Biochemical Pharmacology, Vol. 45, No. 2, pp. 393–400, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INTERACTION OF THE PYRIDOINDOLE STOBADINE WITH PEROXYL, SUPEROXIDE AND CHROMANOXYL RADICALS

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(*Received* 21 *May* 1992; *accepted* 5 *October* 1992)

Abstract—The pyridoindole derivative stobadine [(−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9*b*-hexahydro-1*H*-pyrido(4,3*b*)indole] has been described as a drug with antihypoxic and antiarrhythmic cardioprotective properties. Here its reactivity with peroxyl radicals in liposomes using a lipid-soluble azo-initiator of peroxyl radicals, 2,2′-azo-bis(2,4-dimethyl-valeronitrile) (AMVN), was examined. Stobadine exerted scavenging as evidenced by the inhibition of: (i) *cis*-parinaric acid fluorescence decay (half-maximal effect at 20 μM), or (ii) luminol-sensitized chemiluminescence (half-maximal effect at 33 μM). In rat liver microsomes, stobadine was equally efficient in inhibiting lipid peroxidation induced by lipid-soluble (AMVN) or water-soluble 2,2′-azo-bis(2-aminopropane)-HCl (AAPH), azo-initiators of peroxyl radicals with half-maximal effect at 17 μM. Stobadine partitions in a two-phase system (octanol–water) with the coefficient log P = 0.57 ± 0.03, explaining its ability to quench peroxyl radicals in both lipid and aqueous phases. Stobadine is not an efficient scavenger of superoxide radicals. The second order rate constant for the reaction of stobadine with superoxide was estimated to be $7.5 \times 10^2\ M^{-1}\ sec^{-1}$ as measured by superoxide-induced lucigenin-amplified chemiluminescence. ESR measurements showed that stobadine in liposomes does not reduce the chromanoxyl radical of a vitamin E homologue with a 6-carbon side-chain, 2,5,7,8-tetramethyl-2-(4′-methylpentyl)chroman-6-ol (chromanol-α-C6), in agreement with pulse-radiolysis results obtained using Trolox in homogeneous solution (Steenken *et al.*, *Chem Res Toxicol* **5**: 355–360, 1992). Stobadine increased the magnitude of the chromanoxyl and ascorbyl radical ESR signal generated by lipoxygenase + arachidonate. This was interpreted to be due to the interaction of stobadinyl radicals with the chromanol ring and ascorbate, respectively. It is suggested that high reactivity of stobadine radicals requires the presence of reducing antioxidants (vitamin E, vitamin C) to exhibit its antioxidant effects in physiological systems.

A pyridoindole derivative, stobadine (Fig. 1), recently has attracted interest regarding its antioxidant properties and potential pharmacological use [1, 2]. Pulse-radiolysis studies showed that stobadine efficiently interacts with $C_6H_5^{\cdot}$, $CCl_3O_2^{\cdot}$, $Br^{\cdot}$ and $HO^{\cdot}$ radicals with reaction rate constants of 10^8 to $10^{10}\ M^{-1}\ sec^{-1}$ undergoing one-electron oxidation to the radical cation [3]. No data on the reactivity of stobadine with lipid peroxyl radicals and superoxide radicals are available yet.

The radical cation of stobadine reacts with Trolox C with a rate constant of $1.2 \times 10^7\ M^{-1}\ sec^{-1}$ yielding the phenoxyl-type radical of Trolox [3]. On the other hand, in experiments with biological membranes, i.e. rat liver microsomes, the inhibition of lipid peroxidation by stobadine was not observed in tocopherol-deficient microsomes whereas it was found in tocopherol-sufficient control microsomes [4]. This suggests that the antioxidant effect of stobadine depends on the presence of tocopherol in the membrane. In these experiments the process of membrane lipid peroxidation was initiated with iron-complexes and NADPH or ascorbate as reductants, opening the possibility that stobadine might also interfere with the initiating reagents.

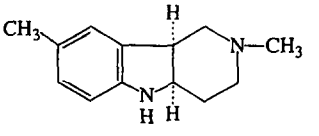

Fig. 1. Structural formula of stobadine.

The goal of the present work, therefore, was to study the antioxidant interactions of stobadine in peroxidation systems independent of iron/reductant initiators. We employed peroxyl radicals generated by thermal decomposition of azo-initiators, 2,2′-azo-bis(2,4-dimethyl-valeronitrile (AMVN)‖ or 2,2′-azo-bis(2-aminopropane)-HCl (AAPH), at a constant

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‖ Abbreviations: AMVN, 2,2′-azo-bis(2,4-dimethyl-valeronitrile); AAPH, 2,2′-azo-bis(2-aminopropane)-HCl; chromanol-α-C6, 2,5,7,8-tetramethyl-2-(4′-menthylpentyl)chroman-6-ol; DOPC, dioleoylphosphatidylcholine; SOD, superoxide dismutase; NBT, nitro-blue tetrazolium; and TBARS, thiobarbituric acid-reactive substances.

rate [5, 6] in dioleoylphosphatidylcholine (DOPC) liposomes or in rat liver microsomes. In liposomes the process was monitored by oxidation of: (i) *cis*-parinaric acid, followed by its fluorescence decay [7], or (ii) luminol, followed by its chemiluminescence [8]. AMVN- or AAPH-induced accumulation of lipid peroxidation products (TBARS) was assayed in microsomes.

