Thiols and disulphides can aggravate peroxynitrite-dependent inactivation of α_1-antiproteinase

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Abstract Peroxynitrite ($ONOO^-$) is a cytotoxic species formed in vivo. There is considerable interest in the development of $ONOO^-$ 'scavengers' as therapeutic agents; several thiols have been suggested to fulfil this role. One protein inactivated by $ONOO^-$ is α_1-antiproteinase (α_1AP), the major inhibitor of serine proteinases in human body fluids. At low thiol:$ONOO^-$ concentration ratios, several thiols (captopril, penicillamine, cysteine, cystine and penicillamine disulphide) aggravated inactivation of α_1AP by $ONOO^-$, whereas GSH, GSSG, homocysteine, ergothioneine, *N*-acetylcysteine, lipoate and dihydrolipoate did not. We suggest that sulphur-containing radicals are produced by reaction of certain thiols/disulphides with $ONOO^-$ or $ONOO^-$-derived products and could mediate biological damage, including inactivation of α_1AP. This must be considered in attempts to use thiols as 'peroxynitrite scavengers'.

Key words: Peroxynitrite; α_1-Antiproteinase; Thiol; Disulfide; Thiyl radical; Homocysteine

1. Introduction

Although the free radical gas nitric oxide (nitrogen monoxide, NO•) has many important physiological functions, its production in excess may contribute to the pathology of several diseases [1–3]. Part of the toxicity of NO• involves its fast [4] reaction with $O_2^{\bullet-}$ to give peroxynitrite, $ONOO^-$.

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

At pH 7.4, $ONOO^-$ protonates and decomposes by a series of complex reaction pathways: addition of $ONOO^-$ to biological systems can result in peroxidation of lipids, oxidation of methionine and -SH residues in proteins, depletion of antioxidants, displacement of metals from metalloproteins, DNA oxidation and nitration, and nitration of protein tyrosine residues (reviewed in [1,5,6]).

There is therefore considerable interest in the design of peroxynitrite 'scavengers' for therapeutic use [3]. Peroxynitrite and/or its breakdown products react with many -SH compounds, including GSH and penicillamine [5,7–12], whereas their reactions with disulphides appear generally slower [13], although lipoic acid is an exception [12]. In the present paper, we show that certain thiols and disulphides, at low concentrations relative to those of $ONOO^-$, can *aggravate* damage to a protein caused by $ONOO^-$ addition. The protein investigated is α_1-antiproteinase, a physiologically important protein that is the major inhibitor of serine proteases (such as elastase) in human body fluids. α_1-Antiproteinase has already been shown to be inactivated upon addition of $ONOO^-$ in vitro [14]. α_1-Antiproteinase is inactivated at sites of inflammation in vivo [15], and attack by $ONOO^-$ provides a plausible explanation of the inactivation of α_1-antiproteinase in the inflamed rheumatoid joint [8].

2. Materials and methods

2.1. Reagents

N-Succinyl (ala)$_3$ *p*-nitroanilide (SANA), elastase (E0258), α_1-antiproteinase (A9024), thiols, disulphides, D,L-tyrosine and all other reagents were from Sigma Chemical Corp., London, UK, except that lipoic and dihydrolipoic acids were a gift from Asta Medica, Germany. Peroxynitrite was synthesised as described in [1]. Concentrations of stock $ONOO^-$ were re-determined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1} [1]. Concentrations of 250–300 mM were usually obtained.

2.2. Analysis of nitrotyrosine

Nitration of tyrosine on addition of peroxynitrite was measured by HPLC as described in [13].

