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Scavenging of hypochlorous acid by lipoic acid

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Upon stimulation, neutrophils are able to produce the reactive oxygen species, superoxide ($O_2^{\cdot -}$) and hydrogen peroxide. Moreover, neutrophils release the enzyme myeloperoxidase. This enzyme catalyses the conversion of chloride (Cl^-) to the powerful oxidant hypochlorous acid (HOCl*) [1, 2]. The reactive oxygen species and HOCl contribute to the bactericidal action of neutrophils. However, the damaging effect of these products is not limited to bacteria, also the surrounding tissue is vulnerable. An important target for HOCl is the α_1-antiproteinase (α_1-AP). α_1-AP is the most important inhibitor of elastase [1, 2]. HOCl oxidizes a critical methionine residue of α_1-AP to a sulphoxide derivative with the consecutive loss of activity of the inhibitory protein [3]. In addition, activated neutrophils excrete elastase. A resulting imbalance between elastase and anti-elastase activity in the respiratory tract may cause the enzymatic destruction of the elastic fibers in the lung, a process believed to be central in the development of certain types of emphysema [1–3].

Lipoate is an 8-carbon fatty acid with, in its reduced form, two thiol groups on the 6th and 8th carbon atom. Oxidized lipoate contains an intramolecular disulfide bridge in a 5-membered ring. Lipoate has been shown to be a potent antioxidant [4, 5]. In the present study the ability of lipoate to scavenge HOCl was determined. The scavenging activity is compared to that of the potent scavengers *N*-acetylcysteine and GSH.

* Abbreviations: HOCl, hypochlorous acid; α_1-AP, α_1-antiproteinase; GSH, glutathione; GSMe, *S*-methyl glutathione; GSSG, oxidized glutathione.

Materials and Methods

N-Acetylcysteine, GSH, GSSG, *S*-methyl glutathione (GSMe), *N-t*-BOC-L-alanine *p*-nitrophenol ester, α_1-antiproteinase (α_1-AP, code A 9024) and elastase (code E 0258) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Reduced lipoate (dihydrolipoate) and oxidized lipoate were gifts from Asta Pharma A.G. (Frankfurt am Main, Germany).

Elastase activity was determined according to the assay described by Wasil *et al.* [2], with minor modifications. All reagents were dissolved in phosphate buffer used (19 mM KH_2PO_4-KOH) pH 7.4, containing 140 mM NaCl, unless otherwise noted. Twenty micrograms of α_1-AP (unless otherwise noted) were mixed with the compound under investigation (0–100 μM) and preincubated at 25°. After 5 min, HOCl was added (50 μM unless otherwise noted). The final volume was 100 μL. The concentration of the test compounds (0–100 μM) and HOCl (usually 50 μM) indicated in the text and the figures, refers to the concentration in the 100 μL incubation mixture. After an additional 5 min, 200 μL buffer containing 5 μg elastase was added. Again after 5 min, 700 μL buffer and 50 μL of

a 10 mM solution of *N-t*-BOC-L-alanine *p*-nitrophenol ester in methanol was added. Immediately after the addition of *N-t*-BOC-L-alanine *p*-nitrophenol ester, the increase in absorption at 410 nm was determined, which represents the activity of elastase. In some experiments, the test compound and HOCl were mixed before α_1-AP was added. In other experiments, α_1-AP and HOCl were mixed before the test compound was added. None of the test compounds affected the elastase activity directly, nor the ability of α_1-AP to inhibit elastase (data not shown). All reactions were carried out at 25°. Determination of elastase activity was reproducible within 5%. HOCl was determined by adding iodate free potassium iodide to a solution of HOCl in 10% acetic acid, and titrating the liberated iodine with thiosulfate [6].

Results

As shown in Fig. 1, α_1-AP inhibited elastase activity in a dose-dependent manner. Twenty micrograms of α_1-AP gave a complete inhibition of proteolytic activity of 5 μg elastase. In the following experiments 20 μg of α_1-AP was used.

Preincubation of α_1-AP with HOCl destroyed the ability of α_1-AP to inactivate elastase. At a HOCl concentration of 50 μM, no inhibition of elastase activity by α_1-AP was observed (Fig. 2). This indicates that at this concentration HOCl inactivated all α_1-AP. The concentration of 50 μM HOCl was used in the experiments where the scavenging of HOCl by the compounds was determined.

