On the Mechanism of Inhibition of Lipid Peroxidation by Manganese

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The antioxidant action of Mn(II) on different peroxidizing systems was studied. Mn(II) inhibits lipid peroxidation induced by free radical producing systems but not the one induced by singlet oxygen.

A close relationship between the action of Mn(II) and that of the classical antioxidant BHA was found. This indicates that Mn(II), likewise BHA, may act by scavenging the free radicals formed during initiation and propagation of lipid peroxidation.

Introduction

Manganese is an essential component of several enzymes and in some of them (superoxide dismutase, pseudocatalase and the photosynthetic oxygen-evolving center) is involved in redox processes [1]. Manganese has been shown to inhibit lipid peroxidation induced *in vitro* with the various systems routinely used for this type of study [2–9]. On the other hand $MnCl_2$ intraperitoneally given to rats significantly inhibits the potential peroxidation of brain phospholipids [10]. Cell membrane integrity seems to be sensitive to manganese deficiency and in particular the mitochondrial membrane in which abnormalities similar to those induced by hyperbaric oxygen were shown [11]. These alterations were related to the role of manganese as an essential component of the mitochondrial superoxide dismutase which protects membranes from the peroxidative damage produced by superoxide radicals [11].

Lipid peroxidation has been generally linked to a variety of pathological conditions [12–14] and, on the other hand, excessive intake of manganese leads to toxic effects at the level of the central nervous system, which have been related at least in part to Mn(II) inhibition of lipid peroxidative processes which could play a role in the remodelling of membranes [15].

In spite of the large use of manganese as an inhibitor of the *in vitro* lipid peroxidation, its mechanism of action has not been hitherto clarified.

In this paper we report a study on the effects of Mn(II) on different lipid peroxidation systems. We found that Mn(II) might both inhibit the initiation step(s) and the subsequent chain reaction probably acting as a free radical scavenger.

Experimental

Materials and Methods

Rat liver microsomes were prepared essentially as described by Ernster *et al.* [16] and washed in 0.125 *M* KCl, 10 mM HEPES at pH 7.4, centrifuged at 105.000 × g for 1 hour and resuspended in the same medium. Liposomes from soybean phospholipids (1 mg phospholipids/ml) were obtained by homogenization followed by ultrasonic treatment at 90 Watt, in an ice bath, under nitrogen stream for four 30 seconds periods, separated by 1 minute intervals, to reduce heating. The medium used was 0.125 *M* KCl and 10 mM HEPES at pH 7.4.

Protein content was estimated by the biuret method [17]. Rat liver microsomes were incubated at a final concentration of 1 mg/ml in 0.125 *M* KCl buffered with 15 mM HEPES and 6.5 mM Tris, at pH 7.4 and 25 °C. Oxygen uptake was followed with a platinum electrode assembly of the Clark type [18]. Malondialdehyde (MDA) was assayed by the thiobarbituric acid method as described by Buege and Aust [19]. Lipid hydroperoxides (LOOH) were measured using the thiocyanate method [20] modified as in [21]. The calibration curve for LOOH determination was obtained with a cumene hydroperoxide solution titrated as described by Jocelyn and Dickson [22]. Photoactivation in the presence of rose bengal (10 μg/ml) was performed in a thermostatted vessel; the reaction was started by illumination with a 200 Watt incandescent lamp at a distance of 15 cm and through a 1% K-bichromate filter solution of 1 cm thickness.

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Abbreviations: BHA, butylated hydroxyanisole; CHP, cumene hydroperoxide; EDTA, ethylenediaminetetraacetic acid; LOOH, lipid hydroperoxides; MDA, malondialdehyde.

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TABLE I. Effect of $MnCl_2$ and EDTA on MDA and LOOH Formation Induced by Different Peroxidizing Systems.[a]

	Control		$MnCl_2$		EDTA	
	MDA	LOOH	MDA	LOOH	MDA	LOOH
NADPH-$FeCl_2$-ADP (Microsomes)	22.9	9	0.8	0	0.7	0.9
Cumene hydroperoxide (Microsomes)	18.6		1.8		17.1	
Rose bengal + light (Liposomes)	1.2	99	0.9	98	1.0	98

[a]Incubation conditions and MDA and LOOH estimation are described in the Materials and Methods section. MDA and LOOH values are expressed as nanomoles/mg protein for microsomes and nanomoles/mg phospholipid for liposomes. $MnCl_2$ and EDTA concentrations are 1 mM. Microsomes were incubated for 15 minutes (in the presence of 0.5 mM CHP) and for 10 minutes (in the presence of 0.3 mM NADPH, 12 μM $FeCl_2$ and 1 mM ADP). Soybean liposomes (1 mg/ml) were photoactivated with rose bengal (10 μg/ml) for 10 min at 25 °C in the same incubation medium used for microsomes.

