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AMINOETHYLCYSTEINE KETIMINE DECARBOXYLATED DIMER PROTECTS SUBMITOCHONDRIAL PARTICLES FROM LIPID PEROXIDATION AT A CONCENTRATION NOT INHIBITORY OF ELECTRON TRANSPORT

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Abstract: In contrast with other inhibitors of the NADH dehydrogenase of the respiratory chain, the decarboxylated dimer of aminoethylcysteine ketimine protects bovine heart submitochondrial particles (SMP) from the NADH-Fe^{+3}-ADP-induced lipid peroxidation. This effect, measured as inhibition of malondialdehyde formation, is concentration-dependent in the range 0.02- 0.2 mM. This range of concentration is not inhibitory on NADH-oxidase activity of SMP. Furthermore the dimer is able to counteract the malondialdehyde formation stimulated by the Complex I inhibitors rotenone and N-methyl-4-phenylpyridinium (MPP^+).

It is known (1, 2) that mitochondria represent a major intracellular source of oxygen radicals (O_2^-, $^{\cdot}OH$) and hydrogen peroxide. The genesis of these reactive oxygen species is imputable to the leakage of some electrons passing through the respiratory chain. The first reduction product of O_2 is the superoxide anion radical (O_2^-) derived by the capture of a single electron. H_2O_2 is then formed by the spontaneous or catalysed (3) dismutation of O_2^-. The production of hydroxyl radical ($^{\cdot}OH$) is due to the reaction of the ubiquitous iron ions with H_2O_2 (the Fenton reaction) or with H_2O_2 plus O_2^- (the Haber-Weiss reaction) (4). Slowing down or blocking the electron flux by various inhibitors increases the oxyradical production in mitochondria leading to the deterioration of membrane layers. This is demonstrated by the detection of lipid peroxidation products (5-8) and by inactivation of some enzymes (9,10). Deterioration of the mitochondrial structure by certain parkinsonism related neurotoxins (11) has been suggested to contribute to the destruction of the neuro-cellular stucture of the *substantia nigra* leading to the generation of the Parkinson's disease (12). In a recent note from our laboratory (13) we have demonstrated the inhibitory activity of the decarboxylated dimer of aminoethylcysteine ketimine (simply named the dimer afterward) on the mitochondrial respiration due to the blockade of the

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Abbreviations: SMP, submitochondrial particles; MDA, malondialdehyde; MPP^+, N-methyl-4-phenylpyridinium; BHT, butylated hydroxytoluene.

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electron flux at Complex I level. The presence in the dimer structure of two sulfur atoms, known to react easily with oxidizing agents (14), makes this compound particularly sensitive to oxygen reactive species (15). Therefore it appeared of interest to ascertain whether the dimer inhibition was accompanied or not by membrane oxyradical damage and superoxide generation as done by non sulfur containing inhibitors of Complex I.

MATERIALS AND METHODS

The dimer has been prepared as previously described (15). N-methyl-4-phenylpyridinium (MPP^+) was purchased from Research Biochemicals, Inc (Maryland, MA); rotenone, catalase and superoxide dismutase (SOD) from Sigma Chemical Co (St Louis, MO). All other reagents were of the highest quality available from Fluka (Switzerland). Bovine heart mitochondria and bovine heart submitochondrial particles (SMP) were prepared as previously reported (13).

The lipid peroxidation experiments were performed essentially as described by Hasegawa et al. (6): the control system of 1 ml containing submitochondrial particles (0.3-0.5 mg protein), 0.2 mM $FeCl_3$, 2 mM ADP and 50 mM Tris/acetate buffer, pH 7.0, was preincubated for 5 min at 37°C. The reaction was started by the addition of NADH to 0.1 mM final concentration and after 5 min the samples were analyzed for malondialdehyde (MDA) formation by the thiobarbituric acid assay as described by Buege and Aust (16) with butylated hydroxytoluene (BHT) added during the boiling step.

The superoxide anion production was followed by monitoring the oxidation of adrenaline to adrenochrome (17) at 485 nm. Reaction conditions are specified in Table II.

Protein content was determined by the method of Lowry et al. (18).

RESULTS

Effect of the dimer on lipid peroxidation in heart SMP. When SMP are incubated with the dimer at concentration inhibiting completely the NADH oxidase activity, the NADH-Fe^{3+}-ADP induced lipid peroxidation (measured as MDA formation) is inhibited too. In a representative experiment, performed as described in Materials and Methods, the MDA formation decreases from 2.4 to 0.1 nmol/ min/ mg of protein with 4 mM dimer present during the preincubation step. This effect is completely different with that exerted by other Complex I inhibitors which, on the contrary, enhance the lipid peroxidation (5-8). Fig.1 shows that the inhibition of MDA formation by the dimer is concentration-dependent in the range 0.02-0.2 mM. Despite inhibiting lipid peroxidation, this range of concentrations has no effect on NADH oxidase activity of SMP. Thus the potency of the dimer on the inhibition of lipid peroxidation is more than one order of magnitude higher than that on the inhibition of NADH oxidase activity (I_{50}= 1.3 mM) (13). Furthermore the dimer is able to protect SMP from lipid damage stimulated by other Complex I inhibitors. Fig. 1 shows the inhibitory effect of the dimer on MDA formation when SMP are preincubated with rotenone or MPP^+.

