Free Rad. Res., Vol. 29, pp. 435–440
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Antioxidant Properties of the Decarboxylated Dimer of Aminoethylcysteine Ketimine: Assessment of its Ability to Scavenge Peroxynitrite

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Accepted by Prof. M. Grisham

(Received 20 June 1998; In revised form 16 July 1998)

The natural sulfur compound aminoethylcysteine ketimine decarboxylated dimer (AECK dimer) has been investigated for its ability to act as peroxynitrite scavenger. It has been found that the product efficiently protects against the nitration of tyrosine and the inactivation of α_1-antiproteinase by peroxynitrite. The tyrosine nitration can be completely prevented by 100 μM AECK dimer which appears as effective as the antioxidants glutathione and N-acetylcysteine. The AECK dimer was also found to limit surface charge alteration of low density lipoprotein induced by peroxynitrite. These findings indicate that the AECK dimer is a strong protective agent against peroxynitrite damage and that it could play an important role in the defence against oxidative stress in human diseases.

Keywords: Aminoethylcysteine ketimine dimer, free radicals, nitric oxide, peroxynitrite, nitrotyrosine, α_1-antiproteinase, LDL

Abbreviations: $ONOO^-$, peroxynitrite; $^{\bullet}NO$, nitric oxide; AECK dimer, aminoethylcysteine ketimine dimer; LDL, low density lipoprotein; α_1AP, α_1-antiproteinase; REM, relative electrophoretic mobility

INTRODUCTION

Peroxynitrite ($ONOO^-$), a reaction product of nitric oxide ($^{\bullet}NO$) and superoxide radical ($O_2^{\bullet-}$),[1] is an endogenous mediator of various forms of tissue injury including neurodegenerative diseases, chronic inflammation, atherosclerosis and many other pathological events.[2–5] Peroxynitrite and/or its decomposition products cause DNA damage,[6] induce peroxidation of lipids,[7] oxidize protein and non protein thiol groups,[8] and react with tyrosine residues to yield 3-nitrotyrosine.[9,10] Nitration of tyrosine has been demonstrated to occur *in vivo* and has been proposed as an indicator of damage induced by peroxynitrite and/or its derived species.[11,12] In addition $ONOO^-$ can oxidize free methionine[13] and methionine in proteins e.g. in the α_1-antiproteinase, where oxidation of critical methionine residues destroys the antiproteinase activity.[14] Damage to this protein has been

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shown to occur at sites of inflammation where $ONOO^-$ is generated.[15,16] Peroxynitrite has been also implicated in the oxidation of low density lipoprotein[17] and this could be an important damaging effect of peroxynitrite generation *in vivo* because oxidative modification of blood lipoprotein is thought to be a critical event in the development of cardiovascular diseases including atherosclerosis.[18,19]

The increasing evidence for the toxicity of peroxynitrite has generated interest in a possible defense against this reactive nitrogen species. We have recently found that the decarboxylated dimer of aminoethylcysteine ketimine (Figure 1), hereafter simply named AECK dimer, is able to interact with reactive oxygen species,[20] to protect mitochondrial membranes from oxyradicals damage,[21] to exert antioxidant activity towards the lipid peroxidation of microsomes and to act as a scavenger of hydroxyl radicals.[22] The AECK dimer has been recently detected in normal human urine,[23] in bovine cerebellum[24] and in a fraction of human plasma proteins (work in progress). Its formation in biological tissues can occur by spontaneous dimerization of aminoethylcysteine ketimine (AECK), a natural sulfur cyclic compound found in bovine brain and cerebellum.[25]

In this paper, we show that the dimer of aminoethylcysteine ketimine is a powerful scavenger of $ONOO^-$ able to protect tyrosine against nitration, α_1-antiproteinase against inactivation and human low-density lipoprotein (LDL) against modification.

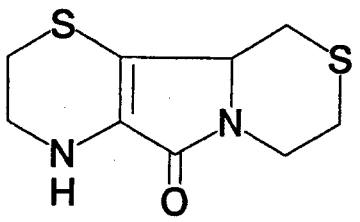

FIGURE 1 Structure of AECK dimer.

