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Free and Peptide-bound DOPA Can Inhibit Initiation of Low Density Lipoprotein Oxidation

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Hydroxyl radicals have been shown to convert free tyrosine to 3,4-dihydroxyphenyl-alanine (DOPA) which has reducing properties. During protein or peptide oxidation such reducing species are also formed from tyrosine residues. Free DOPA or peptide-bound DOPA (PB-DOPA) is able to promote radical-generating events, facilitating the damage of biomolecules such as nucleic acids. Radical induced lipid oxidation in low density lipoprotein (LDL) transforms the lipoprotein into an atherogenic particle. As PB-DOPA has been found in atherosclerotic plaques, we tested the ability of free and PB-DOPA to influence LDL oxidation. Free DOPA, in contrast to tyrosine had strong inhibitory action on both, the copper-ion initiated and metal ion independent (AAPH-induced) lipid oxidation. Free DOPA also inhibited LDL oxidation induced by the copper transport protein ceruloplasmin. To test if PB-DOPA was also able to inhibit LDL oxidation, DOPA residues were generated enzymatically in the model peptides insulin and tyr-tyr-tyr, respectively. PB-DOPA formation substantially increased the ability of both molecules to inhibit LDL oxidation by copper or AAPH. We hypothesize that DOPA-peptides and -proteins may have the potential to act as efficacious antioxidants in the atherosclerotic plaque.

Keywords: LDL oxidation; DOPA; Atherosclerosis; AAPH; Copper; Cu^{++}

INTRODUCTION

The oxidative modification of low density lipoprotein (LDL) may play an important role in the early events of atherogenesis.[1] The *in vivo* pathophysiological relevant pathway(s) of atherogenic LDL alterations, however, have not been elucidated at present. *In vitro* studies have shown that LDL can be altered by a variety of biochemical reactions, e.g. modification by endothelial cells, smooth muscle cells, peroxynitrite, peroxidases, metmyoglobin, copper ions, NO radicals, tocopheryl radicals, ceruloplasmin and reaction products of the myeloperoxidase system of activated neutrophils.[2–11] Virtually all of these studies have focused on the alterations of the lipid moiety of the particle during the oxidative process. As a consequence, less is known about the apoprotein B-100 (apo B) modifications induced by these reactions. The work of Davies, Dean and Heinecke has brought some insight in the reactions which might *in vivo* lead to protein modifications in atherosclerosis and other human diseases.[12–18] However, the basic mechanisms of protein modifications by free radicals have been investigated and identified by Karam, Fong, Simpson, Gebicki and Gieseg.[19–23] Such modifications can lead to the formation of oxidizing (protein hydroperoxides) species which can consume cellular reductants such as ascorbate and glutathione and reducing species which can participate in further radical generation catalyzed by copper and iron ions. Many of the radical- and oxidant-damaged amino acids have been proposed as stable markers of oxidative protein attack. For a comprehensive recent review on this matter, see Davies *et al.*[12] For example, myeloperoxidase reaction products can modify protein tyrosine

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residues generating mono- and di-chlorotyrosine, 3-nitro- and 3,5-dinitrotyrosine as well as dityrosine.[17,18] These tyrosine compounds are chemically inert in contrast to the tyrosine reaction product formed by hydroxyl radical ($HO^{\bullet}$) attack, namely 2,4- and 3,4-dihydroxy- phenylalanine (DOPA) which is susceptible to further oxidation.[12] Protein- or peptide-bound DOPA (PB-DOPA) in presence of redox active metal ions (Fe^{3+} or Cu^{2+}) may have the potential to trigger further radical/oxidative damage to biomolecules.[24] As PB-DOPA has been found in advanced atherosclerotic plaques[25] as well as redox active metal ions[26] one may speculate that DOPA-peptides may facilitate lipid oxidation in LDL initiated by copper ions leading to enhanced atherogenic modification of the lipoprotein.

