Biochemical Pharmacology, Vol. 45, No. 7, pp. 1477–1482, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

IRON-INITIATED TISSUE OXIDATION: LIPID PEROXIDATION, VITAMIN E DESTRUCTION AND PROTEIN THIOL OXIDATION

INHIBITION BY A NOVEL ANTIOXIDANT, U-78517F

KELLY L. LINSEMAN, PHILIP LARSON, J. MARK BRAUGHLER and JOHN M. MCCALL*
The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

(*Received* 25 *September* 1992; *accepted* 2 *December* 1992)

Abstract—Oxidative injury was initiated by addition of ferrous ammonium sulfate (FAS) to a suspension of whole rat brain homogenate in Krebs buffer. After FAS addition, tissue vitamin E dropped sharply over a 30-sec interval and then recovered marginally for 5 min. After 5 min, vitamin E levels dropped to a low and constant level. Also after 5 min, TBARS (thiobarbituric acid reactive substances, a color test for lipid peroxidation) showed a statistically significant ($P \leq 0.05$) increase that continued through the remainder of the 30-min experiment. Reduced protein thiols decreased significantly ($P \leq 0.05$) at 15 min post FAS addition. This suggests that, in this model of iron-initiated lipid peroxidation (LP), the endogenous antioxidant vitamin E is first depleted before membrane lipids and membrane bound proteins show evidence of oxidative injury. A novel antioxidant, U-78517F, inhibited the destruction of vitamin E, LP and protein thiol oxidation in this model. The efficacy of the compound after different times of addition is described.

Reactive oxygen species including oxygen radicals are commonly produced in normal cell metabolism. However, in traumatic or ischemia/reperfusion injury, reactive oxygen overwhelms the normal cellular defense systems. The role of oxygen radicals in the pathophysiology of traumatic or ischemic damage to the central nervous system has been reviewed extensively [1, 2]. Free radical attack of polyunsaturated fatty acids in the cell membrane has been experimentally assessed by the formation of thiobarbituric acid reactive substances (TBARS)† [3–5]. Free radicals also attack cellular proteins and cause: (1) amino acid modification [6], (2) fragmentation [7], and (3) cross-linking [7]. Transition metals, like ferrous ion, have an integral role in the generation of reactive species that initiate oxidative damage via a Fenton-like reaction [8, 9]. In fact, metal ion-catalyzed oxidation of polyunsaturated fatty acids forms lipid alkoxyl radicals ($LO^{\cdot}$) and lipid peroxyl radicals ($LOO^{\cdot}$) [10] which further propagate lipid peroxidation (LP). Reactive oxygen species (e.g. hydroxyl radical and ferryl ion) can also form at metal binding sites on proteins and modify amino acid side chains [6].

Cells can normally defend themselves against oxidative damage by utilizing important endogenous antioxidants such as membrane bound vitamin E. Vitamin E can terminate lipid peroxidative chain reactions by scavenging lipid peroxyl and alkoxyl radicals, thus limiting LP chain reactions [10]. A link between decreasing vitamin E concentration, initiation of LP and protein oxidation has been implied by the work of Takenaka *et al.* [11]. This group looked at both an aqueous [2,2′-azobis(2-amidinopropane)dihydrochloride] and a lipid-soluble [2,2′-azobis(2,4-dimethylvaleronitrile) radical generating system and their respective effects on vitamin E depletion, initiation of lipid peroxidation and protein damage. They found that when radicals were initiated in a lipid region, thiol oxidation and TBARS formation were delayed until vitamin E fell below a critical level. The link between vitamin E concentration and further oxidative cell injury suggests that exogenous antioxidants that can augment and protect endogenous vitamin E may limit subsequent lipid and protein damage. We have developed compounds that are potent inhibitors of free radical-mediated LP [12]. The results reported here show that one representative compound, U-78517F [(−)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol, dihydrochloride], can block lipid peroxidation, preserve vitamin E, and prevent further oxidative damage to the membranes and proteins. This paper describes: (1) the relationship between vitamin E concentration, initiation of LP and protein oxidation caused by ferrous ion, and (2) the inhibition of LP by a novel antioxidant compound.

