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Loss of α-tocopherol upon exposure to nitric oxide or the sydnonimine SIN-1

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SIN-1 which spontaneously decomposes to yield nitric oxide (NO•) and superoxide anion ($O_2^{\bullet-}$) radicals caused a loss of microsomal α-tocopherol paralleled by the formation of α-tocopheryl quinone. The loss was partially prevented by superoxide dismutase but not by catalase. The SIN-1-induced loss of α-tocopherol also occurred when tocopherol was dissolved in ethanol/potassium phosphate buffer (20/80, v/v). Likewise, addition of authentic NO• to α-tocopherol dissolved in ethanol resulted in loss of the vitamin and quinone formation. These results suggest that NO• or its products such as peroxynitrite or nitrogen dioxide react with α-tocopherol, the quinone derivative being a major oxidation product. Depletion of vitamin E by NO• may contribute to tissue injury, e.g. in neuronal tissues.

Vitamin E; Nitric oxide; Sydnonimine

1. INTRODUCTION

Nitric oxide (NO•) contributes to the regulation of blood pressure as EDRF [1] and has been suggested to play a role in neuronal signal transduction [2]. NO• also constitutes the cytotoxic potential of macrophages against tumor cells [3], pancreatic β-cells [4] and microorganisms [5] and is considered to be involved in glutamate neurotoxicity [6] and in tissue injury due to deposition of immune complexes [7]. NO• may also be released pharmacologically. An example is SIN-1 (3-morpholino-sydnonimine) which is formed in the liver from molsidomine (*N*-ethoxy-carbonyl-3-morpholino-sydnonimine) by enzymatic cleavage of the ethoxy carbonyl group, decomposing to yield NO• and $O_2^{\bullet-}$) [8].

Tocopherols (vitamin E) act as biological antioxidants accounting for most, if not all, of the lipid-soluble chain-breaking antioxidant capacity [9]. The antioxidant reaction involves a peroxyl radical and the phenolic hydroxyl group of tocopherol to generate the corresponding organic hydroperoxide and the tocopheroxyl radical [10]. At least in vitro tocopherol can be regenerated by water-soluble hydrogen donors like ascorbate [10,11]. If regeneration does not occur, the chromanoxyl radical may be further oxidized to tocopheryl quinone.

Due to the cytotoxic potential and the radical nature of nitric oxide the question arises whether NO• may also interfere directly with the antioxidant defense system, especially with vitamin E. Here, we examined the effects of SIN-1 and authentic NO• on liver microsomal α-tocopherol and on α-tocopherol dissolved in ethanol/water or pure ethanol.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Superoxide dismutase and catalase were purchased from Boehringer (Mannheim, Germany) and NO• was from Linde (Düsseldorf, Germany). SIN-1 and tocopherols were kind gifts of Cassella-Riedel Co. (Frankfurt, Germany) and Henkel Co. (Düsseldorf, Germany), respectively.

2.2. *Incubations*

Three incubation systems were used: (a) microsomal tocopherol with SIN-1; (b) tocopherol dissolved in a mixture of ethanol and buffer with SIN-1; and (c) a pure ethanolic system, where tocopherol was dissolved and authentic NO• added. Microsomes (1 mg protein/ml), prepared from male Wistar rats according to [12], were incubated in 0.1 M potassium phosphate buffer, pH 7.4. For the experiments in ethanol, the problem was that α-tocopherol is insoluble in water, and SIN-1 is hardly soluble in ethanol. Therefore, we used a mixture of 20% ethanol and 80% 0.1 M potassium phosphate buffer (v/v), pH 7.4. Since aqueous solutions of SIN-1 are light-sensitive and unstable, they were prepared freshly in the dark. For the experiments with authentic NO•, the ethanol was pregassed with nitrogen for 2 h to remove oxygen, and the NO•, dissolved according to a procedure described in [13], was added through a pump, the added volume adjusted to give an NO• supply of 2 μM/min. Incubations were performed at 37°C under aerobic conditions.