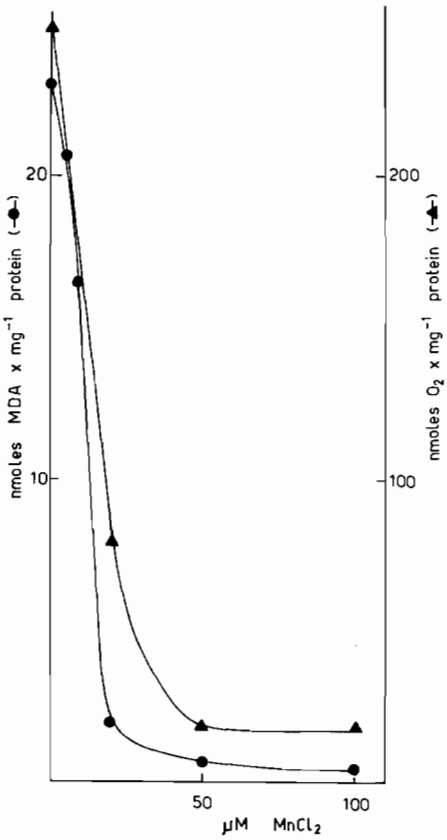

Fig. 1. Effect of increasing concentration of Mn(II) on lipid peroxidation in microsomes. Microsomes were incubated for 10 minutes at 30 °C in the presence of 0.5 mM NADPH, 40 μM $FeCl_3$ and 0.2 mM ADP. Other conditions, determinations of O_2 uptake and MDA formation are described in Materials and Methods.

Results

Figure 1 shows the inhibitory action of increasing concentrations of Mn(II) on MDA production and O_2 uptake in microsomal lipid peroxidation stimulated by NADPH-$FeCl_3$-ADP. Under our conditions, 15 μM Mn(II) inhibits by about 50% both MDA production and O_2 consumption while the inhibition is almost complete when Mn(II) concentration is over 50 μM. Mn(II) exhibits the same inhibitory action when hematin is used as peroxidizing agent in an aged suspension of linolenic acid (not shown).

Lipoperoxidation induced by the system NADPH–$FeCl_3$–ADP in microsomes is dependent on the cyclic reduction of iron by the flavoprotein NADPH–cytochrome P-450 reductase [23]. In fact, reduced iron acts as an inducer of lipid peroxidation and is being oxidized in the process. In this case both Mn(II) and EDTA show a strong inhibition (Table I). On the contrary, EDTA is ineffective on CHP-induced lipid peroxidation while Mn(II) is still a strong inhibitor (Table I). In this case lipid peroxidation depends on CHP activation by cytochrome P-450 and is completely insensitive to free iron ions catalysis [21, 24].

Rose bengal mediated photoactivation of molecular oxygen to the excited state of singlet oxygen gives rise to the formation of lipid hydroperoxides through a direct 'ene' reaction with polyunsaturated fatty acids [25]. This process does not occur through a free radical mechanism [25]. Table I shows that, when liposomes are peroxidized with rose bengal and visible light, MDA formation is only slightly inhibited by Mn(II) and EDTA. This means that metal catalysis is only partially responsible for the MDA production and that another pathway of formation, probably through the direct formation of endoperoxides, could be followed. In the case of rose bengal, Mn(II) and EDTA do not decrease the amount of estimated hydroperoxides. As previously reported [26], Mn(II) by itself does not quench singlet molecular oxygen.

The hypothesis of an antioxidant action of $MnCl_2$ was further confirmed by following its effect on MDA or preformed lipid hydroperoxides, and comparing it with that of the well known antioxidant BHA. The effect of EDTA in this system is also shown. As reported in Fig. 2, hydroperoxides were photochemically formed by the action of rose bengal and light in microsomes for 10 minutes; the reaction

