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Regeneration of vitamin E in rat polymorphonuclear leucocytes

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The objectives of this study were to determine whether the recycling of tocopherol occurs in elicited rat polymorphonuclear leukocytes and if so, whether the recycling process is enzymic or chemical. When incubated with hemoglobin, tocopherol was oxidized in cell homogenates in a time- and concentration-dependent manner. The oxidized tocopherol could be regenerated by addition of ascorbate, glutathione or nordihydroguaiaretic acid. Time course studies showed a rapid regeneration of tocopherol which peaked at 1 min after the addition of reductants. Determination of the regeneration reaction in the presence of $CHCl_3$ and MeOH indicated that under these enzyme-denaturing conditions, a considerable amount of tocopherol was still regenerated, suggesting that the regeneration reaction is predominantly a chemical reaction. This study provided direct evidence from mass analysis that oxidized vitamin E can be regenerated by cellular water-soluble reductants such as ascorbate and glutathione.

1. INTRODUCTION

α-Tocopherol is a membrane-bound antioxidant which functions as a major peroxyl radical scavenger with powerful chain-breaking properties [1,2]. This membrane antioxidant pool operates in concert with antioxidant enzymes and other soluble antioxidants to maintain cellular protection against free radical-induced cellular damages, which have been implicated in the cause of many pathophysiological conditions [3–5].

The interaction of cellular antioxidants was proposed in 1961 by Tappel who postulated that oxidized vitamin E could be regenerated by ascorbate [6]. While it is difficult to conceive that this could occur in vivo due to distinct cellular locations of these two vitamins, a direct reduction of tocopheroxyl radical by ascorbate was detected in a homogenous solution in 1979 [7]. Subsequently, numerous studies consistently showed that the oxidantion of vitamin E can be spared by ascorbate or reduced glutathione (GSH) in a number of pure or biological systems [8–23]. The protective role of GSH in diminishing vitamin E oxidation has led McCay to propose a GSH-dependent reductase in hepatic microsomes which acts to recycle vitamin E [24].

During the course of studying how dietary vitamin E could influence 5-lipoxygenase activity in rat polymorphonuclear leukocytes (PMNL), we noted that the vitamin E content in intact PMNL decreased dramatically when 5-lipoxygenase was activated by the addition of ionophore and arachidonic acid [25]. In order to further investigate the interactions of soluble and membrane-bound antioxidants, the objectives of this study were to determine whether the recycling of tocopherol occurs in PMNL and if so, whether the recycling process is enzymic or chemical.

Abbreviations: EDTA, ethylenediaminetetraacetic acid disodium dihydrate; GSH, reduced glutathione; NDGA, nordihydroguaiaretic acid; PMNL, polymorphonuclear leukocyte; Vitamin E, in this paper, it represents α-tocopherol.

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2. MATERIALS AND METHODS

2.1. *Animals and cell preparation*

Male Sprague–Dawley rats (250–350 g) were used in all experiments. Glycogen-elicited peritoneal polymorphonuclear leucocytes were isolated as described previously [25]. Briefly, 16–18 h after intraperitoneal injection of 20 ml of 0.2% oyster glycogen in saline, the rat peritoneal cavity was rinsed with 40 ml of Ringer–Tyrodes' buffer (Ca^{2+}- and Mg^{2+}-free, EDTA 2 mM; heparin, 5000 IU/litre). Cells from this peritoneal exudate were sedimented by centrifugation (750 × *g*, 5 min) and the cell pellet was suspended in 3 ml of buffer, layered over 3 ml of Ficoll–Paque (Pharmacia), and centrifuged at 1000 × *g* for 30 min. This procedure removed contaminating lymphocytes which migrate as a single layer mid-way up the centrifuge tube. The 'purified' PMNL pellet was taken up in 2 ml of a solution containing 137 mM NH_4Cl in 15 mM Tris-HCl and incubated at 37°C for 15 min to lyse contaminating red blood cells. The PMNL were sedimented again by centrifugation and resuspended in Tyrodes' buffer. After determination of cell number by a hemocytometer, they were adjusted to 20 × 10^6 cells/ml. Routinely, 25 × 10^6 cells yielded 0.5 nmol of tocopherol. Immediately before use, cells were sonicated with a Microson ultrasonic cell disruptor at 60% output volume for 20 s × 3.