The interaction of stobadine with phenoxyl (chromanoxyl) radicals was monitored by ESR spectroscopy using an α-tocopherol homologue with a shorter 6-carbon side chain (chromanol-α-C6) in dioleoylphosphatidylcholine liposomes [9].

MATERIALS AND METHODS

Reagents. Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9*b*-hexahydro-1*H*-pyrido(4,3*b*)indole was provided by Dr. L. Horakova (Institute of Experimental Pharmacology, Slovak academy of Sciences, Bratislava, CSFR). Arachidonic acid, soybean 15-lipoxygenase (101,000 U/mg protein), ascorbate, luminol, 2-thiobarbituric acid, and Tris–HCl were from the Sigma Chemical Co. (St. Louis, MO); and AMVN and AAPH from PolySciences, Inc. (Warrington, PA). HPLC grade ethanol was from Fischer Scientific (Fair Lawn, NJ), and *cis*-parinaric acid from Molecular Probes Inc. (Eugene, OR). The α-tocopherol homologue with a 6-carbon side chain, 2,5,7,8-tetramethyl-2-(4′-methylpentyl)-chroman-6-ol (chromanol-α-C6) was a gift from Prof. R. P. Evstigneeva (Institute for Fine Chemical Technology, Moscow, Russia).

Partition coefficient determination. The partition coefficient for stobadine was determined in the octanol–phosphate buffer (pH 7.4) system [10]. Stobadine (final concentration of 30 μM) was dissolved in a 1:1 (v/v) octanol:phosphate buffer mixture (6 mL), vortexed for 2 hr at 25°, and centrifuged at 3000 *g* for 30 min. Concentrations in both phases were determined at 239 nm on a Shimadzu UV 160U spectrophotometer. Preliminary measurements of concentration dependencies for stobadine absorbance in the buffer and in octanol were taken to accurately perform these determinations. Using these standard curves the stobadine concentration in each phase was calculated. The partition coefficient was calculated as $P = C_{org}/C_{aq}$.

Preparation of liposomes. DOPC liposomes containing stobadine were made by sonication of DOPC dispersions in 20 mM Tris–HCl buffer (pH 7.4 at 40 or 45°) with stobadine under nitrogen.

Fluorescence-based assay for peroxyl radical scavenging activity in the membrane. Peroxyl radicals were generated at a constant rate ($3.2 \times 10^{-6}\ M^{-1}\ sec^{-1}$) by thermal decomposition of the lipid-soluble azo-initiator AMVN according to a method described earlier [6, 7]. DOPC liposomes (800 μM) with incorporated *cis*-parinaric acid (80 μM) were sonicated for 1 min after addition of AMVN (330 μM) to provide for better incorporation and more uniform distribution of the azo-initiator in the lipid bilayer. The oxidation of *cis*-parinaric acid in DOPC liposomes by peroxyl radicals thus generated was monitored by detecting a decay of fluorescence ($\lambda_{ex} = 328$ nm, $\lambda_{em} = 415$ nm) at 45° with Perkin–Elmer MPF-44A spectrofluorimeter [11]. The change in the fluorescence decay rate by the addition of stobadine is a measure of scavenging activity.

Chemiluminescence assay for peroxyl radical scavenging activity in the membrane. The scavenging effect of stobadine on AMVN-generated peroxyl radicals in DOPC liposomes was measured by monitoring the luminol-amplified chemiluminescence in the presence of various concentrations of stobadine with Pharmacia-LKB Wallac 1250 luminometer at 40° [9]. The AMVN-induced chemiluminescence response was not observed in the absence of DOPC liposomes indicating that interaction of AMVN-derived radicals with luminol occurred in DOPC membranes.

