Sulphite enhances peroxynitrite-dependent α_1-antiproteinase inactivation. A mechanism of lung injury by sulphur dioxide?

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Abstract Sulphite is toxic to the lung and can cause allergic reactions, the most common of which is bronchoconstriction in asthmatics. We show that sulphite can considerably potentiate the inactivation of α_1-antiproteinase caused by peroxynitrite. Addition of peroxynitrite to sulphite generated inactivating species that persisted at pH 7.4 and 37°C for at least 30 min. We propose that formation of protein-modifying sulphite radicals from SO_3^{2-} exposed to $ONOO^-$ is a mechanism by which SO_2 could cause lung injury, both by enhancing proteolysis and by creating new antigens that could provoke an immune response.

Key words: Sulfite; Sulfur dioxide; Peroxynitrite; α_1-Antiproteinase; Lung injury; UV spectroscopy

1. Introduction

Sulphur dioxide is an air pollutant released into the atmosphere from the combustion of fossil fuel [1]. Sulphur dioxide can be converted to sulphite[1] upon contact with fluids lining the air passages [2]. In addition, sulphite enters the body in foods, beverages and drugs because sulphiting agents (sulphur dioxide, metabisulphite, bisulphite and sulphite) are widely used as preservatives [3]. Endogenous sulphite is generated during the normal processing of sulphur-containing amino acids [4–6] and can be formed by the metabolism of sulphur-containing drugs, including *N*-acetylcysteine [7].

Sulphite can cause allergic reactions in humans [8], the most common of which is bronchoconstriction in asthmatics [9]. Sulphite also produces a variety of toxic effects in animals [10] and may act as a cocarcinogen [11]. In humans, deficiency of sulphite oxidase, an enzyme which catalyses the oxidative detoxification of sulphite, leads to mental retardation, neurological symptoms such as spastic quadriplegia, and early death [4,5,12,13]. Little information is available about the mechanism of sulphite toxicity, but it may involve formation of sulphur- and oxygen-centred free radicals, such as $SO_3^{\bullet-}$, $SO_4^{\bullet-}$ and $SO_5^{\bullet-}$ [14,15]. Sulphite radicals can damage nucleic acids [16] and induce mutation [17,18]. Moreover, these radicals can react with proteins [19] and lipids [20].

Peroxynitrite, formed from the reaction between superoxide and nitric oxide [21,22], is a potent oxidant that also can contribute to tissue injury in human disease. It is well known to be generated in the inflamed lung [23,24]. Peroxynitrite and its decomposition products induce peroxidation of lipids, cause DNA damage, deplete antioxidants and oxidise methionine and -SH residues in proteins (reviewed in [22,25,26]). $ONOO^-$ also inactivates α_1-antiproteinase (α_1-AP) [27,28], the major inhibitor of serine proteinase enzymes (such as elastase) in human body fluids [29]. Lung is especially susceptible to damage by elastase [29] and recent studies establish a connection between low activity of α_1-AP and asthma [30,31]. α_1-AP inactivation is an important contributor to lung damage in emphysema and possibly in cigarette smokers [32,33] and is widely used as a model system for protein damage, which is an important consequence of oxidative stress in vivo[25].

In the present study, we examine the influence of sulphite in combination with peroxynitrite on the inactivation of α_1-AP.

2. Materials and methods

2.1. Reagents

N-Succinyl (ala)$_3$ *p*-nitroanilide (SANA), elastase (E0258), and α_1-AP (A9024) were from Sigma, Poole, Dorset, UK. Sodium sulphite (anhydrous) was obtained from Fisons, Loughborough, Leics, UK, sodium hypochlorite solution from Aldrich, Steinheim, Germany, and Chelex 100 resin (200–400 mesh, sodium form) from BioRad, California, USA. All other reagents were of the highest quality available from BDH Chemicals Ltd, Poole, UK. Solutions of sodium sulphite were made up fresh daily in distilled water.

2.2. Peroxynitrite synthesis

Peroxynitrite was synthesised essentially as described by Beckman et al. [22]. For full details see [34,35].

