Paradoxical Protective Effect of Aminoguanidine toward Low-Density Lipoprotein Oxidation: Inhibition of Apolipoprotein B Fragmentation without Preventing Its Carbonylation. Mechanism of Action of Aminoguanidine[†]

Iness Jedidi,[‡,§] Patrice Thérond,[§,||] Stéphanie Zarev,[§,⊥] Claudine Cosson,[§] Martine Couturier,[§] Christian Massot,[#] Daniel Jore,[‡] Monique Gardès-Albert,[‡] Alain Legrand,[⊥,○] and Dominique Bonnefont-Rousselot*[,⊥,△]

CNRS UMR8601, Laboratoire de Chimie-Physique, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France, INSERM U347, 80 rue du Général Leclerc, 94276 Le Kremlin Bicêtre, France, Laboratoire de Biochimie, Hôpital André Mignot, 177 rue de Versailles, 78157 Le Chesnay Cedex, France, Laboratoire de Biochimie Métabolique et Clinique (EA 3617), Faculté de Pharmacie, 4 avenue de l'Observatoire, 75270 Paris Cedex 06, France, INSERM CRI-IFR69, 16 avenue Paul Vaillant Couturier, 94807 Villejuif Cedex, France, Laboratoire de Biochimie 1, Hôpital de Bicêtre AP-HP, 78 rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex, France, and Laboratoire de Biochimie B, Hôpital de la Salpêtrière AP-HP, 47 boulevard de l'Hôpital, 75651 Paris Cedex 13, France

Received April 4, 2003; Revised Manuscript Received July 7, 2003

ABSTRACT: Oxidative modification of low-density lipoproteins (LDLs) is an important feature in the initiation and progression of atherosclerosis. Aminoguanidine (AMG), classically described as an inhibitor of advanced glycation end products, turned out to be also efficient in animal models as an antioxidant against lipid peroxidation. The originality of the present study was based on the simultaneous assessment of the oxidation of LDL lipid and protein moieties in order to characterize the molecular sites of AMG protection. Oxidation of the LDL lipid moiety was monitored by measuring conjugated dienes (CD) and hydroperoxide molecular species from cholesteryl esters (CEOOH) and phosphatidylcholines (PCOOH). LDL protein oxidative modifications were assessed by evaluating apoB carbonylation and fragmentation. The LDL oxidation was mediated by water γ radiolysis, which has the advantage of being quantitative and highly selective with regard to the free radicals produced. Here, we reported that AMG resulted in a protection of LDLs against lipid peroxidation (both in the lag phase and in the propagation phase) and against apoB fragmentation in a concentration-dependent manner, due to the scavenging effect of AMG toward lipid peroxyl radicals. Paradoxically, AMG was poorly efficient against apoB carbonylation that began during the lag phase. We hypothesize that, even in the presence of AMG, a nonnegligible proportion of $^{\bullet}$OH radicals remained able to initiate oxidation of the LDL protein moiety, leading to apoB carbonylation.

Among the pathological processes which are mainly implicated in early stages of atherosclerotic lesion formation are biochemical modifications that affect the functional integrity of low-density lipoproteins (LDLs)[1] (*1*). Diabetes mellitus is often associated with a dyslipidemia which is partly characterized by defective lipoprotein uptake and metabolism. At long term, vascular complications including accelerated atherosclerosis can develop (*2*−*4*). There is still debate regarding compounds that are responsible for the oxidative modification of LDLs. The chemical modifications that render LDL particles more atherogenic could be induced both by reactive oxygen species (ROS) and by advanced glycation end products (AGEs) (*5*). Glycoxidation and lipoxidation seem to be intrinsically linked and implicated in atherogenesis (*6*−*8*).

Several natural antioxidants such as vitamin E, vitamin C, and β-carotene have been tested for their ability to protect LDLs from oxidation (*9*). Among the synthetic compounds, aminoguanidine (AMG), a nucleophilic hydrazine, has first been used for its ability to prevent chronic complications of diabetes by inhibiting AGE formation (*10*, *11*). Kinetic and mechanistic studies have shown that AMG acted by reacting with Amadori-derived fragmentation products (*12*, *13*). In some studies, it has also been suggested that AMG could have a potential antioxidant role. In a model of copper-catalyzed oxidation of LDLs, AMG has been described as an inhibitor of reactive aldehyde formation during lipid peroxidation, preventing their subsequent conjugation to

[†] This work was supported by a grant from the MENRT (Ministère de l'Education Nationale, de la Recherche, et de la Technologie).

* Address correspondence to this author at the Laboratoire de Biochimie Métabolique et Clinique (EA 3617), Faculté de Pharmacie. Tel: 01 53 73 96 10. Fax: 01 53 73 97 08. E-mail: dominique.rousselot@psl.ap-hop-paris.fr.

[‡] CNRS UMR8601, Laboratoire de Chimie-Physique.

[§] INSERM U347.

[||] Laboratoire de Biochimie, Hôpital André Mignot.

[⊥] Laboratoire de Biochimie Métabolique et Clinique (EA 3617), Faculté de Pharmacie.

[#] INSERM CRI-IFR69.

[○] Laboratoire de Biochimie 1, Hôpital de Bicêtre AP-HP.

[△] Laboratoire de Biochimie B, Hôpital de la Salpêtrière AP-HP.

[1] Abbreviations: AGE, advanced glycation end product; AMG, aminoguanidine; apoB, apolipoprotein B; CD, conjugated diene; CE, cholesteryl ester; CEOOH, cholesteryl ester hydroperoxide; 2,4-DNPH, 2,4-dinitrophenylhydrazine; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; LDL, low-density lipoprotein; OVA, ovalbumin; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; TBARS, thiobarbituric acid-reactive substances.

