic signals (cytokines, chemokines, antimicrobial peptides, etc.) [2, 3]. When this interaction occurs in an uncontrolled manner, the result is excessive cell and tissue damage that gives rise to an inflammatory process [4, 5].

The importance of exogenous and endogenous reactive oxygen and nitrogen species (ROS, RNS) [5, 6] in airway inflammation lies in the lesions caused by the oxidation of proteins, DNA and lipids [7], which also leads to greater defensive mucus secretion with edema and extracellular matrix remodeling [6]. The increased production of ROS and RNS by activated neutrophils, and the concomitant decrease in antioxidant defensive capacity, give rise to oxidant/antioxidant imbalance that leads to oxidative stress, which plays a pivotal role in the pathogenesis of a number of upper and lower respiratory diseases [6, 8].

The first point of contact between the airways and the external environment is respiratory tract lining fluid, which also represents the first defense by providing an antioxidant screen that protects the underlying respiratory tract epithelial cells against inhaled environmental oxidants and the endogenous oxidants produced by activated neutrophils. The respiratory tract lining fluid contains an array of defensive antioxidant molecules, such as soluble low molecular weight antioxidants, metal-binding proteins, antioxidant enzymes, sacrificial reactive proteins and unsaturated lipids [9, 10]. It also contains high concentrations of reduced glutathione (in the lung and nose respectively about 140 and 40 times higher than those in normal plasma) [11–14], which has led to glutathione (a molecule bearing an HS group) being recognized as a fundamental part of the antioxidant defensive system of the airways in oxidant-induced respiratory tract injury and inflammation [15].

The various useful activities of this soluble nonprotein thiol molecule have been mainly attributed to the presence of a reducing HS (sulfhydryl or thiol) group belonging to the cysteine amino acid, present in the gluthatione, and the important role played by HS groups in the respiratory tract has been confirmed by the similarly protective effects of other low-weight soluble molecules bearing an HS group and used as drugs such as N-acetylcysteine, mesna, thiopronine and dithiothreitol, or prodrugs such as stepronine and erdosteine [16–18].

HS groups can be therapeutically administered not only by means of thiolic molecules, but also by means of sulphurous thermal waters, which contain HS groups in different concentrations. A number of clinical studies have demonstrated the efficacy of inhaling sulphurous thermal waters to reduce the clinical signs of inflammation in various respiratory tract regions, but there is still a lack of data concerning the direct activity of such waters on the release of ROS and RNS during the bursts of human neutrophils. The aim of this study was to investigate this particularly important aspect of the generation and progression of airways phlogogenic phenomena.

Materials and Methods

Human Polymorphonuclear Neutrophil Harvesting

Peripheral venous blood (5 ml) drawn from healthy adult donors was stratified on 3 ml of a Polymorphprep cell separation medium (Axis-Shield, Oslo, Norway), and the polymorphonuclear neutrophils (PMNs) were separated by means of density gradient centrifugation. After centrifugation, the upper mononuclear cell band was discarded, and the lower PMN band was washed in RPMI 1640 medium containing glutamine (Sigma Chemical Co., Mo., USA). When necessary, any residual erythrocytes in the granulocyte preparation were lysed using a 0.15 M 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 by means of counting in a Burker chamber (interference contrast microscopy).

Measurement of Burst Responses by Luminol-Amplified Chemiluminescence

PMN bursts are associated with the generation of superoxide anions, hydrogen peroxide, oxygen radicals, hydroxyl radicals and hypochlorous acid. These ROS are not only microbicidal, but also extremely toxic to human tissue. As luminol degradation by ROS is associated with luminescence, the inclusion of luminol in the reaction medium provides a sensitive means of detecting PMN respiratory bursts. In order to yield light, luminol has to undergo two-electron oxidation to form an unstable endoperoxide, which decomposes to an excited state (3-aminophthalic acid) and then relaxes to the ground state by emitting photons [19–21] that are amplified by the phototube of the luminometer.