MATERIALS AND METHODS

Ceruloplasmin (human) was from Calbiochem. L-DOPA, mushroom tyrosinase (EC 1.14.18.1 6,300 units/mg protein), insulin (porcine), tyr-tyr-tyr, sodium borohydride, bovine serum albumin and nitro blue tetrazolium chloride (NBT) were obtained from Sigma. AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) was from Polysciences. All other chemicals used were of analytical grade.

Lipoprotein Isolation

LDL preparations were isolated by ultracentrifugation as reported previously.[27] The final preparations were dialyzed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA and filter sterilized. Protein was estimated by a modified Lowry method[28,29] using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml. Four different LDL preparations were used in this study.

LDL Oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in phosphate buffered saline (PBS) pH 7.4 using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (0.2 mg of protein/ml) was incubated with the respective compound at 37°C as indicated in the figure legends. Routinely, oxidation was initiated by the addition of Cu^{++} (5 μmol/l) or AAPH (10 mmol/l). All experiments were done in duplicate. Means and standard deviations (SD) are given.

Ceruloplasmin Preparation

Lyophilized ceruloplasmin was dissolved in distilled water and applied to a small Sephadex-column (Nick-column, Pharmacia) equilibrated in PBS and the eluted protein fraction was used in the experiments.

Estimation of LDL Oxidation

Conjugated dienes: Conjugated diene (CD) formation in LDL was measured as increase in absorbance at A_{234} nm. Untreated LDL served as blank. When CD were estimated in presence of DOPA or tyrosine values were corrected for DOPA and tyrosine absorbance.

Thiobarbituric acid reactive substances (TBARS): After incubation, reactions were stopped by the addition of 20 μmol/l EDTA. TBARS were estimated spectrophotometrically essentially according to Gutteridge and Wilkins:[30] 0.5 ml sample was mixed with 0.5 ml 1% TBA in 0.05 N NaOH followed by 0.5 ml of acetic acid and incubated at 100° for 45 min. A molar extinction coefficient of 156 000 mol/l/cm was used to calculate malondialdehyde (MDA) formed.

Lipoprotein electrophoresis: Aliquots (10 μl) of treated or untreated LDL were applied to agarose gels (1% in veronal buffer) and run for 90 min and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Technoclone). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation,[31] setting the electrophoretic mobility of native (untreated) LDL arbitrarily as one.

DOPA Generation in Insulin and Tyr-tyr-tyr

Peptide-bound DOPA was generated enzymatically based on the method reported by Simpson *et al.*[32] Insulin (1 mg/ml) or 2 mmol/l tyr-tyr-tyr was incubated with 400 U/ml of tyrosinase in 0.1 mol/l phosphate buffer pH 7.4 up to 6 h at 30°C and subsequently, DOPA formation was estimated as given below.

Estimation of PB-DOPA

DOPA formation in the respective compound was measured by the redox-cycling assay (NBT assay) according to Paz *et al.*[33] A measured quantity of 100 μl of insulin sample or 100 μl of tyr-tyr-tyr (diluted 1:20) was used for DOPA estimation. Free DOPA was used as a standard.

RESULTS

As seen in Fig. 1, Cu^{2+} initiated LDL oxidation as measured by CD formation was strongly inhibited in presence of free DOPA (10 μmol/l). Tyrosine showed