10.1021/bi034539w CCC: $25.00
Published on Web 08/26/2003

apoB (*14*, *15*). This evidence included the ability of AMG to inhibit formation of LDL lipid peroxidation products (TBARS) and LDL fluorescence at 420 nm (resulting from imino group formation) and to prevent enhanced mobility on agarose gel electrophoresis and macrophage uptake. Measurement of conjugated dienes (CD) in LDLs oxidized by Cu^{2+} in the absence or in the presence of AMG showed that AMG prevented CD formation in a concentration-dependent manner, suggesting a potential antioxidant role of AMG by inhibition of lipid peroxidation (*15*). Nevertheless, a prooxidant effect of AMG has been described at low concentrations (~0.01 mM) (*16*). To explain this, Fruebis et al. (*17*) proposed a concerted reaction between the amino group of a lysine residue of apoB and a lipid-derived peroxy radical ($RO_2^{\bullet}$) which could thus release the protonated form of $O_2^{\bullet-}$, that is, the $HO_2^{\bullet}$ hydroperoxyl radical. The latter radical would in turn propagate the lipid peroxidation. AMG, as a strong nucleophilic compound, could be able to make a similar concerted attack on a polyunsaturated fatty acid, which could result in a subsequent liberation of $HO_2^{\bullet}$. However, if AMG was present at concentrations higher than 0.05 mM, it could scavenge $HO_2^{\bullet}$ to form a nitrone, leading to an antioxidant activity of AMG (*16*). In contrast, other studies reported an in vitro antioxidant effect of AMG by trapping hydroxyl radicals ($^{\bullet}OH$) secondary generated both by addition of ferrous iron (*18*) and by the xanthine−xanthine oxidase (*19*) (at [AMG] > 5 mM). On the basis of these different studies, the underlying mechanism of AMG protection is not clear. Recently, Lisfi et al. reported a protective effect of AMG (0.2 < [AMG] < 5 mM) toward LDL peroxidation induced by $^{\bullet}OH/O_2^{\bullet-}$ free radicals generated by water γ radiolysis (*20*). This work described a concentration-dependent protective effect of AMG toward consumption of LDL endogenous antioxidants (vitamin E and β-carotene), formation of lipid peroxidation products (conjugated dienes and TBARS), and oxidative modification of apoB (relative electrophoretic mobility and fluorescence). However, these methods for assessing protein or lipid damage are indirect and lack specificity.

A recent report evaluated the effects of AMG on lipid peroxidation and protein oxidation in diabetic rat kidneys and showed that AMG treatment decreased both lipid peroxidation and carbonyl content (*21*). The present study aimed at evaluating more precisely the mechanism of protection of AMG toward both lipid and protein moieties upon LDL oxidation by water γ radiolysis. This oxidation method has the advantage of being quantitative and highly selective with regard to the free radicals produced, which allows to propose mechanisms for the antioxidant effect of AMG in this biological system. This oxidation process is physiologically relevant since it has been proposed (*22*) that hydroxyl radical damage might occur in advanced human atherosclerotic plaques and that the basal level of free radicals generated under physiological conditions could be involved in the atherogenic process. Our findings show that AMG protects LDLs against (i) lipid peroxidation by inhibiting formation of conjugated dienes (CD) and hydroperoxide molecular species from cholesteryl esters (CEOOH) and phosphatidylcholines (PCOOH) and (ii) apoB fragmentation, in a concentration-dependent manner. In contrast, apoB carbonylation was poorly inhibited by AMG.

EXPERIMENTAL PROCEDURES

Isolation of LDLs. LDLs were isolated from normolipidemic human plasma ($n = 20$) collected on EDTA (1.08 mM) by sequential ultracentrifugation (*23*). LDLs were then dialyzed against 10^{-2} M sodium phosphate buffer (pH = 7) and stored overnight at 4 °C. For the radiolysis experiments, the dialyzed solutions of LDLs were adjusted to a concentration of 3 $g{\cdot}L^{-1}$ by dilution in the same buffer. The purity of LDL preparations was checked by their chemical composition. LDL cholesterol, choline phospholipids, and triacylglycerols were assayed by enzymatic methods (*24*−*26*), and protein concentration was determined by a pyrogallol technique (*27*).

Oxidation of LDLs by Water γ Radiolysis. γ irradiations were carried out with a ^{137}Cs source (CIS BioInternational). The dose rate was 0.22 $Gy{\cdot}s^{-1}$ as determined by Fricke's dosimetry (*28*). Under these conditions, hydroxyl radicals ($^{\bullet}OH$) and superoxide anions ($O_2^{\bullet-}$) were simultaneously produced with yields of 2.8×10^{-7} $mol{\cdot}J^{-1}$ and 3.4×10^{-7} $mol{\cdot}J^{-1}$, respectively (*29*). Irradiations were performed on 1.5 mL of aerated aqueous LDL solutions in 10^{-2} M sodium phosphate buffer at pH 7 in the absence or presence of AMG at three different concentrations (0.2, 0.5, and 1 mM). For each set of experiments (including LDLs with and without AMG), 1.5 mL of aerated nonirradiated LDL solution was taken as control. The radiation doses varied from 20 to 1000 Gy.

Conjugated Diene Measurement. Conjugated diene formation during LDL oxidation is classically used to monitor LDL lipid peroxidation and is characterized by three phases, i.e., (i) the lag phase resulting from the presence of endogenous antioxidants (vitamin E, β-carotene, etc.), able to inhibit LDL oxidation, (ii) the propagation phase resulting from lipid peroxyl radical formation ($LOO^{\bullet}$) by a chain mechanism that propagates oxidative degradation by abstraction of another labile allylic hydrogen atom from lipid, and (iii) the termination and decomposition phase (*30*−*32*).

The formation of conjugated dienes (CD) was immediately measured after irradiation by monitoring LDL differential absorbance at 234 nm, and the results were expressed as moles per mole of LDLs using a molar extinction coefficient of 27000 $M^{-1}{\cdot}cm^{-1}$ (*33*) and a mean LDL molecular mass of 2.5×10^6 Da (*34*). Note that, under our experimental conditions (water γ radiolysis), very low concentrations of 7-ketocholesterol (absorbance at 234 nm) were formed, which did not interfere with CD measurement (*34*).

Identification of Molecular Species of Cholesteryl Esters and Phosphatidylcholines and of Their Corresponding Hydroperoxides. Total lipids were isolated from native and irradiated LDLs incubated in the absence or in the presence of AMG by hexane/methanol extraction (5/2 v/v). After separation of the hexane layer containing cholesteryl esters (upper phase) and the methanol layer containing phospholipids (lower phase) by centrifugation at 1500*g* for 10 min, the samples were evaporated to dryness under a nitrogen stream and stored at −80 °C. The dried lipid residues containing cholesteryl esters and phospholipids were then dissolved in hexane and/or methanol, respectively, and injected into the high-performance liquid chromatography (HPLC) system. Cholesteryl ester separation was carried out at 30 °C with a 150 × 4.60 mm C18 Spherisorb column.