2.3. α_1-Antiproteinase inactivation

Elastase and α_1-antiproteinase (α_1AP) activities were measured essentially as described in [16]. α_1AP was dissolved in phosphate-buffered saline, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4) to a concentration of 4 mg/ml and elastase in the same buffer to 5 mg/ml. The volume of α_1AP needed to inhibit elastase 80–90% (typically 60–70 μl) was added to buffer (500 mM K_2HPO_4-KH_2PO_4 pH 7.4) with or without 0.1 ml of thiol/disulphide to be tested to give a volume of 0.945 ml (final α_1AP concentration 0.3 mg/ml) and incubated in a water bath at 37°C for 15 min, when peroxynitrite (typically 5 μl) was added to give a final concentration of 0.5 mM. The sample was vortexed for 10 s and incubated for 5 min. Then elastase (usually 50 μl) was added and the mixture incubated for 15 min. This was followed by addition of 2.0 ml of buffer and incubation at 37°C for a further 15 min. Finally, 0.1 ml of elastase substrate (SANA) was added and the rate of reaction followed at 410 nm for 30 s. A similar procedure was followed for inactivation of α_1AP by hypochlorous acid (HOCl); α_1AP was incubated with HOCl (10 μM) for 60 min since under these reaction conditions the degree of inactivation was similar to that produced by 0.5 mM $ONOO^-$ for 5 min incubation. HOCl was prepared fresh before use and its concentration determined, both as described as in [17].

3. Results

3.1. Inactivation of α_1AP by $ONOO^-$ addition

Addition of $ONOO^-$ to α_1AP at pH 7.4 causes loss of its elastase-inhibitory capacity [13,14]. The reaction is complete within 5 min and the extent of inactivation depends upon peroxynitrite concentration; 0.5 mM was chosen on the basis of previous studies to achieve a substantial, but not complete, inactivation [13]. Previous studies [13] have shown that other constituents of the $ONOO^-$ preparation [1] do not contribute to the inactivation.

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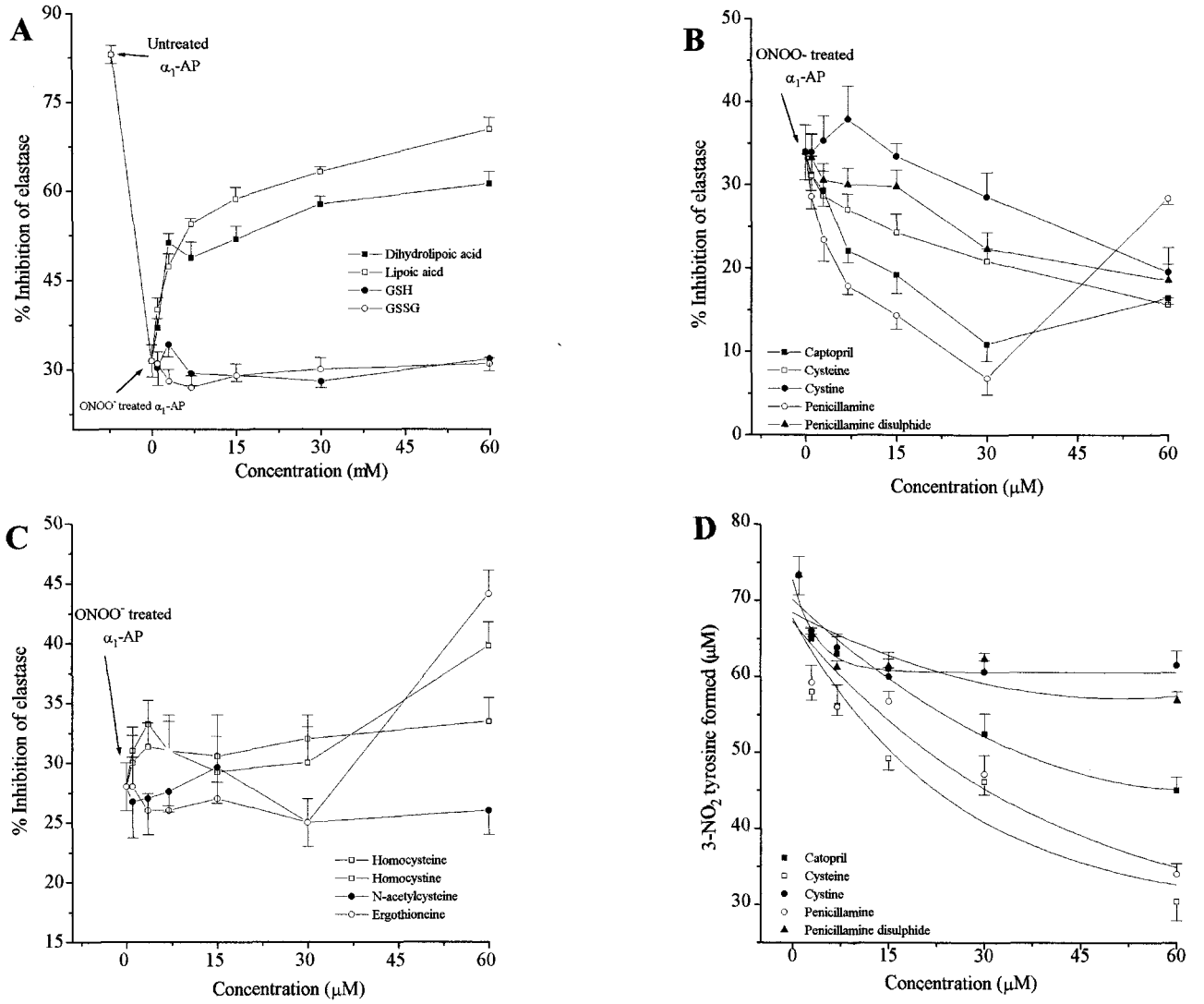

