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Original Research Communication

Low-Density Lipoprotein Oxidation and Its Prevention by Amidothionophosphate Antioxidants

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ABSTRACT

Amidothionophosphates (AMTPs) are a novel group of antioxidants that are lacking in pro-oxidant activity. In this paper, we compare two different amidothionophosphates: 2-hydroxy-ethyl amido, diethyl thionophosphate (AMTP-B), which contains a single primary amido group, and *N,N',N''*-tripropylamidothionophosphate (AMTP-3A), which contains three primary amido groups. The lipoprotein/medium partition coefficients of AMTP-3A and AMTP-B are 74 and 38, respectively. Both protected isolated human low density lipoprotein (LDL) against oxidative damage induced by copper sulfate. Oxidative damage to polyunsaturated acyl chains was determined by gas chromatography (GC), and oxidation kinetics were monitored by following the accumulation of conjugated dienes spectrophotometrically at 234 nm. The AMTP antioxidants significantly protected the LDL against Cu^{2+}-induced oxidation. However, if the LDLs were already partially oxidized, protection against oxidation by the AMTPs was reduced. AMTP-3A was more effective in protecting LDL than was AMTP-B. The difference in antioxidant activity was attributed to the 15-fold higher reactivity of AMTP-3A toward peroxides. Oxidizability of plasma lipoproteins from guinea pigs injected with AMTPs was strongly reduced. Antiox. Redox Signal. 1, 325–338.

INTRODUCTION

HUMAN PLASMA LOW-DENSITY LIPOPROTEIN (LDL) is composed of an envelope consisting of apolipoprotein B 100, phospholipids, and free cholesterol, and a core composed of cholesterol esters and triglycerides. The particle has an average diameter of 22 nm and an average mass of 2.5 million daltons. Both the envelope and core are rich in polyunsaturated acyl chains. The core contains about 1,500 esterified cholesterol molecules. The most common acyl chain in the core is linoleate, followed by arachidonate. In the envelope, about 40% of the phospholipid molecules have at least one polyunsaturated fatty acid (PUFA) (Quinn *et al.*, 1987; Bowry and Stocker, 1993; McCarthy, 1993; Fruchart and Duriez, 1994; Berliner and Heinecke, 1996). Therefore, the LDL particle is highly susceptible to oxidation, as has been demonstrated *in vitro* and *in vivo* (Quinn *et al.*, 1987; Bowry and Stocker, 1993; McCarthy, 1993; Fruchart and Duriez, 1994; Berliner and Heinecke, 1996).

In vitro, LDL can be oxidatively modified by several agents, including cupric ions (Steinbrecher *et al.*, 1984), azo compounds (Bowry and Stocker, 1993), and enzymes such as lipoxygenase (Sparrow *et al.*, 1988; Belkner *et al.*, 1993; Berliner and Heinecke, 1996) and

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myeloperoxidase (Savenkova *et al.*, 1994). Antioxidants present in an LDL particle include several lipophilic antioxidants: α-tocopherol (6–12 molecules), γ-tocopherol (0.5 molecule), ubiquinol (0.5–1.2 molecules), and carotenoids (0.4 molecule) (Esterbauer *et al.*, 1992; Bowry and Stocker, 1993). However, in many cases the total antioxidant content of the LDL and that of α-tocopherol (the dominant antioxidant) did not correlate well with the oxidizability of LDL (Jessup *et al.*, 1990).

Maiorino *et al.* (1995) and Berliner and Heinecke (1996) addressed this problem and raised several points concerning LDL oxidizability:

(i) No correlation was found between the amount of antioxidants in the LDL or in the plasma and the oxidizability of the LDL particles *in vitro*.
(ii) The lag phase in the kinetics of LDL oxidation and the kinetics of consumption of tocopherols are not overlapping.
(iii) Tocopherols added to the LDL *in vitro* do not affect the lag phase extensively.
(iv) LDLs were shown to accumulate lipid hydroperoxides prior to the consumption of the tocopherols in the LDL particle.

The structure of the LDL particles is complex, and the nature of their oxidation kinetics, including the molecular mechanism responsible for the lag phase, is still a controversial issue. The conclusion of many investigators (reviewed by Berliner and Heinecke, 1996) suggests that factors other than antioxidant level contribute to the kinetics of oxidation. The molecular explanation for the sigmoid shape of LDL oxidation kinetics may be important for the development of effective compounds to slow down or to prevent oxidative damage in cardiovascular disease.

In this study, we addressed the issue of LDL oxidation kinetics using a unique group of antioxidants synthesized and characterized in our laboratory. These compounds (Fig. 1) possess a wide spectrum of activity toward a variety of reactive oxygen species (ROS), including lipid hydroperoxides, NaOCl, and hydrogen peroxide (H_2O_2). However, their reactivity with peroxyl radicals is extremely low (Tirosh *et al.*, 1996). These unique antioxidant properties allowed us to investigate the mechanism of LDL oxidation by copper ions and to elucidate various stages in the oxidation of these particles.

MATERIALS AND METHODS

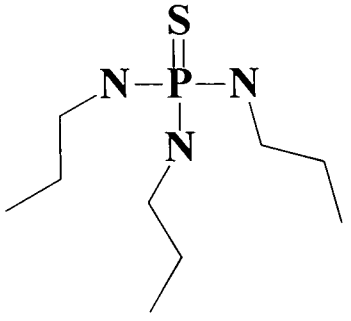
Synthesis of amidothionophosphates

The amidothionophosphates (AMTPs) were synthesized in our laboratory and characterized by ^{31}P nuclear magnetic resonance (NMR), ^{1}H NMR, and thin-layer chromatography (TLC). All reagents for the synthesis were of analytical grade or better (Aldrich, Milwaukee, WI).

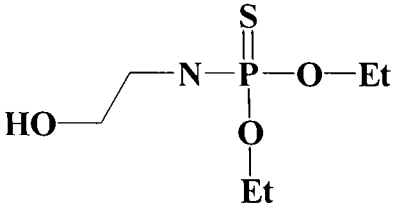
Synthesis of 2-hydroxy-ethyl amido, diethyl thionophosphate (AMTP-B) was prepared by coupling of a chlorodiethylthionophosphate with ethanolamine according to an already published procedure (Tirosh *et al.*, 1996).

Synthesis of *N,N',N"*-tripropylamidothionophosphate (AMTP-3A) was as follows.

Trichlorothiophosphate (1 equiv) was dissolved in 50 ml of dry dichloromethane. The



AMTP-3A



AMTP-B

FIG. 1. Chemical structures of AMTP-B and AMTP-3A.

solution was added dropwise with stirring to a solution of propylamine (9 equiv) in 50 ml of dichloromethane. After stirring for 1 hr 25 ml of acidified water (HCl), pH 2.0, was added and the organic layer was washed five times to extract all the free amine. The organic layer was dried on anhydrous magnesium sulfate and evaporated to dryness. The white powder residue was collected and dried under reduced pressure for 5 hr. Yield 85%.^{31}P NMR ($CDCl_3$, 121.42 MHz)—Singlet 63 ppm. ^{1}H NMR ($CDCl_3$, 299.9 MHz)—9H (t, 0.8–1 ppm), 6H (m, 1.4–1.6 ppm), 6H (dt, 2.8–3 ppm).

Preparation of LDL

LDL in various degrees of purity was prepared by two different methods. In the first, an LDL-rich fraction was prepared from a pool of fresh blood from healthy donors by a single ultracentrifugation (100,000 × *g* for 24 hr). This fraction also contained albumin and other plasma proteins. The LDL-containing fraction was dialyzed twice for 1 hr and then overnight at 4°C under a stream of nitrogen against 50 m*M* phosphate buffer pH 7.4 containing 10 μM EDTA. In the second method, pure LDL (*d* 1.019–1.063) was prepared from a pool of fresh blood from healthy donors, as described elsewhere (Havel *et al.*, 1955).