The mobile phase was methanol. Phospholipid separation was performed at 40 °C with a 250 × 4.6 mm C18 Kromasil column. The mobile phase was composed of 6% 10 mM ammonium acetate and 94% methanol. Hydroperoxides from each molecular species of phosphatidylcholines (PC) [linoleate (16:0/18:2, 18:0/18:2), arachidonate (16:0/20:4, 18:0/20:4), and docosohexaenoate (16:0/22:6, 18:0/22:6)] or cholesteryl esters (CE) (linoleate and arachidonate) have been detected by chemiluminescence (CL) (*35*) and identified by their retention times. Polyunsaturated fatty acids (PUFA) were determined at 205 nm by an ultraviolet (UV)−visible light detector (Thermo Separation Products). Each hydroperoxide was quantified on the basis of the relative sensitivity of the CL assay for 15-hydroperoxyeicosatetraenoic (15-HPETE) and 13-hydroperoxyoctadecadienoic (13-HPODE) acids obtained from BIOMOL (TEBU, France). The limit of hydroperoxide detection was 30 pmol, and no hydroperoxide was detected in isolated LDLs.

HPODE Assay. To check that hydroperoxides are not decomposed in the presence of AMG (and reciprocally) under our conditions, 13-HPODE was incubated for 1 h with AMG or oxidized AMG; 10^{-5} M 13-HPODE was resuspended in 10^{-2} M sodium phosphate buffer containing 0.5 mM AMG or 0.5 mM AMG solutions irradiated at 503 and 600 Gy.

Preparation and Oxidation of 16:0/18:2 PC Liposomes. To study a phospholipidic model without protein (apoB), 0.25 × 10^{-3} M 16:0/18:2 PC liposomes (concentration close to that of LDL PC) were prepared by sonication according to the procedure of Cubillos et al. (*36*). The size of the unilamellar vesicles obtained was 60−75 nm. Oxidation of these liposomes and analysis of the corresponding hydroperoxides were performed as described above for LDLs.

Quantification of Oxidatively Modified LDL ApoB Using Western Blot Immunoassay. Oxidative modifications of apoB by oxygen free radicals result from carbonyl group formation into protein side chains (protein C═O). To detect apoB carbonyls, the carbonyl groups into the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH), using an Oxyblot oxidized protein detection kit from Oncor (Quantum Appligene, Illkirch, Bas-Rhin, France) (*37*). Briefly, 20 μL of LDL native or oxidized solutions (3 g·L^{-1}) was dissolved in 9 μL of 20% SDS and vortex-mixed with an equal volume of DNPH derivatization solution. Controls were treated with an equal volume of control solution without DNPH from the kit. The mixture was incubated at room temperature for exactly 15 min and after neutralizing, β-mercaptoethanol was added to the non-DNPH-derivatized and to the DNPH-derivatized preparations (*38*). This treatment denatured apoB, rendering apoB carbonyls equally accessible to DNPH.

The treated samples (native and oxidized LDLs) and negative control were separated on a 4−10% exponential gradient SDS−PAGE according to Fairbanks et al. (*39*) for 3 h at 40 V. Samples were then transferred to nitrocellulose membranes for 2 h at 150 mA. Membranes were immunoblotted with the rabbit anti-dinitrophenyl (DNP) antibody at 1:150 dilution, incubated with horseradish peroxidase-conjugated secondary antibody at 1:300 dilution. Carbonyl groups were visualized by chemiluminescence with Supersignal West Pico chemiluminescent substrate from Pierce Chemical Co. (Perbio Science, France) after exposure to radiographic film. To quantify apoB carbonyl groups, an external standard consisting of irradiated ovalbumin (OVA) at 50 Gy has been chosen, considering its absence in the human plasma and its molecular weight (45 kDa) that is lower than the apoB molecular weight (∼500 kDa). At this radiation dose, we checked whether OVA was carbonylated and for the absence of carbonylated OVA fragments. Quantification of carbonyl groups from oxidized OVA has been performed with a spectrophotometric method, using the carbonyl-specific reagent DNPH (*38*, *40*, *41*). Carbonyl content was calculated from the absorbance at 365 nm using a molar absorption coefficient of 22000 M^{-1} cm^{-1} (*40*). The variation coefficient of the method, based upon eight experiments, was 8.4%. For western blot, irradiated OVA has also been subjected to the derivatization reaction by 2,4-DNPH in parallel with native and oxidized LDLs. The repeatability and reproducibility of the carbonylated signal band related to OVA were determined, and variation coefficients were 12.4% and 15.1%, respectively. In our conditions, the intensity of western blot scans was linear over the ranges used.

After exposure to a radiographic film, the scanned films were quantified using the 45 kDa carbonylated signal band of irradiated OVA.

Data Analysis. CD, PCOOH, and CEOOH formation as a function of radiation dose was fitted by nonlinear regression analysis to the Hill equation:

$$E = E_{max} D^n / (DE_{50} + D)^n$$

where E was the formation of CD or PCOOH or CEOOH, E_{max} was the maximum of CD or PCOOH or CEOOH formation, D was the radiation dose, DE_{50} was the radiation dose at which CD or PCOOH or CEOOH formation was half-maximal, and n was the Hill coefficient, which was directly related to the slope parameters.

Statistical Analysis. The significance of differences between the mean values of various parameters was analyzed by ANOVA, followed by Student's *t*-test to assess differences among mean values. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

AMG Acts as an Inhibitor of Hydroperoxide Formation. (A) In LDLs. Figure 1 describes conjugated diene (CD) and total hydroperoxide formation from phosphatidylcholines (linoleate, arachidonate, and docosohexaenoate) (PCOOH) and cholesteryl esters (linoleate and arachidonate) (CEOOH) in 3 g·L^{-1} LDLs (physiological plasma LDL concentration) as a function of the radiation dose, in the absence or presence of AMG at the three concentrations studied (0.2, 0.5, and 1 mM). These data are fitted to the Hill equation, which is characterized by sigmoidal concentration effect curves (that is, in our model, radiation dose−LDL oxidation curves).

Comparison of the kinetics of CD (panel A) and of PCOOH and CEOOH (panels B and C) formation indicates that hydroperoxide formation was concomitant with the beginning of the CD propagation phase in the absence or presence of AMG. In LDLs without AMG, the propagation phase for CD began at about 80 Gy (end of the lag phase). Addition of AMG to the LDL solutions resulted in a marked prolongation of the lag dose in a concentration-dependent

