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Effect of Stobadine on Cu^{++}-Mediated Oxidation of Low-Density Lipoprotein

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ABSTRACT. The pyridoindole derivative stobadine [(−)-cis-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido(4,3b) indole] has been described as a drug with antihypoxic and antiarrhythmic cardioprotective properties. The antioxidative properties of this compound were studied during Cu^{++}-mediated low-density lipoprotein (LDL) oxidation. Stobadine (concentration 0–5 μM) prolonged the lag phase (in min produced by one molecule antioxidant per LDL particle) as measured by diene formation more effectively than did ascorbate, trolox, or alpha-tocopherol. It also has the ability to decrease the rate of diene formation during the propagation phase very efficiently. Diene formation, Trp destruction, and alpha-tocopherol consumption were measured in the presence and absence of stobadine. Stobadine (10 μM) did not influence tocopherol consumption during oxidation and the Trp fluorescence quenching of Cu^{++} was not influenced by this compound. From these results, as well as polarographic measurements, we conclude that the antioxidative effect of stobadine is not simply a result of Cu^{++}-ion complexation. In contrast to ascorbate, this compound is stable in the presence of Cu^{++}. Stobadine inhibits the oxidation of LDL-Trp residues very efficiently *via* its radical scavenging properties, and may even have the ability to reduce Trp radicals to tryptophan. The concentration of stobadine used for LDL oxidation was in the range found in plasma (stobadine given p.o. in human and rats results in plasma concentrations between 0.2–3.9 μM). BIOCHEM PHARMACOL 51;10:1277–1282, 1996.

KEY WORDS. LDL; stobadine; lipid peroxidation; tryptophan; antioxidant

Stobadine, a pyridoindol derivative, has been shown to be a potent antioxidant. It scavenges hydroxyl radicals [1, 2], inhibits lipid peroxidation [2–4], quenches singlet molecular oxygen [5], and scavenges peroxyl radicals [6]. Protective effects of stobadine against reperfusion damage were reported for isolated rat heart [7], sympathetic neurons [8], and rat brain tissue [9]; it is also described as a cardioprotective drug with antihypoxic and antiarrhythmic effects [7].

Antioxidant properties of stobadine were also found under *in vivo* conditions, using the model of alloxan-induced diabetes in mice [10].

The hypothesis that oxidation processes render LDL‖ more atherogenic [11–13] has led to an intensive search for natural and synthetic antioxidants that could prevent LDL oxidation [12, 14, 15]. Consequently, it appeared of interest to investigate whether or not stobadine can protect LDL against oxidative modification. Using a standardized *in vitro* model, we show here for the first time that micromolar concentrations of stobadine increase the oxidation resistance of LDL in a dose-dependent manner.

MATERIAL AND METHODS

Plasma Preparation

Plasma was prepared from 10 healthy normolipidemic volunteers 25–35 years of age. Blood was drawn by venipuncture after overnight fasting. The blood was collected into polypropylene tubes containing a 10% (w/w) EDTA solution pH 7.4 to give a final concentration of EDTA in the blood of 0.1% (w/w). The plasma was immediately separated by low spin centrifugation (1800 *g*, 10 min). The supernatant was removed and recentrifugated (5000 *g*, 5 min with an increase in rotor speed to 15,000 g, 10 min). Sucrose was then added to give a final concentration of 6 g/L plasma. Aliquots of the plasma were stored at −80°C not longer than 5 weeks before LDL isolation.

LDL Preparation

LDL was prepared as described [16]. In brief, the plasma density was adjusted to 1.24 g/mL by adding 0.3816 g of solid KBr per mL of plasma. EDTA solution (d = 1.006 g/mL, pH = 7.4, 1 mg EDTA/mL) was added to a centrifuge

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‖ *Abbreviations:* LDL, low-density lipoprotein; Ex, excitation wavelength; Em, emission wavelength; o-LDL, oxidized LDL; apoB, apolipoprotein B-100; vit E, vitamin E; CD, conjugated diene; Trp, tryptophan.

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tube. This solution was underlaid with plasma (up to 4 mL). Centrifugation was performed at 10°C for 2 hr at 60,000 rpm with an NVT 65 rotor and a Beckman L70 ultracentrifuge. After centrifugation, the LDL was collected from the centrifuge tube (1.0–1.5 mL per tube) with a bent needle, sterile filtered through a Corning disposable syringe filter (0.2 μm) and stored in a sterile vial at 4°C under argon in the dark.