Normalization of results obtained for the LDL-rich fraction was done according to protein core the same way it has been done for purified LDL.

Preparation of oxidatively "modified" LDL

Partially oxidized, "modified" LDL (m-LDL) was obtained by leaving the LDL particles under air at 4°C for 14 days. This treatment increased the UV absorbance at 234 nm of LDL (indicative of conjugated dienes) by 25–33%.

Measurements of α-tocopherol and γ-tocopherol in LDL

α-Tocopherol and γ-tocopherol content were determined by HPLC-EC (column, C-18 RP Merck; mobile phase, ethanol/water 1:1). Tocopherols were eluted at a flow rate of 1 ml/min with retention times of 3.74 and 3.55 min for alpha and gamma, respectively. LDL cholesterol was determined simultaneously by a UV diode array at a retention time of 4.44 min.

Determination of LDL/medium partition coefficient (Kp)

The determination of the LDL/medium *Kp* is based on quantification of AMTPs by their phosphorus. Purified LDL, which was dialyzed three times against 50 volumes of bidistilled water, was used. Coefficient of AMTPs partition between LDL and water was determined using a two-compartment cell especially designed for equilibrium dialysis. Following 72 hr of incubation under continuous shaking at room temperature, samples from the LDL and LDL-free compartments were removed and their phosphorus determined (Barenholz and Amselem, 1993).

The *Kp* was determined as described by Samuni and Barenholz (1997), using an average LDL partial specific volume of 0.9891.

AMTP protection of LDL against Cu^{2+}-induced oxidation

Oxidative modification of LDL was determined by the following procedures:

Acyl chain composition of LDL. One milliliter of LDL (0.1 mg protein/ml) before and after exposure to oxidation (100 μM $CuSO_4$ for 600 min), with and without 1 m*M* AMTP-B, was extracted from the aqueous phase by adding 1 ml of ethanol and 1 ml of chloroform and vortexing for 1 min. Two phases were formed, and the organic phase was collected and dried by a stream of nitrogen, followed by 2 hr of lyophilization to remove all traces of water. The dry lipids were dissolved in 50 μl of toluene, 10 μl of methanol, and 20 μl of Meth-Prep II methanol esterification reagent (Alltech). The mixture of lipids was analyzed by gas chromatography using a Perkin Elmer 1020 plus GC, with a Silar 10C chromatographic column (Alltech), using a temperature gradient of 5°C/min from 140°C to 240°C (Barenholz and Amselem, 1993).

Conjugated diene accumulation in LDL. Solutions of LDL at a protein concentration of 0.1 mg/ml in a 50 m*M* phosphate, 10 μM EDTA

buffer, pH 7.4, were exposed to Cu^{2+}-induced oxidation at 31°C. The final concentration of Cu^{2+} was 100 μM. The accumulation of conjugated dienes was monitored spectrophotometrically at 234 nm.

Peroxide-removing capacity of AMTPs

AMTP-B, which possesses only one primary amino group, was compared to AMTP-3A, with three amino groups (100 mM final concentration). This experiment was aimed to reveal different reactivities of the two AMTP molecules toward an oxidizing species. The activity of these compounds was tested by their rate of reaction with 1 M H_2O_2 in a solution of dioxane:water, 1:9 (vol/vol), having a dielectric constant of 9.6. The reaction rates were monitored by following the disappearance of the thiophosphate using ^{31}P NMR. ^{31}P NMR spectra were used to evaluate both the disappearance of the AMTP compounds and to elucidate the oxidation product following exposure to hydrogen peroxide (Tirosh *et al.*, 1996).

Lipid hydroperoxide accumulation/ decomposition assay

Small unilamellar liposomes (vesicles) (SUV) were prepared from 25 mM egg-phosphatidylcholine (EPC2)/7 mM cholesterol in 50 ml of HEPES (20 mM) buffer, pH 7.2, using the high-pressure homogenizer Model Minlab type 8.30 H (APV Rannie, Albertslund, Denmark) according to a published procedure (Barenholz and Amselem, 1993). EPC2 was obtained from Lipoid KG (Ludwigshafen, Germany) and cholesterol from Sigma (St. Louis, MO). The liposomes were used following 1 year storage at 4°C in Vacutainer tubes. This storage condition resulted in accumulation of hydroperoxides; however, most of the PUFA in the liposomes was not oxidized. The liposomes were exposed to oxidation conditions under air at 37°C for 96 hr in the presence and absence of 2 mM AMTP-3A. Lipid hydroperoxides were monitored using a spectroscopic method that was modified to the micromolar range as follows: 50 μl of liposomal dispersion was dissolved in 1 ml of ethanol. Fifty microliters of a 50% KI solution was added, and the mixture was incubated for 20 min in the dark. Absorbance at 400 nm was measured (Tirosh *et al.*, 1996). Liposome acyl chain composition was evaluated by GC at the beginning and end of the incubation period (Barenholz and Amselem, 1993).

Peroxyl radical trap assay

The reaction mixture consisted of 1.5×10^{-8} M R-phycoerythrin (R-PE) in phosphate-buffered saline (PBS), pH 7.4, at 37°C. The oxidation reaction was started by adding 2,2′-diazobis(2-amidinopropane) 2 HCl (AAPH) (a temperature-dependent peroxyl radical generator) to a final concentration of 4.0 mM, and the decay of R-PE was monitored every 30 sec for 30 min (Ghielli *et al.*, 1995). Scavenging the peroxyl radicals was done by 5 μM uric acid and by AMTP-B at 10 and 100 μM.

Effect of antioxidants on oxidation of guinea pig lipoproteins

Four groups of three guinea pigs (each $\approx$ 500 grams) were injected intraperitoneally (i.p.) with one of three different antioxidants: AMTP-B (400 mg/kg), AMTP-3A (200 mg/kg), and α-tocopherol (200 mg/kg). Each antioxidant was dissolved in ethanol/water, 2:1 (vol/vol). The control group was injected with 1.5 ml of the ethanol/water solution. Two hours following the treatment, the animals were anesthetized with ether, and 5 ml of blood was withdrawn directly from the heart and placed in heparinized tubes. These were centrifuged at 3,000 rpm for 15 min, and the plasma samples of all the animals of the same group were combined (about 4.5 ml of plasma). Plasma was centrifuged overnight at 4°C at 100,000 $\times$ g and the yellow (lower) fraction was collected (about 1 ml). Samples were dialyzed overnight against 30 ml of 50 mM phosphate buffer, pH 7.2, and then, to reduce the level of nonlipoprotein proteins, this fraction was filtered through a Sephadex G 200 column. The yellow void volume (1 ml) was used for oxidizability measurements.

The samples were diluted to a final protein concentration of 0.25 mg/ml, oxidation was induced by adding $CuSO_4$ to a final concentration of 200 μM at 31°C, and conjugated diene accumulation was determined.

NMR measurements to evaluate copper chelation by AMTP

NMR spectra were recorded in D_2O solution on a Varian VXR 300S spectrometer. Chemical shifts are reported in ppm downfield from 1% H_3PO_4 in D_2O as external standard for ^{31}P spectra (121 MHz). Positive chemical shifts are at low field with respect to the standard. AMTP-B and potassium hydrogen phosphate (as an internal reference phosphate), each at 38 m*M*, were dissolved in D_2O. pH was then adjusted to 7.4. Varied amounts of $CuSO_4$ were added to this solution, and the effect of the Cu^{2+} on the chemical shifts of the phosphates and on the broadness of the peaks at the middle of their highs was recorded.