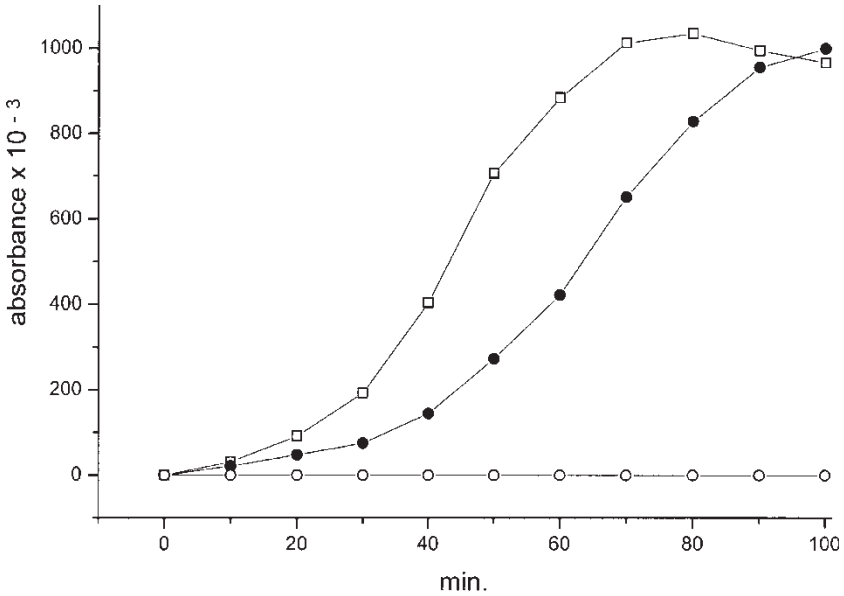

FIGURE 1 Influence of free DOPA and tyrosine on copper-initiated CD formation in LDL. LDL (0.2 mg/ml PBS) was incubated with 5 μmol/l Cu^{++} in the absence or presence of DOPA or tyrosine (10 μmol/l both) at 37°C and CD formation was monitored as the increase in absorbance A_{234} nm. One representative experiment out of three is shown. □ Cu^{2+}, ● Cu^{2+} + tyrosine, ○ Cu^{2+} + DOPA.

some antioxidant activity at the same concentration as monitored by an increase in lag time[34,35] from 32 to 46 min. In presence of DOPA (up to 20 μmol/l), copper-ion induced LDL oxidation (2 h at 37°C) as measured by MDA-formation was strongly inhibited (Fig. 2). The alteration of the apoprotein as measured by REM was also inhibited by DOPA (control: 1, Cu^{++}: 2.4, Cu^{++} + DOPA: 1.5). In contrast, the parent amino acid tyrosine showed no inhibitory action (Fig. 2A). Extending the incubation time to 4 h still resulted in diminished lipid oxidation in LDL (not shown). The copper-transport protein ceruloplasmin has been shown to induce LDL oxidation in cell free and cell mediated systems.[8] DOPA (0.5 and 5 μmol/l) also inhibited ceruloplasmin (25 μg/ml) induced lipoprotein oxidation (Fig. 2B). It should be noted that Chelex treatment of buffers did not alter the finding that DOPA, in contrast to tyrosine, inhibits LDL oxidation (data not shown). This indicates that the effect of DOPA is not due to trace metal contaminations. Next we tested the ability of DOPA (and tyrosine) to inhibit metal ion independent LDL oxidation using the thermolabile azo-compound AAPH as a free radical generating system.