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Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by oxidized and reduced lipoic acid

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Abstract Peroxynitrite, formed by combination of superoxide radical with nitric oxide, is a reactive tissue-damaging species apparently involved in the pathology of several human diseases. Peroxynitrite nitrates tyrosine residues and inactivates α_1-antiproteinase. We show that both lipoic acid and dihydrolipoic acid efficiently protect against damage by peroxynitrite. By contrast, other disulphides tested did not. The biological antioxidant effects of lipoate/dihydrolipoate may involve scavenging of reactive nitrogen species as well as reactive oxygen species.

Key words: Lipoic acid; Peroxynitrite; Nitrotyrosine; α_1-Antiproteinase; Nitric oxide; Superoxide

1. Introduction

The metabolic role of lipoamide as a cofactor in the pyruvate and α-ketoglutarate multienzyme complexes is well-known [1,2]. More recently, however, considerable attention has been given to possible antioxidant functions for α-lipoic acid and its reduced form, dihydrolipoate [3]. It is well-known that various reactive oxygen species such as superoxide radical, hydrogen peroxide, hydroxyl radical and hypochlorous acid are formed in vivo and contribute to tissue injury in human disease [4]. Hence the established ability [3,5–8] of lipoate/dihydrolipoate to scavenge some of these species, plus its relative lack of toxicity, may make it a therapeutically-useful antioxidant [3].

Recently, there has been considerable interest in reactive *nitrogen* species as mediators of tissue injury [9,10]. Although nitric oxide ($NO^{\bullet}$) has many important physiological functions [11], its production in excess may contribute to the pathology of neurodegenerative disease, chronic inflammation, acute respiratory distress syndrome, atherosclerosis and septic shock [9–14]. Part of the toxicity of $NO^{\bullet}$ involves its reaction with superoxide radical ($O_2^{\bullet-}$) to give peroxynitrite, $ONOO^-$

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^- \quad (1)$$

Peroxynitrite induces peroxidation of lipids, oxidizes methionine and –SH residues in proteins, depletes antioxidants and causes DNA damage [9,10,15,16]. In particular, addition of peroxynitrite to biological fluids leads to nitration of tyrosine residues, and the presence of these appears to be a 'marker' of peroxynitrite-dependent damage in vivo [10,12–16]. Tyrosine nitration can interfere with signal transduction mechanisms involving phosphorylation/dephosphorylation [17]. Peroxynitrite also inactivates α_1-antiproteinase, the major inhibitor of serine proteases (such as elastase) in human body fluids [15,18]. Hence peroxynitrite generation in vivo can facilitate both oxidative and proteolytic damage [9,10,18].

In the present paper, we show that both α-lipoic acid and dihydrolipoic acid are very powerful scavengers of $ONOO^-$, able to protect tyrosine against nitration and α_1-antiproteinase against inactivation by $ONOO^-$.

2. Materials and methods

2.1. Reagents

N-Succinyl(Ala)$_3$ *p*-nitroanilide (SANA), DL-tyrosine, elastase (E2058), α_1-antiproteinase (A0924), DL-tyrosine (type T-3379), GSH (type G-4241), *N*-acetylcysteine (A-8199), GSSG (G-6654), methionine (M-9500), DL-penicillamine (P-5125) and penicillamine disulphide (P-1271) were from Sigma, Poole, Dorset, UK. Dihydrolipoic (DHLA) and lipoic acids were a kind gift from Asta Medica.

2.2. Peroxynitrite synthesis

An acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for one second and the reaction quenched with ice-cold NaOH (1.2 M). The concentration of the resultant stock was measured spectrophotometrically at 302 nm [10]. The stock was then frozen overnight (−20°C) and the top layer of the solution collected for the experiment [10].

2.3. Measurement of tyrosine nitration

A stock concentration (10 mM) of DL-tyrosine was prepared in 10 ml by adding 8 ml of water to 250 μl of 10% (w/v) KOH followed by 250 μl of 5% phosphoric acid with 1.5 ml of water. 0.1 ml of tyrosine solution together with 0.1 ml of a solution of the compound to be tested was added to a plastic test tube containing 0.795 ml of buffer (500 mM K_2HPO_4/KH_2PO_4 pH 7.4) and incubated in a water bath at 37°C for 15 min. After this time peroxynitrite (typically 5 μl) was added to a final concentration of 1 mM, the tubes vortexed for 15 s and incubated for a further 15 min. The pH was measured after the addition of peroxynitrite and found to be 7.4–7.5.