Error of analyses

The oxidation run was carried out twice. Results of conjugated diene accumulation represent a mean of the two experiments (variation <5%). A statistical analysis was done by Student's *t*-test on the points following the propagation phase (plateau levels of conjugated dienes) $p < 0.05$.

RESULTS

Protection of LDL by AMTPs

Accumulation of conjugated dienes. An LDL-rich fraction, which was isolated by a single ultracentrifugation, was subjected to Cu^{2+} at a final concentration of 100 μM. The oxidation kinetics was monitored by following the accumulation of conjugated dienes at 234 nm, as described in Materials and Methods. AMTP-B in this system was effective in inhibiting formation of conjugated dienes. The efficiency of the protection was found to be dependent on AMTP concentration (Fig. 2). At a concentration of 0.05 m*M*, AMTP-B increased the lag phase in the LDL oxidation from 250 min to 400

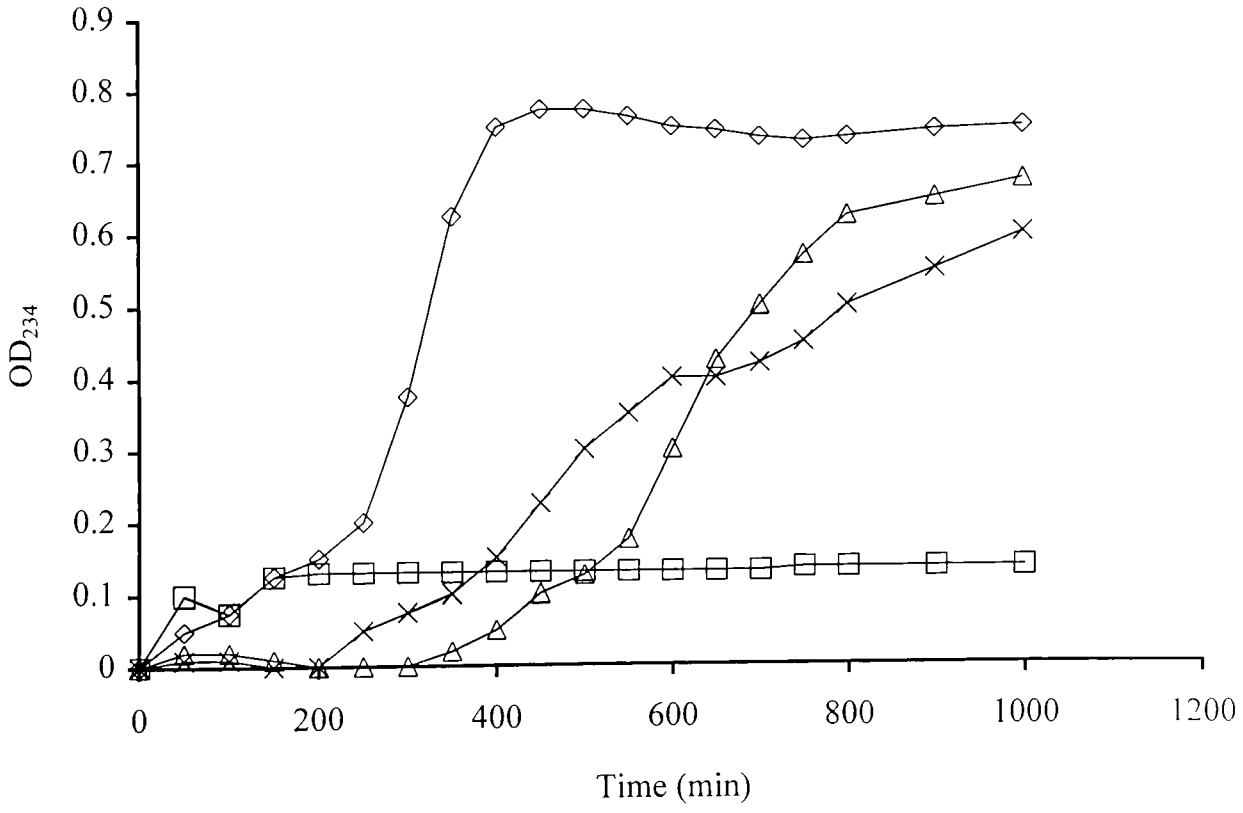


FIG. 2. Kinetics of conjugated diene accumulation in LDL following incubation with Cu^{2+} (100 μM). LDL (prepared according to method 1; see Materials and Methods) were incubated with AMTP-B at concentrations of 1 m*M* (□), 0.1 m*M* (δ), and 0.05 m*M* (△), and without AMTP-B (◇). (1 m*M* of AMTP-B significantly inhibited conjugated diene accumulation compared to the oxidized control.)

min; at a concentration of 0.1 m*M*, to 550 min; and at 1 m*M*, the presence of AMTP-B inhibited the LDL oxidation to a period longer than 1,000 min (Fig. 2). The rate of conjugated diene accumulation in the fast oxidation phase following the lag period was also affected by the AMTP-B and was slower than in the control (Fig. 2).

Acyl chain composition of LDL. The accumulation of conjugated dienes alone is not sufficient to evaluate oxidative damage in lipids (Halliwell and Gutteridge, 1989). LDL were incubated with Cu^{2+} for 600 min at 31°C (about two to three times longer than the lag-phase period), and the loss of PUFA was evaluated by gas chromatography. Fifty percent of the linoleic acid and almost all of the arachidonic acid were lost (Fig. 3). However, in the presence of AMTP-B (1 m*M*), no change in the level of these two PUFA occurred following exposure to Cu^{2+}. The ratio of PUFA/palmitic acid was even higher in the presence of the AMTP-B (and Cu^{2+}) than in the control that was incubated for 600 min at 31°C (without Cu^{2+}).

Reactivity of AMTPs with H_2O_2. We assume that the basis of the protection mechanism of AMTP is its ability to decompose lipid hydroperoxides without eliciting pro-oxidative effects. The ability to react with peroxides was compared for the two AMTP compounds. The compounds were dissolved in a mixture of dioxane: 30% H_2O_2, 9:1 (vol/vol). The dioxane was used to lower the dielectric constant to that of an LDL interface or of a membrane bilayer. The rates of oxidation of AMTP thionophosphate to phosphate by H_2O_2 was followed by ^{31}P NMR (Tirosh *et al.*, 1996). AMTP-3A was consumed 15 times faster than AMTP-B (Fig. 4A); in 3 hr, 20% and 95% of AMTP-3A and AMTP-B, respectively, were left, and after 30 hr, 30% of AMTP-B remained. The decomposition products of the original AMTPs had chemical shifts between 10 and −5 ppm, which means that sulfur was replaced by oxygen (data not shown). In the control mixtures of dioxane and water, both AMTPs were stable.