Chemiluminescence assay for superoxide radical scavenging activity. The apparent oxidation rate constant of stobadine with superoxide was determined from the competition between stobadine and lucigenin for superoxide. The rate constant of lucigenin with superoxide was estimated from the competition between lucigenin and superoxide dismutase (SOD) for superoxide [12]. Since the catalytic reaction of SOD is first order with respect to both the enzyme and superoxide [13, 14], this procedure is applicable and commonly used [15–18]. In separate experiments we found that the second order rate constant for the reaction between cytochrome *c* and superoxide measured using the above described procedure was $4.3 \times 10^5\ M^{-1}\ sec^{-1}$. This is in fair agreement with the values for this rate constant reported in the literature (in the range from 1 to $6 \times 10^5\ M^{-1}\ sec^{-1}$) [19]. The reaction of lucigenin with superoxide was monitored by superoxide-induced lucigenin-amplified chemiluminescence in 20 mM Tris–HCl buffer (pH 7.8) containing 40 μM lucigenin, 20 μM diethylenetriaminepentaacetic acid (DETAPAC), 4 mM xanthine, and 20 mU/mL xanthine oxidase at 25° using a Pharmacia-LKB Wallac 1250 chemiluminometer. SOD concentration was confirmed by the inhibition of superoxide-induced nitro-blue tetrazolium (NBT) reduction using the rate constant values for SOD ($2.3 \times 10^9\ M^{-1}\ sec^{-1}$) [16] and NBT ($5.9 \times 10^4\ M^{-1}\ sec^{-1}$) [20] for superoxide in 20 mM Tris–HCl buffer (pH 7.8) containing 10 μM NBT, 20 μM xanthine, 40 mU/mL xanthine oxidase at 25° (Shimadzu UV 160U spectrophotometer). NBT and lucigenin concentrations were determined by their absorbance at 257 and 369 nm with extinction coefficients for NBT of $61{,}000\ M^{-1}\ cm^{-1}$, [20] and for lucigenin, $37{,}300\ M^{-1}\ cm^{-1}$ [21], respectively.

Xanthine oxidase activity was determined by the formation of uric acid from xanthine followed at 295 nm in 20 mM Tris–HCl buffer (pH 7.8) containing 20 μM xanthine and 40 mU/mL xanthine oxidase at 25° (Shimadzu UV 160U spectrophotometer) [22].

Isolation of microsomal membranes. The livers of Sprague–Dawley female rats (120–150 g) were perfused with ice-cold 1.15% KCl and homogenized in a Potter homogenizer. Microsomal fractions were obtained by a differential centrifugation: 10 min 10,000 *g* followed by centrifugation of the supernatant at 105,000 *g* for 60 min.

Lipid peroxidation determination. Accumulation of lipid peroxidation products reacting with 2-

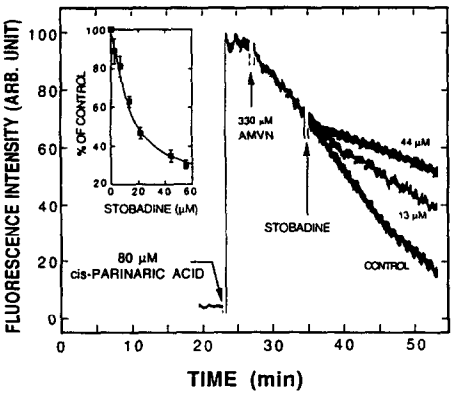

Fig. 2. Protection by stobadine against loss of *cis*-parinaric acid fluorescence induced by AMVN in DOPC liposomes. The reaction mixture contained 80 μM *cis*-parinaric acid, 330 μM AMVN and 800 μM DOPC in 20 mM Tris–HCl buffer (pH 7.4) at 45°. Fluorescence intensity was monitored at 328 nm excitation and 415 nm emission with a 5 and 10 nm slit, respectively. Inset: Dependence of *cis*-parinaric acid fluorescence decay inhibition on stobadine concentration. Fluorescence intensity in the absence of stobadine = 100%. Values are means ± SD, N = 4.

thiobarbituric acid (TBARS) was measured spectrophotometrically by the absorbance at 535 nm, as described by Buege and Aust [23]. Lipid peroxidation was induced in rat liver microsomes by AMVN or AAPH (10 mM) at 40° in the incubation medium containing 1 mg/mL protein in 0.1 M phosphate buffer (pH 7.4 at 40°). Stobadine was added to the incubation medium in ethanolic solutions and was preincubated with the microsomal suspension for 5 min. Incubation time was 40 min. Protein concentration was measured by the Bio-Rad Protein Assay kit.

Generation of chromanoxyl radicals. Chromanoxyl radicals from an α-tocopherol homologue with a 6-carbon side-chain, chromanol-α-C6, were generated using: (1) UV-irradiation, or (2) an enzymic oxidation system (soybean 15-lipoxygenase + arachidonate). When the enzymic oxidation system was used, the incubation medium (60 μL) contained liposomal suspension (20 mg/mL DOPC) in 0.1 M phosphate buffer, pH 7.4, at 37°. Arachidonate (2.0 mM) and lipoxygenase (3 U/μL) were added subsequently to the liposomal suspension.

