TYROSINE · COPPER(II) INHIBITS LIPID PEROXIDATION IN RAT LIVER MICROSOMES

Christoph RICHTER, Theres PRIPFL and Kaspar H. WINTERHALTER
Eidgenossische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, Universitätsstrasse 16, CH-8092 Zürich, Switzerland

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1. Introduction

Rat liver microsomes catalyze an NADPH-dependent peroxidation of endogenous unsaturated fatty acids [1,2]. Several reactive oxygen species, the superoxide radical, hydrogen peroxide, the hydroxyl radical and singlet oxygen have been implicated in the lipid peroxidation mechanism (reviewed [3]). Complexed and free superoxide radicals have been proposed to participate in the initiation and propagation of lipid peroxidation [4,5]. The possible involvement of the superoxide radical in the lipid peroxidation mechanism has been investigated to date mainly using the enzyme superoxide dismutase (EC 1.15.1.1) which catalyzes the dismutation of superoxide radicals according to the following equation [6]:

$$O_2^- + O_2^- + 2\,H^+ \rightleftharpoons H_2O_2 + O_2$$

Rat liver microsomes produce superoxide radicals by an NADPH-dependent pathway. The main source of O_2^- is the enzyme NADPH-cytochrome *c* (cytochrome P450) reductase (EC 1.6.2.4) [7]. There is indirect evidence that cytochrome P450 itself can also generate O_2^- [8–10].

The highly unsaturated fatty acid residues of membrane phospholipids are located for the most part in the hydrophobic midzone of the membrane. Furthermore, oxygen is 7–8-times more soluble in an apolar environment than in water. Therefore, the hydrophobic inner part of the membrane might be particularly susceptible to oxidative damage. Due to the large size (mol. wt 32 000) of superoxide dismutase it is questionable whether this enzyme can react with superoxide radicals localized within the hydrophobic domains of the membrane.

Small hydrophobic Cu(II)-complexes exhibiting high superoxide dismutase activity have successfully been used as probes for the participation of the superoxide radical in the microsomal hydroxylation reaction at the level of cytochrome P450 [9,10], in microsomal γ-carboxylation of the prothrombin precursor and a related synthetic peptide [11], and in the formation of hydrogen peroxide by intact hepatocytes [12]. Here we show that the small and hydrophobic copper(II) · tyrosine complex, a very efficient scavenger of superoxide radicals [13], inhibits at low concentrations NADPH-dependent lipid peroxidation in rat liver microsomes.

2. Materials and methods

Chemicals were obtained from the following sources: Boehringer, (Mannheim), isocitrate dehydrogenase (pig heart, grade III), cytochrome *c* (horse heart, salt free), nicotinamide adenine dinucleotide phosphate (reduced form, tetrasodium salt); Fluka, (Buchs), 2-thiobarbituric acid. Tyrosine · copper(II) and tyrosine · zinc(II) was a generous gift of Dr A. Wendel, University of Tübingen. All other chemicals were obtained from Merck, Darmstadt.

Male Wistar rats (150–200 g body wt) were injected i.p. with phenobarbital (80 mg/kg body wt) daily for 2 consecutive days, were starved for 24 h, then killed. Liver microsomes were obtained by the method in [14]. Peroxidation of microsomes was performed in buffer containing 50 mM potassium phosphate (pH 7.2), 150 mM KCl, 12 μM EDTA, 12 μM $FeSO_4$, 7 mM isocitrate, 10 mM nicotinamide and 0.1 mg isocitrate dehydrogenase/ml. Microsomal protein was 2 mg/ml. The suspension was placed in beakers on a shaking water bath (100 cycles/min) kept at 37°C. After a preincubation of 10 min 0.5 mM