2.3. *Analysis of α-tocopherol and of α-tocopheryl quinone*

α-Tocopherol and α-tocopheryl quinone were analyzed by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection [14–16]. RRR-δ-tocopherol was used as an internal standard. A standard of α-tocopheryl quinone was produced by incubation of α-tocopherol with $FeCl_3$ and subsequent chromatographic purification.

2.4. *Determination of nitric oxide and of lipid peroxidation*

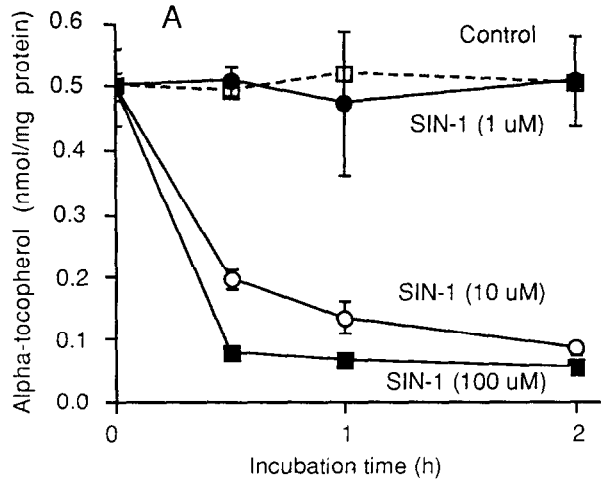
NO• release from SIN-1 in potassium phosphate buffer and the content of NO• dissolved in ethanol were determined by the oxyhemo-

globin method [17,18]. The formation of thiobarbituric acid-reactive substances was measured according to [16]. Results were expressed in malondialdehyde equivalents using an extinction coefficient of 156 $mM^{-1} \cdot cm^{-1}$.

3. RESULTS

3.1. *Nitric oxide release from SIN-1 in potassium phosphate buffer*

At 100 μM SIN-1, NO• was released at a rate of 26.4 ± 2.4 μM/30 min. At 10 μM SIN-1, NO• was still released at a rate of 6.5 ± 1.0 μM/30 min, and at 1 μM SIN-1, NO• release decreased to 1.0 ± 0.2 μM/30 min. Thus, the relation between liberation of NO• and concentration of SIN-1 is nonlinear, as reported previously [13].



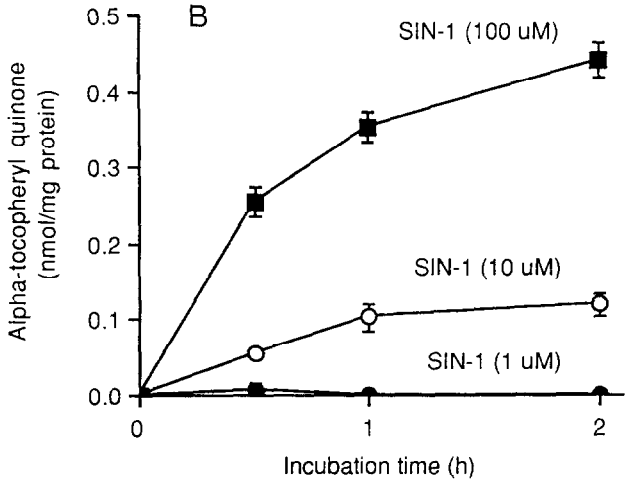


Fig. 1. Loss of microsomal α-tocopherol (A) and formation of α-tocopheryl quinone (B) upon incubation with SIN-1. Microsomes (1 mg microsomal protein/ml) were incubated in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C. SIN-1 was added as indicated. α-Tocopherol and α-tocopheryl quinone were determined by HPLC and electrochemical detection. Data presented are means ± S.E. (n = 6).