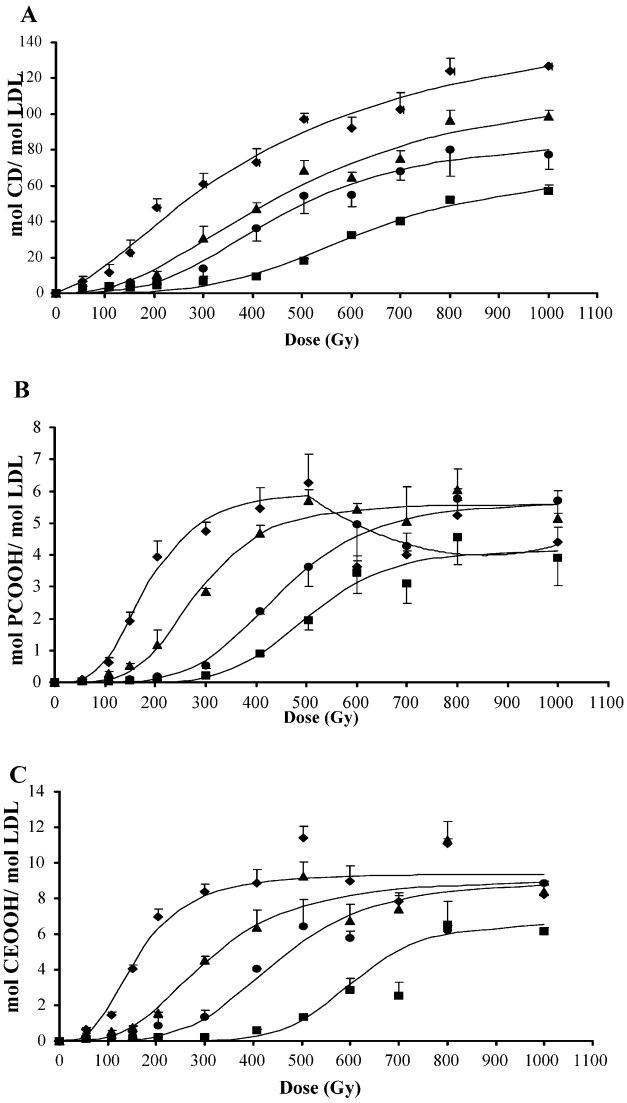

FIGURE 1: AMG prevents lipid peroxidation in LDLs upon γ radiolysis. Before irradiation, 3 g·L^{-1} LDLs were incubated in sodium phosphate buffer at pH = 7 alone (◆) or in the presence of 0.2 mM AMG (▲), 0.5 mM AMG (●), or 1 mM AMG (■). CD formation (panel A) was monitored at 234 nm; total hydroperoxides (PCOOH, panel B; CEOOH, panel C) were analyzed by HPLC as described in Experimental Procedures. The curves are the result of four to eight separate experiments. All control LDLs without AMG have been represented on a single curve.

manner. Indeed, with 0.2, 0.5, and 1 mM AMG, the propagation phase of CD formation began at about 130, 200, and 300 Gy, respectively. The lipid oxidative modifications monitored by PCOOH and CEOOH in the absence of AMG showed an increase of PCOOH and CEOOH formation as a sigmoidal curve until 400 Gy. A PCOOH degradation was observed after 500 Gy (not observed with CEOOH) and was likely due to a secondary PCOOH attack by free radicals. In the presence of AMG, the hydroperoxide formation from CE and PC was inhibited in a concentration-dependent manner, and no secondary PCOOH degradation was observed. The shape of the curves, as indexed by the Hill coefficient, was similar for PCOOH and CEOOH, in the absence of AMG ($n \approx 3.5$), and for each of the three AMG concentrations studied ($n \approx 4$ for 0.2 mM AMG; $n \approx 4.7$ for 0.5 mM AMG; $n \approx 7$ for 1 mM AMG). Nevertheless, it can be noted that the kinetics of CD formation as a function

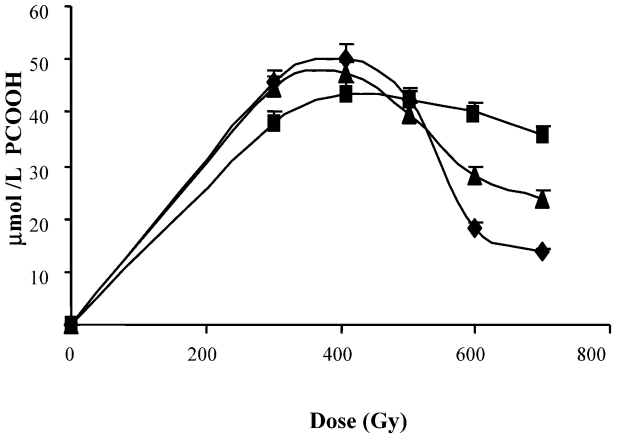

FIGURE 2: AMG protects against PCOOH degradation. Total PCOOH formation in 16:0/18:2 PC liposomes as a function of the radiation dose is represented in the absence of AMG (◆) or in the presence of AMG at 0.2 mM (▲) or 1 mM (■).

of the radiation dose were not similar to that of PCOOH and CEOOH in the absence ($n \approx 1.5$), as well as in the presence of AMG ($n \approx 2.3$ for 0.2 mM AMG; $n \approx 3.1$ for 0.5 mM AMG; $n \approx 3.9$ for 1 mM AMG).

In the absence or in the presence of 0.2 and 0.5 mM AMG, a plateau was observed at about 600 and 700 Gy, respectively, both for PCOOH and CEOOH ($E_{max} = 5.5$ for PCOOH and $E_{max} = 9$ for CEOOH). With 1 mM AMG, the plateau was obtained at about 800 Gy for PCOOH and CEOOH; this plateau was significantly lower than that obtained in the absence or in the presence of 0.2 and 0.5 mM AMG ($E_{max} = 4$ for PCOOH and $E_{max} = 6$ for CEOOH). It can be noted that the Hill coefficient and the E_{max} obtained with 1 mM AMG differed from those obtained in the absence or presence of AMG at 0.2 or 0.5 mM.

It is interesting to highlight that AMG protected against oxidation all PUFA molecular species derived from PC [i.e., 16:0/18:2 (105 mol/mol of LDLs) and 16:0/(20:4 + 22:6) (40 mol/mol of LDLs)] and from CE [i.e., 18:2 (585 mol/mol of LDLs) and 20:4 (74 mol/mol of LDLs)] in the same proportion, although these PUFA were present at different concentrations in LDLs (data not shown).

(B) In Liposomes. To tentatively explain the absence of drop in PCOOH concentration observed in LDLs irradiated with AMG, hydroperoxide measurements have been performed in 0.25×10^{-3} M irradiated PC linoleate liposomes (action of $^{\bullet}OH/O_2^{\bullet-}$ free radicals) in the absence and in the presence of AMG (Figure 2). These kinetics were characterized by a propagation phase with a sudden decrease in PCOOH concentrations for radiation doses higher than 408 Gy. Indeed, in the absence of AMG, we showed a degradation of PCOOH after 408 Gy. In the presence of increasing concentrations of AMG, this degradation was decreased. In addition, no change in 13-HPODE concentration was observed in the presence of native or irradiated AMG, which suggests that (i) AMG did not induce hydroperoxide decomposition, (ii) hydroperoxides did not spontaneously decompose to form a carbonyl able to react with native or irradiated AMG, and (iii) AMG was not destroyed upon incubation with hydroperoxides. AMG does not seem to form a Schiff base with oxidized lipid because hydroperoxide lipids produced seemed to be stable in our conditions.