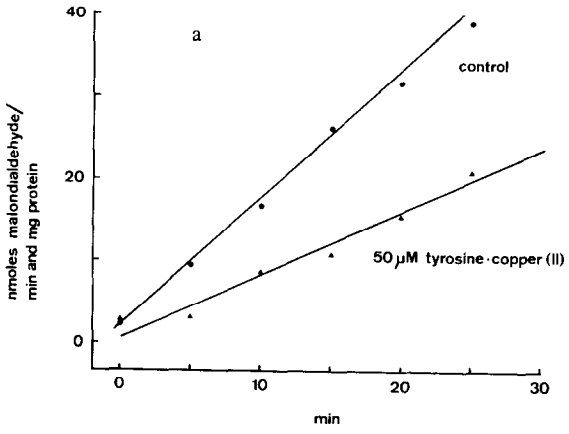
NADPH was added to start the peroxidation. Malondialdehyde was determined by the thiobarbituric acid method in [15]. NADPH-cytochrome *c* reductase activity was assayed as in [9] using 20 μg microsomal protein/ml. Protein was determined by the biuret method.

3. Results

3.1. *Inhibition of malondialdehyde formation*

The NADPH-dependent production of malondialdehyde, an indicator of lipid peroxidation, was determined in the presence and absence of the superoxide dismutase-active tyrosine · copper(II) complex. Fig.1a reports the time course of malondialdehyde production in control buffer and in buffer containing 50 μM tyrosine · copper(II), a concentration which inhibits microsomal dealkylation and γ-carboxylation reactions effectively [9–11]. Fig.1b shows that the inhibition of malondialdehyde production is dependent on the concentration of tyrosine · copper(II) present during peroxidation. Control experiments showed that tyrosine · copper(II) did not interfere with the thiobarbituric acid assay for malondialdehyde, nor that it reacted with already formed malondialdehyde.

Table 1 shows that various concentrations of superoxide dismutase do not inhibit the NADPH-dependent lipid peroxidation. 80 μM tyrosine · zinc(II), which is devoid of superoxide dismutase activity, inhibits lipid peroxidation very slightly. Two 'classical' inhibitors of cytochrome P450-dependent microsomal hydroxylation reactions, metyrapone and SKF 525-A, inhibit the NADPH-dependent lipid peroxidation to



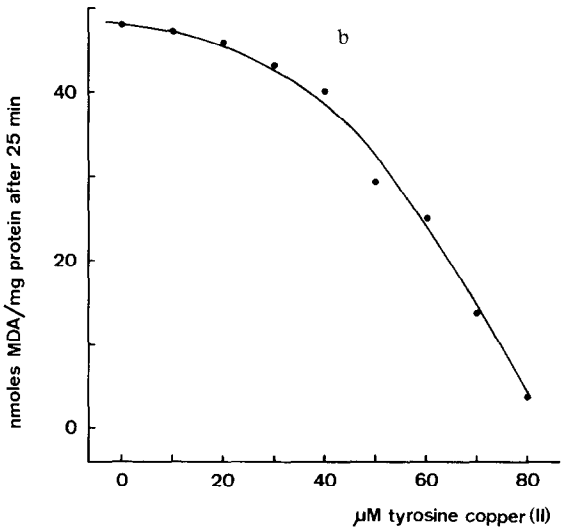


Fig.1. Inhibition by tyrosine · copper(II) of NADPH-dependent lipid peroxidation in rat liver microsomes. Malondialdehyde was used as indicator for lipid peroxidation. (a) Time course of malondialdehyde production. (b) Malondialdehyde determined after 25 min peroxidation.

60% at 0.5 and 0.01 mM, respectively. At higher concentrations of these compounds no further inhibition was observed. $CuCl_2$ at 80 μM inhibits the peroxidation to 82%.