Lipid hydroperoxide decomposition by AMTP-3A

Lipid hydroperoxides accumulated in liposome following incubation at 37°C (from 0.07 m*M* to 0.42 m*M* after 96 hr) (Fig. 4B). However, in the presence of AMTP-3A, no such accumulation was observed. The content of lipid hydroperoxide was even reduced to the lowest detection threshold of the method (Fig. 4B). To evaluate whether the decomposition of the lipid hydroperoxides observed was due to an oxidative process (such as metal-induced decomposition) or due to a nonoxidative decomposition process, we measured the acyl chain composition of the liposomes. The results showed that the control liposomes (without AMTP) lost 66% of linoleic acid, 100% of arachidonic acid, and 100% of docosahexaenoic acid. In contrast, the liposomes containing AMTP-

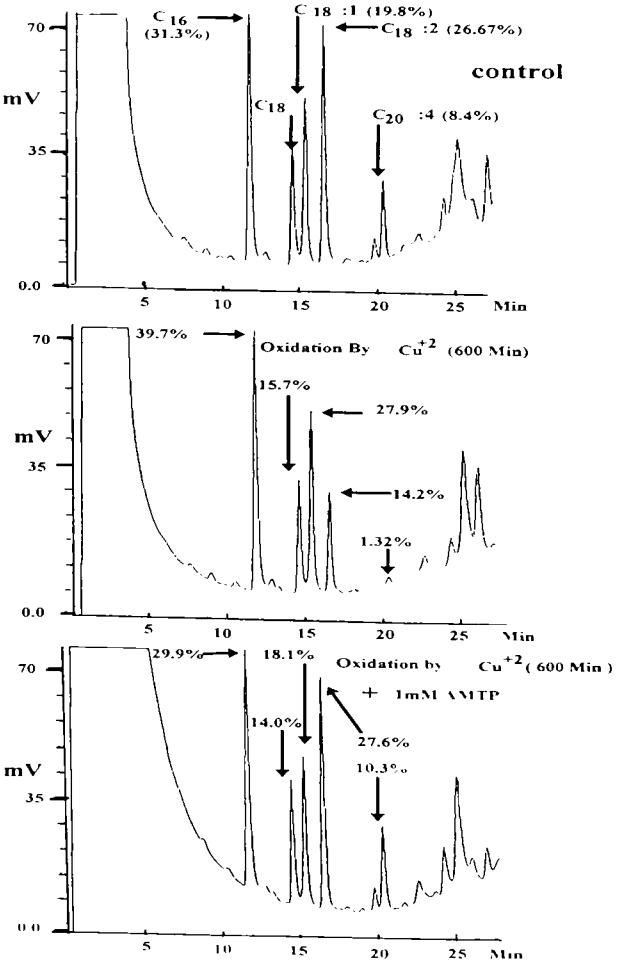


FIG. 3. Acyl chain composition in LDL (upper chromatogram), oxidized LDL (middle chromatogram), and oxidized LDL protected by 1 m*M* AMTP-B (lower chromatogram). The oxidation procedure included incubation of the LDL with Cu^{2+} for 600 min as described in Materials and Methods. The LDL-rich fraction was used at a concentration of 0–1 mg protein/ml.

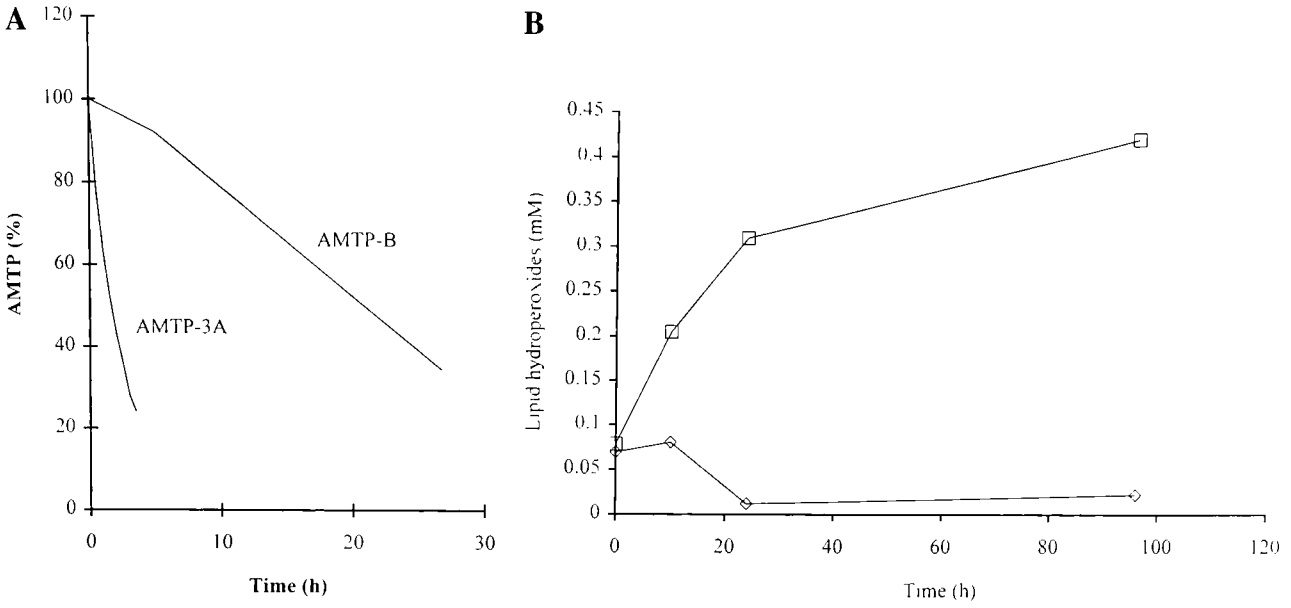


FIG. 4. Decomposition of peroxides by AMTPs. A. Oxidation kinetics (followed by ^{31}P NMR) of AMTP-B (100 m*M*) or AMTP-3A (100 m*M*) with H_2O_2 (1 *M*). **B.** Lipid hydroperoxide content of SUV in the presence and absence of AMTP-3A. The liposomes were incubated for 96 hr at 37°C under air. (□) Without protection; (◇) AMTP-3A, 2 m*M*.

3A lost only 9% of linoleic acid, 10% of arachidonic acid, and 10% of docosahexaenoic acid. These results indicate the decomposition of the lipid hydroperoxides was not accompanied by any acceleration of the oxidative damage to the PUFA.

Comparison of AMTP-3A and AMTP-B in preventing LDL oxidation. The capability of the two AMTP compounds to inhibit oxidative damage induced by Cu^{2+} to LDL was tested on purified LDL, which was exposed to 100 μM $CuSO_4$ at 31°C in the presence of 1 m*M* AMTP-B, 1 m*M* AMTP-3A, and without any addition. AMTP-B has only a minimal protective effect, because the lag period was extended by only 50 min (Fig. 5) and the rate of conjugated diene accumulation following the lag phase was similar to that obtained in LDL lacking AMTPs. On the other hand, 1 m*M* AMTP-3A completely prevented the oxidation (Fig. 5).

LDL/water partition coefficients of AMTPs

The LDL/water *Kp* for both AMTPs are in favor of the LDL. The values for both AMTPs are relatively low, although the value for AMTP-3A is about twice that of AMTP-B (74 vs. 38).

Peroxyl radical trap assay

AMTPs are antioxidants donating two electrons simultaneously (Tirosh *et al.*, 1996). Therefore, it is expected that when interacting with peroxyl radicals (which react preferentially with one electron at a time, as is the case with tocopherol), these compounds will have very low reactivity. This was proven using a flux of peroxyl radicals produced by AAPH, which induced constant loss of R-PE fluorescence due to fluorophore oxidation (Ghielli *et al.*, 1995). In the presence of 5 μM uric acid, an inhibition lag of 8 min was observed in the oxidation of R-PE (Fig. 6). AMTP-B at concentrations of 10 and 100 μM did not induce any lag period in the loss of fluorescence (Fig. 6), indicating that it did not scavenge peroxyl radicals.