LDL Oxidation

LDL was desalted to remove EDTA by gel filtration with an Econo-Pac 10 DG column supplied by Biorad, using PBS (160 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, pH 7.4) as eluent. The LDL concentration was determined by the CHOD-PAP method using the cholesterol kit supplied by Boehringer-Mannheim (Mannheim, Germany). In a cuvette, the LDL was diluted to a concentration of 0.25 mg LDL total mass/mL (= 0.1 μM LDL) and mixed with $CuSO_4$ to start the oxidation. The final concentration of $CuSO_4$ was 1.66 μM or 3.33 μM. Stobadine (dissolved in PBS pH = 7.4) was added prior to $CuSO_4$ in final concentrations between 1–15 μM. The kinetics of the oxidation process was followed at 25°C or 37°C by measuring the increase in the conjugated diene absorbance at 234 nm [17].

Polarographic Measurement of Free Cu^{++}

The concentration of free Cu^{++} was determined using a polarographic analyzer (EG&G Princeton Applied Research, Princeton, NJ; Model 264A) with an SMDE working electrode and an Ag/AgCl reference electrode. The settings used for measurement were: initial potential −1.0V; final potential 0.0V; deposition time 30 sec; equilibration time 15 sec; scan rate 10 mV/sec; drop time 0.2 sec; pulse height 50 mV; drop size medium.

Additional Methods

Alpha-tocopherol was determined by HPLC (ODS-2, methanol/water 95:5 (v/v)) using fluorescence detection (Ex 292/Em 335 nm) according to Esterbauer *et al.* [12]. The kinetics of tryptophan destruction was measured by fluorescence spectroscopy (Shimadzu RF 5001 PC spectrofluorimeter) at an emission wavelength of 331 nm, with excitation of 282 nm. Details can be found in Ref. 16.

RESULTS

Effect of Stobadine on Lipid Peroxidation

The kinetics of lipid peroxidation of LDL can be measured by monitoring the change in the 234 nm absorption, characteristic of lipid hydroperoxides with conjugated double bonds [18]. This conjugated diene (CD) assay is also frequently used to investigate effects of antioxidants on oxidation resistance of LDL. Figure 1 shows a representative experiment on the kinetics of CD formation. In the absence

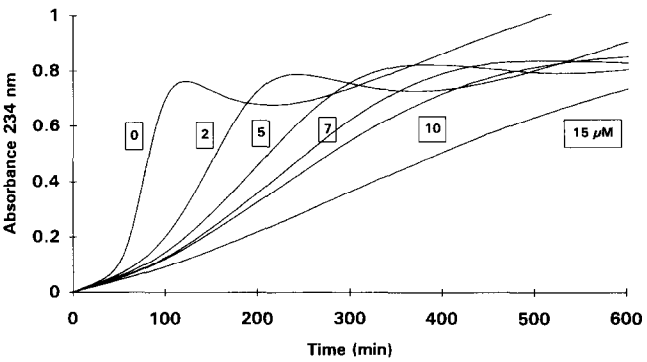

FIG. 1. Effect of stobadine on Cu^{++}-mediated oxidation of LDL as measured by the increase in the diene absorption. 0.1 μM LDL in PBS was incubated with 1.66 μM Cu^{++} at 37°C. Stobadine was added to the LDL solution at final concentrations of 0, 2, 5, 7, 10, and 15 μM after addition of $CuSO_4$.

of stobadine, the classical type of curve with lag, propagation, and decomposition phases was observed. Stobadine led to a dose-dependent increase in lag-time (t_{lag}) and significantly reduced the rate of propagation as evidenced by the flattening of the curves.

This strong inhibition of propagation can clearly be seen in the rate-versus-time plots, where the peaks represent maximal propagation rate (Fig. 2). The time required to reach the peak maximum ($t_{1/2}$) increased from 90 min (absence of stobadine) to approximately 300 min (10 μM stobadine). All effects shown in Fig. 1 and 2 were reproducable and mean values ± SD obtained from 3 to 4 independent experiments are given in Fig. 3 and Table 1.