3.2. *Activity of NADPH-cytochrome* c *reductase*

NADPH-dependent microsomal lipid peroxidation involves the activity of the flavoprotein NADPH-cytochrome *c* (cytochrome P450) reductase [4,16]. We therefore tested the effect of tyrosine · copper(II) on the activity of this enzyme in peroxidizing microsomes. Table 2 shows that the activity does not

Table 1
Inhibition of NADPH-dependent lipid peroxidation by various compounds

Compound	% inhibition
Superoxide dismutase, 1 μM	0
Superoxide dismutase, 5 μM	0
Superoxide dismutase, 10 μM	0
Tyrosine · zinc(II), 80 μM	5
Metyrapone, 0.5 mM	60
SKF 525-A, 10 μM	60
$CuCl_2$, 80 μM	82

Rat liver microsomes were peroxidized for 25 min and then assayed for malondialdehyde production

Table 2
Activity of NADPH-cytochrome *c* reductase in peroxidizing rat liver microsomes

Incubation condition	NADPH-cytochrome reductase activity (nmol cytochrome *c* reduced per min and mg protein)
Control before peroxidation	132
Control at 25 min peroxidation	136
Control + 80 μM tyrosine · copper(II) before peroxidation	137
Control + 80 μM tyrosine · copper(II) at 25 min peroxidation	135
Control + 80 μM $CuCl_2$ at 1 min peroxidation	31
Control + 80 μM $CuCl_2$ at 2 min peroxidation	31

Microsomes were peroxidized in the absence (control) and presence of tyrosine · copper(II) and $CuCl_2$

change in peroxidizing microsomes over 25 min, regardless whether tyrosine · copper(II) is absent or present. The presence of 80 μM $CuCl_2$ leads to an immediate depression of the enzyme activity by 77%.

4. Discussion

This report shows that tyrosine · copper(II), a small lipophilic compound with high superoxide dismutase activity, inhibits the NADPH-dependent lipid peroxidation in rat liver microsomes. The copper complex does not inhibit the enzyme NADPH-cytochrome *c* (P450) reductase, the main generator of superoxide radicals in microsomes. We have shown that tyrosine · copper(II) does not inhibit the electron flow from the reductase to cytochrome P450, and that it does not cause a transformation of cytochrome P450 to cytochrome P420 [9,10]. We conclude that tyrosine · copper(II) inhibits NADPH-dependent lipid peroxidation by scavenging superoxide radicals which participate in the peroxidation reaction.

We find no inhibition of microsomal lipid peroxidation by superoxide dismutase in agreement with [5,17]. More than 90% of the malondialdehyde formed during NADPH-dependent lipid peroxidation was reported [5] due to the propagation reaction which most likely proceeds within the membrane at sites which are not accessible to superoxide dismutase.

The small lipophilic tyrosine · zinc(II) complex which has no superoxide dismutase activity inhibits microsomal lipid peroxidation only slightly. Copper (II) alone inhibits the peroxidation and NADPH-cytochrome *c* reductase to about the same extent. Thus the decreased malondialdehyde production in the presence of copper(II) alone must be attributed to an inhibition of the electron transfer by the enzyme from NADPH to oxygen and cytochrome P450, and not to a direct inhibition of lipid peroxidation.

Inhibition of lipid peroxidation by metyrapone and SKF 525-A indicates a participation of cytochrome P450 in this process. A similar inhibition by SKF 525-A, albeit at higher concentration, has been shown in [1,5].

The experiments suggest that superoxide radicals participate in the NADPH-dependent peroxidation of microsomal unsaturated fatty acids and that these radicals react in domains of the microsomal membrane which are not accessible to superoxide dismutase. Due to its small size and lipophilic character tyrosine · copper(II) scavenges O_2^- in these domains and thus prevents lipid peroxidation. It remains to be established whether the tyrosine · copper(II)-sensitive O_2^- acts directly on unsaturated fatty acids and lipid peroxides as proposed in [18], whether it forms perferryl complexes with ADP-bound iron, or whether it acts indirectly as a reductant of heavy metal ion complexes like ADP · Fe^{3+}, EDTA · Fe^{3+}, and oxidized cytochrome P450.

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