Effect of initial oxidation state on LDL, level of conjugated dienes, endogenous antioxidant content, and oxidizability

To evaluate how the initial level of oxidation affects LDL oxidation kinetics, we compared the kinetics of conjugated diene accumulation in fresh LDL and in LDL samples which were partially oxidized by their incubation in air at 4°C for 14 days. This modified LDL is referred

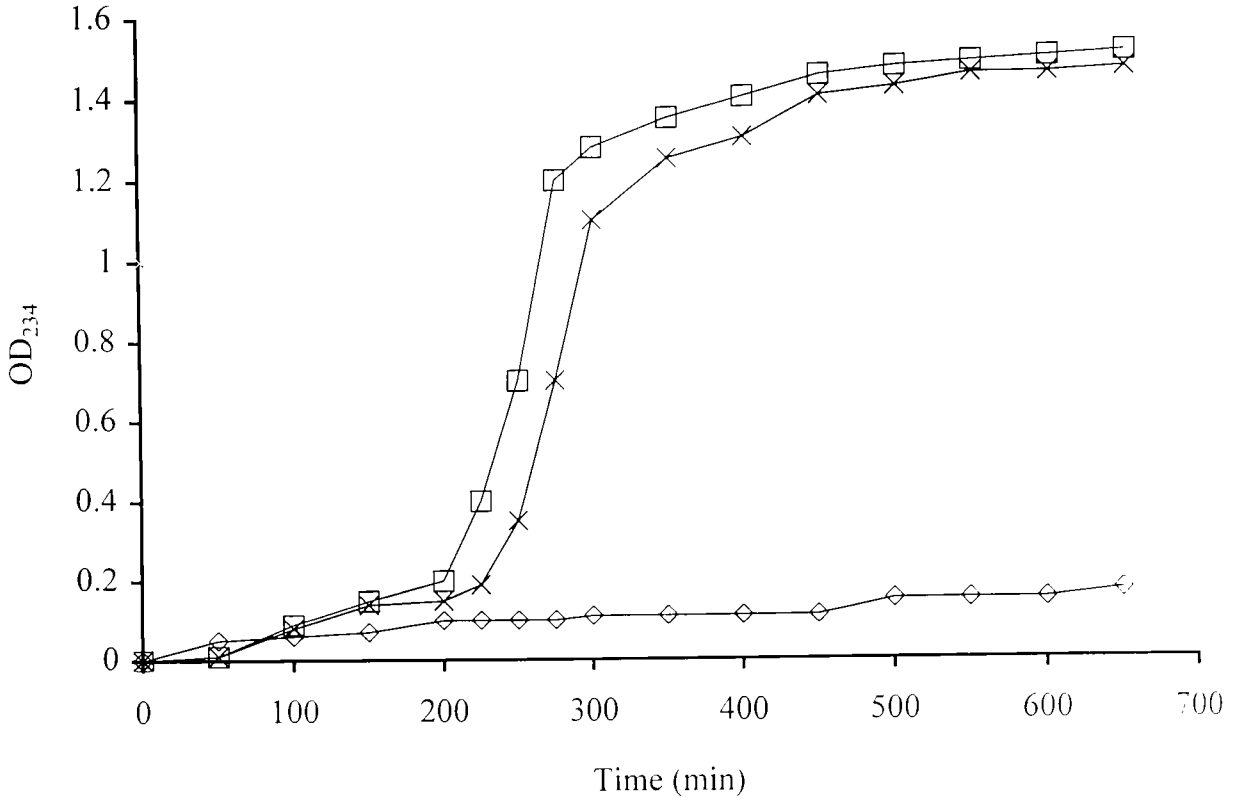


FIG. 5. Activity of AMTP-3A and AMTP-B in protecting LDL (purified by method 2; see Materials and Methods) against oxidation induced by 0.1 m*M* copper sulfate. (□) Without protection; (×) AMTP-B, 1 m*M*; (◇) AMTP-3A, 1 m*M*. (1 m*M* of AMTP-3A significantly inhibited conjugated diene accumulation compared to the oxidized control.)

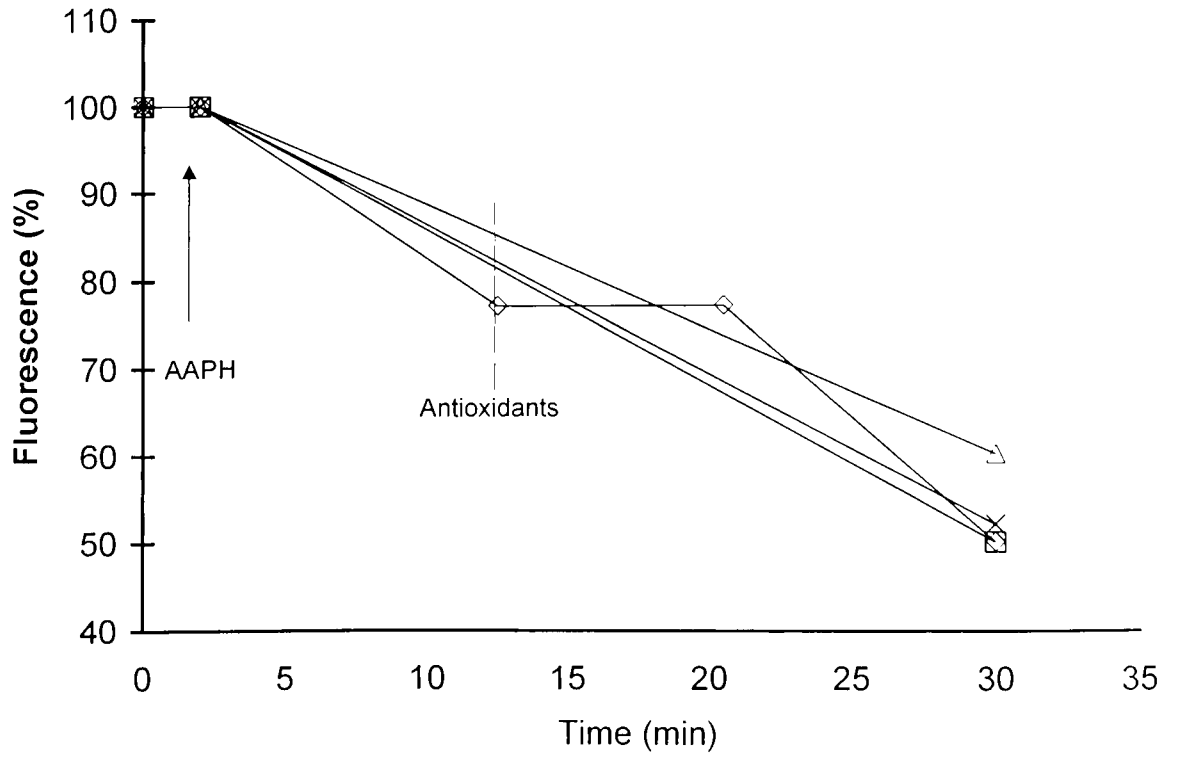


FIG. 6. Fluorescence decay of R-PE following its oxidation by 20 m*M* AAPH (peroxyl radical generator) at 37°C in the presence of 10 μM AMTP-B (×), 100 μM AMTP-B (△), and uric acid 5 μM (◇).

to as m-LDL. The m-LDL has elevated levels of conjugated dienes (as indicated by its higher absorbance at 234 nm) by 25–33% compared to the freshly produced LDL. The antioxidant content of the fresh and m-LDL was analyzed as described in Materials and Methods. α-Tocopherol concentrations were 17.35 ± 0.37 pmol/nmol cholesterol (11,173 pmol/mg protein) for the fresh LDL and 15.45 ± 0.34 pmol/nmol cholesterol (10,209 pmol/mg protein) for the m-LDL. γ-Tocopherol concentrations were 0.24 ± 0.02 pmol/nmol cholesterol and 0.24 ± 0.019 for the fresh- and m-LDL, respectively.

The Cu^{2+}-induced oxidation kinetics of the m-LDL differed from that of fresh LDL. The lag phase in the m-LDL completely disappeared and the fast oxidation rate started immediately (Fig. 7).

Both AMTP-B and AMTP-3A protect fresh LDL in a similar way. However, only AMTP-3A is capable of giving significant protection to m-LDL against Cu^{2+}-induced conjugated diene accumulation (Fig. 8).