Fig. 1. Effect of various compounds on the inactivation of α_1AP by peroxynitrite in a strongly buffered solution at pH 7.4. Concentrations given on the x-axis are the final concentrations in the first reaction mixture (containing α_1AP and buffer). Results are mean ± S.E., $n \geq 4$. Before treatment with $ONOO^-$, the α_1AP inhibited elastase by 85 ± 4% (A). A: No effect of GSH or GSSG on inactivation; protection by lipoic acid and dihydrolipoate. B: Aggravation of inactivation by low concentrations of captopril, penicillamine, cysteine, cystine and penicillamine disulphide. The scale on the x-axis has been expanded to make the results clearer. C: No effect of homocysteine, homocystine, *N*-acetylcysteine or ergothioneine (scale also expanded). D: Effect of some of the above compounds on the nitration of tyrosine by $ONOO^-$. No stimulation of nitration was shown by any compound.

3.2. *Aggravation of $ONOO^-$-dependent α_1AP inactivation by certain thiols and disulphides*

Fig. 1 shows the effect of adding low concentrations of thiols and disulphides. Captopril, penicillamine, cysteine, cystine and penicillamine disulphide reproducibly aggravated α_1AP inactivation by $ONOO^-$ (Fig. 1B). By contrast, homocysteine, dihydrolipoate, ergothioneine, lipoic acid, GSH, GSSG and *N*-acetylcysteine at similar concentrations did not (Fig. 1A,C). Indeed, dihydrolipoate and lipoate were protective (Fig. 1A).

Control experiments showed that none of the thiols/disulphides had any direct effect on elastase, or on the ability of α_1AP to inhibit elastase, i.e. they do not interfere with the assay procedures. If any of the thiols or disulphides were added to the reaction mixture *after* 5 min incubation of α_1AP with $ONOO^-$, they had no effect (Fig. 2A). Hence these compounds cannot alter the residual activity of α_1AP after damage by $ONOO^-$. Incubation of the thiols or disulphides with $ONOO^-$ for 5 min before adding α_1AP gave no observable inactivation of α_1AP subsequently added (Fig. 2B), except for a small effect in the case of penicillamine. Hence the products of reaction of thiols/disulphides with $ONOO^-$ that cause inactivation of α_1AP do not persist in the reaction mixture.

3.3. *Lack of effect of thiols/disulphides on nitration of tyrosine by $ONOO^-$*

Addition of peroxynitrite to cells and tissues leads to nitration of tyrosine residues [1]. Hence another assay often used to assess peroxynitrite 'scavengers' in vitro is to examine their effect on tyrosine nitration [1,8,18]. None of the thiols or disulphides at concentrations that aggravated α_1AP inactivation stimulated the nitration of tyrosine. Either they had no effect (cystine, penicillamine disulphide) or they inhibited ty-

rosine nitration (captopril, ergothioneine, cysteine, penicillamine) (Fig. 1D).