Measurement of 3-nitrotyrosine was performed essentially as in [19] using a Spherisorb 5 μm ODS2 C_{18} column (HPLC Technology, Wellington House, Cheshire, England, UK) with a guard column C_{18} cartridge (Hibar from BDH, Poole, England, UK). The eluent was 500 mM KH_2PO_4/H_3PO_4 pH 3.01, with 20% methanol (v/v) at a flow rate of 1 ml · min^{-1} through a Polymer Laboratories pump (Essex Road, Church Strotten, Shropshire, England, UK) and UV detector set at 274 nm (sensitivity 0.02). The 3-nitrotyrosine detected was confirmed by spiking with standards. Peak heights of tyrosine and 3-nitrotyrosine were measured and concentrations calculated from a standard curve.

2.4. Prevention of α_1-antiproteinase inactivation

Elastase and α_1-antiproteinase were measured essentially as described in [20]. α_1-Antiproteinase 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 of ml compound to be tested to give a volume of 0.945 ml and incubated in a water bath at 37°C for 15 min, when peroxynitrite (typically 5 μl) was added to a final concentration

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of 0.5 mM. The sample was vortexed for 10 seconds and incubated for 5 min. Then elastase (typically 50 μl) was added and the sample incubated at 37°C for a further 15 min. Then 0.1 ml of elastase substrate (SANA) was added and the rate of reaction followed at 410 nm for 30 s.

3. Results

3.1. Inactivation of α_1-antiproteinase

As expected [18], addition of $ONOO^-$ to α_1-antiproteinase (α_1AP) led to inactivation of the ability of α_1AP to inhibit elastase. Inactivation increased with $ONOO^-$ concentration and the reaction was complete within 5 min at pH 7.4 (data not shown). A 5 min incubation time with 0.5 mM $ONOO^-$ was selected for further studies. If the $ONOO^-$ solution was added to the buffer and incubated for 5 min at 37°C before adding α_1AP the resulting 'decomposed $ONOO^-$' [10] solution had no effect on α_1AP.

Fig. 1 (column A) shows the activity of uninhibited elastase. Addition of α_1AP markedly decreased elastase activity (column B) but pre-incubation of α_1AP with $ONOO^-$ largely destroyed its elastase-inhibitory capacity (column C). Several thiols and disulphides were tested, at a concentration two-fold excess over $ONOO^-$, for ability to prevent inactivation of α_1AP. Methionine was protective, probably because inactivation of α_1AP involves damage to an essential methionine residue. Thiols (penicillamine, GSH, *N*-acetylcysteine) were generally protective whereas disulphides (GSSG, penicillamine disulphide) were much less so. By contrast, both dihydrolipoic acid (DHLA) and lipoic acid (LA) were highly protective. Fig. 2 shows the concentration-dependence of this effect. LA and DHLA could protect α_1AP effectively even at concentrations much lower than those of $ONOO^-$, indicating that one molecule of these compounds can scavenge several $ONOO^-$ molecules.

3.2. Inhibition of tyrosine nitration

When tyrosine is exposed to $ONOO^-$ at physiological pH, nitration occurs [10,12,13,16,17]. Fig. 3 shows that several thiol

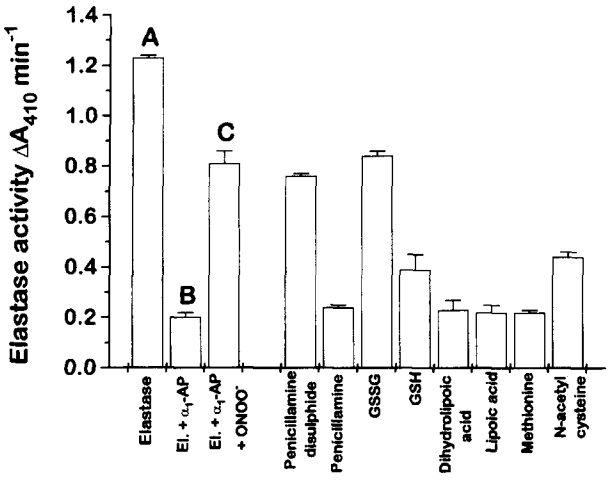

Fig. 1. A screen of thiols and disulphides for ability to protect α_1AP against inactivation by peroxynitrite. The test compound at a final concentration of 1 mM was mixed with α_1AP before addition of $ONOO^-$ (final concentration 0.5 mM). For further details see section 2. Control experiments showed that none of the compounds had any effect on elastase itself, nor on the ability of α_1AP to inhibit elastase, nor could they reactivate α_1AP after it had been inactivated by $ONOO^-$. Results are mean ± S.E.M. of 4 or more experiments.