[32] DOPA, but not tyrosine (both up to 100 μmol/l) was an effective inhibitor of $ROO^{\bullet}$ initiated lipid oxidation (Fig. 2C). REM were increased by AAPH treatment from 1 to 1.8 and suppressed by coincubation with DOPA to 1.2. Increasing the incubation time to 4 h still resulted in strong inhibition (40%) of DOPA (12.5 μmol/l) by AAPH (10 mmol/l) (data not shown). It should be noted that AAPH does not generate DOPA from tyrosine,[32] which is in accordance with the present observation that prolonged incubation of AAPH/LDL with tyrosine did not result in inhibition of LDL oxidation. It could be possible that the observed inhibitory action of DOPA on the AAPH/LDL oxidation may be due to direct oxidation of DOPA by AAPH, thus lowering the radical flux to LDL. To test this possibility, DOPA (100 μmol/l) was incubated in absence or presence of AAPH (1 mmol/l) for 60 min at 37° C and UV/VIS-spectra were recorded. As seen in Fig. 3A AAPH caused increased oxidation of DOPA to DOPA-chrome[36] as indicated by the appearance of compounds absorbing at 475 nm. Essentially the same was found when DOPA was incubated with Cu^{++} indicating that transition metal ion can directly oxidize DOPA (Fig. 3B). Next the influence of PB-DOPA on LDL oxidation was tested. In the present study, insulin and tyr-tyr-tyr were used as model peptides to investigate the role of PB-DOPA residues on metal ion dependent (Cu^{2+}) or independent (AAPH) LDL oxidation. Tyrosinase treatment was used to generate DOPA because this method specifically modifies tyrosine residues but not other amino acids as found by irradiation or metal-catalyzed treatment.[32] Incubation of insulin with tyrosinase (400 U/ml) resulted in increasing DOPA-reactivity as measured in the redox-cycling (NBT) assay for the detection of quinoproteins reported by Paz *et al.*[33] Maximal DOPA formation was found after 5–6 h (not shown). This is in accordance to Simpson *et al.*[32] who reported near maximal "peptide-bound reducing moieties" after 250 min of insulin/tyrosinase treatment as monitored by a Cu^{2+}-reduction/neocuproine assay. In contrast to insulin, tyr-tyr-tyr was rapidly converted to DOPA-reactive material within 2 h (not shown).