2.3. Measurement of α_1-AP inactivation

Inactivation of α_1-AP was followed by its loss of elastase-inhibitory capacity essentially as described before [28,36]. The volume of α_1-AP solution needed to inhibit elastase activity 80–90% (usually 50–60 μl) was added to buffer (500 mM K_2HPO_4-KH_2PO_4, pH 7.4) with or without sulphite (5 μM–10 mM), copper or iron ($CuSO_4$, $FeSO_4$, $FeCl_3$ 5–10 μM) to give a volume of 0.983 ml. The mixture was incubated in a water bath at 37°C for 15 min. Peroxynitrite (typically 5 μl) was added to give the desired final concentration (in most experiments 0.5 mM). The sample was vortexed for 10 s and incubated for a further 15 min. The final pH was 7.4–7.5. Then elastase (usually 12 μl, 0.8 μM final conc.) was added followed by 2.0 ml of buffer (500 mM K_2HPO_4-KH_2PO_4, pH 7.4), and the sample incubated at 37°C for another 15 min. Finally, 0.1 ml of elastase substrate (SANA, 0.5 mg/ml) was added and the rate of reaction followed at 410 nm for 30 s. A similar procedure was followed for inactivation of α_1-AP by HOCl. HOCl (typically 5 μl) was added to α_1-AP to give a final concentration of 9 μM. HOCl was diluted in ice-cold distilled water freshly before use and its concentration determined spectrophotometrically as in [37].

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[1] The term sulphite has been used throughout this manuscript to indicate both the unprotonated (SO_3^{2-} or sulphite) and protonated (HSO_3^- or bisulphite) forms of this molecule. At pH 7.4 approximately 75% of the molecules are unprotonated.

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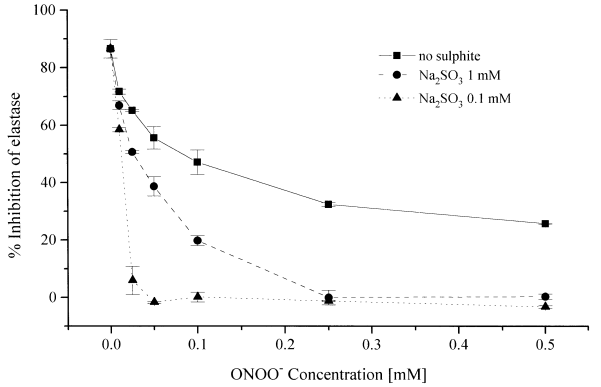

Fig. 1. Aggravation of the peroxynitrite dependent α_1-AP inactivation by sulphite. Dependence on $ONOO^-$ concentration. α_1-AP was preincubated with or without Na_2SO_3 (0.1 or 1 mM) for 15 min when $ONOO^-$ was added at the final concentration stated and incubated at pH 7.4 and 37°C for a further 15 min. Residual elastase inhibitory capacity was measured as described in Section 2. Data are mean ± S.E.M. ($n = 4$).

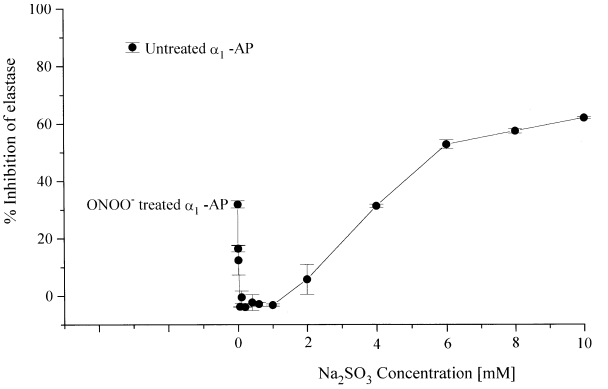

Fig. 2. Aggravation of the peroxynitrite dependent α_1-AP inactivation by sulphite at pH 7.4. Dependence on sulphite concentration. α_1-AP was preincubated with the final Na_2SO_3 concentration stated for 15 min when $ONOO^-$ 0.5 mM was added and incubated at pH 7.4 and 37°C for a further 15 min. Residual elastase inhibitory capacity was measured as described in Section 2. Data are mean ± S.E.M. ($n = 4$).