A dose of 5 μM stobadine led to a 1.7- and 2.2-fold increase in lag time and $t_{1/2}$, respectively (Fig. 3) and reduced the propagation rate 4-fold (equivalent to a 75%

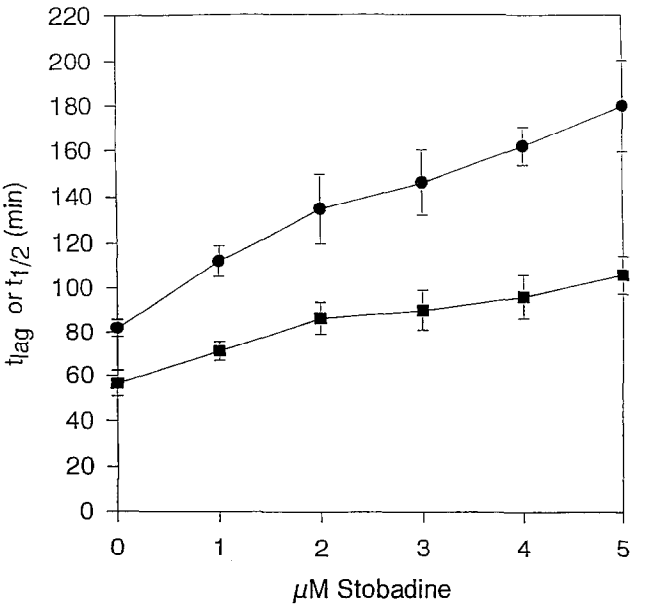

FIG. 2. Dependence of t_{lag} (■) or $t_{1/2}$ (■) on stobadine concentration. Experimental conditions as described in Fig. 1. Each point is the mean of at least three experiments.

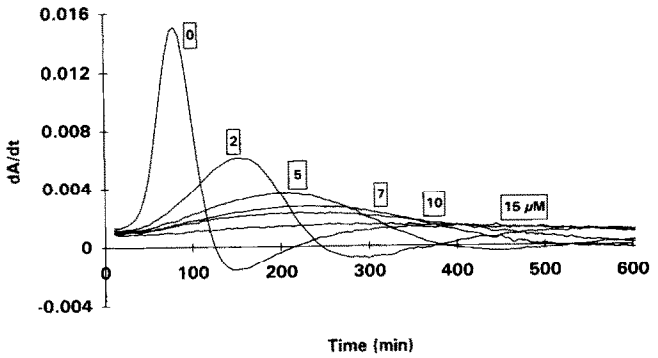

FIG. 3. First derivative of diene vs time curve (the change in the rates of oxidation as a function of time). The data used are from the same measurements as shown in Fig. 1.

inhibition). Expressed on a molar base, the increase in lag time given by one stobadine molecule per single LDL particle was on average 1.5 min; values for other antioxidants are published in Ref. 19 (ascorbate 1.21 min, urate 6.92 min, trolox 0.38 min, α-tocopherol 0.48 min).

We were interested in examining whether or not the inhibitory effect of stobadine is due to an α-tocopherol sparing effect, and measured tocopherol consumption in the presence and absence of stobadine. Figure 4 clearly shows that stobadine had no effect whatsoever on the α-tocopherol consumption rate, even at a relatively high concentration of 10 μM. Repetition of the experiment shown in Fig. 4 with another batch of LDL gave the same result. Similarly, stobadine had no influence on γ-tocopherol loss (data not shown). This excludes the possibility that stobadine has a protective effect on vitamin E. If the rate of tocopherol loss is directly proportional to the rate of initiation R_i (i.e. rate by which lipid peroxidation is initiated in LDL), it is also unlikely that stobadine affects the rate of initiation. In the experiment shown in Fig. 4, R_i was 18.3 nM/min. This is equivalent to 1 strike (initiation) per LDL particle every 11 min.

Effect of Stobadine on Destruction of Tryptophan Residues in apo B

Apolipoprotein B-100 contains 37 Trp residues and it has been shown [16] that their destruction during Cu^{++}-mediated oxidation of LDL is an early and vitamin E-independent event. Loss of Trp residues was followed by measuring the decrease in its characteristic fluorescence at 331 nm (excitation 282 nm). Consistent with previous observations, we found (Fig. 5) that addition of Cu^{++} to an LDL solution resulted in a rapid drop in Trp fluorescence due to the quenching effect. Then followed a period where Trp fluorescence steadily decreased for approximately 200 min more or less linearly at a rate of 0.15%/min, corresponding to an apparent rate of 1 Trp decomposed in each LDL particle every 18 min. After 200 min, Trp loss accelerated to a rate of 0.41%/min: this acceleration has been ascribed to lipid peroxidation-mediated destruction of Trp residues.