The Yield of Hydroperoxide Formation Is 1 Order of Magnitude Lower Than the Yield of Free Radical Production from γ Radiolysis. During the propagation phase, yields of CD, PCOOH, and CEOOH formation [i.e., G(CD), G(PCOOH), G(CEOOH)] have been determined from the slopes of the linear part of the curves exhibiting the CD, PCOOH, and CEOOH formation expressed in moles per liter as a function of the radiation dose. These yields, which are equivalent to rates of CD, PCOOH, or CEOOH formation, were compared to G(•OH) and G($O_2^{\bullet-}$) obtained by radiolysis. In the absence of AMG, the yield of CD formation was $(3.4 \pm 0.5) \times 10^{-7}$ mol·J^{-1}, that is, of the same order of magnitude as G(•OH) or G($O_2^{\bullet-}$) (see Experimental Procedures). However, it has been shown that superoxide radicals were poor initiators of lipid peroxidation (*42*). Therefore, •OH radicals were the main initiators of peroxidation processes in LDLs, so we will compare our yield values to G(•OH) and not to G($O_2^{\bullet-}$). The yields of CD formation were lowered by the presence of 0.2 and 0.5 mM AMG [$(2.2 \pm 0.2) \times 10^{-7}$ mol·J^{-1} for both AMG concentrations]. These yield values are slightly lower than G(•OH). However, the yield of CD formation was only $(1.3 \pm 0.1) \times 10^{-7}$ mol·J^{-1} in the presence of 1 mM AMG. A correlation appeared between the yield of CD formation and AMG concentrations ($r = 0.92$) (Figure 3, panel A).

Comparison of yields of CD formation (Figure 3, panel A) with those of PCOOH and CEOOH formation (Figure 3, panels B and C, respectively) shows that the yields of hydroperoxide formation in the absence or in the presence of AMG were 1 order of magnitude lower than the yields of CD formation. In the absence of AMG, the yields of PCOOH and CEOOH obtained, i.e., $(3.3 \pm 0.1) \times 10^{-8}$ and $(6.3 \pm 0.2) \times 10^{-8}$ mol.J^{-1}, respectively, are about 1 order of magnitude lower than G(•OH). These respective yield values dropped to $(2.6 \pm 0.4) \times 10^{-8}$ and $(2.9 \pm 0.7) \times 10^{-8}$ mol.J^{-1}, $(2.1 \pm 0.4) \times 10^{-8}$ and $(3.4 \pm 0.6) \times 10^{-8}$ mol.J^{-1}, and $(1.5 \pm 0.1) \times 10^{-8}$ and $(1.4 \pm 0.1) \times 10^{-8}$ mol·J^{-1} in the presence of 0.2, 0.5, and 1 mM AMG, respectively. Panels B and C of Figure 3 show the relationship between the yields of PCOOH and CEOOH formation and AMG concentration ($r = 0.98$ and $r = 0.85$, respectively).

AMG Does Not Prevent LDL ApoB Carbonylation. Carbonylated apoB and carbonylated apoB fragments have been assayed in native LDLs and in oxidized LDLs after radiolysis from 54 to 503 Gy. ApoB carbonylation was assessed by western blot (Figure 4). Typical immunoblots of apoB from nonoxidized LDLs and LDLs oxidized by γ radiolysis are shown after revelation with anti-DNP antibodies (Figure 4A). The quantification of the immunoblots is depicted in Figure 4B. Beyond 408 Gy, we did not distinguish between carbonylated apoB and carbonylated apoB fragments in the absence or presence of AMG.

In native LDLs, a slight signal of carbonylated apoB is detected which is subtracted from the signal observed with irradiated LDLs. In LDLs oxidized by γ radiolysis, carbonylated apoB appeared from 54 Gy during the lag phase of CD. This apoB carbonylation slightly but significantly increased as a function of the radiation dose. Revelation by Coomassie blue also showed that apoB disappeared as a function of the radiation dose (data not shown). The presence of AMG during LDL oxidation seemed to poorly protect apoB against carbonylation whatever the AMG concentration.

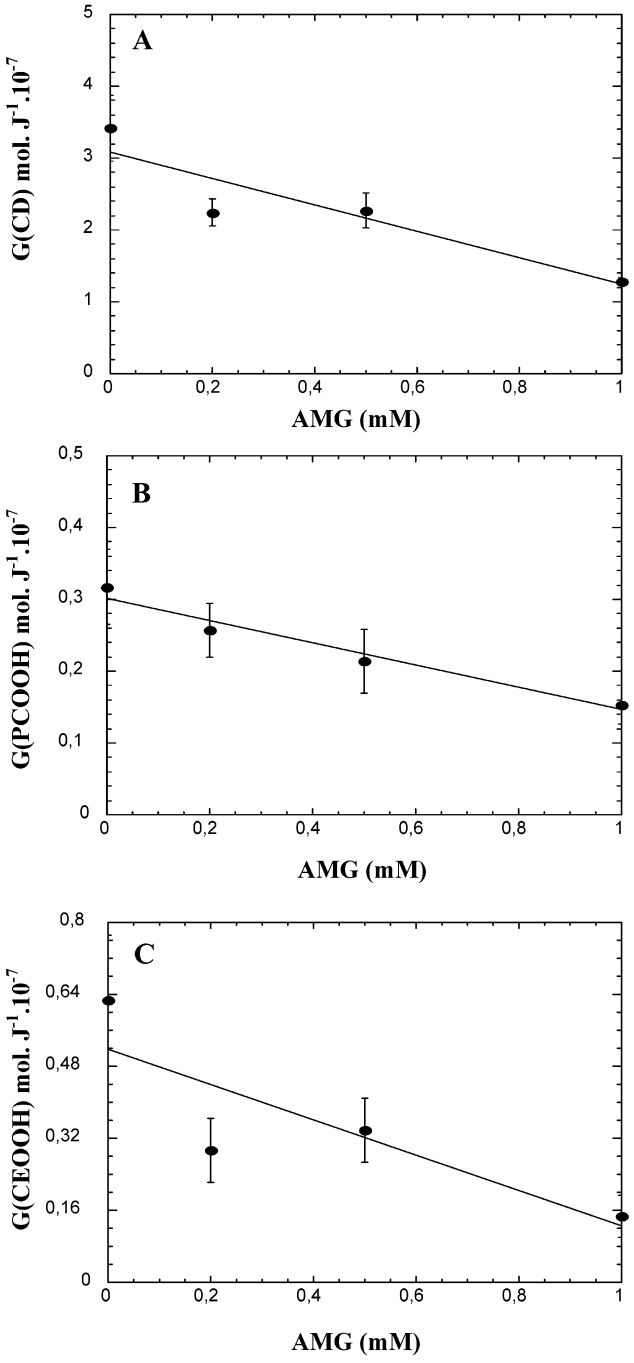

FIGURE 3: CD and hydroperoxide formation inversely correlate with AMG concentrations. Yields of CD, PCOOH, and CEOOH formation have been determined from slopes of the linear part of the curves exhibiting the CD, PCOOH, and CEOOH formation in moles per liter as a function of the radiation dose. Yields of CD (panel A), PCOOH (panel B), and CEOOH (panel C) formation in 3 g·L^{-1} LDLs are shown as a function of AMG concentrations. The data are derived from Figure 1, panels A−C.