3. Results

3.1. Potentiation of the peroxynitrite-dependent α_1-AP inactivation by sulphite

The activity of α_1-AP was measured by its ability to inhibit the serine protease elastase. α_1-AP caused $\geq 80\%$ inhibition under our experimental conditions (Fig. 1). As expected [27,35], addition of $ONOO^-$ to α_1-AP led to a concentration-dependent inactivation of the ability of α_1-AP to inhibit elastase (Fig. 1). Addition of sulphite (0.1 mM and 1 mM) to the reaction mixture with α_1-AP and $ONOO^-$ markedly potentiated the α_1-AP inactivation although SO_3^{2-} itself had no effect. For example, 0.05 mM $ONOO^-$ without sulphite decreased the capacity of α_1-AP to inhibit elastase from $\geq 80\%$ to 58%, whereas the same peroxynitrite concentration with 0.1 mM sulphite resulted in a complete inactivation of α_1-AP, i.e. the capacity of α_1-AP to inhibit elastase was reduced to zero. The effect of sulphite on the $ONOO^-$ induced inactivation of α_1-AP was concentration-dependent (Fig. 2). At a constant peroxynitrite concentration of 0.5 mM, sulphite concentrations in the physiological range and above (5 μM to ≤ 4 mM) increased α_1-AP inactivation by $ONOO^-$ whereas only non-physiological concentrations (> 4 mM) decreased the inactivation.

Control experiments showed that the activity of elastase itself was not significantly affected by inclusion of sulphite (0.1–10 mM) or peroxynitrite (0.5 mM) in the reaction mixture at the reaction conditions used. Sulphite (0.1–10 mM) had no significant effect on the ability of α_1-AP to inhibit elastase and decomposed $ONOO^-$ (0.5 mM) had no effect, either on elastase, or on α_1-AP (Table 1, row D and Fig. 3).

3.2. Reactive species resulting from the reaction between sulphite and peroxynitrite persist in the reaction mixture

If the $ONOO^-$ solution (0.5 mM) was added to the buffer, vortexed for 10 s and incubated for 1 min at 37°C before adding α_1-AP the resulting decomposed $ONOO^-$ solution had no effect on α_1-AP (Table 1, row D and Fig. 3). However, if the 0.5 mM solution of $ONOO^-$ was added to buffer containing 1 mM sulphite and incubated for 1 min at 37°C before adding α_1-AP the resulting reaction mixture abolished the ability of α_1-AP to inhibit elastase (Table 1, row E). This

Table 1
Reaction between sulphite and $ONOO^-$: formation of reactive species that persist in the reaction medium

	Time point of addition to reaction mixture			Elastase activity [ΔA_{410}/min]
	Sulphite	α1-AP (0.2 mg/ml)	$ONOO^-$ (0.5 mM)	
A	none	none	none	1.20 ± 0.025
B	none	at time 0	none	0.20 ± 0.094
C	none	at time 0	after 15 min	0.81 ± 0.080
D	none	after 16 min (1 min after $ONOO^-$)	after 15 min	0.20 ± 0.026
E	1 mM at time 0	after 16 min (1 min after $ONOO^-$)	after 15 min	1.19 ± 0.013
F	1 mM at time 0	after 25 min (10 min after $ONOO^-$)	after 15 min	1.23 ± 0.036
G	1 mM at time 0	after 35 min (20 min after $ONOO^-$)	after 15 min	1.10 ± 0.056
H	1 mM at time 0	after 45 min (30 min after $ONOO^-$)	after 15 min	0.81 ± 0.041
I	0.1 mM after 20 min	at time 0	after 15 min	0.81 ± 0.073
J	1 mM after 20 min	at time 0	after 15 min	0.85 ± 0.029
K	10 mM after 20 min	at time 0	after 15 min	0.79 ± 0.059