In the absence of Cu^{++}, stobadine had no effect on LDL Trp fluorescence (data not shown). Similarly, stobadine had no influence on Cu^{++}-mediated quenching, which indicates that it did not interfere with the Cu^{++} binding sites responsible for Trp fluorescence quenching and that no significant Cu^{++}-stobadine complex formation occurred. A polarographic measurement of the free copper concentration in the presence and absence of 10 μM stobadine indicated that at the Cu^{++} concentrations we used for LDL oxidation only a weak complexation of Cu^{++} occurred (Fig. 6). However, stobadine had a very pronounced protective effect on lipid peroxidation-dependent Trp destruction. As can be seen in Fig. 5, a dose of 10 μM stobadine did not inhibit early destruction of Trp (0–120 min) but completely blocked destruction at later stages, where heavy lipid peroxidation occurred.

According to the CD curves, LDL was fully oxidized with 3 μM stobadine after 550 min, whereas Trp fluorescence decreased only to 34% of the initial value. The diene maximum of LDL oxidized without stobadine was reached after 335 min and Trp fluorescence decreased to a value of 18.8% of its initial value. The dose-dependence of stobadine on the maximal rate of Trp destruction (% fluorescence decrease/min) and the maximal rate of diene formation during the propagation phase are very similar (Fig. 7).

DISCUSSION

In this study, we have shown that stobadine inhibits LDL oxidation initiated by copper ions in a dose-dependent manner, as evidenced by a prolongation of the lag phase and a decrease in the rate of diene formation during the propagation phase. Moreover, stobadine also protects Trp residues of apoB against lipid peroxidation-dependent destruction.

The various experiments reported here allow us to make some firm conclusions as to the underlying mechanism. First, from polarographic measurements with 10 μM stobadine and different Cu^{++} concentrations, we were able to show that only a low percentage of Cu^{++} is complexed in the presence of stobadine (Fig. 6) and that Cu^{++} complexation does not, in itself, cause the effect of stobadine on LDL oxidation.

Second, stobadine does not spare α-tocopherol. The fact that the rate of consumption of α-tocopherol is not changed by addition of 10 μM stobadine indicates that there is no interference between the chromanoxyl radical and stobadine. This agrees very well with the suggestion that stobadine is not able to reduce chromanoxyl radicals out of their one-electron oxidation potentials (stobadine: $E_{7.0}$ = 0.58V [5], vitamin E: $E_{7.0}$ = 0.48V [20]). Indeed, it was shown by ESR measurements [6] that stobadine in liposomes does not reduce the chromanoxyl radical of a vitamin E homologue. This is in contrast to the fact that inhibition of lipid peroxidation by stobadine was not observed with tocopherol-deficient microsomes [4]; the authors suggest that the antioxidant effect of stobadine depends on the vitamin E in the membrane.

TABLE 1. Effect of stobadine on the maximal rate of propagation and the lag phase

Stobadine (μM)	Maximal rate of propagation (μM/min) mean ± SD	Inhibition %	Lag phase (min) mean ± SD	Prolongation %
0	0.567 ± 0.053	0	82 ± 4.0	100
1	0.386 ± 0.053	32	112 ± 6.9	136
2	0.261 ± 0.042	54	135 ± 14.9	165
3	0.242 ± 0.015	58	146 ± 14.1	178
4	0.196 ± 0.018	65	162 ± 8.5	197
5	0.145 ± 0.019	75	180 ± 20.6	219
7	0.103 ± 0.014	82	n.d.	n.d.
10	0.080 ± 0.028	86	n.d.	n.d.

n.d., lag phase not determined, because the differences between the rate of diene formation during lag and propagation phases became very small.

LDL was oxidized under conditions described in Fig. 1. Maximal rate of oxidation was determined from the peaks of the first derivative of the CD curves (see Fig. 2). The rate in μM dienes/min is given by ΔA_{234nm}/min × 33.9 [17]. The values are mean ± SD from 3 to 4 independent experiments. (All measurements were performed at 37°C and 1.66 μM $CuSO_4$.)