2.2. *Determination of cellular tocopherol*

All reactions were stopped with $CHCl_3$ and MeOH and total lipid was extracted from cell homogenates by the method of Bligh and Dyer [26] after the addition of 0.25 μg of tocopherol acetate as internal standard. Tocopherol was determined by reversed-phase high pressure

liquid chromatography using a C-18 column with a solvent system containing 99% methanol/1% H_2O/0.1% trifluoroacetic acid as described in earlier publications [25,27].

2.3. *Materials*

All solvents were of reagent grades from BDH, Toronto; and all glassware was silanized before use. Heparin, ascorbate, glutathione, DL-α-tocopherol, glycogen (type II) and bovine hemoglobin (H-2625) were purchased from Sigma and nordihydroguaiaretic acid (NDGA) was from Aldrich. Desferrioxamine (Desferal) was from Ciba.

3. RESULTS

When PMNL homogenates were incubated with 10 μM of hemoglobin, there was a rapid consumption of cellular tocopherol which was essentially complete within the first min; extending the incubation time up to 12 min did not cause any further tocopherol oxidation (Fig. 1A). This hemoglobin-induced oxidation of PMNL tocopherol was dependent on the concentration of hemoglobin added. Fig. 1B shows that the amount of cellular tocopherol oxidized was proportionately increased in the range of hemoglobin concentration from 2.5 μM to 10 μM. Therefore, all our subsequent experiments were conducted with the addition of 10 μM of hemoglobin and 1 min of oxidation time. We routinely terminated these reactions with the addition of desferrioxamine in order to chelate the potential iron ions that may be dissociated from the hemoglobin molecule. Iron ions can react with trace amount of residual peroxides to generate peroxyl or alkoxyl radicals which could oxidize the regenerated tocopherol. The mechanism by which hemoglobin caused the oxidation of tocopherol is not investigated in the present study. It is known, however, that superoxide anion is released when oxyhemoglobin undergoes decomposition to methemoglobin. Conversely, the presence of trace amounts of hydrogen peroxide commonly found in phygocytes could react with methemoglobin to form a ferryl species that can cause hydrogen abstraction [5]. Both superoxide anion and the ferryl intermediate are sufficiently reactive to induce tocopherol oxidation.

Recent experimental data from our laboratory and others [28–31] have demonstrated that in mammalian tissues, cellular tocopherol is first oxidized to tocopheroxyl radical which can readily be reduced by physiologically relevant soluble reductants such as vitamin C or reduced glutathione to regenerate tocopherol. We have recently documented that this recycling pathway exists in human platelets [28]. In order to determine whether this tocopherol recycling system is operative in rat PMNL, we conducted experiments in which PMNL homogenates were first incubated with hemoglobin to cause oxidation of tocopherol. This was immediately followed by the addition of various reductants and cellular tocopherol was monitored over various time points. Fig. 2 illustrates this regeneration time course experiment in which we tested the ability of ascorbate, GSH and NDGA to regenerate the oxidized tocopherol. The addition of hemoglobin to cell homogenates caused a dramatic disappearance of cellular tocopherol from 0.3 nmol to 0.04 nmol within the first minute. Following the addition of various reductants, the amount of toco-