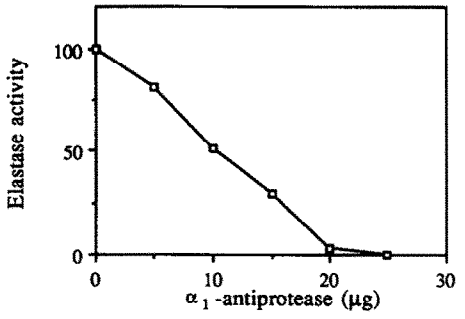

Fig. 1. Dose-dependent inhibition of elastase by α_1-antiproteinase (α_1-AP). Five micrograms of elastase were used. Elastase activity was measured according to the method described in Materials and Methods, and it is expressed as percentage of that in the absence of α_1-AP.

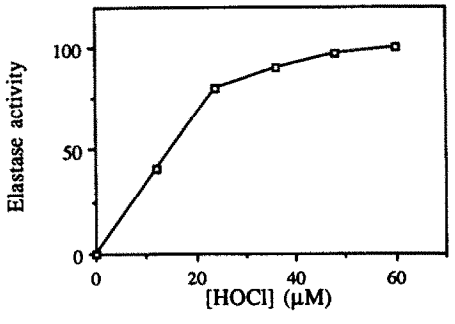

Fig. 2. Concentration-dependent inactivation of α_1-antiproteinase (α_1-AP) by HOCl. Twenty micrograms of α_1-AP were preincubated with several concentrations of HOCl. After 5 min, 5 μg elastase was added. The inactivation of α_1-AP results in an increase in elastase activity. Elastase activity was measured according to the method described in Materials and Methods, and it is expressed as percentage of that in the absence of α_1-AP.

GSH (Fig. 3) and *N*-acetylcysteine (Fig. 4) prevented the inactivation of 20 μg α_1-AP by 50 μM HOCl very efficiently. At a concentration of 80 μM of either GSH or *N*-acetylcysteine almost no activity of elastase could be detected, indicating that at this concentration both GSH and *N*-acetylcysteine almost completely protect against the inactivation of α_1-AP by HOCl. The protective effect was not due to the regeneration of α_1-AP after it has been inactivated by HOCl. This can be concluded from the experiment where α_1-AP was first inactivated by 50 μM HOCl. Addition of 100 μM GSH or *N*-acetylcysteine to the inactivated α_1-AP did not restore the inhibitory effect of α_1-AP on elastase (data not shown). Therefore, the most likely mechanism by which GSH and *N*-acetylcysteine protect α_1-AP is that they scavenge HOCl before it can react with α_1-AP.

The effect of GSMe was comparable to that of GSH, although GSMe was slightly less potent (Fig. 3). GSSG was not able to protect efficiently against the inactivation of α_1-AP by HOCl (Fig. 3). Preincubation of 50 μM HOCl with 20 μM GSSG before the addition of α_1-AP did protect against the inactivation of α_1-AP (data not shown). Apparently GSSG scavenges HOCl, but the rate of the reaction of HOCl with GSSG is much lower than the rate of the reaction of HOCl with the methionine residue of α_1-AP.

The reduced form of lipoate, dihydrolipoate, appeared to be a very effective scavenger of HOCl (Fig. 4), its potency is comparable to that of GSH and *N*-acetylcysteine.

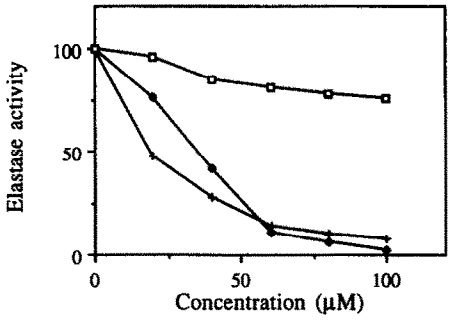

Fig. 3. Concentration-dependent protection of α_1-antiproteinase (α_1-AP) by GSH(+), GSMe (◇) and GSSG (□) against the inactivation by HOCl. The compounds were mixed with 20 μg α_1-AP before 50 μM HOCl was added. Elastase activity was measured according to the method described in Materials and Methods, and it is expressed as percentage of that in the absence of α_1-AP.