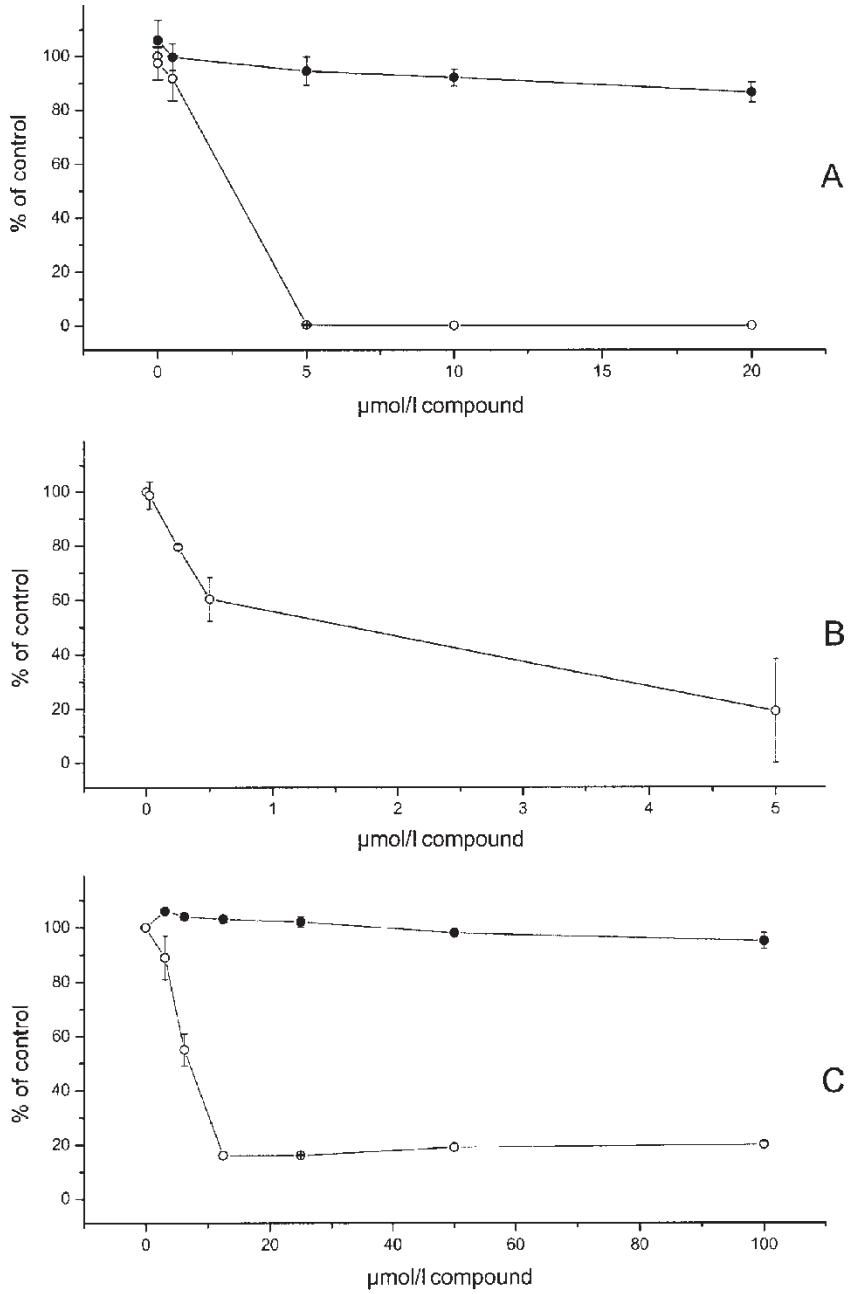

FIGURE 2 Influence of free DOPA and tyrosine on copper-initiated (panel A, 100% = 2.01 ± 0.11 µmol/l MDA), ceruloplasmin (B, 100% = 0.5 ± 0.08 µmol/l MDA) or AAPH-initiated (C, 100% = 2.39 ± 0.04 µmol/l MDA) LDL oxidation. LDL (0.2 mg/ml PBS) was incubated in the absence or presence of increasing concentrations of free DOPA or tyrosine for 2 h (copper, AAPH) or 4 h (ceruloplasmin) at 37°C. Lipid oxidation was initiated by Cu^{++} (5 µmol/l), ceruloplasmin (25 µg/ml) or AAPH (10 mmol/l). Reactions were terminated by addition of EDTA (20 µmol/l) and MDA formation was estimated as given in Methods. ○ DOPA, ● tyrosine.

Figure 4 depicts the influence of PB-DOPA (DOPA-insulin) on Cu^{2+}-initiated LDL oxidation. DOPA generation in insulin substantially increased the ability of the protein to inhibit LDL oxidation. Whereas the antioxidant property of untreated insulin declined after 2 h, DOPA-insulin still inhibited LDL oxidation even after prolonged incubation (up to 7 h). The observation that untreated insulin exerts some inhibitory effect on LDL oxidation is due to its tyrosine content and unspecific antioxidant effect of proteins (like BSA) and peptides in general.[37] The antioxidant activity of the tyrosine tripeptide tyr-tyr-tyr strongly increased after tyrosinase treatment (DOPA-tyr-tyr-tyr) as seen in Fig. 5. As low as 6.2 µmol/l DOPA-tripeptide effectively inhibited LDL oxidation in contrast to untreated tyr-tyr-tyr. The metal ion independent, AAPH-induced LDL oxidation was also stronger inhibited by DOPA-insulin than by unmodified insulin (Fig. 6). Thus the above results indicate that DOPA residues in proteins or peptides are also effective antioxidant moieties as found for free DOPA. Next we tested