Third, stobadine does not act as a preventative antioxidant by scavenging initiating radicals. Stobadine has no influence on the rate of early Trp destruction (0–120 min, Fig. 5) nor on that of α-tocopherol [16]. It has been shown that early Trp destruction is a Cu^{++} concentration-dependent process [16], and it is also known that Cu^{++} is involved in the initiation process of lipid peroxidation. From the observation that Cu^{++}, but not stobadine, affects early Trp destruction, we assume that stobadine does not scavenge the initiating radicals of lipid peroxidation during lag time.

From our observations, we conclude that stobadine acts as a scavenger of peroxyl radicals ($LOO^{\bullet}$). The effect of stobadine on the rate of diene formation and Trp destruction during the propagation phase (Fig. 7) can be explained from its $LOO^{\bullet}$ radical scavenging properties. The efficient peroxyl radical-scavenging properties of this compound with AMVN- or AAPH-derived peroxyl radicals have already been described by others [6]. The fact that this compound has no influence on tocopherol consumption during the lag phase can be explained by the fact that the reaction:

$$LOO^{\bullet} + \text{stobadine} \rightarrow \text{stobadinyl radical} + LOOH$$

has a much lower rate constant than tocopherol for this reaction.

The rate constant for α-tocopherol with $LOO^{\bullet}$ (methyl linoleoylperoxy radical) as measured by others [21] is approximately $5 \times 10^5\ M^{-1}\ s^{-1}$. The rate constants for reactions of lipid peroxyl radicals with stobadine have not been published until now, but our results suggest that they are approximately 2 orders of magnitude lower.

Fig. 5 shows that Trp destruction can be totally inhibited by 10 μM stobadine (LDL:stobadine ratio 1:100), whereas lipidperoxidation still goes on at a much lower rate. It is possible that stobadine and Trp compete for $LOO^{\bullet}$ radicals (parallel reaction) and that a surplus of stobadine slows down the reaction of Trp destruction. Decrease in Trp destruction during the propagation phase may also be according to the one electron oxidation potential of stobadine

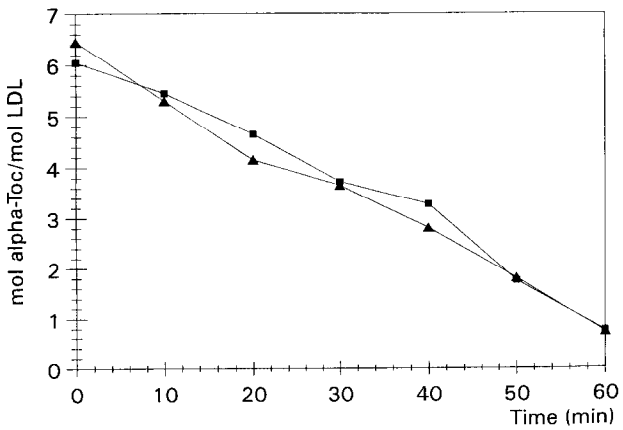

FIG. 4. Consumption of alpha-tocopherol during Cu^{++}-mediated LDL oxidation. 0.2 μM LDL was incubated in PBS with 3.3 μM $CuSO_4$ at 25°C. Stobadine (10 μM) was added prior to Cu^{++}, control (▲)/stobadine (■).

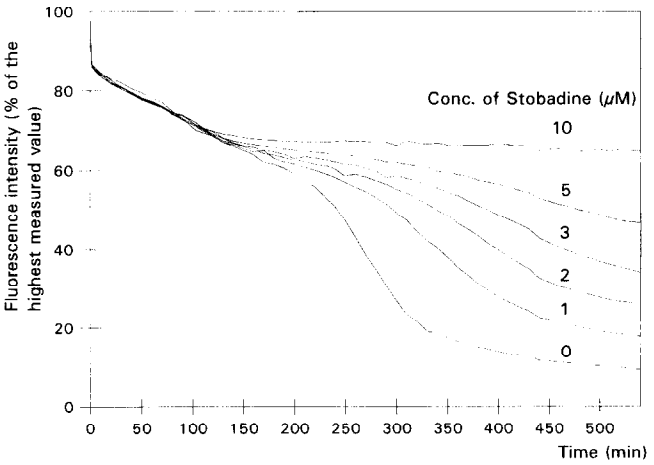

FIG. 5. Decrease of Trp fluorescence during Cu^{++}-mediated oxidation of LDL with different concentrations of stobadine. 0.1 μM LDL was incubated in PBS with 3.3 μM $CuSO_4$. Fluorescence (Ex/Em = 282/331 nm) was measured at intervals of 10 min at 25°C. Stobadine 0, 1, 2, 3, 4, 5, 10 μM was added prior to Cu^{++}.