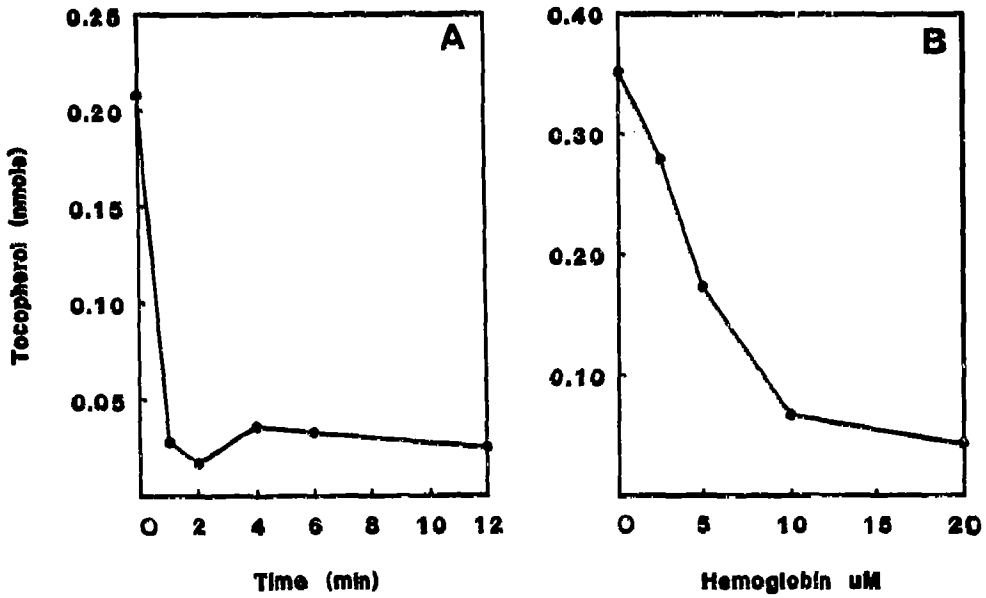

Fig. 1. Time-course and dose-dependent oxidation of tocopherol in rat PMNL. Sonicated cells were incubated with 10 μM of hemoglobin at 37°C for indicated times (A) or they were incubated with increasing concentrations of hemoglobin for 5 min. Reactions were terminated with the addition of $CHCl_3$/MeOH in the presence of desferrioxamine (100 μM). After total lipid extraction, tocopherol was determined by HPLC as described in section 2. Values are from one of three representative experiments.

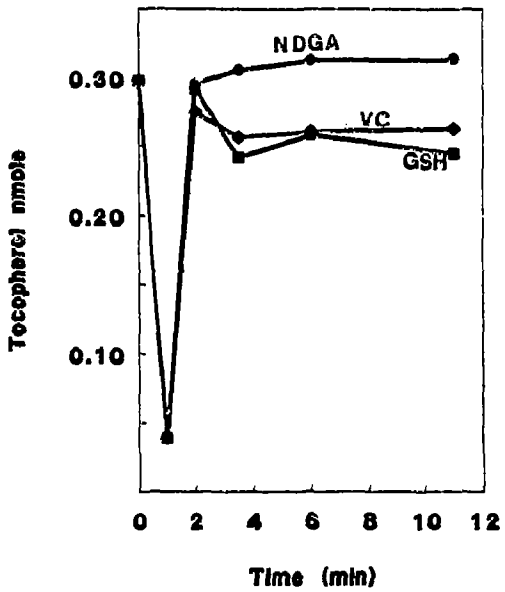

Fig. 2. Time-course of tocopherol regeneration by various reductants. Cell homogenates were incubated with hemoglobin (10 μM) for 1 min after which they were further incubated with NDGA (50 μM), vitamin C (2 mM) and GSH (2 mM) in the presence of desferrioxamine (100 μM) for indicated times. Values are from one of three representative experiments.

pherol regenerated was striking. Over 95% of the oxidized tocopherol reappeared within the first min after the addition of reductants. NDGA caused a sustained and higher tocopherol level which was maintained over the 10 min of regeneration time course. In contrast, after the initial rapid regeneration, the addition of either ascorbate or GSH showed a slight decline in tocopherol level at 2 min. However, these levels were generally maintained throughout the regeneration time course (Fig. 2).