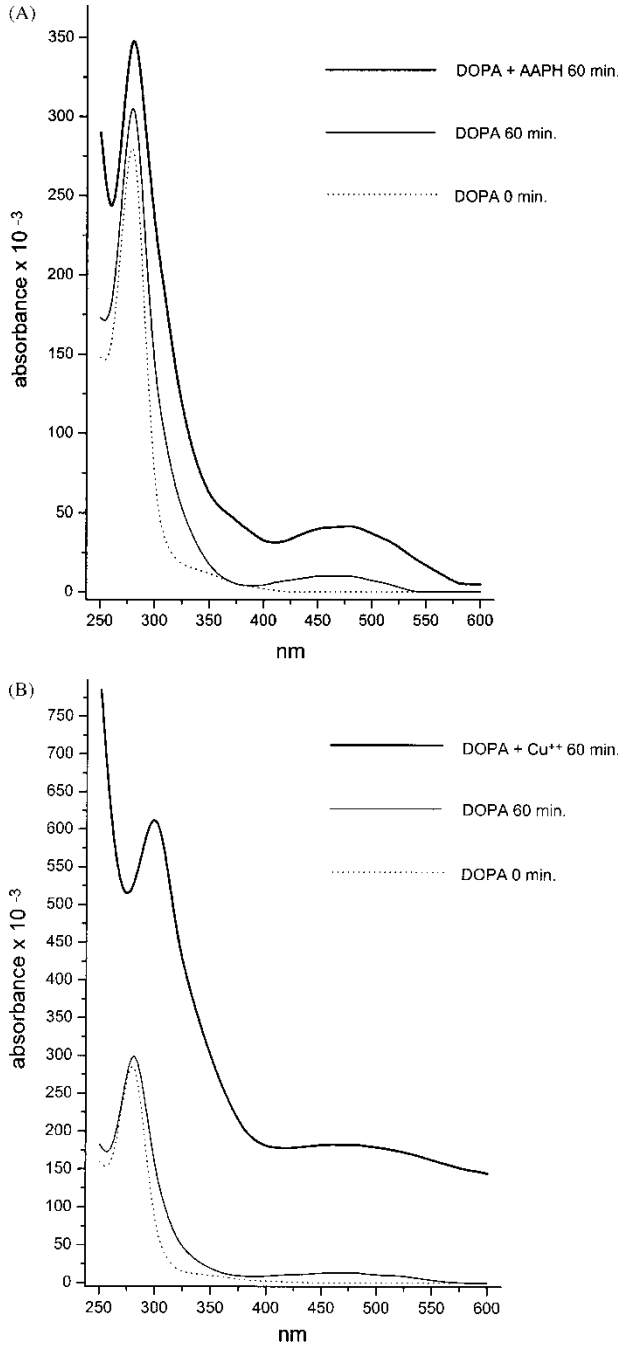

FIGURE 3 (A) Oxidation of DOPA by AAPH. DOPA (100 μmol/l in PBS) was incubated in the absence or presence of AAPH (1 mmol/l) for 60 min at 37°C. Spectra were recorded between 250 and 600 nm. (B) Oxidation of DOPA by Cu^{++}. DOPA (100 μmol/l in PBS) was incubated in the absence or presence of Cu^{++} (50 μmol/l) for 60 min at 37°C. Spectra were recorded between 250 and 600 nm.

the ability of DOPA to act as an antioxidant of LDL oxidation already in progress. LDL oxidation was initiated by Cu^{2+} (5 μmol/l). DOPA (50 μmol/l) was added immediately (0 min) or after 5, 10, 15, 20 or 30 min during oxidation. After a total incubation time of 60 min, MDA formation was measured. When DOPA was added at 0 min, the compound strongly inhibited LDL oxidation (about 10% of control, see Fig. 7). Addition of DOPA 5 min after initiation of lipid oxidation still resulted in inhibition of MDA formation (about 40% of control). However, when DOPA was added to LDL oxidation in progress 10, 15, 20 and 30 min from initiation by Cu^{2+}, DOPA showed a pro-oxidant effect causing a 1.7–2.6-fold increase in LDL oxidation compared to controls (LDL oxidation by Cu^{2+} for 60 min at 37°C). This effect was dependent on the presence of copper ions as concomitant addition of EDTA (see Fig. 7) or bathocuproine disulfonate (not shown) to DOPA completely abolished the stimulating effect. In contrast to the metal ion mediated LDL oxidation, DOPA was still