MATERIALS AND METHODS

Reagents Aminoethylcysteine ketimine decarboxylated dimer (AECK dimer) was prepared by reacting equimolar amounts of cysteamine hydrochloride and bromopyruvic acid and crystallizing the precipitate from boiling water according to Antonucci *et al.*[20] Cysteamine hydrochloride, bromopyruvic acid, N-Succinyl-$(Ala)_3$ *p*-nitroanilide (SANA), L-tyrosine, 3-nitro-L-tyrosine were from Fluka (Buchs, CH); elastase (E0258), α_1-antiproteinase (A9024) were from Sigma Chemical Co. (St. Louis, MO, USA).

Peroxynitrite synthesis Peroxynitrite was synthesized essentially as described by Beckman *et al.*[26] Five milliliters of an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with 5 ml of KNO_2 (0.6 M) on ice for one second and the reaction quenched with 5 ml of ice-cold NaOH (1.2 M). The solution was then frozen overnight (−20°C) and the yellow liquid layer on top of the ice crystals collected for the experiments. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm using a molar absorption coefficient of $1670\,M^{-1}\,cm^{-1}$.

Tyrosine nitration assay Peroxynitrite (final concentration 100 μM) was added to a solution containing L-tyrosine (100 μM) in the presence of varying concentration (5–200 μM) of the AECK dimer in 0.5 M K-phosphate buffer, pH 7.4, giving a final volume of 1 ml. The tubes vortexed for 15 s were incubated for 10 min at 25°C. The pH was measured after the addition of peroxynitrite and found to be 7.4–7.5. Appropriate controls, without the AECK dimer, were carried out to estimate levels of tyrosine nitration.

The samples were then analyzed for 3-nitrotyrosine formation by HPLC. Analyses were carried out with a Waters Chromatograph equipped with two model 501 pumps, a model 680 gradient controller, a U6K sample injector and a model 996 photodiode array detector linked to a Millenium 2010 data station. The column was a Nova-pak C18 (3.9 mm × 150 mm), 4 μm. The mobile phase

was: A, 50 mM K-phosphate/H_3PO_4, pH 3.0; B, acetonitrile : water (50 : 50, v/v). A linear gradient from A to 33% B for 10 min was used at a flow rate of 1 ml/min. Tyrosine and 3-nitrotyrosine were monitored at 274 nm and 360 nm respectively and concentrations were calculated from standard curves. The detection limit for 3-nitrotyrosine was 10 pmol. AECK dimer did not interfere with the analysis of tyrosine and 3-nitrotyrosine.

Prevention of α_1-antiproteinase inactivation Elastase and α_1-antiproteinase (α_1AP) activity were measured as described in.[27] Briefly, 60 µl α_1AP (4 mg/ml) was incubated without or with AECK dimer (1 µM to 0.5 mM) in 0.5 M K-phosphate buffer, pH 7.4 to give a volume of 0.945 ml and incubated at 37°C for 15 min, when peroxynitrite (typically 5 µl) was added at a final concentration of 0.5 mM. After 10 seconds vortexing and incubation for 5 min, 50 µl elastase (5 mg/ml) was added and the sample further incubated for 15 min followed by addition of 2.0 ml of buffer (0.5 M K-phosphate, pH 7.4). Then after 15 min, 0.1 ml of 1 mM elastase substrate (SANA) was added and the reaction followed at 310 nm for 30 s. Appropriate control experiments showed that AECK dimer did not affect the elastase activity or the α_1AP capacity to inhibit elastase.

Isolation of LDL LDL was isolated from human plasma by sequential ultracentrifugation through a potassium bromide density gradient.[28] LDL protein concentration was measured by the Lowry assay.[29] The freshly prepared LDL was stored under nitrogen at 4°C in 0.01 M K-phosphate buffer, containing 0.15 M NaCl, pH 7.4.

Gel electrophoresis of LDL Modification of LDL by peroxynitrite was assessed by measuring changes in electrophoretic mobility by using agarose-gel electrophoresis. Varying concentrations of the AECK dimer (10 µM to 1 mM) were added to LDL (125 µg/ml) in 0.2 M K-phosphate buffer, pH 7.4 and incubated for 15 min at 37°C, before peroxynitrite (0.6 mM final concentration) addition. A control sample without the AECK dimer and a sample without peroxynitrite were included for each experiment. Samples were incubated for 2 h at 37°C prior to gel electrophoresis. An aliquot (5 µl) of each sample was subsequently applied to the 0.5% agarose gels at 100 V for 50 min in 50 mM barbital buffer, pH 8.6 (Beckman Paragon Lipo Gel electrophoresis system). Lipoprotein were visualized by staining with Sudan Black B.