The regeneration of tocopherol observed in Fig. 2 suggests that the mechanism of tocopherol regeneration

Table I

Effects of reductants on non-enzymic tocopherol regeneration in rat PMNL homogenates

Reductants	Tocopherol (nmol)	% of Tocopherol regenerated
None	0.154	0
NDGA (50 μM)	0.325	96
Vitamin (2 mM)	0.257	58
GSH (2 mM)	0.239	48

Cell homogenates (0.8 ml) containing 0.331 nmol of tocopherol were incubated with hemoglobin (10 μM) for 1 min at which time 1 ml of MeOH was added to terminate enzymic activities. This was followed by immediate addition of reductants in the presence of desferrioxamine (100 μM) and tocopherol regeneration was allowed to proceed for 5 min. % tocopherol regenerated was calculated from the amount regenerated over the amount oxidized. Values are means of duplicates from one of two representative experiments.

may be a chemical reaction. In order to test this hypothesis, we conducted experiments in which we induced tocopherol oxidation by incubating PMNL-homogenates with 10 μM of hemoglobin for 1 min at which times a mixture of chloroform and methanol was added to terminate any enzymic activities. This was followed by immediate addition of reductants in the presence of desferrioxamine and tocopherol regeneration was allowed to process for 5 min. Table I shows that under this enzyme-denaturing condition, 96% of the oxidized tocopherol was regenerated by the addition of NDGA whereas only 58 and 48% were recovered by the addition of ascorbate and GSH, respectively. These data support the hypothesis that tocopherol regeneration occurs via a chemical reaction. However, these results do not preclude the existence of an enzymic pathway because the amount of regeneration afforded by ascorbate and GSH in this protein-denaturing condition was lower than that observed in Fig. 2.

4. DISCUSSION

In PMNL, depletion of cellular tocopherol occurs when its 5-lipoxygenase activity is activated [25] or when cells come in direct contact with hemoglobin as shown in this study. While the activation of lipoxygenase and the release of hemoglobin through red blood cell hemolysis are well controlled in normal conditions, they are, however, not uncommon in certain disease states. Therefore, depletion of membrane reductants such as tocopherol is likely to occur during pathological conditions. Since the present study demonstrates that these soluble reductants can regenerate oxidized tocopherol in cell membranes, cellular defense against this form of oxidative stress is highly dependent on the presence of other cytosolic reductants such as ascorbate and the thiols.

The synergistic effect of ascorbate and tocopherol in retarding oxidative rancidity of dietary fats was noted half a century ago when Golumbic and Mattill reported that ascorbate acts to reduce the rate of tocopherol consumption [32]. Subsequently, Willson et al. [7] and Niki et al. [33] demonstrated that in organic solvents, tocopheroxyl radicals disappeared rapidly when ascorbic acid was added. These findings indicate that the reduction of oxidized tocopherol can occur in a chemical system. More recently, a number of studies from Packer's laboratory reported the successful generation of tocopheroxyl radicals in rat hepatic organelles incubated with a combination of soybean lipoxygenase and arachidonic acid [20,29–31]. These studies not only permit a detailed analysis of the ESR decay kinetics of tocopheroxyl radicals, they also provide an additional repair mechanism for oxidized tocopherol that is closely linked with the mitochondrial electron transport and extended the list of soluble reductants to include the pyridine nucleotides [20,30]. Collectively, these findings

lent strong support to the view that repairing mechanisms for oxidized tocopherol do exist in vivo.

Results from the present study clearly demonstrate that a recycling pathway for oxidized vitamin E exists in rat PMNL which utilizes physiologic soluble reductants such as ascorbate and GSH as electron donors. We also have demonstrated that this regeneration occurs predominantly via a chemical reaction since it proceeds in enzyme-denaturing conditions. This vitamin E repair mechanism may exist in most mammalian tissues in vivo. This finding illustrates the intricate dependency of membrane redox potential on cytosolic reductants and further reinforces the notion that, in estimating the need of vitamin E, other dietary factors such as ascorbate, GSH and unsaturated fatty acids should also be considered. Whether a tocopheroxyl radical reductase exists and the magnitude it may contribute to the overall regeneration of tocopherol in mammalian tissues remain to be determined.

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