Row A: activity of the uninhibited elastase. Addition of α_1-AP inhibits the elastase (row B). Treatment of the α_1-AP with $ONOO^-$ decreases this inhibitory effect (row C) but treatment with 'decomposed' $ONOO^-$ does not (row D). Inclusion of 1 mM sulphite in the reaction mixture results in persistent, reactive species that abolish or decrease the inhibitory effect of α_1-AP even if it is added 10 or 30 min after $ONOO^-$ (rows E–H). Addition of sulphite 5 min after $ONOO^-$ does not further increase damage to α_1-AP (rows I–K). Results are mean ± S.D. ($n = 4$). Incubations with the complete reaction mixture (before addition of the elastase) were for 15 min at 37°C. Residual elastase activity was then measured as described in Section 2.

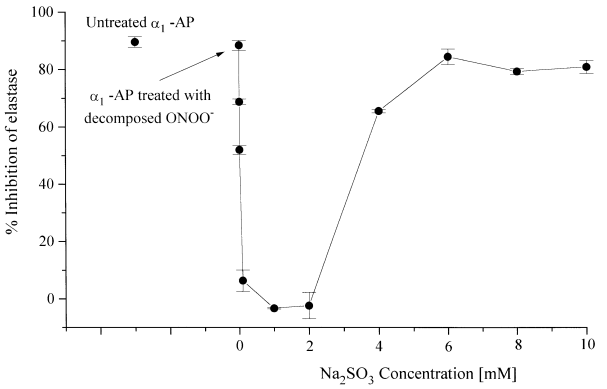

Fig. 3. Reaction between sulphite and $ONOO^-$ at pH 7.4. Formation of reactive species that persist in the reaction medium. Na_2SO_3 at the stated final concentration was preincubated at pH 7.4 and 37°C for 15 min when $ONOO^-$ 0.5 mM was added, vortexed for 10 s and incubated for 1 min at 37°C. α_1-AP (0.2 mg/ml) was then added and incubated for a further 15 min at 37°C. Residual elastase inhibitory capacity was measured as described in Section 2. Data are mean ± S.E.M. ($n = 4$).

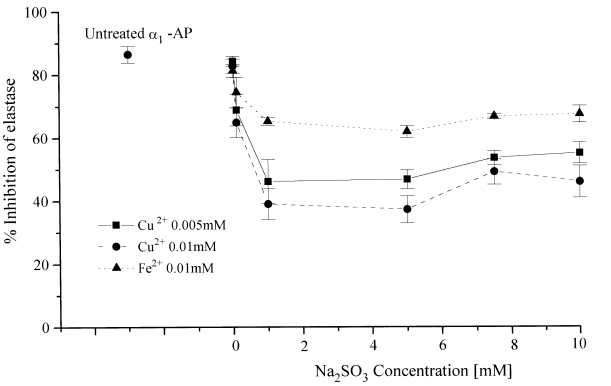

Fig. 4. Inactivation of α_1-AP by sulphite plus copper or iron. α_1-AP was incubated with Na_2SO_3 plus copper or iron at the final concentrations stated for 30 min at pH 7.4 and 37°C. Residual elastase inhibitory capacity was measured as described in Section 2. Data are mean ± S.E.M. ($n = 4$).

marked inactivation was observed even after an incubation period of 30 min before α_1-AP addition (Table 1, rows F–H). Hence the reaction between sulphite and peroxynitrite results in damaging species that persist for at least 20–30 min in the reaction mixture. Fig. 3 shows that the existence of such reactive species was again dependent on the ratio of sulphite to $ONOO^-$. Only sulphite concentrations about the same order of magnitude or lower than the $ONOO^-$ concentration caused inactivation of α_1-AP if the $ONOO^-$ solution was added 1 min before α_1-AP. Sulphite concentrations higher than 6 mM did not influence the elastase inhibitory capacity of α_1-AP under these reaction conditions.

If sulphite (10 μM to 10 mM) was added to the reaction mixture 5 min after incubation of α_1-AP with $ONOO^-$, it did not further increase damage to α_1-AP (Table 1, rows I–K). Hence sulphite did not act by further potentiating or by repairing the already existing damage caused to α_1-AP by $ONOO^-$.