ESR spectroscopy. ESR measurements were made on a Varian E-109E spectrometer at room temperature, in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan, NJ). The permeable tube (approximately 8 cm in length) was filled with 60 μL of the mixed sample, folded into quarters, and placed in an open 3.0-mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. The sample was flushed with oxygen. Spectra were recorded at 100 mW power and 2.5 G modulation at 100 KHz, and 25 G/min scan time. Spectra were recorded at room temperature, under aerobic conditions by flowing oxygen through the ESR cavity.

HPLC measurements of chromanol-α-C6 content. Chromanol-α-C6 content was assayed by reverse phase HPLC using a C-18 column (Waters, Inc.) with an in-line electrochemical detector. The eluent was 1:9 methanol:ethanol (v/v), 20 mM lithium perchlorate. Chromanol-α-C6 was extracted into hexane from sodium dodecyl sulfate-treated samples as described earlier [24]. Incubation conditions: DOPC liposomes (20 mg/mL) in 0.1 M phosphate buffer, pH 7.4 at 37°, lipoxygenase (3 /mL), arachidonic acid (2.0 mM), stobadine (0.4 mM) and chromanol-α-C6 (3.2 mM).

Irradiation. Irradiation was by a solar simulator (Solar Light Co., Philadelphia, PA, model 14S), whose output closely matches the solar spectrum in the wavelengths 290–400 nm. The samples were illuminated directly in the ESR resonator cavity; the distance between the light source and the sample was 30 cm. The intensity of the light at the sample surface in the spectral region 310–400 nm was 1.5 mW/cm^2, and it was 10% of this value at 290 nm.

RESULTS

Partitioning of stobadine in a two-phase system. To evaluate the ability of stobadine to partition into membranes from the aqueous phase, we measured the partition coefficient in a two-phase octanol–water system [10]. Our results showed that stobadine partitions efficiently into the hydrophobic phase with log P = 0.57 ± 0.03.

Azo-initiator-generated peroxyl radicals. Fluorescence of *cis*-parinaric acid decayed following the addition of AMVN (Fig. 2), in accordance with previous observations [7]. The addition of stobadine caused a protection, indicated by a decreased slope in the fluorescence trace. The half-maximal stobadine concentration for this inhibitory effect was 20 μM. The effect was compared to that of other antioxidants, as shown in Table 1. At a 50 μM concentration, the protection afforded by stobadine was similar to that reached by butylated hydroxytoluene (BHT), whereas that of other lipid-soluble antioxidants such as α-tocopherol or ubiquinol Q_{10} was less.

With luminol as a reporting molecule, a qualitatively similar pattern was observed. The photoemission resulting from luminol was depressed

Table 1. Effects of antioxidants on AMVN-induced *cis*-parinaric acid fluorescence decay

Antioxidant	v/V at 50 μM*	Relative efficiency
Stobadine	0.30 ± 0.025	1.00
BHT	0.30 ± 0.032	1.01
α-Tocopherol	0.42 ± 0.038	0.73
Q_{10} (reduced form)	0.54 ± 0.016	0.56

* V: fluorescence decay rate in the absence of antioxidant; v: fluorescence decay rate in the presence of antioxidant. Values are means ± SD, N = 5.

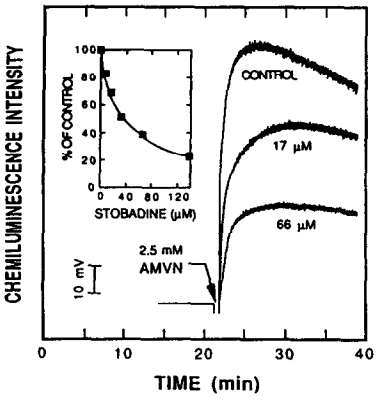

Fig. 3. Effect of stobadine on AMVN-induced luminol-amplified chemiluminescence in DOPC liposomes. The reaction mixture contained 150 μM luminol, 2.5 mM AMVN and 2.5 mM DOPC in 20 mM Tris–HCl buffer (pH 7.4) at 40°. Inset: Dependence of the chemiluminescence inhibition on stobadine concentration. Chemiluminescence intensity in the absence of stobadine = 100%.

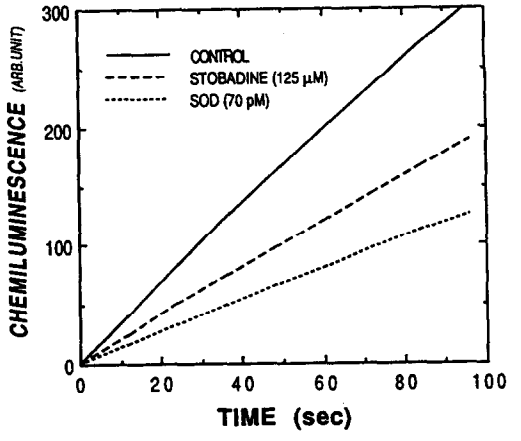

Fig. 5. Effects of stobadine and SOD on the superoxide-induced lucigenin-amplified chemiluminescence. The reaction mixture contained 40 μM lucigenin, 20 μM DETAPAC, 4 mM xanthine, and 20 mU/mL xanthine oxidase in 20 mM Tris–HCl buffer (pH 7.8) at 25°. Chemiluminescence is shown as integrated photoemission per second.