MATERIALS AND METHODS

Reagents. Ferrous ammonium sulfate (FAS), thiobarbituric acid, dithio-bis-nitrobenzoic acid, and Tris–HCl were purchased from the Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA)

* Corresponding author: John M. McCall, Ph.D., Discovery Research, 7245-267-3, The Upjohn Company, Kalamazoo, MI 49001. Tel. (616) 385-7632; FAX (616) 384-9308.

† Abbreviations: TBARS, thiobarbituric acid reactive substances; LP, lipid peroxidation; FAS, ferrous ammonium sulfate; and TCA, trichloroacetic acid.

was purchased from the Mallinckrodt Specialty Chemical Co. (Paris, KY).

Homogenate preparation, incubations, and TBARS measurement were as described elsewhere [4]. Time-course incubations for TBARS measurement were conducted in triplicate in individual assay tubes containing homogenate (20 μL) and Krebs buffer (q.s. to 100 μL). Samples were pre-warmed to 37° and reactions were started by the addition of FAS (10 μL, 200 μM final concentration). Samples (100 μL) for TBARS determination were removed and added to ice-cold TCA (12.5%)/desferal (5 mM) either before FAS addition or at the following time points after FAS addition: 0.5, 1, 2, 5, 10, 15, 20, 30, 45, and 60 min. Control tubes containing no iron were incubated for 60 min. Where indicated, the effects of U-78517F were investigated by adding the compound (10 μL, 3 μM final concentration) to incubations either prior to FAS addition, or at various times after FAS as indicated (0.5, 2, 5, 15 or 30 min after FAS). The procedure for measuring TBARS has been described [4].

Time–course incubations for vitamin E determination. Vitamin E was detected by a modified method of VonVoigtlander *et al.* [13]. Briefly individual assay tubes (in duplicate) containing homogenate (100 μL) and Krebs buffer (q.s. to 500 μL) were incubated with FAS (50 μL, 200 μM final concentration) in the presence or absence of U-78517F (50 μL, 3 μM final concentration) as above except that reactions were terminated by the addition of 500 μL of ethyl acetate. Samples were then transferred to 1.5 mL plastic Eppendorf conical tubes with 2–3 4-mm glass beads, capped, and placed on ice. Samples were vortexed and microcentrifuged at 15,900 *g* for 5 min; then 300 μL of the top layer were transferred to HPLC vials. Samples (20 μL) were injected onto a reverse phase column (Supelco C18, 250 × 4.6 mm; Waters) with a Perkin–Elmer ISS-100 autosampler. Mobile phase consisted of degassed 100% methanol. Vitamin E was detected via a Waters fluorescence detector at 285 nm excitation and 335 nm emission. Peaks were integrated with Waters Maxima 820 software.

Time–course incubations for SH measurement. Individual assay tubes (in duplicate) containing homogenate (80 μL) and Krebs buffer (q.s. to 400 μL) were incubated with FAS (40 μL, 200 μM final concentration) in the presence or absence of U-78517F (40 μL, 3 μM final concentration) as above. Detection of protein SH was determined by the method of Di Monte *et al.* [14].

Detection of U-78517F. Time–course assays were conducted as above with U-78517F added at the beginning of the assay before FAS addition and at 5, 15, or 30 min after FAS addition. U-78517F was extracted by adding 2 mL acetonitrile to samples that were supplemented with U-78218 [1-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-yl)carbonyl]-4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl-piperazine)] as an internal standard. The extracts were evaporated to dryness and redissolved in mobile phase (300 μL) consisting of 60% (v/v) acetonitrile, 40% (v/v) water containing 25 mM triethylamine and 1 mM EDTA adjusted to an apparent pH of 5.0 with glacial acetic acid.