LDL tocopherol content of fresh- and m-LDL was virtually identical. That is, oxidation of LDL occurred without influencing its tocopherol content (see discussion below). These results are in agreement with previous studies (Bowry *et al.*, 1992; Frei and Gaziano, 1993; Maiorino *et al.*, 1995).

Effect of AMTPs and α-tocopherol on oxidation of guinea pig lipoproteins

Lipoprotein-enriched fractions obtained from the plasma of guinea pigs injected with α-tocopherol, AMTP-B, and AMTP-3A (see Materials and Methods) were subjected to Cu^{2+} (200 μ*M*) -induced oxidation.

The rates and plateau levels of conjugated diene accumulation in the oxidizability test of these fractions were in the following order: control guinea pigs > > α-tocopherol-treated > AMTP-3A-treated > AMTP-B-treated (Fig. 9). Thus, all three compounds are shown (to varying degrees) to have a protective antioxidant effect.

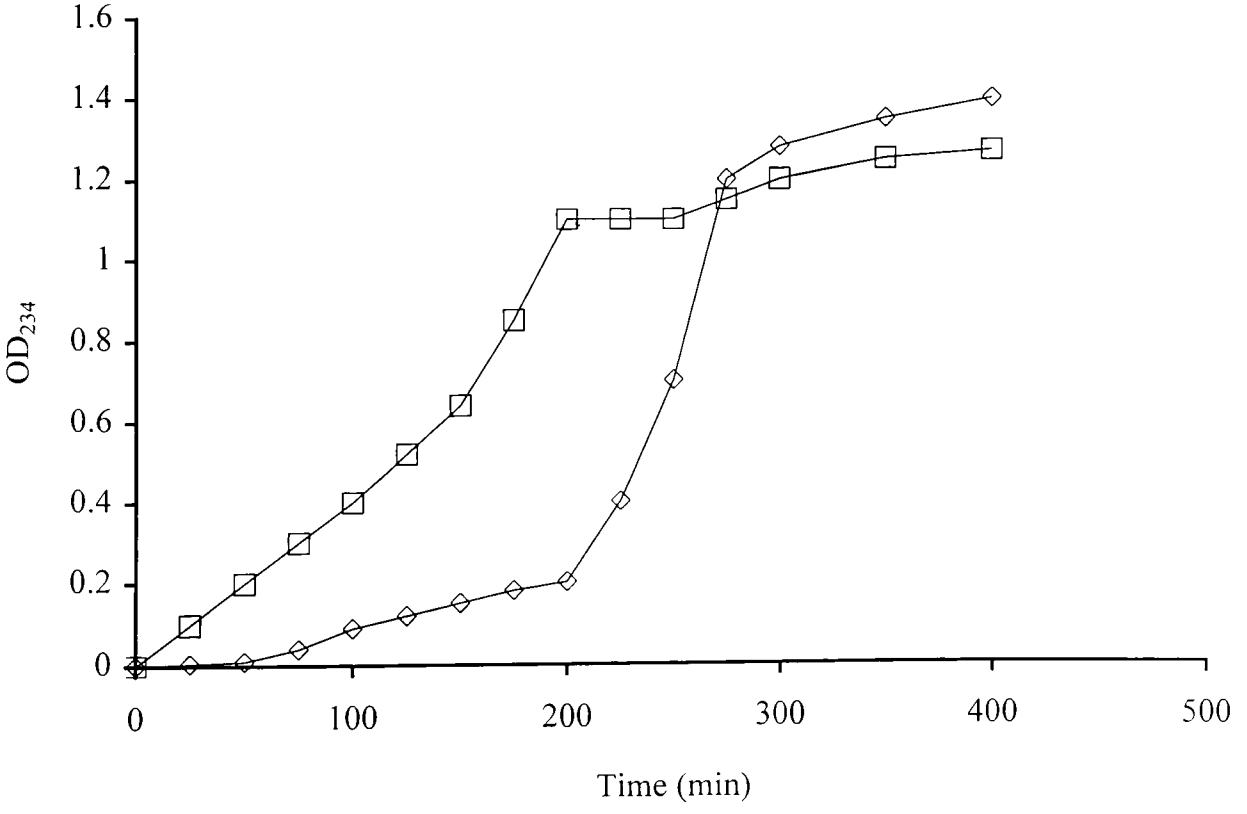


FIG. 7. Oxidation kinetics after exposure to 0.1 m*M* copper sulfate, of oxidatively modified LDL (m-LDL) (□) and of freshly prepared LDL (◇).

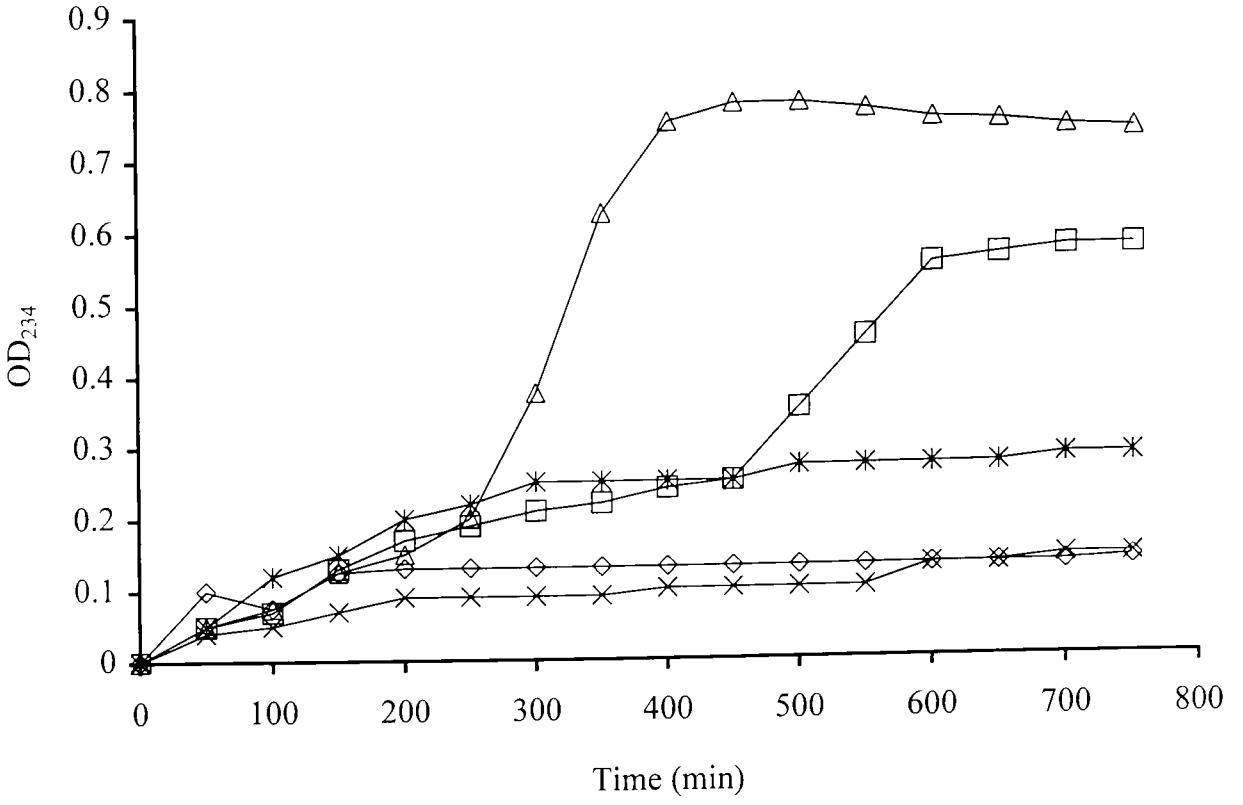


FIG. 8. Oxidation kinetics after exposure to 0.1 m*M* copper sulfate of freshly prepared LDL without AMTP protection (△), with 1 m*M* AMTP-B (◇), and with 1 m*M* AMTP-3A (×); and of m-LDL with 1 m*M* AMTP-B (□) and with 1 m*M* AMTP-3A (✱).