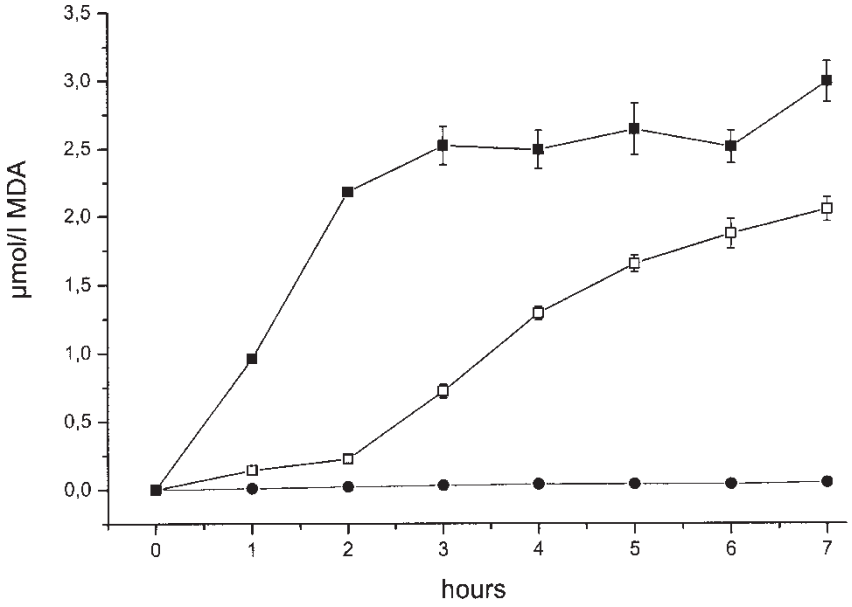

FIGURE 4 Influence of insulin or DOPA-insulin on copper-initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated in the absence or presence of insulin or DOPA-insulin (60 µg/ml both) up to 7 h at 37°C. Lipid oxidation was initiated by Cu^{++} (5 µmol/l). Reactions were terminated by addition of EDTA (20 µmol/l) and MDA formation was estimated as given in Methods. ■ Cu^{2+}, □ insulin, ● DOPA-insulin.

an antioxidant when added up to 30 min after initiation of LDL oxidation by AAPH generated radicals (Fig. 8).

DISCUSSION

The attack of free tyrosine and tyrosine residues in proteins and peptides by hydroxyl radicals ($HO^{\bullet}$) formed by ionizing reactions, fenton type systems, UV photolysis and certain reactive nitrogen species (HOONO) can lead to the formation of DOPA.[19–24,38] In contrast to tyrosine-chlorination and -nitration reaction products formed by other oxidative insults, protein-bound DOPA has been shown to be further chemically reactive. In presence of redox-active metal ions like Fe^{3+} and Cu^{2+} so called "oxychelates"[24] are formed and could trigger further oxidative damage to macromolecules like DNA or proteins. In presence of DOPA facilitated

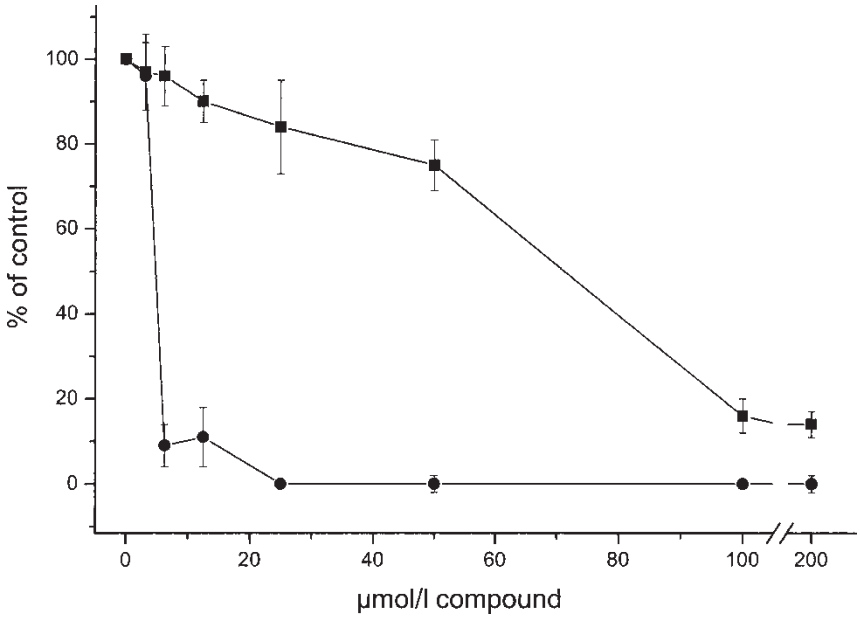

FIGURE 5 Influence of tyr-tyr-tyr or DOPA tyr-tyr-tyr on copper-initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated in the absence or presence of increasing concentrations of tyr-tyr-tyr or DOPA tyr-tyr-tyr (up to 200 µmol/l) for 2 h at 37°C. Lipid oxidation was initiated by Cu^{++} (5 µmol/l). Reactions were terminated by addition of EDTA (20 µmol/l) and MDA formation was estimated as given in Methods. 100% = 3.15 ± 0.5 µmol/l MDA. ■ tyr-tyr-tyr, ● DOPA tyr-tyr-tyr.