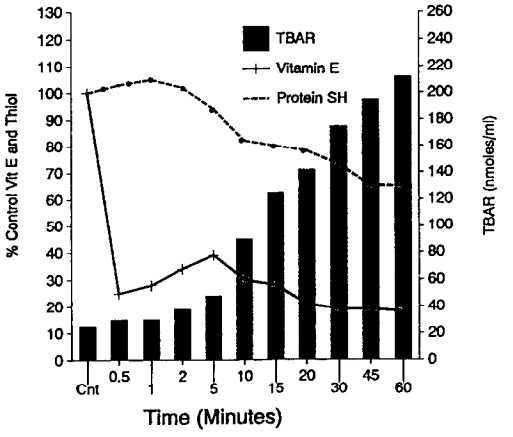

Fig. 1. Percent of control values for vitamin E and SH, and TBARS values from 60-min iron time–course studies in rat brain homogenate (N = 6). Assay conditions are described under Materials and Methods. Vitamin E and SH levels prior to the addition of FAS were 16 ng/mg tissue and 50 nmol/mg protein, respectively. Vitamin E concentration in FAS-treated samples was significant ($P \leq 0.05$) from control values at 30 sec, TBARS values were significant ($P \leq 0.05$) from control values from 5 min on, and SH levels were significant ($P \leq 0.05$) from 15 min on.

Samples were then chromatographed on a reverse phase column (Zorbax C8, 250 × 4.6 mm; Dupont) using an auto sampler for sample injection (150 μL) and a flow rate of 1.25 mL/min. The amperometric electrochemical response of the column effluent was monitored using a glassy carbon electrode surface with an applied potential of +400 mV versus the Ag/AgCl reference electrode. The concentration of U-78517F in unknown samples and standard curve statistics were calculated by using peak height measurements of U-78517F and U-78218 (internal) standards. U-78517F standards were prepared by spiking rat brain homogenate (500 μL) with various concentrations of compound.

The electrochemical properties of U-78517F were evaluated by cyclic voltametry in water: acetonitrile (30:70) with pH adjusted to 5.0 with triethylamine and glacial acetic acid. U-78517F demonstrated two independent peak oxidation potentials centered at +370 mV (the trolox ring) and +870 mV (the bis-pyrrolopyrimidine ring). Cyclic voltametric measurements were performed at room temperature with a glassy carbon working electrode, Ag/AgCl reference electrode and platinum auxiliary electrode.

Statistical analysis. Values represent the means ± range of duplicate (SH and vitamin E) samples or the means ± SEM of triplicate (TBARS) samples from representative experiments. All data were analyzed by Student's *t*-test, with $P \leq 0.05$, considered to be statistically significant.

RESULTS AND DISCUSSION

The addition of FAS resulted in a sharp decrease in the vitamin E concentration of brain homogenate

Table 1. Comparison of vitamin E levels vs U-78517F levels when U-78517F (92 ng/mg tissue) was added at different time points over a 60-min time–course incubation following the addition of FAS in rat brain homogenate

Incubation time (min)	Vitamin E, no U-78517F	U-78517F, 0 min		U-78517F, 5 min		U-78517F, 15 min	
		Vit E (ng/mg tissue)	78517F (ng/mg tissue)	Vit E (ng/mg tissue)	78517F (ng/mg tissue)	Vit E (ng/mg tissue)	78517F (ng/mg tissue)
0	24	22	85	22	—	28	—
0.5	5	10*	83	5	—	6	—
1	6	9*	83	6	—	8	—
2	7	11*	83	7	—	9	—
5	9	14*	83	13*	85	12	—
10	8	16*	83	17*	52	9	—
15	7	15*	83	18*	46	14*	92
20	6	15*	88	18*	43	16*	76
30	7	15*	76	17*	40	15*	70
45	6	17*	83	18*	43	16*	60
60	6	17*	88	18*	43	16*	60

* Iron + U-78517F-treated samples were significantly different from iron-treated samples at $P \leq 0.05$.

within 30 sec. This was followed by a transient recovery of vitamin E until 5 min, when vitamin E again decreased, but more slowly, to a constant basal level. No significant TBARS formation was observed until 5 min of incubation after which vitamin E concentrations began falling again despite the presence of some remaining vitamin E. After 5 min, TBARS increased continuously throughout the remaining incubation period. Protein thiol concentrations were preserved until 15 min after the introduction of FAS, then decreased gradually, but significantly with time (Fig. 1).