The yields of carbonylated apoB formation [G(carbonyl apoB)] confirmed these results, since their values were $(1.8 \pm 0.1) \times 10^{-8}$ mol.J^{-1} in the absence of AMG vs $(1.5 \pm 0.1) \times 10^{-8}$, $(1.2 \pm 0.1) \times 10^{-8}$, and $(1.1 \pm 0.1) \times 10^{-8}$ mol·J^{-1} for 0.2, 0.5, and 1 mM AMG, respectively (insert, Figure 4B). It is noteworthy that these yields were 1 order of magnitude lower than those of free radicals produced by γ radiolysis. We have checked that no false positive reaction occurred in the carbonyl detection due to the presence of

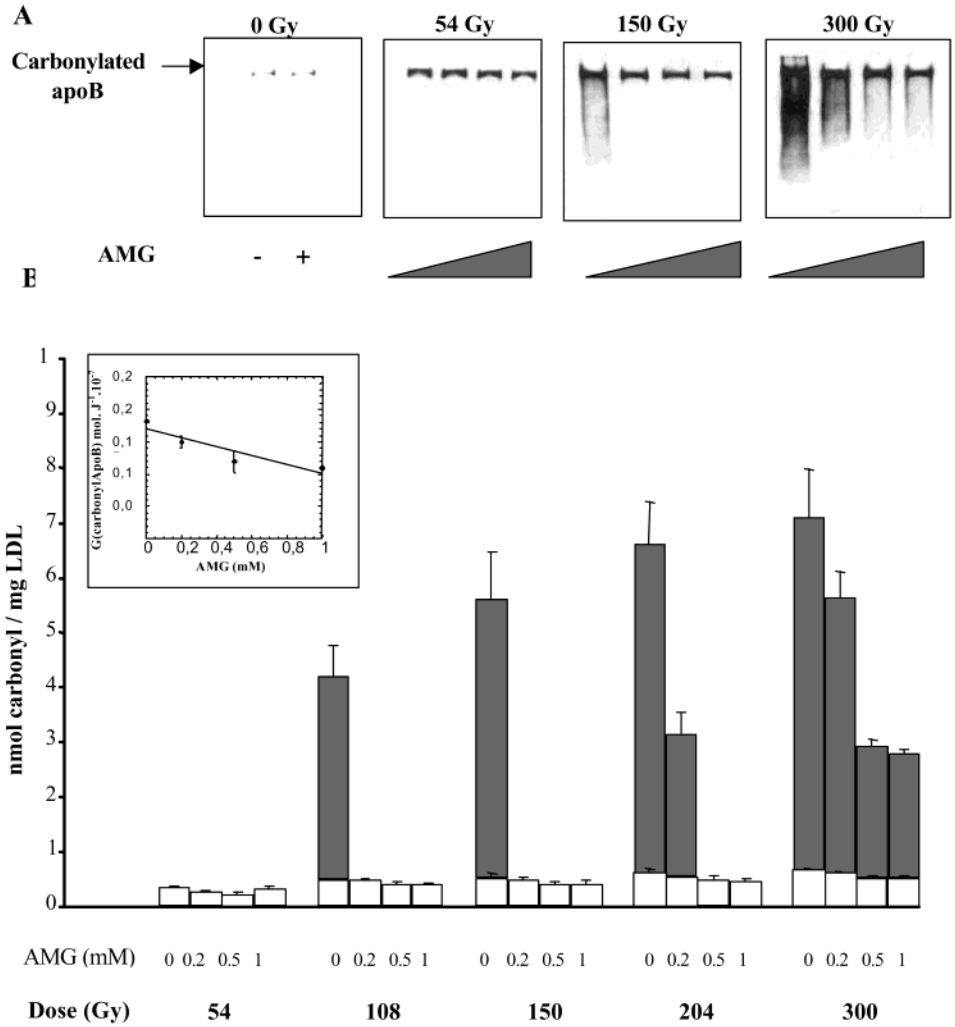

FIGURE 4: AMG inhibits fragmentation of carbonylated apoB. Panel A: Three typical immunoblot analyses of apoB from LDLs oxidized by γ radiolysis (0, 54, 150, and 300 Gy) in the absence or in the presence of increasing concentrations of AMG from 0.2 to 1 mM are shown. Panel B: Quantification of western blot analysis of carbonyl apoB (open bars) and carbonylated apoB fragments (solid bars) of irradiated LDLs (3 g·L^{-1}). The detection by immunoassay was described in Experimental Procedures. Insert: Yield of apoB carbonylation formation in 3 g·L^{-1} LDLs as a function of AMG concentration. These results are the mean of four to eight separate experiments.

AMG. Indeed, AMG itself is an amine compound that could compete with amine targets of the revelation system used (DNPH) for carbonylation and could contribute to the generation of reactive intermediate, via a Michael addition reaction. However, in the absence of DNPH, no signal was detected by immunoblot, which suggested that AMG was not involved in the apoB carbonylation.

In the absence of AMG, fragmentation of carbonylated apoB was detected from 108 Gy, just after the beginning of the propagation phase assessed by CD. This fragmentation increased in a dose-dependent fashion. In the presence of AMG, carbonylated apoB fragments also appeared in a dose-dependent manner and were detected from 204 Gy with 0.2 mM AMG and from 300 Gy with 0.5 and 1 mM AMG, concomitant with the beginning of the propagation phase assessed by CD. Therefore, in the presence of AMG, apoB fragmentation is totally inhibited until 150 Gy whatever the AMG concentration. However, the effectiveness of AMG to prevent this fragmentation decreased in a dose-dependent fashion. Thus, at 204 Gy, 57 ± 5% inhibition was obtained with 0.2 mM AMG vs 100% with 0.5 or 1 mM AMG. At 300 Gy, only 21 ± 1% inhibition was obtained with 0.2 mM AMG vs 58 ± 7% and 67 ± 1% with 0.5 or 1 mM AMG, respectively. It is noteworthy that, at 300 Gy, no difference of inhibition was noted between 0.5 and 1 mM AMG, whereas the formation of carbonylated apoB fragments with 0.2 mM AMG was significantly higher than with 0.5 and 1 mM AMG ($p < 0.0005$).