3.3. Inactivation of α_1-AP in the presence of sulphite and copper or iron

Incubation of α_1-AP with sulphite and Cu^{2+} (5 μM or 10 μM) for 30 min at 37°C and pH 7.4 caused a marked reduction in its elastase inhibitory capacity (Fig. 4). A similar inactivation of α_1-AP was observed when it was incubated with sulphite and Fe^{2+} (10 μM). Control experiments showed that Cu^{2+} (10 μM), Fe^{2+} (10 μM) and Fe^{3+} (10 μM) had no significant effect on elastase or α_1-AP at the reaction conditions used.

4. Discussion

In the present paper, we show that sulphite can considerably potentiate the inactivation of α_1^-AP caused by addition of $ONOO^-$. The effect of sulphite on peroxynitrite-induced α_1-AP inactivation was dependent on the ratio of sulphite to $ONOO^-$. When the sulphite concentration was about the same order of magnitude or lower than the $ONOO^-$ concentration, sulphite aggravated the damage to α_1-AP whereas if it was 10–100 times higher, it had a protective action. The inactivating species persisted in the reaction mixture for at least 20–30 min. Compared to $ONOO^-$, which was completely inactive after 1 min at pH 7.4, the damaging power remained for a relatively long period of time. The reaction between sulphite and $ONOO^-$ may initiate a chain propagation reaction during which sulphur- and oxygen-centred free radicals are formed [38]. These radicals can then damage α_1-AP. At higher levels of sulphite, the $SO_3^{\bullet-}$, $SO_4^{\bullet-}$ and $SO_5^{\bullet-}$ radicals formed may well disappear by further reactions with SO_3^{2-} rather than attacking α_1-AP. The protection observed at high sulphite concentrations may thus be due to scavenging of $ONOO^-$ and/or $ONOO^-$-derived species. Sulphite alone had no direct effect on α_1-AP and addition of sulphite to α_1-AP already damaged by $ONOO^-$ did not restore or further diminish its activity. Consistent with the assumption that the potentiation of the $ONOO^-$-dependent inactivation of α_1-AP by sulphite involves sulphite radicals, no sulphite potentiation of the inactivation of α_1-AP by HOCl was observed even at low sulphite concentrations (data not shown). In contrast to $ONOO^-$, which can act as a one-electron and a two-electron oxidant [38,39], HOCl mainly acts as a two-electron oxidant [40] and does not therefore generate sulphite radicals. Another mechanism of generating sulphite radicals is by exposure of SO_3^{2-} to transition metal ions [41,42]. Indeed, mixtures of copper or iron ions with SO_3^{2-} also inactivated α_1-AP. The transition metal catalyzed autoxidation of sulphite is an oxygen-consuming chain reaction [41,42].

The data show that sulphite in combination with peroxynitrite or transition metal ions leads to considerable damage of α_1-AP in vitro. It seems likely that α_1-AP and other proteins can be damaged by sulphite radicals in vivo, since SO_2 can form SO_3^{2-} at low levels in lung lining fluids, and nitric oxide and superoxide can be generated in the lung. Peroxynitrite formation has often been demonstrated in the injured/inflamed lung [23,24], and $ONOO^-$ concentrations as low as 10 μM could interact with SO_3^{2-} to inactivate α_1-AP. This may be a potential mechanism by which SO_2 could aggravate injury to the inflamed lung, as in asthma. Another possibility is that reaction of the sulphite radical with proteins could generate new antigens, which might be a trigger for the allergic responses to sulphite shown by some subjects.

Living organisms are equipped with sulphite oxidase to detoxify sulphite, although this enzyme may not be present in

lung lining fluids. The deleterious consequences of sulphite oxidase deficiency [4,5,12,13] indicate the noxious nature of SO_3^{2-}. Copper and iron, as well as other transition metals are ubiquitous in living organisms and can be released from damaged tissues to provide another mechanism of damage by sulphite. Indeed, iron can be released into lung lining fluids during lung injury [43], and $ONOO^-$ can degrade caeruloplasmin, a copper-containing protein present in lung lining fluids, to release Cu ions [44].

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