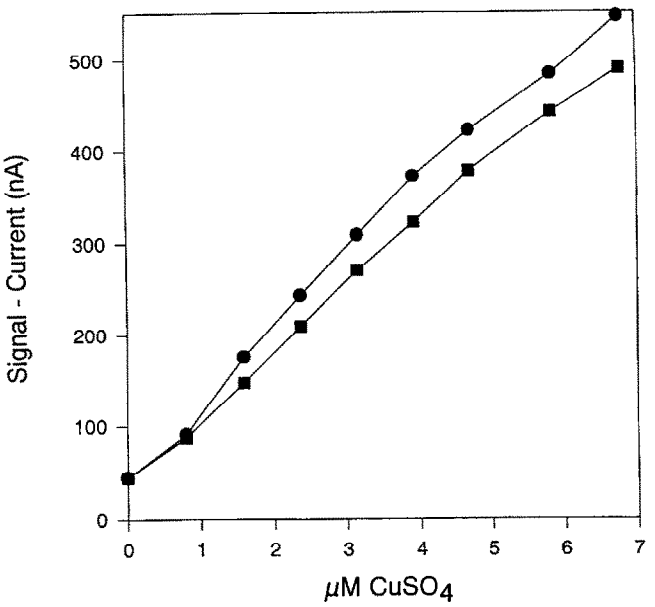

FIG. 6. Polarographic measurement of Cu^{++} complexation by stobadine. To a PBS buffer pH = 7.4 without (●) and 10 μM (■) stobadine, a $CuSO_4$ solution was added.

($E_{7.0}$ = 0.58V) [5] and tryptophan ($E_{7.0}$ = 1.03V) [22], a result of the reduction of Trp radicals (formed by the reaction with $LOO^{\bullet}$) by stobadine:

$$Trp^{\bullet} + \text{stobadine} \rightarrow \text{stobadinyl radical} + Trp$$

This ability of stobadine to repair oxidized amino acids by one electron donation has already been discussed by others [5] and may, in fact, occur during LDL oxidation with higher concentrations of stobadine (10 μM) (Fig. 5) because beginning at 120 min until 550 min no more Trp destruction can be measured. Stobadine concentrations giving efficient inhibition of LDL oxidation are in the range expected to be reached *in vivo*. Serum concentrations of stobadine in humans after oral intake (0.27–2.5 mg/kg p.o. stobadine administered p.o. as dipalmitate) were 0.2–1.1 μM [23]. Higher doses of this compound were administered to dogs (2.9–4.7 mg/kg p.o. stobadine dipalmitate) and the plasma concentration was found to be 3.4 μM. After oral intake of stobadine dihydrochloride by rats (1 mg/kg) for 25 consecutive days, the concentration of stobadine in plasma was 3.9 μM [24].

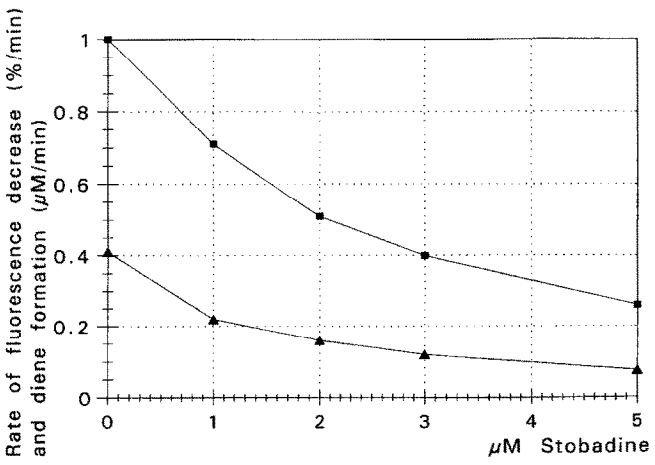

FIG. 7. Rate of tryptophan fluorescence decrease (▲) and diene formation (■) in the propagation phase. Experimental conditions as described in Fig. 5.

In conclusion, stobadine is a promising new pharmaceutical compound because of its cardio- and neuroprotective properties [7, 8, 9] and its ability to protect LDL lipids and apoB Trp residues against oxidative damage.

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