We have checked that carbonyl detection did not result from the presence of 4-hydroxynonenal (HNE), a lipid carbonyl. In our system, the concentration of HNE produced at 408 Gy and determined by HPLC was only 0.8 nmol of carbonyl/mg of LDL (data not shown), so it was negligible compared to the concentration of carbonylated apoB plus fragments obtained (≈8 nmol of carbonyl/mg of LDL). We have also check that fragmentation in the presence of AMG did not result from hydroperoxide degradation. 13-HPODE was incubated with native AMG or irradiated AMG. No change in 13-HPODE concentration was observed, suggesting that the hydroperoxide did not spontaneously decompose to form a carbonyl able to react with native or irradiated AMG (data not shown).

DISCUSSION

The originality of the present study was based on the simultaneous assessment of the oxidation of LDL lipid and

protein moieties in order to characterize the molecular sites of AMG protection. The main observation under our experimental conditions was that AMG inhibited LDL hydroperoxide formation and apoB fragmentation but did not protect apoB against carbonylation.

Inhibition of Lipid Peroxidation by AMG. Our data gave evidence for prevention of LDL lipid peroxidation and particularly hydroperoxide formation by AMG in a concentration-dependent manner. AMG acted at two levels, the lag phase and the propagation phase. Indeed, it extended the lag phase in a concentration-dependent manner for CD as well as for PCOOH/CEOOH formation, which is likely related to protection of LDL endogenous antioxidants (*20*). AMG also inhibited the propagation phase (as shown by the AMG-induced decrease in the yield values) in the same way for PCOOH and CEOOH. This antioxidant effect is in agreement with previous data obtained on conjugated dienes (*20*), suggesting that AMG acts by trapping lipid peroxyl radicals (LOO$^\bullet$).

Superoxide radicals are very poor initiators of LDL peroxidation, and their effect is totally inhibited in the presence of superoxide dismutase (SOD) (*43*, *44*). Thus, $^\bullet$OH radicals are the main efficient oxidants, and the yield of CD formation allows us, by comparing it to the yield of $^\bullet$OH production, to evaluate the proportion of these radicals attacking LDL particles. In the absence of AMG, the yield of G(CD) obtained was slightly higher than G($^\bullet$OH) due to the presence of a short chain reaction, which is in agreement with previous results of our group (*20*, *45*). In the presence of 0.2, 0.5, and 1 mM AMG, the yield of CD formation decreased, expressing a progressive inhibition of the propagation chain by scavenging of lipid peroxyl radicals. Moreover, the yield of CD formation became lower than G($^\bullet$OH), suggesting that a part of initiating $^\bullet$OH radicals were also scavenged by AMG. At high radiation doses, AMG clearly lowered the plateaus of PCOOH, CEOOH, and CD, which implies that AMG protected a part of the LDL oxidizable sites, by scavenging both a part of initiator $^\bullet$OH radicals and propagator peroxyl radicals. It is noteworthy that the yields of CD formation were 10 times higher than the yields of hydroperoxide formation, which relies on the fact that CD other than hydroperoxides (such as alcohols, epoxides, and endoperoxides) were taken into account.

Besides, our results showed that G(CEOOH) was about twice as high as G(PCOOH) in LDLs without AMG. These data could result from the PC/CE ratio present in LDLs but could also depend on their localization and on the three-dimensional structure of LDLs upon γ radiolysis. If we consider the number of PC and CE molecules per LDL particle, PC/CE $\approx$ 600/1600 = 0.4 (*31*), we could expect to obtain at high radiation doses a plateau of PCOOH/CEOOH close to this value, whereas the experimental value was close to 0.7 (see Figure 1). This difference could be explained by a better accessibility of PC targets (localized on the outer surface of LDL particle) than CE to free radicals.

No Inhibition of ApoB Carbonylation by AMG. The changes occurring in apoB during LDL oxidation (i.e., carbonylation and fragmentation) are usually considered to result from lipid peroxidation, at least upon a classical copper-induced oxidation (*46*). However, our study shows that apoB carbonylation appeared during the lag phase in the absence of detectable amounts of hydroperoxides and while vitamin E was not still consumed. This is in agreement with the observation of Zarev et al. (*38*), who reported that lipid peroxidation in radiolyzed LDLs did not precede nor induce apoB oxidation. Furthermore, our results showed that AMG did not clearly prevent apoB carbonylation whatever the radiation dose and AMG concentration. These data could let us hypothesize that apoB carbonylation would result from the action of a part of nonscavenged $^\bullet$OH free radicals (*47*). Stadtman (*48*) proposed that $^\bullet$OH radicals could initiate the carbonylation and the fragmentation of polypeptidic chains. Under our conditions, it would remain enough nonscavenged hydroxyl radicals to competitively attack the apoB protein generating carbonylation, due to the relatively poor reactivity of $^\bullet$OH free radicals toward the AMG molecule (*47*). Indeed, we had shown that AMG was not a good scavenger of $^\bullet$OH free radicals because hydrogen peroxide could compete with AMG in order to eliminate hydroxyl radicals (*47*). Given that the order of magnitude of the rate constant of $^\bullet$OH free radicals with hydrogen peroxide is 10^7 $M^{-1}\cdot s^{-1}$(*49*), it can be supposed that the rate constant of $^\bullet$OH free radicals with AMG is of the same order of magnitude ($k_{(^\bullet OH+H_2O_2)} \approx k_{(^\bullet OH+AMG)} \approx 10^7$ $M^{-1}\cdot s^{-1}$). Such k values are 2 or 3 orders of magnitude lower than those usually admitted for $^\bullet$OH reactions [10^9-10^{10} $M^{-1}\cdot s^{-1}$ (*49*)]. In the case of LDL solutions, $^\bullet$OH free radicals react on all of the molecular components of LDLs (lipid moiety, protein moiety, antioxidants, etc.) with a rate constant which is likely diffusion-controlled, that is, $\approx 10^{10}$ $M^{-1}\cdot s^{-1}$. Hence, it can be possible to write the equations of the rates of $^\bullet$OH free radicals with AMG and LDLs, respectively:

$$v_{(^\bullet OH+AMG)} = k_{(^\bullet OH+AMG)}[AMG][^\bullet OH] \approx 10^7[AMG][^\bullet OH]$$

$$v_{(^\bullet OH+LDLs)} = k_{(^\bullet OH+AMG)}[LDLs][^\bullet OH] \approx 10^{10}[LDLs][^\bullet OH]$$