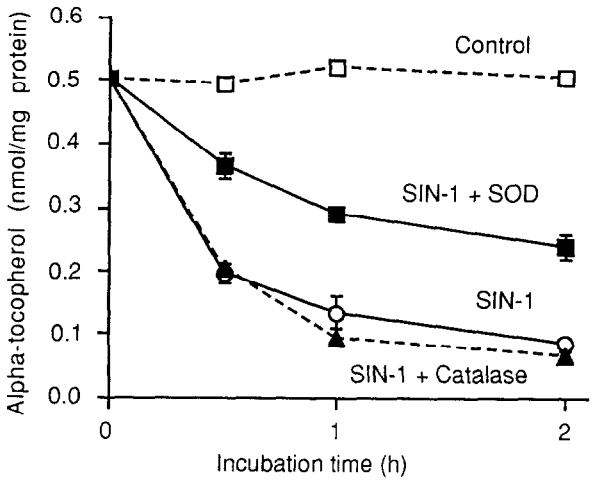


Fig. 2. Effects of superoxide dismutase and catalase on SIN-1-induced loss of microsomal α-tocopherol. SIN-1 was 10 μM and superoxide dismutase (SOD) and catalase were 5 μg/ml. Data presented are means ± S.E. (n = 5).

3.2. *Effect of SIN-1 on microsomal α-tocopherol*

Addition of 100 μM SIN-1 to rat liver microsomes resulted in an almost complete loss of α-tocopherol within 30 min (Fig. 1A), slightly less so upon addition of 10 μM SIN-1. At 1 μM, SIN-1 was without effect on microsomal α-tocopherol.

Parallel to the decrease in α-tocopherol, α-tocopheryl quinone was formed (Fig. 1B). At 100 μM SIN-1, α-tocopheryl quinone was formed almost stoichiometrically. At 10 μM SIN-1, the ratio of α-tocopheryl quinone formed to α-tocopherol lost was lower. At 1 μM SIN-1, no α-tocopheryl quinone was formed. The loss of α-tocopherol (Fig. 2) and the formation of its quinone (not shown) were significantly diminished by added superoxide dismutase but were unaffected by catalase.

SIN-1 caused a moderate but significant increase in microsomal lipid peroxidation (Fig. 3) suppressed by superoxide dismutase but not by catalase.

3.3. *Effect of SIN-1 and of authentic nitric oxide on α-tocopherol in solution*

Tocopherol dissolved in ethanol/potassium phosphate buffer was also lost upon addition of SIN-1 (Table I) but less pronounced than in the microsomal system (compare Fig. 1A). Formation of α-tocopheryl quinone was not detectable at 10 μM SIN-1, but did occur at 100 μM SIN-1 (Table I).

Upon addition of authentic NO• at a rate of 2 μM/min to α-tocopherol dissolved in pure ethanol there was a marked decrease in the vitamin (Table I). The decrease was about 70% after 90 min and was accompanied by the formation of α-tocopheryl quinone.

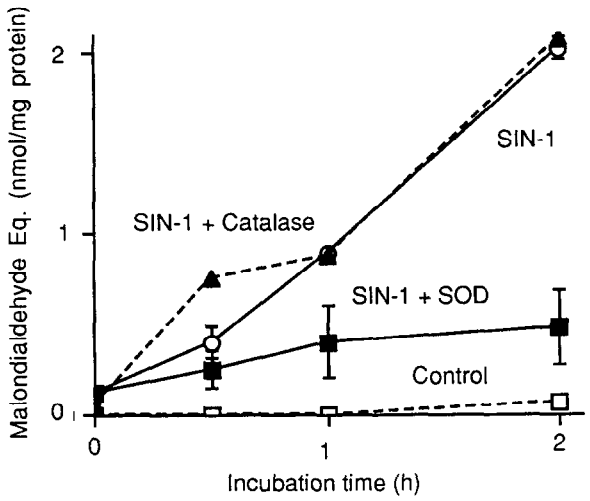


Fig. 3. Induction of lipid peroxidation by SIN-1 (10 μM) in microsomes. Inhibition by superoxide dismutase (5 μg/ml). Thiobarbituric acid-reactive substances were measured and expressed as malondialdehyde equivalents. Data presented are means ± S.E. (n = 5).