These results are consistent with those of Takenaka *et al.* [11] who reported similar observations using RBC ghost membranes from Wistar rats. The present data, along with that of Takenaka, suggest that initially vitamin E may be converted rapidly to its stable radical form following the addition of FAS to rat brain homogenate. Apparently vitamin E and its radical form can recycle to some extent between 0.5 and 5 min, perhaps until antioxidants like ascorbate and glutathione are consumed. The fact that TBARS formation did not increase significantly until vitamin E began to decline again at 5 min suggests that multiple mechanisms may interplay initially (between 0 and 5 min) to limit membrane lipid damage in part by maintaining critical levels of vitamin E in the membrane.

If an endogenous antioxidant inhibited the LP chain reaction or spared or recycled vitamin E, then the time course for LP and protein oxidation could be altered. U-78517F is an effective antioxidant with an oxidation potential (+400 mV) similar to that of vitamin E* [15]. The effects of U-78517F on vitamin E, TBARS and loss of protein sulfhydryls were examined by adding U-78517F at different time points during homogenate incubation. When compound was added before FAS or at later times up to 5 min after FAS addition, vitamin E was markedly preserved (Table 1) and returned to near control levels. Despite the presence of U-78517F at the beginning of these assays, vitamin E still dropped sharply upon addition of FAS, though not quite to the extent observed in the absence of U-78517F. These findings suggest that vitamin E is the first line of defense against an oxidant challenge even in the presence of an exogenous antioxidant like U-78517F. Despite the significant preservation of vitamin E and inhibition of LP by U-78517F, significant loss of U-78517F was not observed unless the compound was added at later times in the assay (e.g. 5 min and later). When added at 30 min after FAS, U-78517F was rapidly consumed in the short time between its addition and the first sampling (approximately 5 sec). Associated with the loss of U-78517F on its addition at 5 or 15 min was an increase in vitamin E levels. This was in marked contrast to what is typically seen with vitamin E in the absence of U-78517F where vitamin E continues to decrease after 5 min. The preservation and apparent restoration of vitamin E by U-78517F are not surprising in view of its structural similarity to vitamin E and its similar oxidation potential* [15, 16].

LP, as measured by TBARS, was inhibited almost completely when U-78517F was added at 0–5 min after FAS addition (Fig. 2). Furthermore, when U-78517F was added at later time points after FAS addition, such as 15 min, TBARS formation was essentially halted although some modest increase in TBARS was observed at long incubation times under these conditions (Fig. 2). The fact that U-78517F could halt LP when added to an ongoing reaction suggests termination of the LP chain reaction. Coincident with the halting of LP was a loss of U-78517F and a partial restoration of vitamin E (Table 1).

Loss of protein SH in the absence of U-78517F was gradual over time and became statistically significant after 15 min when vitamin E concentration began its second loss phase (Fig. 1). As seen for

* Padbury G, The Upjohn Company, personal communication, July 1992. Cited with permission.