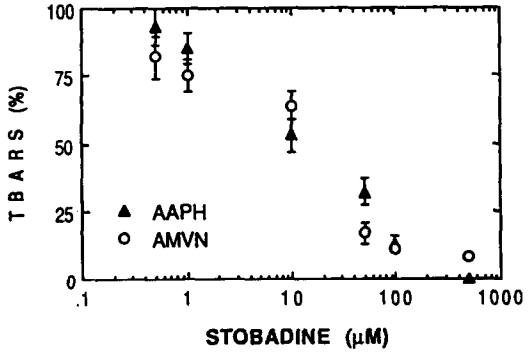

Fig. 4. Inhibition of TBARS accumulation by stobadine in rat liver microsomes challenged by AMVN and AAPH. Incubation conditions: microsomes (1 mg protein/mL) in 0.1 M phosphate buffer (pH 7.4 at 40°), AMVN or AAPH (10 mM). The rates of AMVN- and AAPH-induced lipid peroxidation were 5.7 ± 0.5 and 7.6 ± 0.6 nmol MDA/mg protein/20 min, respectively. Values are means ± SD, N = 3.

in the presence of stobadine (Fig. 3). The half-maximal inhibitory effect was produced by 33 μM stobadine.

The antioxidant effect of stobadine in rat liver microsomes was demonstrated using AAPH or AMVN as peroxyl radical generators. The rates of radical generation at 40° by these two azo-initiators are known to be very close: 1.36×10^{-6} [AAPH] M/sec [25] and 3.2×10^{-6} [AMVN] M/sec [11], respectively. We found that the rates of AMVN- and AAPH-induced lipid peroxidation in microsomes were 5.7 ± 0.5 and 7.6 ± 0.6 nmol MDA/mg protein/20 min, respectively. The accumulation of thiobarbituric acid-reactive substances (TBARS) was inhibited in the presence of stobadine, the effect being half-maximal at 17 μM with both AAPH and AMVN (Fig. 4).

Superoxide radicals. Stobadine did not have any significant effect on the xanthine oxidase catalyzed formation of uric acid from xanthine, suggesting that no inhibition of the enzyme occurred. Lucigenin-amplified chemiluminescence in the xanthine–xanthine oxidase system was inhibited completely by the addition of 1.0 nM SOD, indicating that the chemiluminescence response was due solely to the superoxide production. The second order rate constant for the reaction of lucigenin with superoxide was estimated to be $3.5 \times 10^5\ M^{-1}\,sec^{-1}$. This was determined by the lucigenin chemiluminescence inhibition by SOD (Fig. 5) using the known kinetic constant for the reaction of SOD with superoxide, $2.3 \times 10^9\ M^{-1}\,sec^{-1}$ [16]. Based on the measured competition between lucigenin and stobadine for superoxide in the same system and on the above calculated rate constant for the reaction of lucigenin with superoxide, the second order rate constant for the reaction of stobadine with superoxide was estimated to be $7.5 \times 10^2\ M^{-1}\,sec^{-1}$.

Chromanoxyl radicals. When generation of chromanoxyl radicals from chromanol-α-C6 in DOPC liposomes was performed by UV-irradiation [26], addition of stobadine did not elicit changes in the level of the chromanoxyl radical ESR signal, identified by the characteristic 7-line spectrum (Fig. 6B). This indicates that stobadine is not capable of reducing the tocopheroxyl radical, in agreement with the pulse-radiolysis data obtained with Trolox [3].

In the presence of both chromanol-α-C6 and ascorbate, the typical ESR spectrum of the chromanoxyl radical was not observed. Instead a characteristic ESR signal of ascorbyl radical was