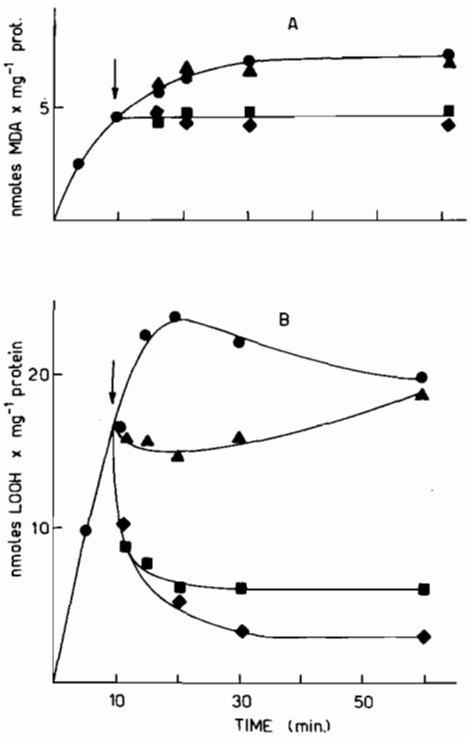

Fig. 2. Effects of Mn(II), BHA and EDTA on lipid hydroperoxides preformed by photoactivation in microsomes. Rat liver microsomes were incubated with rose bengal as described in Materials and Methods. The reaction lasted 10 minutes, then light was turned off (indicated by the arrow) and, in the dark, the suspension was divided into four aliquots treated in the following ways: (●—●) control, no addition; (▲—▲) 1 mM EDTA; (◆—◆) 1 mM $MnCl_2$ and (■—■) 0.3 mM BHA. MDA and LOOH were determined as described in Materials and Methods.

was then stopped by turning the light off and the indicated additions were made. As shown in Fig. 2a, EDTA is unable to stop the formation of MDA occurring after the reaction with rose bengal is over, while Mn(II) and BHA stop it effectively (Fig. 2a). In order to know the fate of lipid hydroperoxides which are at the midpoint of the peroxidative degradation of lipids, the change with time in the concentration of lipid hydroperoxides after irradiation with rose bengal and light was followed (Fig. 2b). As reported, Mn(II) and BHA behave in a similar way: they decrease the content of LOOH by completely inhibiting the formation of new lipid hydroperoxides and consequently of MDA. Moreover cytochrome P-450, present in the microsomal fractions, is able to spark lipid peroxidation by decomposing the preformed lipid hydroperoxides [21] and this peroxidation is blocked by both Mn(II) and BHA. On the contrary, EDTA only lowers the rate of formation of new lipid hydroperoxides.

Discussion

According to some authors [10, 27], the antiperoxidative action of manganese can be explained as a competition of Mn(II) for the binding sites of iron, so preventing the iron-dependent lipid peroxides formation. Nevertheless a free radical scavenging activity of Mn(II) can also be assumed. In fact our results indicate that Mn(II) completely blocks MDA formation and oxygen uptake in microsomes incubated with CHP while EDTA is completely ineffective in both cases. The Mn(II) inhibitory effect should not be ascribed to a competition with iron but more probably to a direct removal of the activated species formed by the interaction of CHP with cytochrome P-450.

Other authors [28] have proposed that Mn(II) might be forming a complex with unsaturated lipids making them more resistant to attack by peroxide. We found that lipoperoxidation induced by singlet oxygen is not inhibited by Mn(II) ruling out a mechanism based on a physical-chemical interaction of Mn(II) with polyunsaturated lipids.

The results of Fig. 2 clearly show that Mn(II), like BHA, inhibits the free radical chains which follow the formation of hydroperoxides and that lead to the formation of MDA.

There are some reports in the literature describing the interaction of Mn(II) with free radicals species [29, 30]. Mn(II) can reduce the superoxide anion to hydrogen peroxide with the concomitant formation of Mn(III) [31, 32]. The possibility of oxidation of Mn(II) by the superoxide anion has been challenged [30] on the ground that Mn(II) is able to form complexes both with $O_2^{\cdot-}$ and ·OH giving rise to species like MnO_2^+ and $Mn(OH)^{2+}$. In a recent report [33] Archibald and Fridovich have confirmed that complexed Mn(II) is able to scavenge the superoxide anion, while hexaquo Mn(II) is a poor scavenger of $O_2^{\cdot-}$. The interaction of Mn(II) with the reported free radical species supports the hypothesis of a general antioxidant action that might occur through the reduction of lipid free radicals (RO· and ROO·) making them unable to carry on the process of lipid peroxidation.

The concentration of Mn(II) in mammalian liver has been reported to be about 2.5 10^{-5} *M* [34]. Although this concentration is quite low compared with that of other biological metal ions, it is nevertheless in the range where significant antioxidant action has been observed (Fig. 1).

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