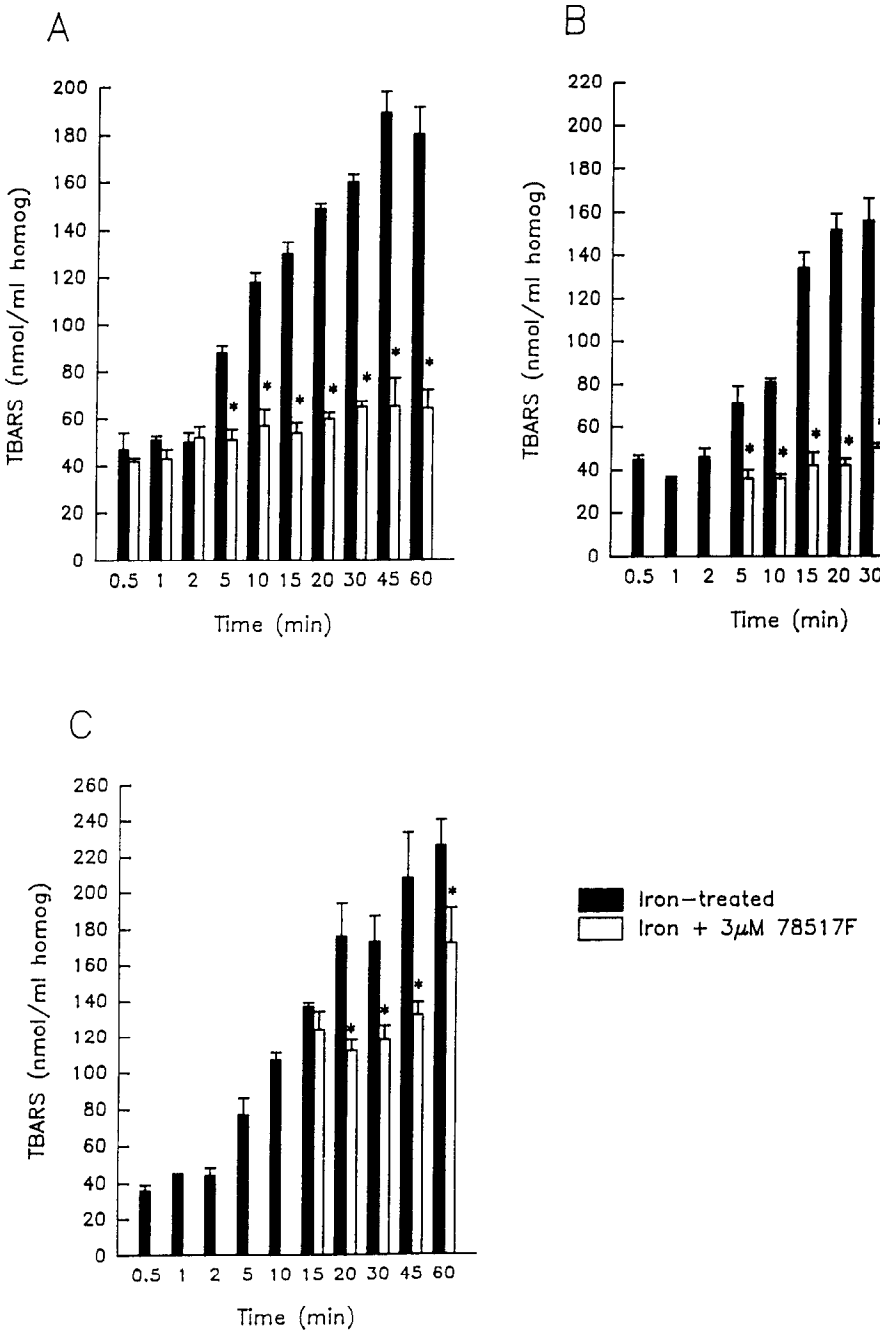

Fig. 2. TBARS during a 60-min time-course study in rat brain homogenate. (A) U-78517F was added before FAS, (B) U-78517F was added 5 min after addition of FAS, and (C) U-78517F was added 15 min after FAS. Values are means ± SEM of triplicate determinations from one experiment. Control samples were incubated for 60 min in the absence of FAS. Control samples incubated in the absence of FAS (not shown) contained: 35 ± 4 (A), 32 ± 2 (B), and 34 ± 5 (C) nmol TBARS/mL homogenate, respectively. Details of the assay are described in Materials and Methods. Key: (*) significantly different from iron-treated samples at $P \leq 0.05$.

TBARS formation, U-78517F completely inhibited protein oxidation (Fig. 3).