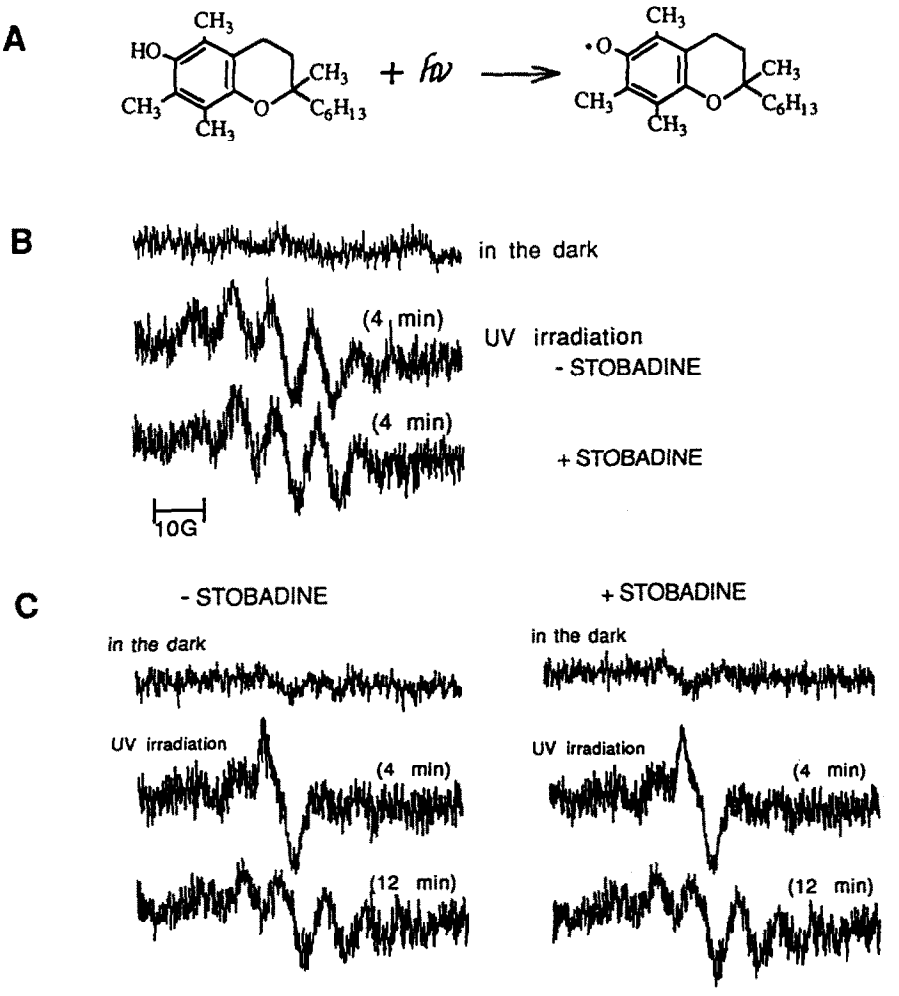

Fig. 6. ESR spectra of UV-induced chromanoxyl radicals generated from chromanol-α-C6: Effects of ascorbate and/or stobadine. (A) Scheme of UV-induced generation of the chromanoxyl radical from chromanol-α-C6. (B) Chromanol-α-C6 ± stobadine. (C) (Chromanol-α-C6 + ascorbate) ± stobadine. Incubation conditions: DOPC liposomes (20 mg/mL) in 0.1 M phosphate buffer, chromanol-α-C6 (20 mM), stobadine (0.4 mM), ascorbate (15 μM). For other details, see Materials and Methods.

recorded (Fig. 6C). This signal, however, was transient and was then followed by the appearance of the chromanoxyl radical signal. The magnitude and time course of the ascorbyl radical signal and the chromanoxyl radical signal were not changed when stobadine was present in the incubation medium. This suggests that stobadine did not affect the ascorbate-driven recycling of chromanol-α-C6.

In further attempts to elucidate the interaction of stobadine with peroxyl and chromanoxyl radicals, peroxyl radicals of arachidonic acid produced by its lipoxygenase-catalyzed oxidation were used to generate chromanoxyl radicals from chromanol-α-C6. Arachidonate + lipoxygenase was not able to generate steady-state concentrations of a stobadine radical high enough to be detected by ESR under the conditions used (Fig. 7B). When chromanol-α-C6 or ascorbate was added to the arachidonate + lipoxygenase oxidation system, the chromanoxyl radical or ascorbyl radical ESR signals were observed, respectively. The addition of stobadine increased rather than decreased these characteristic ESR signals. This suggests that stobadine does not reduce these radicals, but rather that the steady-state concentration of radicals generated via lipoxygenase-catalyzed oxidation is augmented by the addition of stobadine. This may be interpreted as indicating the generation of stobadinyl radicals by arachidonate + lipoxygenase. Generation of ESR-detectable stobadinyl radicals was shown earlier in a chemical oxidation system [27]. Our HPLC measurements demonstrated that stobadine enhanced chromanol-α-C6 consumption by lipoxygenase + arachidonate: during a 10-min incubation, 18% of the initial chromanol-α-C6 was oxidized by lipoxygenase + arachidonate in the absence of stobadine and 41% when stobadine was added to the incubation system.