Antioxidant activity of the dimer compared with traditional scavengers. The protective effect of the dimer on lipid peroxidation has been compared with other scavengers of oxygen reactive species. As reported in Table I, the reaction is partially prevented by catalase but cannot be inhibited by SOD or by the hydroxyl radical scavenger mannitol. Ascorbic acid acts as both an antioxidant and a prooxidant depending on the concentration. Glutathione acts as prooxidant in the range of concentration used. On the other hand the peroxidation can be prevented by the

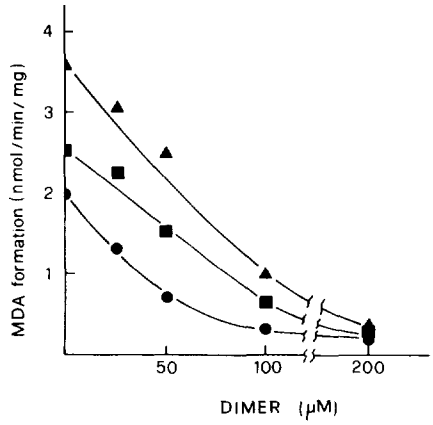

Fig.1. Inhibition of NADH-dependent MDA formation of SMP by the dimer. The assay conditions were as described in Materials and Methods except that SMP were preincubated in the presence of varying concentrations of dimer. Without other additions (●); with 20 mM MPP^+ (■) or 1 μM rotenone (▲) during the preincubation step. All values are means of at least three independent determinations for which S.E. was less than 10 %.

Table I. Dimer antioxidant activity compared with traditional scavengers

conditions	malondialdehyde formation (as % of the control)
control system	100
+ dimer (0.2 mM)	10
+ mannitol (100 mM)	100
+ formate (10 mM)	62
+ ascorbic acid (0.05 mM)	278
+ ascorbic acid (0.1 mM)	160
+ ascorbic acid (0.5 mM)	47
+ ascorbic acid (1mM)	13
+ glutathione (0.1mM)	107
+ glutathione (1mM)	189
+ glutathione (10 mM)	264
+ catalase (100 μ/ml)	76
+ SOD (100 μg/ml)	100
+ vitamin E (10 μM)	16
+ butylated hydroxytoluene (BHT) (10 μM)	4

The assay conditions were as described in Materials and Methods with the indicated additions during the preincubation step. The MDA formation of the control was 2.2 nmol/min/mg of protein. Values are the means of two independent determinations.

dimer which exhibits an excellent inhibiting effect compared with the lipid-soluble antioxidants vitamin E and BHT.

Effect of the dimer on superoxide anion generation by SMP. Rotenone as well as other Complex I inhibitors stimulates NADH dependent O_2^- generation in SMP (7,19). Table II shows that in the presence of 4 mM dimer, a concentration inhibiting almost completely the NADH oxidase activity of SMP, the production of superoxide (measured as adrenochrome formation) is lower compared to that induced by rotenone or by MPP^+ under similar inhibitory conditions of SMP activity. This result cannot be ascribed to a possible ability of the dimer to react with O_2^- because the dimer added to rotenone or MPP^+ incubations produces additional (or even more) oxyradicals.

DISCUSSION

The results obtained clearly illustrate the protective effect of the dimer toward lipid peroxidation of bovine heart SMP. This is indicated by the ability of the dimer to decrease the MDA formation when SMP are incubated with NADH in the presence of ADP-Fe^{+3} chelate. Moreover the dimer is able to counteract the MDA formation enhanced by some Complex I inhibitors (Rotenone and MPP^+). This result is in line with the recent finding of the ability of the dimer to interact with reactive oxygen species suggesting a radical scavenging role for this compound (15). Of interest is to compare the concentration of the dimer as inhibitor of SMP electron flux with that of antioxidant agent. The I_{50} on NADH oxidase activity is 1.3 mM (13)

Table II. Effect of the dimer on NADH oxidase activity and on O_2^- formation of SMP

conditions	activity (μmol/min/mg)	Adrenochrome (nmol/min/mg)
control	1.5	0.3
+ Dimer (4 mM)	0.1	2.2
+ Rotenone (1μM)	0	7.6
+ MPP^+ (20 mM)	0.13	4.5
+ Dimer (4 mM) + Rotenone (1μM)	0	11.2
+ Dimer (4 mM) + MPP^+ (20 mM)	0.09	10.4

NADH oxidase activity was measured spectrophotometrically at 350 nm in 0.1 mM EDTA, 20 mM K-phosphate, pH 7.4. After preincubation for 5 min at 37°C in the indicated conditions the assay was started by the addition of NADH to 0.15 mM final concentration. The blank cuvette received the same additions with the exception of NADH.

O_2^- production was followed by oxidation of adrenaline to adrenochrome: the reaction mixture containing SMP (0.75 mg/ml) and 1 mM adrenaline in 50 mM K-phosphate, pH 7.5, was preincubated for 5 min at 37°C in the indicated conditions. The reaction was started by the addition of NADH to 0.1 mM final concentration and the rate of adrenochrome formation was determined at 485 nm in the linear portion of the time course (5-10 min).

All values are means of at least three independent determinations for which S.E. was less than 10 %.

while the concentration of dimer required for 50% protection against MDA production is 0.04 mM. This difference, being more than one order higher in favour of the antioxidant activity, assigns to the dimer a major role of protecting agent of SMP structure than that of inhibitor of the electron transport. Furthermore the result that the dimer at concentration even higher than that inhibiting the lipid peroxidation does not prevent the O_2^- production (adrenochrome formation) induced by rotenone or MPP^+ could be explained by the lower reactivity of the dimer towards O_2^- than towards the hydroxyl radical (15) and by the lipophilic properties of the dimer able to react with radicals inside of the membrane (the socalled crypto-radicals).

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