The results of this study are consistent with those reported by others [11], demonstrating that lipid peroxidation, vitamin E depletion and loss of protein thiol occur with a definite chronology after an oxidative insult. Vitamin E is the first membrane component that is rapidly depleted. However, the slight increase of vitamin E that was seen over the first 5 min after FAS addition may be explained by

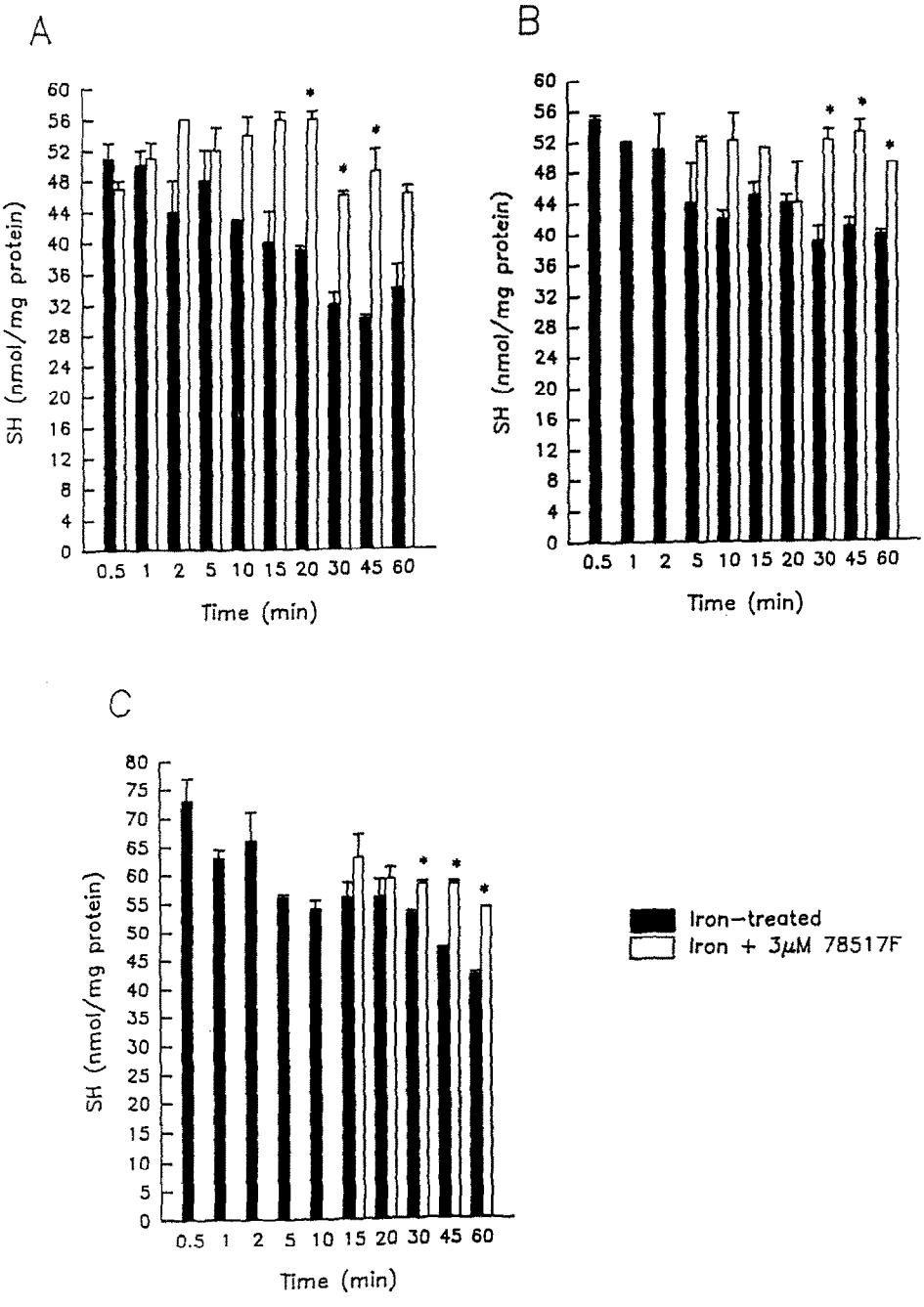

Fig. 3. Protein sulfhydryl content during a time-course study in rat brain homogenate. See legend of Fig. 2 for description of panels A–C. Values are means ± range of duplicate determinations from one experiment. Control samples (not shown) contained: 50 ± 0 (A), 50 ± 0.5 (B), and 69 ± 4 (C) nmol SH/mg protein, respectively. Key: (*) significantly different from iron-treated samples at $P \leq 0.05$.