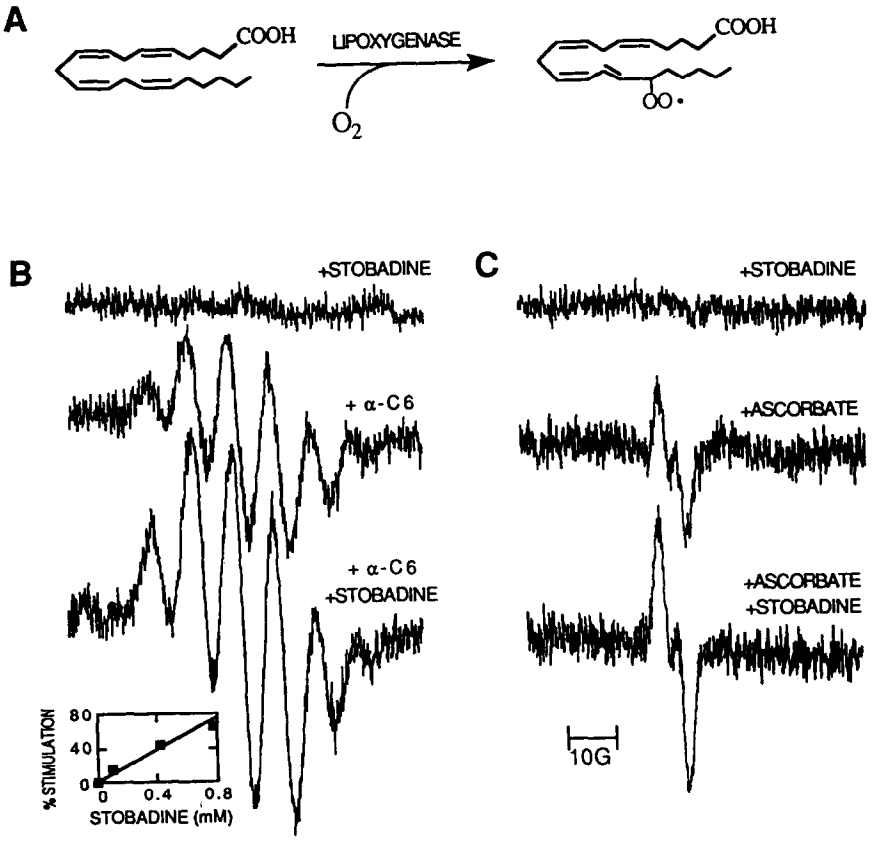

Fig. 7. Effect of stobadine on lipoxygenase + arachidonate-induced ESR signals of chromanoxyl or ascorbyl radicals. (A) Scheme of arachidonate peroxyl radical generation by lipoxygenase. The peroxyl radical generated from linoleate was employed. (B) ESR spectra recorded in the presence of stobadine, chromanol-α-C6, or stobadine + chromanol-α-C6. Inset: Effect of different stobadine concentrations. (C) ESR spectra recorded in the presence of stobadine, ascorbate, or stobadine + ascorbate. Incubation conditions: DOPC liposomes (20 mg/mL) in 0.1 M phosphate buffer, chromanol-α-C6 (20 mM), stobadine (0.4 mM), ascorbate (600 μM). For other details, see Materials and Methods.

DISCUSSION

Interaction with peroxyl radicals. Earlier studies have shown that stobadine acted as an efficient antioxidant in liposomes and in different membranes (heart and brain mitochondria, cerebral synaptosomes and microsomes) [28]. Antioxidant effects of stobadine in preventing lipid peroxidation and/or thiol oxidation were also found in the brain cortex under conditions of ischemia–reperfusion [29] and in the heart subjected to isoprenaline-induced oxidative stress [30]. These antioxidant effects of stobadine may be due to its ability to: (i) scavenge peroxyl radicals, (ii) chelate transition metals (iron), involved in the peroxidation initiation, and (iii) interact with reductants (e.g. ascorbate) necessary for maintaining transition metals in the reduced state.

As mentioned above, it was demonstrated recently that stobadine can donate electrons to $C_6H_5^{\cdot}$, $CCl_3O_2^{\cdot}$, $Br^{\cdot}$ and $HO^{\cdot}$ radicals [3]. However, the efficiency of the stobadine interaction with lipid peroxyl radicals was not addressed thus far. Our results with AMVN-derived peroxyl radicals in the systems where neither transition metals nor reductants were involved demonstrate that stobadine is an efficient scavenger of peroxyl radicals. The high efficiency of the peroxyl radical scavenging by stobadine (in micromolar concentrations) was found to occur in liposomal membranes both for a highly hydrophobic oxidation substrate (*cis*-parinaric acid) and for a less lipophilic compound, luminol. The efficiency of stobadine in inhibiting microsomal lipid peroxidation was similar with both water-soluble (AAPH) or lipid-soluble (AMVN) azo-initiators of peroxyl radicals. This suggests that stobadine acts as an efficient peroxyl radical scavenger predominantly in the lipid phase of microsomal membranes. The concentrations of stobadine which produced half-maximal inhibition of AMVN- or AAPH-induced lipid peroxidation in liver microsomes were comparable to those when iron/NADPH or iron/ascorbate was used as an inducer of microsomal lipid peroxidation [4]. This may indicate that peroxyl radical scavenging was the major contributor to the overall antioxidant activity of stobadine in these different lipid peroxidation systems.