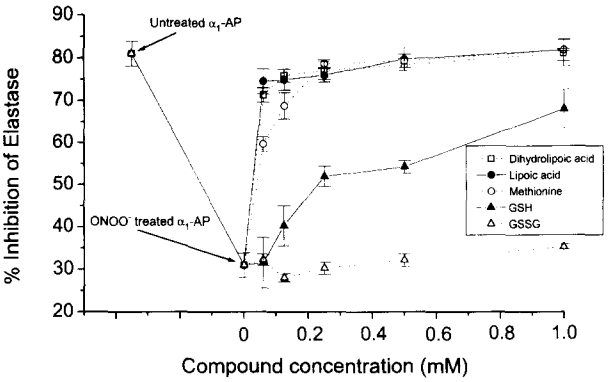

Fig. 2. Concentration-dependence of the protective effects of lipoic acid, DHLA, methionine, GSH and GSSG against inactivation of α_1AP by $ONOO^-$. Compounds were added to give the final concentrations stated. For further experimental details see legend to Fig. 1. Results are mean ± S.E.M. of 4 or more experiments.

compounds (GSH, DHLA and penicillamine) could prevent this nitration whereas the disulphides GSSG and penicillamine were much less effective. By contrast, lipoic acid was almost as protective as DHLA. As observed with the α_1AP studies reported above, LA and DHLA could prevent tyrosine nitration even at concentrations much lower than those of $ONOO^-$, indicating that one molecule of these compounds can scavenge several $ONOO^-$ molecules.

4. Discussion

Peroxynitrite generation in vivo is being implicated in a wide range of human diseases, including atherosclerosis [13], lung disease [12], neurodegenerative disorders [9] and chronic inflammation [14,21]. Hence agents able to protect against $ONOO^-$-dependent damage may be therapeutically useful. Thiols are already known to be good scavengers of $ONOO^-$ [10,15,16] and we show here that DHLA is very effective, able to protect α_1AP against inactivation by $ONOO^-$ and to prevent nitration of tyrosine by this species. The presence of two –SH groups in DHLA may explain why it is more effective, on a molar basis, than GSH or *N*-acetylcysteine in protecting against α_1AP inactivation (Figs. 1 and 2). It is of special interest, however, that (unlike the other disulphides tested), LA is comparably protective. α-Lipoic acid is readily absorbed from the diet and converted to DHLA in vivo, but this conversion may be only partial [3]. The high reactivity of LA may be due to its strained cyclic disulphide moiety [3,22]. Hence LA and DHLA may exert significant antioxidant activities in vivo not only because of their ability to scavenge reactive oxygen species [3] but also because they can prevent damage by reactive nitrogen species.

Our data do not necessarily mean that LA and DHLA are acting by directly scavenging $ONOO^-$: they could instead be combining with reactive intermediates of peroxynitrite decomposition. Although inactivation of α_1AP may involve direct attack on methionine residues by $ONOO^-$ [18], nitration of tyrosine by $ONOO^-$ is a complex reaction that may involve such species as $NO_2^{\bullet}$ and NO_2^+ [10,23]. Hence scavenging of these species could also account for the actions of LA and DHLA.

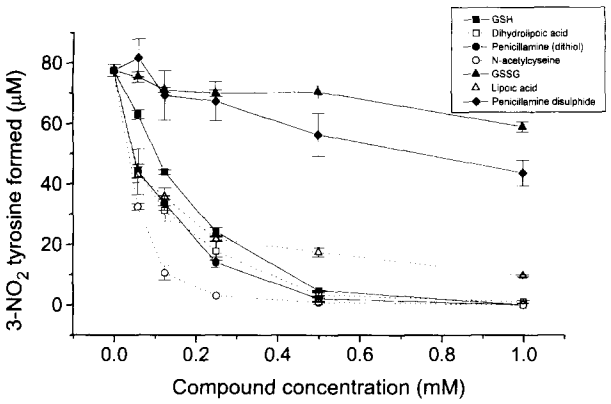

Fig. 3. Prevention of peroxynitrite-dependent nitration of tyrosine by thiols and disulphides. Tyrosine (1 mM) was incubated with 1 mM $ONOO^-$ as described in section 2. Compounds were added to give the final concentrations stated. Results are mean ± S.E.M. of 4 or more experiments.

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