Under our experimental conditions, LDL concentration is equal to 1.2×10^{-6} M (based on a mean LDL molecular mass of 2.5×10^6 Da), and the ratio of the rate $v_{(^\bullet OH+LDLs)}/v_{(^\bullet OH+AMG)}$ can be calculated for both AMG extreme concentrations:

$$[AMG] = (2 \times 10^{-4}\ M) \quad v_{(^\bullet OH+LDLs)}/v_{(^\bullet OH+AMG)} = 6$$

$$[AMG] = (10^{-3}\ M) \quad v_{(^\bullet OH+LDLs)}/v_{(^\bullet OH+AMG)} = 1.2$$

Such rate ratio values involve (i) a poor scavenging capacity of $^\bullet$OH free radicals by AMG for the lowest AMG concentration (2×10^{-4} M), which is in agreement with experimental results, and (ii) a more important but not complete scavenging capacity of $^\bullet$OH free radicals by AMG for the highest AMG concentration (10^{-3} M) corresponding to nearly 50% of $^\bullet$OH free radicals reacting with AMG and 50% with LDLs. Under the latter conditions, a nonnegligible fraction ($\approx$50%) of hydroxyl radicals would directly react with LDLs, which would explain the carbonylation of apoB whatever the AMG concentration.

Inhibition of ApoB Fragmentation by AMG. Carbonylated apoB fragments appeared at the end of the lag phase and from the beginning of hydroperoxide formation. Whereas

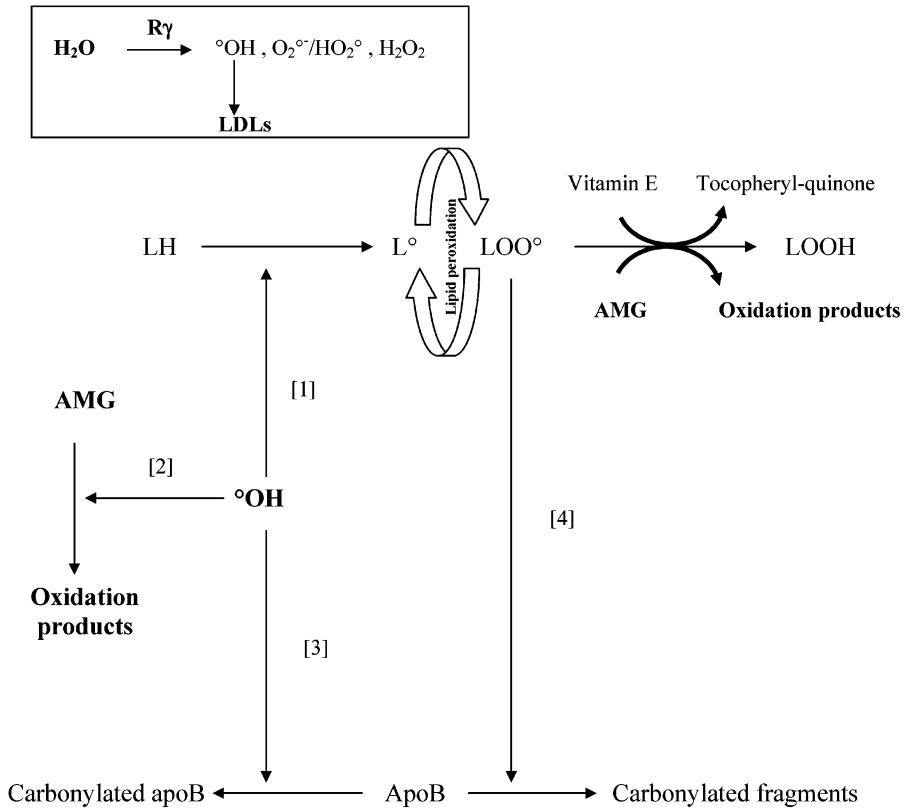

FIGURE 5: Hypothetical mechanism of action of AMG on LDL lipid peroxidation and apoB oxidation. Rγ = γ radiolysis; LH = an unsaturated fatty acid side chain.

AMG was poorly efficient on the apoB carbonylation process, it was able to inhibit apoB fragmentation in a concentration-dependent manner. The absence of reactivity of AMG with hydroperoxides (data not shown) and the inhibition of the propagation phase by AMG (as observed with the decrease of yield of CD formation) suggest that AMG inhibits the fragmentation of apoB by scavenging LOO•.

Mechanism of Action of AMG. Three factors need to be considered in analyzing the results. First, radical scavenging by aminoguanidine would be concentration-dependent. Second, radiolysis can generate radicals that are located adjacent to a target molecule. Third, the hydroxyl radicals do not discriminate among most target molecules. Consequently, as proposed in the scheme of Figure 5, these three factors result in competitive reactions 1, 2, and 3. Our findings suggest that apoB carbonylation is due to a fraction radical species (•OH) generated close to the target and not scavenged by AMG (reaction 3). With regard to apoB fragmentation, it was most likely due to lipid-derived propagating radicals (LOO•) which could be scavenged by AMG (reaction 4).

In conclusion, our results showed that AMG was an in vitro scavenger of hydroxyl and lipid-derived peroxyl free radicals under our experimental conditions, in a concentration-dependent manner. AMG protected LDLs against lipid peroxidation and apoB fragmentation but only poorly against apoB carbonylation, due to the fact that a part of hydroxyl radicals remained available to initiate protein oxidation in LDLs. By contrast, AMG inhibited apoB fragmentation by scavenging lipid-derived peroxyl radicals. This is of interest in the early phases of atherosclerosis where lipid−protein interactions might have important consequences for the mechanisms of LDL oxidation (*50*). It would be interesting to assess whether oxidative modifications of apoB that were described during the lag phase of free radical-mediated LDL oxidation could induce their recognition by receptors such as lectin-like oxidized LDL receptor-1 (LOX-1) that bind to the protein portion of oxidized LDLs (*51*). It will also be necessary to identify the products derived from aminoguanidine to prove the efficacy and safety of this drug (the pharmacological and toxicological study).

ACKNOWLEDGMENT

We are indebted to D. Averbeck for the use of the irradiator of Institut Curie (Paris), C. Corbière and H. Vitrac for help in experiments, S. Guerroui and J. Maccario for help in statistics and the mathematical model, and J. Delattre for helpful discussion.

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BI034539W