RESULTS

Inhibition of tyrosine nitration Tyrosine, when exposed to peroxynitrite at neutral pH, undergoes nitration to form 3-nitrotyrosine.[9,10] In our experimental conditions, exposure of tyrosine (100 µM) to peroxynitrite (100 µM) resulted in the production of 8.4 ± 0.4 µM 3-nitrotyrosine. Figure 2 shows that increasing concentrations of AECK dimer decrease the peroxynitrite-dependent tyrosine nitration. At 20 µM concentration of the compound, reduction of nitration was 56 ± 1.5%. At higher concentration (100 µM) the nitration is completely prevented. Compared with other sulfur compounds with established antioxidant activity, the AECK dimer exerted a protective effect similar to that of glutathione (GSH) and N-acetylcysteine, but higher than that of methionine (Figure 2). On the other hand the AECK dimer sulfoxide, a product of oxidation of the AECK dimer by H_2O_2,[30] had almost no activity, indicating that the protective effect of the compound is linked to the oxidation level of the sulfur atom(s) of the thiol ether functionality of the molecule.

Prevention of α_1-antiproteinase inactivation Treatment of α_1-antiproteinase (α_1AP) with $ONOO^-$ causes a strong reduction in its elastase inhibitory capacity.[14] Figure 3 shows the concentration-dependence of the protection of α_1AP by the AECK dimer. The compound was able to exert complete protection against α_1AP inactivation even at concentrations much lower than those of $ONOO^-$. AECK dimer was not

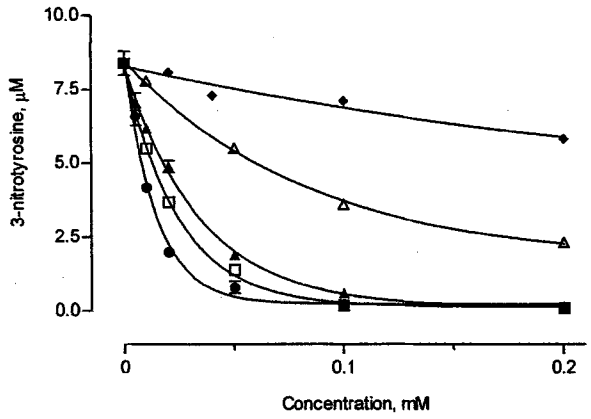

FIGURE 2 Prevention of peroxynitrite-dependent nitration of tyrosine. The assay conditions were as described in Materials and Methods. Compounds, at the indicated concentrations, were mixed with tyrosine (100 μM) before peroxynitrite addition (100 μM). Results are mean ± S.E.M. of three or more experiments. Concentration-dependence of the protective effect of AECK dimer (□) is compared with oxidized AECK dimer (◆), glutathione (▲), N-acetylcysteine (●) and methionine (△).

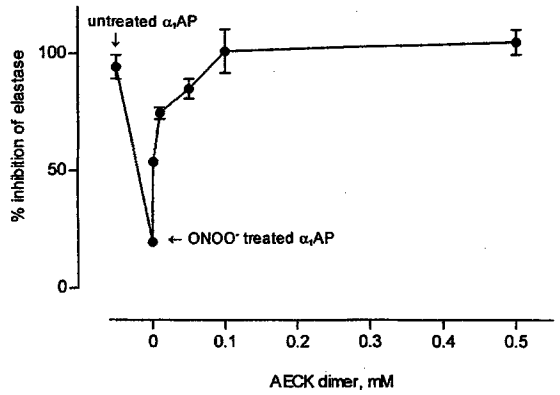

FIGURE 3 Concentration-dependence of the protective effects of AECK dimer against inactivation of α_1-antiproteinase (α_1-AP) by peroxynitrite. The assay conditions were as described in Materials and Methods. The AECK dimer was added to give the final concentrations stated and incubated with α_1-AP before peroxynitrite addition (0.5 mM). Results are the mean ± S.E.M. of four experiments.

protective if added to α_1AP preincubated with $ONOO^-$ for 5 min indicating that the compound did not reverse but prevent α_1AP inactivation by scavenging $ONOO^-$ and/or its decomposition products.