Luminol-amplified chemiluminescence (LACL) was investigated using the soluble stimulants N-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA). fMLP is a bacterial tripeptide that is frequently used to stimulate PMN respiratory bursts and acts via a specific receptor, whereas PMA is another commonly used stimulus that directly activates intracellular protein kinase. The measurements were made using a slightly modified version of the procedure described by Briheim and Dahlgren [22]. Briefly, 0.1 ml of a PMN suspension (1×10^6 cells/ml) plus 0.2 ml of 2×10^{-5} M of luminol (Sigma) were put into a 3 ml flat-bottomed polystyrene vial. The vial

was placed in the light-proof chamber of a Luminometer 1250 (Bio Orbit, Turku, Finland), and the carousel was rotated to bring the sample in line with the photomultiplier tube in order to record background activity. fMLP at a concentration of 5×10^{-7} M or PMA 2.5×10^{-6} M was added to reach a final volume of 1 ml, and the resulting light output was continuously recorded in millivolts on a chart recorder, and simultaneously by means of a digital printout set for recording intervals of 1–10 s. All of the constituents of the mixture were kept at 37°C during the reaction by passing water from a thermostatically controlled circulation system through a polished hollow metal sample holder. No mixing took place during the recordings. The gain control was set to give a recording of 10 mV for a built-in standard. A background subtraction control zeroed the instrument before the addition of fMLP and PMA. The LACL response patterns were identified by calculating peak values (mV) and the times to peak values (min, s).

Effect of Sulphurous Thermal Water Containing an HS Group on the LACL of Human PMNs

A first series of tests were made by incubating PMNs for 15 min at 37°C with sulphurous thermal water collected from the spring called 'Fontanino dell'Acqua Marcia', Acqui Terme (Alessandria) Italy. Using a previously described LACL method, the effects of the water were investigated at HS concentrations ranging from 15.5 μg/ml down to 0.47 μg/ml, as assayed using the iodometric method [23].

In a second series of similar tests, L-arginine (L-Arg) 170 μg/ml (Sigma) was added to the medium as an NO donor before the challenge in order to study the possible interference of HS on the generation of O_2^-, NO and peroxynitrite (as a byproduct of the rapid interaction between O_2^- and NO) during stimulated bursts of human PMNs.

In order to evaluate the presence of a direct effect of HS, a third series of tests were performed in the same manner as the first except for the fact that after incubation with sulphurous water, the PMN were washed with medium and then challenged with fMLP.

Effects on Cell-Free Systems

A final series of tests were made using a cell-free system in order to investigate whether the HS group, present in sulphurous water, acted as a scavenger and directly quenched the chemiluminescence.

SIN-1 (linsidomine or 3-morpholino-sydnonimine) is a molecule whose hydrolysis produces peroxynitrite as a by-product of the NO plus O_2^- reaction. This is due to base catalysis and the opening of the five-membered SIN-1 ring, with the reduction of oxygen to superoxide anions and a radical ring-opened compound that releases NO upon rearrangement [19, 24]. The peroxynitrite formed in this manner reacts with luminol to produce an initial excited state that relaxes to a ground state, and the emitted light can be readily detected by the photomultiplier of the luminometer. The reaction occurs because luminol is oxidized by peroxynitrite [25]. Using a slightly modified version of the procedure of Ginsburg et al. [26, 27] we used SIN-1 in combination with other compounds (SIN-1 cocktail) in order to improve the stability of the reactions generating the chemiluminescence. The SIN-1 cocktail [27] consists of a combination of 200 μl luminol (stock solution 1 mM), 200 μl SIN-1 (stock solution 100 mM), 400 μl sodium selenite (SEL[IV]stock solution 100 mM), 200 μl bovine serum albumin (BSA, stock solution 1 mM), and 200 μl $CoCl_2 \cdot 4H_2O$ (stock solution 1 mM). To generate luminescence, 50 μl of the cocktail was added to plastic vials containing 1 ml of Hank's balanced salt solution (pH 7.4) at 22°C. Antioxidants are able to quench the light generated by the SIN-1 cocktail, and we investigated the effect of the addition of different amounts of sulphurous water.

Statistical Analysis

Four assays were made for each concentration of each test and the statistical significance of the differences was calculated using a paired t test; a p value of ≤ 0.05 was considered statistically significant.

Results

Table 1 shows the effects of the investigated HS concentrations in the sulphurous thermal water from the 'Fontanino dell'Acqua Marcia' on the LACL of fMLP-induced PMN bursts. A final HS concentration of 0.47 μg/ml had no significant effect on LACL; the lowest concentration that had significant LACL inhibitory activity was 0.94 μg/ml and, from this concentration to 15.5 μg/ml (the highest investigated concentration) there was a significant concentration-dependent inhibition of peak chemiluminescence (table 1). The times to peak chemiluminescence were similar at the various HS concentrations, and generally not significantly different from those of the controls.