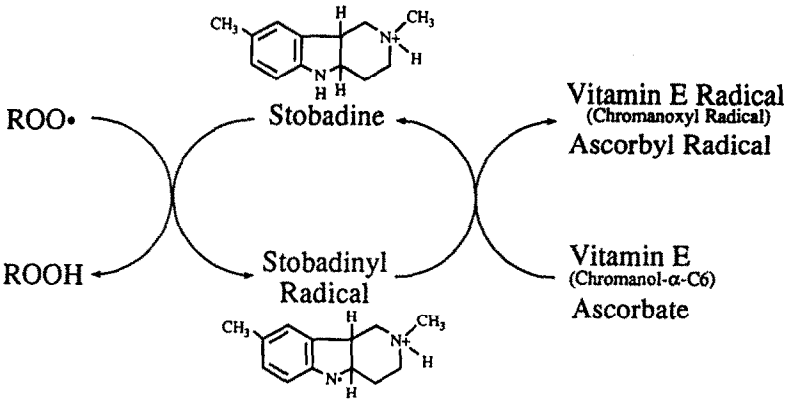

Fig. 8. Scheme of antioxidant interactions of stobadine. Possible reactions of stobadine with peroxyl radicals and of stobadinyl radical with vitamin E (chromanol-α-C6) or ascorbate.

Interaction with superoxide radicals. Our data suggest that stobadine is not an efficient scavenger of superoxide radicals. The second order rate constant for the reaction of stobadine with superoxide ($7.5 \times 10^2\ M^{-1}\ sec^{-1}$) is significantly lower than the reactivity of the vitamin E water-soluble homologue, Trolox ($1.7 \times 10^4\ M^{-1}\ sec^{-1}$) [15] or ascorbate ($2.7 \times 10^5\ M^{-1}\ sec^{-1}$) [18]. This may be due, at least in part, to its limited solubility in the aqueous buffer. In accord with our measurements of this, stobadine partitioning between octanol and water showed that it is localized predominantly in a hydrophobic membranous phase (about 75%) rather than in the aqueous phase (about 25%).

Interaction with chromanoxyl radicals. It was observed [3] that the stobadine antioxidant effect was dependent on vitamin E in membranes. Thus, we tested the hypothesis that stobadine may act as a vitamin E recycler by reducing chromanoxyl radicals. Our ESR data directly demonstrate that stobadine does not reduce chromanoxyl radicals of the vitamin E homologue, chromanol-α-C6, in DOPC liposomes either by itself or in the presence of ascorbate. This finding is in agreement with the report by Steenken *et al.* [3] that stobadine has a more positive redox potential ($E_{7.0} = 0.58$ V) than tocopherol ($E_{7.0} = 0.48$ V) and ascorbate ($E_{7.0} = 0.30$ V). Moreover, our results give an indirect indication for a high reactivity of stobadine radicals as evidenced by the increased level of chromanoxyl or ascorbyl radical generation by arachidonate + lipoxygenase in the presence of stobadine. We did not detect the stobadinyl radical signal in the ESR spectra obtained in liposomal or microsomal suspensions. However, a resolved ESR signal of stobadinyl radical was observed during stobadine oxidation by PbO_2-*tert*-butyl hydroperoxide in benzene [27]. Although stobadine may be very efficient in scavenging peroxyl radicals, its radicals thus formed may be highly reactive [27] and readily interact with other substrates. This suggestion is supported by our data showing that stobadine enhanced consumption of chromanol-α-C6 in the course of lipoxygenase + arachidonate oxidation. High reactivity of stobadine radicals towards unsaturated lipids may cause promotion of lipid oxidation by stobadine radicals. This explains the requirement in vitamin E for efficient inhibition of lipid peroxidation by stobadine in liver microsomes reported earlier [4]. Thus, the antioxidant potency of stobadine may be increased by its interaction with other lipid- or water-soluble antioxidants with more negative redox-potentials (e.g. vitamin E and vitamin C) (Fig. 8).

Acknowledgements—This work was supported by NIH (CA-47597). H. S. gratefully acknowledges a Visiting Miller Research Professorship at UC Berkeley.

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