3.4. Effect of thiols/disulphides on inactivation of α_1AP by HOCl

Another agent known to inhibit α_1AP in vitro is HOCl [19]. None of the compounds that aggravated α_1AP inactivation in the presence of $ONOO^-$ did so in the presence of HOCl: they either protected or had no effect (Fig. 3). Our data are consistent with previous literature reports that thiols and certain disulphides are HOCl scavengers [17,20,21].

4. Discussion

Our data show that certain thiols and disulphides at low concentrations can aggravate the inactivation of α_1AP upon addition of $ONOO^-$. By contrast, addition of any of the thiols (or of lipoic acid) at concentrations greater than 200 μM decreased inactivation of α_1AP (data not shown, as in agreement with previous literature reports [8,12,13]). Our data show the importance of testing 'scavengers' over a wide concentration range.

What mechanism can account for these effects? The thiols/disulphides had no direct effect on α_1AP or elastase. Addition of them to α_1AP already inactivated by $ONOO^-$ did not restore or further diminish activity (Fig. 2A). Hence the effects must involve interaction of the thiols/disulphides with $ONOO^-$ or $ONOO^-$-derived species. However, any damaging products that result do not survive in the reaction mixture when $ONOO^-$ is preincubated with thiols/disulphides before α_1AP addition (Fig. 2B).

Several thiols are known to generate thiyl and thiyl-derived radicals upon exposure to $ONOO^-$ [5,9–11]. It is also known that penicillamine-derived sulphur radicals can inactivate α_1AP [22]. It thus seems logical to propose that $ONOO^-$-dependent formation of sulphur radicals from certain thiols/disulphides is the explanation of aggravated α_1AP inactivation. At higher levels of thiols/disulphides, the sulphur radicals responsible may well disappear by further reactions with their parent compounds [23,24] rather than attacking α_1AP, thereby accounting for loss of the aggravating effect. A mechanism involving sulphur radicals would also explain why no aggravation of HOCl-dependent inactivation of α_1AP is observed; reaction of HOCl with GSH (and presumably other thiols or disulphides) is reported not to generate free radicals [25]. The lack of stability of the species involved (Fig. 2B) is also consistent with free radical formation.

There is growing interest in biological damage by thiyl and oxysulphur radicals [21,23,24,26]. For example, such radicals have been suggested to account for the autoimmunity often seen in arthritis patients treated with penicillamine [22]. If our proposed explanation is correct, then there must be fundamental differences in the ability of sulphur radicals derived from different thiols/disulphides to inactivate α_1AP: cysteine, captopril, cystine, penicillamine and penicillamine disulphide aggravated α_1AP inactivation, but GSH, ergothioneine and homocysteine did not. Of course, sulphur radicals are able to damage biological targets other than α_1AP [26,27]. The use of α_1AP may thus represent a useful 'screen' for potential toxic effects of radicals derived by interaction of $ONOO^-$ and/or $ONOO^-$-derived species with putative '$ONOO^-$ scavengers' proposed as therapeutic agents.