4. DISCUSSION

Upon decomposition of SIN-1, NO• and $O_2^{\bullet-}$) are released [8] and react to form the peroxynitrite anion ($ONOO^-$) [19]. The peroxynitrite anion can protonate to peroxynitrous acid which in turn can homolytically decompose to yield OH• and $NO_2^{\bullet}$) [20,21]. Proton-catalyzed decomposition of peroxynitrite causes oxidation of deoxyribose and dimethyl sulfoxide [20], while the peroxynitrite anion by itself is a potent sulfhydryl oxidizing compound [22]. Because of its reactivity, peroxynitrite may also be responsible for SIN-1-mediated loss of α-tocopherol reported here (Fig. 1A, Table I). Loss of microsomal α-tocopherol may be caused by reaction of peroxynitrite with the vitamin or result from peroxynitrite-induced lipid peroxidation. Loss of vitamin E due to lipid peroxidation is a known phenomenon (e.g. [23]). Induction of lipid peroxidation by peroxynitrite, probably via its protonated form, has recently been shown in phosphatidylcholine liposomes [24]. This interpretation is supported by the inhibitory effect of superoxide dismutase (Fig. 2). However, protection afforded by superoxide dismutase, by acting either against $O_2^{\bullet-}$) or against peroxynitrite [25], was not complete, indicating that the formation of peroxynitrite cannot solely account for the loss of vitamin E even in the microsomal system.

Since $O_2^{\bullet-}$) or H_2O_2 alone did not cause a decrease in vitamin E (data not shown), NO• may also lead to a loss of vitamin E directly. Such a reaction of NO• with vitamin E is also suggested by the experiments with authentic NO• in the ethanolic system (Table I). The mechanism of such a degradation of vitamin E by NO• is presently unclear. It may involve electron transfer from α-tocopherol to NO• and deprotonation, leading to the formation of the α-tocopheroxyl radical and NO^-. By a second one-electron oxidation step the α-tocopherol cation would be formed subsequently, hydrolyzing to α-tocopheryl quinone.

Loss of α-tocopherol may also involve reactive nitrogen intermediates different from NO• or peroxynitrite, e.g. $NO_2^{\bullet}$) which is formed not only upon decomposition of ONOOH but also by reaction of NO• with molecular oxygen. $NO_2^{\bullet}$) is known to exert its tissue-injuring effects through a free radical mechanism, probably lipid peroxidation. Its toxicity is enhanced by vitamin E deficiency and partly prevented by vitamin E supplementation [26,27].

A decrease in vitamin E favors free radical-induced cell and tissue injury. Thus, depletion of vitamin E mediated by NO• or products derived from it, as demonstrated by the present experiments, may be a further way by which NO• may contribute to cell and tissue injury. Vitamin E deficiency is associated with neuronal dysfunction [28,29].

Table I

Loss of α-tocopherol in solution and formation of α-tocopheryl quinone after incubation with SIN-1 and authentic nitric oxide

	α-Tocopherol (μM at 90 min)	α-Tocopheryl quinone (μM at 90 min)
SIN-1 10 μM	0.44 ± 0.03 (88)	n.d.
100 μM	0.26 ± 0.02 (52)	0.05 ± 0.01
Authentic NO 2 μM/min	0.14 ± 0.06 (28)	0.26 ± 0.03

Experiments with SIN-1 were performed in ethanol/potassium phosphate buffer (20/80, v/v), experiments with authentic NO in pure ethanol. In the respective controls no loss of α-tocopherol and no formation of α-tocopheryl quinone were detectable. The initial α-tocopherol concentration was 0.5 μM. Incubations were performed at 37°C for 90 min. α-Tocopherol and α-tocopheryl quinone were determined by HPLC and electrochemical detection. Values presented are means ± S.E.M. (n = 3). Percent of the initial value is given in parentheses. n.d., not detectable.

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