When L-Arg was added to the reaction medium as an NO donor, basal LACL increased about six times in comparison with the corresponding values without L-Arg (table 2). Incubation with the same HS concentrations under these new conditions led to similar behavior as before, but the lowest concentration that significantly reduced LACL was 1.89 μg/ml; there was a significant concentration-dependent inhibition of peak chemiluminescence up to 15.5 μg/ml. The time to peak LACL was not significantly different from that of the controls.

The second series of tests used PMA to stimulate PMN bursts, and led to similar results (table 3). The average mean time to peak was 10 min, which was longer than that of fMLP because of their different stimulation pathways: fMLP is a formylated oligopeptide that is structurally similar to bacteria-derived peptides and binds to specific neutrophil receptors, thus initiating a signal transductional cascade that increases free intracellular calcium concentration; PMA is a chemical that lacks specific PMN cell surface receptors but, after entering the cell, activates the transduction sequence without involving the calcium system.

Table 1. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on the LACL of PMN bursts induced by fMLP (peak = mV; T_{peak} = min·s)

Control		HS											
		15.15 μg/ml		7.57 μg/ml		3.78 μg/ml		1.89 μg/ml		0.94 μg/ml		0.47 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}
67.70	2′20″	15.60	1′20″	22.90	1′10″	35.2	1′50″	44.10	2′30″	57.50	2′30″	65.10	1′20″
54.40	3′30″	18.10	2′50″	25.40	2′10″	25.70	1′40″	31.30	3′10″	43.40	1′30″	54.20	2′50″
76.02	2′40″	36.20	2′10″	46.04	1′40″	48.29	1′50″	56.55	1′40″	67.20	2′40″	70.50	2′10″
39.98	2′30″	7.50	2′10″	12.42	2′10″	14.20	2′40″	23.70	2′50″	27.07	2′00″	33.00	3′00″
59.27	2′45″	19.35**	2′05″	26.69**	1′55″	30.85**	2′00″	38.91**	2′32″	48.79**	2′10″	55.70	2′20″
±8.10	±31″	±6.06	±36″	±7.04	±26″	±7.23	±27″	±7.24	±38″	±8.73	±31″	±8.29	±45″

** $p \leq 0.01$. Values below the line are expressed as mean ± SEM.

Table 2. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on the LACL of PMN bursts induced by fMLP after L-Arg incubation (peak = mV; T_{peak} = min·s)

Control		HS											
		15.15 μg/ml		7.57 μg/ml		3.78 μg/ml		1.89 μg/ml		0.94 μg/ml		0.47 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}
387.20	1′20″	40.10	1′20″	56.40	1′20″	88,50	1′20″	279.00	1′40″	400.90	1′20″	461.20	1′00″
348.28	1′25″	139.00	1′00″	156.76	1′05″	230.61	1′20″	256.60	1′20″	281.50	1′20″	320.50	1′00″
148.40	1′50″	50.10	1′21″	63.00	1′20″	93.80	1′20″	108.30	1′10″	112.10	1′10″	146.00	1′10″
389.00	1′10″	120.30	1′10″	149.10	1′00″	179.70	3′10″	230.10	1′20″	258.70	1′00″	318.60	1′00″
318.22	1′26″	87.37*	1′13″	106.31*	1′11″	148.15*	1′45″	218.40*	1′20″	276.08	1′12″	311.57	1′02″
±57.38	±8″	±28.79	±9″	±26.99	±11″	±34.53	±56″	±38.14	±14″	±73.67	±9″	±64.51	±5″

* $p \leq 0.05$. Values below the line are expressed as mean ± SEM.

When L-Arg was added to the medium, PMA stimulation led to statistically significant inhibition of LACL at HS concentrations of 0.47–15.15 μg/ml with a mean baseline peak time of about 25 min (table 4). None of the HS concentrations used in the fMLP or PMA tests affected PMN viability as assessed by means of trypan blue exclusion.

Table 5 shows the effects of various HS concentrations on the LACL of bursts induced by fMLP after PMN washing. Under these conditions, only the HS concentrations of 15.5 and 7.57 μg/ml were still significantly active in reducing LACL intensity, whereas the lower concentrations showed no differences in comparison with baseline. Furthermore, for these first two concentrations, the extent of the effect was less than the case of no washing: 42 and 24% (washing) versus 67 and 55% (no washing), respectively.