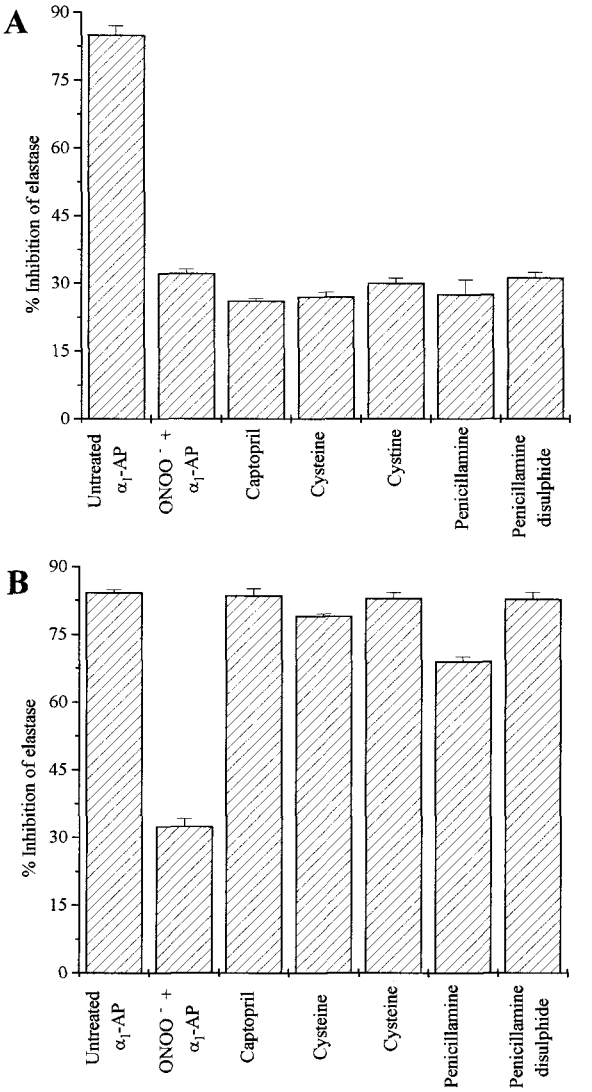

Fig. 2. Control experiments. A: α_1AP was incubated with $ONOO^-$ and buffer for 5 min, then thiols/disulphides were added to give a 30 μM final concentration followed by a further 30 min incubation. None of the compounds restored or further decreased the elastase-inhibitory capacity of α_1AP. B: Peroxynitrite was incubated with thiols/disulphides (30 μM) for 5 min, then α_1AP was added followed by a further 30 min incubation. There was no effect on the elastase-inhibitory capacity of α_1AP, except for a small decrease in the case of penicillamine. Results for both panels are mean ± S.E., $n = 4$.

Another question is whether aggravation of damage to α_1AP by this mechanism could occur in vivo. This protein is inactivated at sites of chronic inflammation, e.g. in the inflamed rheumatoid joint [15], in which $ONOO^-$ is generated at high levels as evidenced by measurements of nitrotyrosine [28] and NO_2^-/NO_3^- [29]. Levels of cysteine and cystine in human body fluids are normally in the range of 8–10 and 90 μM respectively [30], and typical body fluid levels of penicillamine in patients taking this drug are 20–50 μM [31]. These levels are comparable to those shown here to aggravate the effects of $ONOO^-$ on α_1AP Of course, we have used a high $ONOO^-$ concentration to achieve substantial inactivation of α_1AP in a short time and allow accurate measurements. Aggravation of $ONOO^-$-dependent α_1AP inactivation

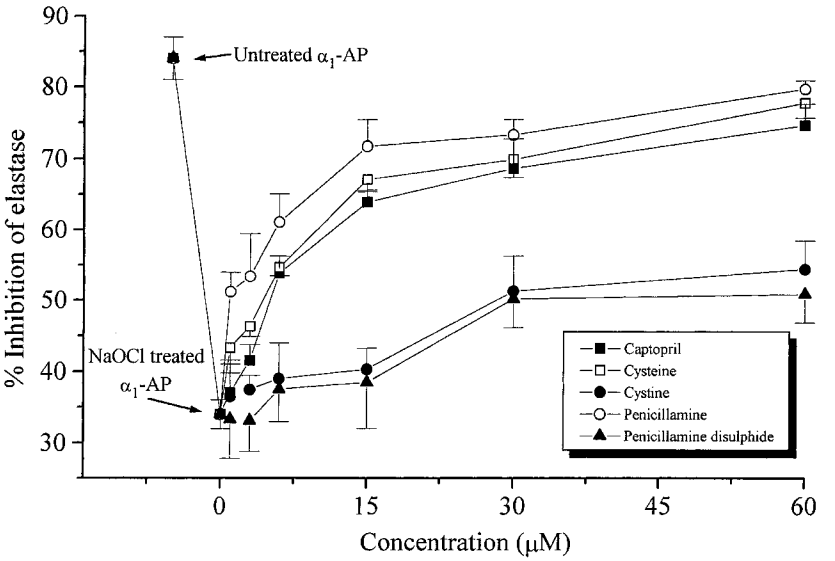

Fig. 3. Effect of various thiols/disulphides on the inactivation of α_1AP by HOCl at pH 7.4. No aggravation of inactivation by HOCl was observed by any of the thiols or disulphides mentioned in this paper.

is still observed at lower $ONOO^-$ concentrations (tested down to 50 μM). Hence it might be feasible in vivo, although more work is needed to establish this.

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