vitamin E radical recycling through endogenous antioxidant stores such as glutathione, vitamin C, and other protein SH groups to regenerate reduced vitamin E. Furthermore, an exogenous antioxidant like U-78517F may assist either directly or indirectly in regeneration of endogenous vitamin E. Further studies of this recycling will be required to test this hypothesis. Logically, once the vitamin E radical can no longer be recycled and the equilibrium shifts in favor of the oxidized form, other cellular components like lipid and protein sulfhydryls become prime targets for oxidation. In such *in vitro* systems, antioxidants like U-78517F may be key to sparing vitamin E and protecting membrane components by

either directly inhibiting LP or perhaps by maintaining vitamin E at a critical level for preventing the propagation of LP.

Either mechanism may result in only minor consumption of the exogenous antioxidant present at the beginning of radical initiation since radical reaction chain lengths would be short and propagation reactions might be kept to a minimum. On the other hand, when antioxidants are added after free radical damage has had time to progress for some time and endogenous vitamin E has been depleted, significant consumption of the exogenous antioxidant may occur. Under these circumstances, radical chains are long and the concentrations of $LOO^{\cdot}$, $LO^{\cdot}$, and LOOH may be high.

REFERENCES

1. Braughler JM and Hall ED, Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radial formation and lipid peroxidation. *Free Radic Biol Med* **6**: 289–301, 1989.
2. Farber JL, Kyle ML and Coleman JB, Mechanisms of cell injury by activated oxygen species. *Lab Invest* **62**: 670–677, 1990.
3. Buege JA and Aust SD, Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302–310, 1978.
4. Braughler JM, Duncan LA and Chase RL, The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. *J Biol Chem* **261**: 10282–10289, 1986.
5. Valenzuela A, The biological significance of malondialdehyde determination in the assessment of tissue oxidative stress. *Life Sci* **48**: 301–309, 1991.
6. Stadtman ER, Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Rad Biol Med* **9**: 315–325, 1990.
7. Wolff SP, Garner A and Dean RT, Free radicals, lipids, and protein degradation. *Trends Biochem Sci* **11**: 27–31, 1986.
8. Aust SD, Morehouse LA and Thomas CE, Role of metals in oxygen radical reactions. *J Free Radic Biol Med* **1**: 3–25, 1985.
9. Wink DA, Nims RW, Desrasiers F, Ford PC and Keefer LF, A kinetic investigation of intermediates formed during the Fenton Reaction: Evidence for an oxidative pathway not involving hydroxyl radical. *Chem Res Toxicol* **4**: 510–512, 1991.
10. Maiorino M, Coassin M, Roveri A and Ursini F, Microsomal lipid peroxidation: Effect of vitamin E and its functional interaction with phospholipid hydroperoxide glutathione peroxidase. *Lipids* **24**: 721–726, 1989.
11. Takenaka Y, Miki M, Yasuda H and Mino M, The effect of α-tocopherol as an antioxidant on the oxidation of membrane protein thiols induced by free radicals generated in different sites. *Arch Biochem Biophys* **285**: 344–350, 1991.
12. Braughler JM, Chase RL and Pregenzer JF, Oxidation of ferrous iron during peroxidation of lipid substrates. *Biochim Biophys Acta* **921**: 457–464, 1987.
13. VonVoigtlander PF, Burian MA, Althaus JS and Williams LR, Effects of chronic haloperidol on vitamin E levels and monoamine metabolism in rats fed normal and vitamin E deficient diets. *Res Commun Chem Pathol Pharmacol* **68**: 343–352, 1990.
14. Di Monte D, Ross D, Bellomo G, Eklöw L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334–342, 1984.
15. Althaus JS, Travis MA, Hall ED and VonVoigtlander PF, Chronic two-fold vitamin E (VE) supplementation antagonizes MPTP-induced neurological and neurochemical deficits in cats. *Neurosci Abstr* **14**: 1217, 1988.
16. Hall ED, Pazara KE, Braughler JM, Linseman KL and Jacobsen EJ, Nonsteroidal lazaroid U-78517F in models of focal and global ischemia. *Stroke* **21**: III–83–III–87, 1990.