Table 6 shows the results of the SIN-1 cocktail test in the cell-free system. Significant progressive inhibition of the chemiluminescence was induced by the addition of sulphurous water from an HS concentration of 2.75 μg/ml (86.90% inhibition) down to 0.34 μg/ml (23.64% inhibition). The effects of this inhibitory activity are also shown by the increased time to peak, which, in this test, indicates the presence of an interfering factor that affects the speed of production of oxidant species.

Table 3. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on the LACL of PMN bursts induced by PMA (peak = mV; T_{peak} = min·s)

Control		HS											
		15.15 μg/ml		7.57 μg/ml		3.78 μg/ml		1.89 μg/ml		0.94 μg/ml		0.47 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}
193.30	9′20″	26.21	8′30″	36.70	7′40″	60.00	7′10″	95.10	8′00″	130.10	8′30″	167.80	10′00″
138.60	11′30″	15.19	10′00″	16.90	8′50″	27.75	10′10″	72.80	11′10″	100.00	11′50″	141.00	11′10″
174.40	9′00″	15.50	6′40″	29.48	6′40″	51.10	8′20″	89.90	7′20″	122.50	8′00″	166.80	7′30″
180.20	7′10″	19.68	6′00″	38.00	5′00″	59.30	7′00″	92.10	7′10″	139.80	5′50″	167.00	7′00″
171.62	9′15″	19.14**	7′47″	30.27**	7′02″	49.54**	8′10″	87.47**	8′40″	123.07**	8′42″	160.65	9′10″
±11.70	±53″	±2.57	±54″	±4.83	±48″	±7.54	±43″	±5.01	±51″	±8.47	±1.07″	±6.55	±1′11″

** $p \leq 0.01$. Values below the line are expressed as mean ± SEM.

Table 4. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on the LACL of PMN bursts induced by PMA after L-Arg incubation (peak = mV; T_{peak} = min·s)

Control		HS													
		15.15 μg/ml		7.57 μg/ml		3.78 μg/ml		1.89 μg/ml		0.94 μg/ml		0.47 μg/ml		0.23 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	T_{peak}	T_{peak}	peak	T_{peak}	peak	T_{peak}
446.70	8′50″	33.20	10′10″	48.39	8′30″	77.22	8′50″	156.10	10′00″	214.08	8′30″	312.20	10′10″	401.40	10′30″
394.30	12′20″	28.00	9′30″	41.72	11′10″	70.10	10′30″	130.80	8′50″	200.10	12′20″	310.36	11′15″	390.10	14′00″
466.00	10′40″	34.28	8′10″	63.10	8′00″	101.30	9′40″	182.30	12′00″	275.30	8′10″	373.30	8′10″	420.00	9′15″
383.70	13′30″	52.30	13′20″	84.50	13′50″	100.90	14′50″	120.86	10′00″	168.52	9′25″	210.10	13′30″	391.10	10′10″
422.67	11′20″	36.94**	10′17″	59.43**	10′22″	87.38**	10′57″	147.51***	10′12″	214.50**	9′36″	301.49**	10′46″	400.65	12′43″
±19.96	±1′00″	±5.30	±1′05″	±9.48	±1′20″	±8.05	±1′20″	±13.76	±39″	±22.39	±56″	±33.79	±1.06″	±6.94	±1′27″

** $p \leq 0.01$. Values below the line are expressed as mean ± SEM.

Discussion

When struck by deleterious exogenous or endogenous substances, airway epithelial cells react by producing and releasing primary proinflammatory mediators, such as nuclear factor-κB, tumor necrosis factor-α and interferon-γ, which in turn cause the production of interleukins and a cascade of secondary lipid mediators, such as prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids and platelet-activating factor [28–31].

This complex traffic of a wide variety of biomolecules leads to the recruitment of infiltrating inflammatory cells, such as eosinophils, neutrophils and macrophages. The last two cell types are particularly organized to release ROS, nitric oxide (NO) and proteolytic enzymes. The recruitment of PMNs, which are the predominant cells leading to acute or chronic inflammation [32–34], is a protective mechanism, but ROS production by PMNs activated by various types of stimuli can damage not only the target but also the surrounding cells, thus triggering a self-sustained phlogogenic loop. The final result of these combined biochemical pathways is the induction of local inflammatory and immune responses [35]. These are not only the responses of host defenses, but also represent the recognized etiopathogenesis of inflammatory airway diseases, including asthma, chronic obstructive pulmonary disease, airway infections, bronchiectasis and cystic fibrosis [35–37].