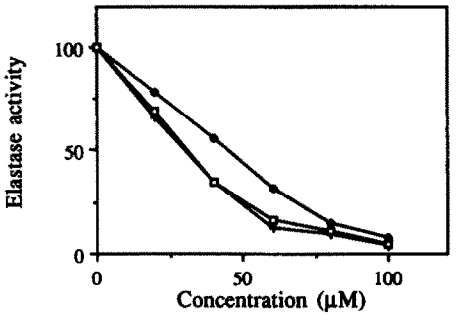

Fig. 4. Concentration-dependent protection of α_1-antiproteinase (α_1-AP) by reduced lipoate (□), oxidized lipoate (+) and *N*-acetylcysteine (◇) against the inactivation by HOCl. The compounds were mixed with 20 μg α_1-AP before 50 μM HOCl was added. Elastase activity was measured according to the method described in Materials and Methods, and it is expressed as percentage of that in the absence of α_1-AP.

To our surprise, the oxidized form of lipoate was equally effective in scavenging HOCl as dihydrolipoate (Fig. 4). In contrast to oxidized glutathione, oxidized lipoate appears to compete with the methionine residue of α_1-AP in the reaction with HOCl.

Discussion

Data from literature and also from this study indicate that several thiol-containing compounds, like cysteamine [7], *N*-acetylcysteine [8, this study] and GSH [this study] are effective scavengers of HOCl. Therefore, it did not come as a surprise that reduce lipoate, a molecule that contains two thiol groups, is also a potent scavenger of HOCl. Moreover, it was found that GSMe was a good scavenger. That a compound with a methylated thiol group can act as a scavenger of HOCl is also not surprising, because it is known that a methionine residue (which also contains a methylated thiol group) in α_1-AP is attacked by HOCl on its sulphur atom [3].

In addition, we found that GSSG is only a poor scavenger of HOCl. GSSG reacts with HOCl, but the reaction rate is too slow to provide an efficient protection of the methionine residue of α_1-AP. Unexpectedly, we found that oxidized lipoate is a potent scavenger of HOCl. In the past, the antioxidant activity of lipoate has been ascribed to its reduced form. Dihydrolipoate is able to elevate the concentration of GSH by reducing GSSG [4]. In addition, it has been stated that dihydrolipoate may protect against lipid peroxidation in an interaction with vitamin E [5]. The oxidized form of lipoate has no protective effect on the process of lipid peroxidation [4, 5]. The findings of this study indicate that, with respect to the scavenging of HOCl, oxidized lipoate, as well as reduced lipoate, acts as a potent antioxidant. This is of special importance because lipoate is used therapeutically in its oxidized form.

The most striking result in this study was the difference between GSSG, not a potent scavenger of HOCl, and oxidized lipoate, a very good scavenger of HOCl. The explanation for this difference may be found in the nature of the disulfide bridge in both molecules. Dihydrolipoate contains an intramolecular S—S bridge. Because this bridge is fixed in a 5-membered ring, some ring strain exists. It has been reported that the angle between both sulphur molecules is energetically not optimal [9]. In GSSG no ring strain exists because it is a flexible molecule. The difference of the S—S bridges probably explains the difference between GSSG and oxidized lipoate in their ability to scavenge HOCl. By scavenging HOCl, oxidized lipoate is probably converted into a sulphoxide. The sulphoxide of dihydrolipoate is also formed in the reaction of dihydrolipoate with hydrogen peroxide [10].

In summary, it was found that reduced GSH, *N*-acetylcysteine and GSMe are potent scavengers of HOCl, while GSSG is not. Surprisingly, not only reduced but also oxidized lipoate is an effective scavenger. The difference in scavenging effect between oxidized GSH and oxidized lipoate is probably caused by the difference in reactivity of the disulfide bridge in both molecules. The present results indicate that lipoate might be effective in the treatment of emphysema caused by the destruction of α_1-antiproteinase.

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Interspecies differences in *in vitro* etoposide plasma protein binding

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Preclinical studies in laboratory animals are used to define initial pharmacologic and toxicologic endpoints of anticancer drugs. Phase I protocols of anticancer drugs initiate doses in humans equal to or less than one-tenth the mouse lethal dose (LD_{10}), and titrate the dose upwards until dose-limiting toxicity is observed. This procedure is based on previous observations that a quantitative relationship exists between toxic doses of anticancer drugs in animals and humans [1]. Despite these similarities, often several dose escalations are required to reach the maximum