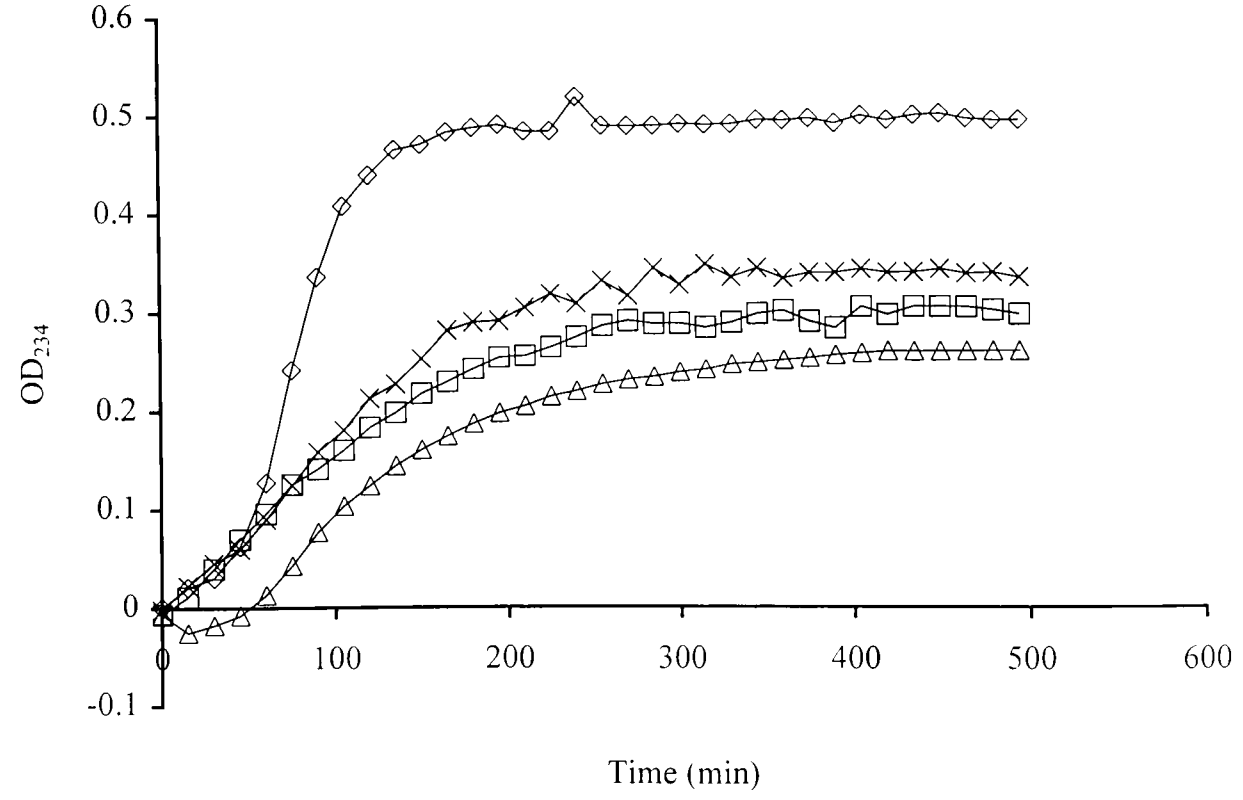


FIG. 9. Lipoprotein oxidation kinetics following i.p. administration to guinea pigs of 200 mg/kg (0.46 nmol/kg) α-tocopherol (×), 200 mg/kg (0.84 nmol/kg) AMTP-3A (□), 400 mg/kg (1.8 nmol/kg) AMTP-B (△), and vehicle (water/alcohol, 1:2) (control) (◇). (The results were taken from two separate experiments.)

Metal-chelating activity of AMTP

^{31}P NMR of a solution of AMTP-B (organic phosphate) in potassium hydrogen phosphate buffer (ionized, inorganic phosphate) in the presence of the paramagnetic Cu^{2+} ions at half the concentration of the inorganic phosphate and above shows elimination of the inorganic ^{31}P peak. The AMTP-B ^{31}P chemical shift was not affected, and only moderate change in the peak broadening at the middle of its height was observed (from 11 to 23 Hz, probably due to the high concentration of copper in the solution) without significant change in the area under the curve. That is, copper was chelated with the ionized phosphate of the buffer but not with the substituted phosphate of the AMTP-B. (AMTPs are not copper chelators, although further study is needed on the nature of their interactions with metals).

DISCUSSION

The role of LDL oxidative modification in atherogenesis is not clear. One possibility for clarifying this controversial issue is to use inhibitors that have defined effects on the oxidative processes, and to relate their effects on the oxidative damage to their ability to affect atherogenesis. This study has two goals: (i) to evaluate the antioxidant activity of AMTP in protecting LDL lipids against oxdiative damage, and (ii) to investigate AMTP use as a tool in elucidating the mechanism of LDL oxidation.

AMTPs protect LDL against Cu^{2+}-induced oxidation

The experiments presented here showed that AMTPs efficiently inhibited LDL oxidation. Exposing LDLs to Cu^{2+} ions leads to the oxidative degradation of the particles lipids and the Apo-B 100. AMTPs protected against the oxidative damage to the LDL lipids (Figs. 2–3, 5, 8). AMTP-3A is a more effective antioxidant than AMTP-B. This is related to its greater ability to reduce peroxides (Fig. 4A). Administration of AMTPs to guinea pigs reduced to a large extent the oxidizability of LDL. AMTP-B and AMTP-3A inhibited the initiation of LDL oxidation by Cu^{2+} in the presence of plasma proteins; however, in the purified LDL fraction, only AMTP-3A was effective as an antioxidant. In the presence of plasma proteins, the initiation rate of oxidation by Cu^{2+} might be reduced, due to protein binding of Cu^{2+} (indeed, in unfractionated dialyzed plasma, no oxidation was induced by Cu^{2+}; data not shown). Therefore, the difference in antioxidant activity of the two AMTPs can be explained by the availability of Cu^{2+} to initiate oxidation. In the LDL-rich fraction where proteins were present (*e.g.*, albumin), the effect of copper-induced oxidation was probably dimmed due to the binding capacity of the present proteins. Therefore, AMTP-B, which is less potent as an antioxidant, can protect. A mechanism of action of lipid hydroperoxides decomposition by AMTPs and preventing Cu^{2+}-dependent initiation of LDL oxidation can explain the lower antioxidant capacity of AMTPs to protect the partially oxidized LDL particles that are more susceptible to Cu^{2+}-induced oxidation. Enhanced protection of AMTP-B compared to AMTP-3A administered to guinea pigs in decreasing the oxidizability of LDL could be due to combination of many factors, *e.g.*, different pharmacokinetic and different *in vivo* distribution of the two compounds. Protection against Cu^{2+}-induced oxidation by the AMTP compounds could be due to neutralization of lipid hydroperoxides in the plasma, thereby reducing LDL oxidizability, and/or due to partitioning into the lipoprotein particle, which was not removed by the Sephadex G-200 gel filtration.

The prevention of LDL oxidation by AMTPs may make them potential antiatherosclerosis agents. A special structural design of AMTPs (attaching them covalently to relevant biological molecules such as phospholipids and cholesterol) might be used to target them to areas such as the intima of the arterial wall.