TABLE I Effects of peroxynitrite (0.6 mM) on the relative electrophoretic mobility (REM) of human LDL (125 μg/ml) in the absence and in the presence of the AECK dimer

	REM[a]	% reduction in REM[b]
Peroxynitrite-treated LDL	2.5	
+AECK dimer 10 μM	2.3	13
+ AECK dimer 100 μM	1.4	73
+ AECK dimer 500 μM	1.2	87
+ AECK dimer 1 mM	1.0	100

[a] The relative electrophoretic mobility (REM) of LDL samples was determined setting the electrophoretic mobility of the untreated LDL as 1.0. [b] calculated as: $(REM_{ONOO^-\,treated} - REM_{sample})/(REM_{ONOO^-\,treated} - 1.0) \times 100$. Data are mean of two experiments.

Inhibition of human low density lipoprotein (LDL) modification It has been reported previously that peroxynitrite is able to oxidatively modify LDL resulting in an increse in electrophoretic mobility.[17] The effectiveness of the AECK dimer (10 μM to 1 mM) in decreasing peroxynitrite-mediated LDL oxidation, as measured by the relative electrophoretic mobility (REM), is shown in Table I. LDL were preincubated with the AECK dimer for 15 min at 37°C before peroxynitrite addition. The results show that the AECK dimer is able to prevent quite completely the modification of LDL at a concentration of 500 μM.

DISCUSSION

The results reported in this study clearly demonstrated that the AECK dimer is a powerful scavenger of peroxynitrite and/or its derived species in that it could efficiently protect tyrosine against nitration, α_1-antiproteinase against inactivation and LDL against modification. These assays have been used in several studies to evaluate the ability of various compounds to protect against peroxynitrite-dependent damage.[27,31–34] Moreover, damage to these important biological targets has been shown to occur in various forms of tissue injury where the generation of peroxynitrite has been implicated.[3,11,12,15,16,18,19]

In previous works,[21,22] we have demonstrated that the AECK dimer is an effective antioxidant agent against lipid peroxidation of mitochondrial membranes and brain microsomes; moreover, the AECK dimer exerts a significant antioxidant activity *in vitro* as a scavenger of hydroxyl radicals. The present results indicate that the AECK dimer may act as an antioxidant not only because of its ability to scavenge reactive oxygen species, but also because it can prevent damage by peroxynitrite.

At present, our data do not give clues about the mechanism of protection; the AECK dimer could act by directly scavenging $ONOO^-$ or by combining with reactive intermediates of peroxynitrite decomposition. It would be of interest to determine the products obtained by reaction of AECK dimer with $ONOO^-$. The identification of these products might enable elucidation of the mechanism of any protective effect exerted by this scavenger. The finding that the AECK dimer but not its product of oxidation (AECK dimer sulfoxide) protects tyrosine from nitration by $ONOO^-$ suggests that the thiol ether functionality of the compound could be involved in the mechanism of protection. Similarly, detailed chemical studies on the reaction of peroxynitrite with methionine have identified the sulfoxide as a major reaction product.[13]

The AECK dimer has been reported to be present in normal human urine[23] at concentration of about 100 nmol/g creatinine (Matarese, personal communication), in bovine cerebellum[24] at concentration of 0.6–1.0 nmol/g wet wt and in human plasma (work in progress indicates a concentration in the μM range). In the experimental conditions used in the present study, the AECK dimer exerts a complete protective effect against peroxynitrite-dependent damage at concentration of 100 μM, but significant scavenging effects are also observed at lower concentrations (Figures 2 and 3) similar to those found in human plasma. Moreover, it is conceivable that the peroxynitrite concentration to which the AECK dimer has been exposed is higher than that produced under pathophysiological conditions.

It is obvious that the physiological significance of AECK dimer as a peroxynitrite scavenger needs *in vivo* experiments which will be the object of further investigations.

Acknowledgments

This work was supported in part by grants from MURST and by Sigma Tau Foundation.

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