Activated PMNs not only produce ROS, but also NO [38, 39], which subsequently combines with superoxide anion (O_2^-) to yield peroxynitrite anions ($ONOO^-$) [39]. It has been observed that NO-derived peroxynitrite is an

Table 5. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on the LACL of PMN bursts induced by fMLP after PMN washing

Control		HS											
		15.15 μg/ml		7.57 μg/ml		3.78 μg/ml		1.89 μg/ml		0.94 μg/ml		0.47 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}
41.78	2′10″	18.90	2′10″	30.90	1′40″	38.60	1′10″	61.50	1′20″	48.50	2′10″	44.20	2′50″
59.60	2′10″	42.90	1′20″	49.90	1′20″	63.50	0′50″	57.60	1′30″	54.10	1′50″	58.30	2′30″
80.70	1′20″	46.56	1′20″	56.77	1′10″	72.04	1′00″	61.66	0′50″	72.00	1′10″	75.80	1′40″
47.44	2′00″	24.74	1′30″	36.60	1′40″	42.14	2′00″	47.50	1′50″	44.10	1′00″	48.20	1′10″
57.38	1′55″	33.27**	1′35″	43.54*	1′27″	54.07	1′15″	57.06	1′22″	54.67	1′47″	56.77	2′02″
±8.62	±11″	±6.76	±11″	±5.94	±13″	±8.13	±15″	±3.32	±12″	±6.13	±25″	±6.96	±22″

* $p \leq 0.05$; ** $p \leq 0.01$. Values below the line are expressed as mean ± SEM.

Table 6. Effects of various concentrations of HS in the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' on LACL in a free-cell system using the SIN-1 cocktail

Control		HS									
		2.75 μg/ml		1.37 μg/ml		0.68 μg/ml		0.34 μg/ml		0.17 μg/ml	
peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}	peak	T_{peak}
30.42	6′30″	0.13	17′00″	5.60	11′20″	10.18	9′10″	23.72	8′40″	28.90	8′40″
51.28	6′40″	9.10	16′00″	28.97	16′00″	35.37	10′30″	41.00	9′50″	51.13	9′10″
69.10	4′10″	10.20	13′00″	23.94	10′20″	34.75	10′10″	52.41	9′00″	72.30	6′40″
26.52	4′12″	3.80	14′20″	8.60	12′10″	13.89	9′00″	18.29	8′30″	26.58	9′30″
44.33	5′20″	5.81*	15′00″**	16.78*	12′30″**	23.55*	9′40″**	33.85*	9′00″**	44.73	8′30″**
±9.88	±42″	±2.35	±53″	±9.88	±1′14″	±6.69	±22″	±7.85	±16″	±10.73	±30″

* $p \leq 0.05$; ** $p \leq 0.01$. Values below the line are expressed as mean ± SEM.

important mediator of free radical-dependent toxicity because of its strongly oxidizing effects [40–42]. All these radicals and their reactive derivatives are also released into extracellular spaces, where they indiscriminately break down biomacromolecules and thus contribute to extending the tissue injury, and broadening the area of the development and maintenance of airway and bronchial hyperreactivity [43].

Different sulphurous thermal waters have different physicochemical properties depending on their different compositions and amounts of ions and salts, but the common denominator is the presence of the sulfide species: hydrogen sulfide (H_2S), hydrosulfide ion (HS^-; also called the thiol group or sulfhydryl group) and sulfide anion (S^{2-}). Until relatively recently, H_2S was primarily considered a toxic environmental pollutant at some concentrations [44]; however, it is also produced endogenously from L-cysteine in mammalian tissues, mainly as a result of the activity of two pyridoxal phosphate-dependent enzymes: cystathionine-γ-lyase and cystathionine-β-synthetase [45–48], in the human mouth by resident bacteria [49], and in the large intestine of mammals as a result of the metabolism of sulfhydryl proteins by anaerobic bacteria [50].

The importance of sulfide compounds in biological processes has been demonstrated, and it is well known that the generation of sulfide is linked to a number of vital chemical, physical and biochemical processes [51].