AMTPs as a tool to assess the mechanism of LDL oxidation

The role of the antioxidant system of LDL is not fully understood. It is already established that the mechanism of LDL oxidation induced by metals is different from the oxidation by a metal-independent system such as lipophilic

and hydrophilic azo compounds (Frei and Gaziano, 1993). When using a metal independent lipid peroxidation initiator such as AAPH for the oxidation of LDL, the rate of radical generation is one of the main factors determining whether α-tocopherol acts as a chain-breaking antioxidant or as a pro-oxidant that imports radicals into the LDL interior (radical-chain-transfer agent) (Bowry *et al.*, 1992; Bowry and Stocker, 1993; Ingold *et al.*, 1993).

It has been suggested that metal-induced oxidation of LDL may be biologically relevant: Catalytic amounts of redox-active metals are required to promote LDL oxidation *in vitro* by all major cell types present in the arterial wall when grown in cell culture (Heinecke *et al.*, 1984; Steinbrecher *et al.*, 1984). However, the role of metal ions in the development of atherosclerotic lesions *in vivo* is still uncertain (Berliner and Heinecke, 1996).

In metal-induced oxidation, the mechanism is even less clear than in the AAPH model. Several attempts have been made to correlate the oxidizability of LDL to their antioxidant content. However, their antioxidant content failed to explain the oxidizability differences in LDL (Berliner and Heinecke, 1996). A few correlation studies have tried to identify components of human LDL that contribute to their susceptibility to oxidation (Frei and Gaziano, 1993; Kontush *et al.*, 1996). It was shown that 65% of the lag phase time (T_{lag}) appears to be determined by unidentified factors; only 18% is determined by the α-tocopherol and 15% by the endogenous peroxides in the LDL (all the other antioxidants had no effect on the LDL oxidizability) (Frei and Gaziano, 1993). Maiorino *et al.* (1995) reported that short incubation of LDL with Cu^{2+} caused a total depletion in LDL tocopherols, but following dialysis and reincubation of LDL with Cu^{2+}, the oxidation kinetics was almost the same as in the LDL without pretreatment. These experiments suggest that in metal-induced oxidation the tocopherol content has only a small effect on the LDL oxidation lag phase.

The AMTP antioxidants do not affect the propagation of lipid peroxidation by scavenging peroxyl radicals (Fig. 6 and Tirosh *et al.*, 1996). However, they do interact, with different degrees of reactivity (depending on their type) with peroxides and thereby inhibit lipid peroxidation initiation (Fig. 4 and Tirosh *et al.*, 1996). We took advantage of this selectivity and used it to investigate the importance of the initial degree of LDL oxidation on its oxidation kinetics. Our results clearly demonstrated that not only chain-breaking antioxidants such as tocopherol, but also antioxidants that inhibit initiation of peroxidation have the ability to control the oxidation kinetics and the length of the lag phase in LDL oxidation. The mechanism may be one of decreasing the level of lipid hydroperoxides or of preventing their formation. When the initial level of the lipid hydroperoxides was high, the protection of the AMTP was reduced, as was shown in the m-LDL, which had a higher level of conjugated diene hydroperoxides than did fresh LDL. Oxidation kinetics of m-LDL was different from that of fresh LDL in lacking the lag phase (Fig. 7), although the α-tocopherol content of fresh and m-LDL was almost identical. Our oxidation experiments demonstrated that in copper-induced oxidation the initial degree of oxidation is a major factor in determining kinetics and level of LDL oxidation.

The reactions that are responsible for the initiation of LDL oxidation are:

$$1.\ Cu^{2+} + LOOH \rightarrow LOO^{\bullet} + Cu^{+}$$

$$2.\ Cu^{+} + LOOH \rightarrow LO^{\bullet} + Cu^{2+}$$

In LDL particles the number of lipid hydroperoxide molecules/LDL is extremely small (0.125) (Frei and Gaziano, 1993). Niki and co-workers (Nagata *et al.*, 1996) showed that lecithin:cholesterol acyl transferase (LCAT) catalyzes the formation of cholesteryl ester hydroperoxides (CEOOH) from phosphatidylcholine having hydroperoxide acyl chains (phosphatidyl hydroperoxide, PCOOH). CEOOH is much more lipophilic than PCOOH (although it is less lipophilic than unoxidized cholesteryl esters). Most of CEOOH is present in the LDL core (although a small part of it is partitioned into the interface between the LDL core and envelope). Therefore, CEOOH is less exposed to metal ions than is PCOOH, which is located entirely in the LDL envelope (Nagata *et al.*, 1996). In addition, plasma glutathione

peroxidase reduces PCOOH to their alcohols (Yamamoto *et al.*, 1993; Nagata *et al.*, 1996), thereby reducing the availability of PCOOH for the oxidation process. In LDL oxidation, accumulation of CEOOH is faster than PCOOH (Gotoh *et al.*, 1996). This fact and the abovementioned physiological mechanisms imply that removal of the initial unstable oxidation product (CEOOH) is of crucial importance in protecting LDL against oxidation.

Pro-oxidant activity in LDL can be due to the α-tocopherol content of the LDL. Recently it was shown that Cu^{2+} cannot oxidize LDL that is free of tocopherol and lipid hydroperoxides (Neuzil *et al.*, 1997). Tocopherol-mediated peroxidation might be due to two reactions (Nagaoka *et al.*, 1990):

$$3.\ Toc^{\bullet} + LH \rightarrow TocH + L^{\bullet}$$

$$4.\ Toc^{\bullet} + LOOH \rightarrow TocH + LOO^{\bullet}$$

The rate of reaction 4 is 1,000-fold faster than reaction 3 (Nagaoka *et al.*, 1990) and both reactions recycle tocopherol. AMTP inhibits the initiation of lipid peroxidation by decomposition of lipid hydroperoxides (Fig. 4B) and therefore prevents reaction 4. This suggests that lipid hydroperoxides are important in tocopherol-mediated peroxidation of LDL, and by removing them oxidation is suppressed even in the presence of intact levels of LDL tocopherols.

In conclusion, this is the first time that antioxidants that operate only as peroxide reducers are evaluated for LDL protection. Several attempts to address this issue have been made in the past using ebselen as the protector. However, recently it was shown that ebselen protects LDL against oxidation via its ability to scavenge peroxyl radicals and not only as a peroxide reducer (Lass *et al.*, 1996). It appears that AMTPs are the only available antioxidants that can be used to study this issue.

AMTP compounds may represent a new approach in the design of antioxidants. The mechanism by which they prevent LDL oxidation implies that oxidative damage to lipids might be inhibited by decomposition of unstable ROS such as lipid hydroperoxides, instead of by reacting with a free radical, *e.g.*, peroxyl radical, in an attempt to break the radical chain. AMTPs' unique qualities might serve as a tool that will allow us to gain a better understanding of the factors involved in biological oxidation processes.

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ABBREVIATIONS

AAPH, 2,2'-diazobis(2-amidinopropane 2 HCl; AMPT, Amidothionophosphate; AMTP-3A, *N,N',N"*-tripropyl-amidothionophosphate; AMTP-B, 2-hydroxy-ethylamido, diethylthionophosphate; CEEOH, cholesteryl ester hydroperoxides; CV, cyclic voltammetry; EPC2, egg-phosphatidylcholine; GC, gas chromatography; H_2O_2, hydrogen peroxide; LCAT, lecithin: cholesterol acyl transferase; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; PBS, phosphate- buffered saline; PCOOH, phosphatidyl hydroperoxidases; PUFA, polyunsaturated fatty acid; R-PE, R-phycoerythrin; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SUV, small unilamellar liposomes; TLC, thin-layer chromatography.

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