Brain H_2S levels have recently been measured in rats, cattle and humans, and the relatively high concentration (up to 160 μM) in comparison with plasma (50 μM) suggests that it has a physiological function, probably as a neuromodulator [52, 53]. It has been suggested that endogenous H_2S may be a new gas signal transmitter in the cardiovascular system [54] and it is known to be a vasodilator and to have relaxant effect on smooth muscle [55, 56].

Our findings show that the sulphurous thermal water of the spring 'Fontanino dell'Acqua Marcia' significantly reduces the LACL of PMNs activated with fMLP and PMA. This is true even after the addition of L-Arg, which contributes to increasing LACL through NO-derived peroxynitrite production [57–59], thus indicating that this sulphurous water also has useful activity against this dangerous molecule.

H_2S is a colorless, flammable gas with a characteristic odor of rotten eggs and, in water, it dissociates to form HS^- and S^{2-} [60]. The H_2S in solution is in dynamic equilibrium at the air-water interface with gaseous H_2S in the air [44]. Characterizing the H_2S reaction intermediates in water and air is very complex, and the chemistry of H_2S is not completely understood. When H_2S is present in solution in natural sulphurous waters, it is generally oxidized to sulfate but, during the oxidation process, a number of S species in an intermediate state of oxidation can be produced, such as polysulfides, elemental S, thiosulfate and possibly other species [61]. When present or released in air, H_2S is oxidized by molecular oxygen and hydroxyl radicals to form the sulfhydryl radical and ultimately sulphur dioxide (SO_2) or sulfate compounds [42, 62].

As H_2S is a diprotic acid, the proportion of the various forms depend on the pH and temperature. At pH values of between 6 and 9 (the pH for most natural waters), about two thirds are HS^- and about one third H_2S (the S^{2-} form is present in negligible concentration) [44, 62, 63].

About 60–70% of the sulfides in the water of the 'Fontanino dell'Acqua Marcia' (pH 7.6) are in the form of HS^- anion, and only about 30–40% in the form of undissociated H_2S, and so the observed inhibitory activity on LACL can be mainly attributed to the HS group. This is in line with the well-known reducing activity of molecules having an HS group [17, 18].

As dissociated species such as HS^- anion do not generally diffuse easily through biological membranes, its effect on LACL can mainly be attributed to its scavenger activity, as the HS groups are mainly confined inside the medium. The presence of a scavenger effect is confirmed by the cell-free findings, as well as by the results obtained when we washed the PMNs after incubation with sulphurous water, thus removing the HS groups. In this case, only the two higher HS concentrations had an effect on LACL, and this was less than that observed without washing. However, this partial effect can still be attributed to the amount of H_2S, present in the water during the incubation time which, being undissociated and thus capable of diffusing more rapidly through the biological membranes of PMNs, entered the cytoplasm and dissociated there into HS^- and H^+. This amount of HS groups released into the cytoplasm can interfere with intracellular ROS, thus explaining why the inhibitory effect on LACL was still present when the PMNs were washed.

One further point concerning this effect on LACL should be considered: cytoplasmic HS groups can open the disulphide bond (S = S) for example in the oxidized form of glutathione (GS = SG), thus leading to the reconstitution of the reduced and active antioxidant form of glutathione (GSH). At the same time, the presence of intracellular free sulfhydryl groups working as sacrificial (reducing) groups can protect GSH from oxidation due to metabolic activation or ROS.

The pharmacological uses of sulphurous water have been traditionally applied to various disorders, and sulphurous water inhalation therapy seems to be particularly useful in phlogistic respiratory disorders. Scientific proof of the activity of sulphurous thermal waters has been partially based on the patients' subjective sense of well-being and partially on symptomatic (or general) clinical improvements that are not always easy to quantify. The albeit relatively few scientific experimental reports confirm the presence of in vitro activity, and our findings indicate that, in addition to their known mucolytic effects and trophic activity on the respiratory mucosa, the HS groups present in the sulphurous thermal water of the 'Fontanino dell'Acqua Marcia' also have useful antioxidant activity, thus contributing to the water's therapeutic effect in upper and lower inflammatory airway diseases. As these effects are similar to those that can be obtained using 'thiolic drugs', this sulphurous thermal water can be called 'thiolic water'; however, each sulphurous thermal water has its own personal 'finger print' arising from differences in the concentration of HS and other ions leading to different chemical characteristics and properties. In order to maximize the available therapeutic possibilities, studies of this type should therefore be extended to other sulphurous thermal waters.

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