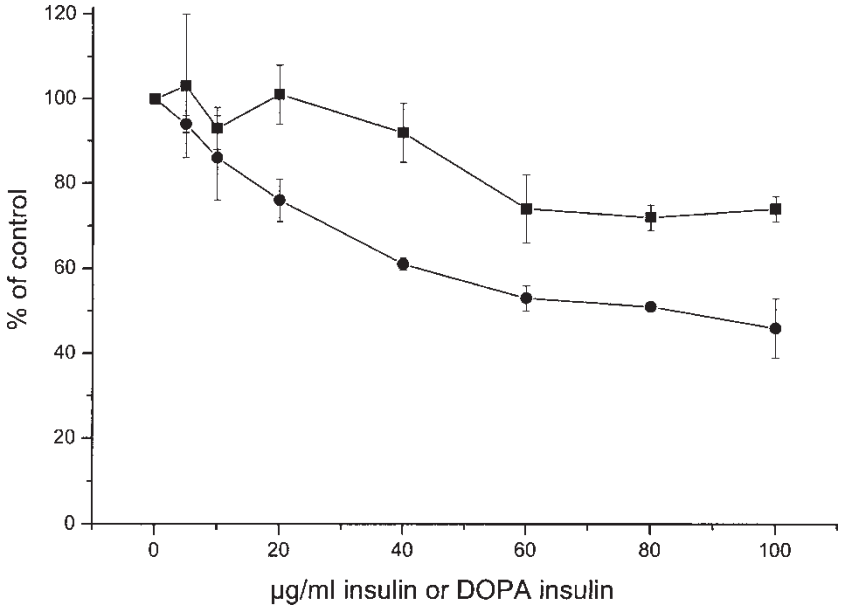

FIGURE 6 Influence of insulin or DOPA-insulin on AAPH-initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated in the absence or presence of increasing concentrations of insulin or DOPA-insulin for 75 min at 37°C. Lipid oxidation was initiated by AAPH (10 mmol/l). Reactions were terminated by addition of EDTA (20 µmol/l) and MDA formation was estimated as given in Methods. 100% = 1.85 ± 0.18 µmol/l MDA. ■ insulin, ● DOPA-insulin.

DNA hydroxylation and fragmentation by copper ions has been reported.[39–41] The reaction of DOPA with Cu^{2+} may lead to the formation of Cu^{+}, DOPA radical, superoxide radical and H_2O_2 and ultimately to hydroxyl radical formation.[39] Increased levels of PB-DOPA have been found in atherosclerotic plaques and also in LDL from vessel lesions.[25] In advanced human atherosclerotic lesions about 400 pmol DOPA residues/mg protein have been measured.[25] It has been speculated that the DOPA-proteins may facilitate further pathological reactions in the plaque. Reduction of Cu^{++} to Cu^{+} may play a role in lipid peroxidation. Cu^{++} reducing factors like lipid hydroperoxides ($Cu^{++} + LOOH \rightarrow Cu^{+} + LOO^{\bullet} + H^{+}$), α-tocopherol ($Cu^{++} + \alpha - Toc{-}OH \rightarrow Cu^{+} + \alpha - Toc{-}O^{\bullet} + H^{+}$), ascorbic acid ($Cu^{++} + AscH^{-} \rightarrow Cu^{+} + Asc^{\bullet -} + H^{+}$) and thiols ($Cu^{++} + RSH \rightarrow Cu^{+} + 1/2\ RSSR + H^{+}$) have been described.

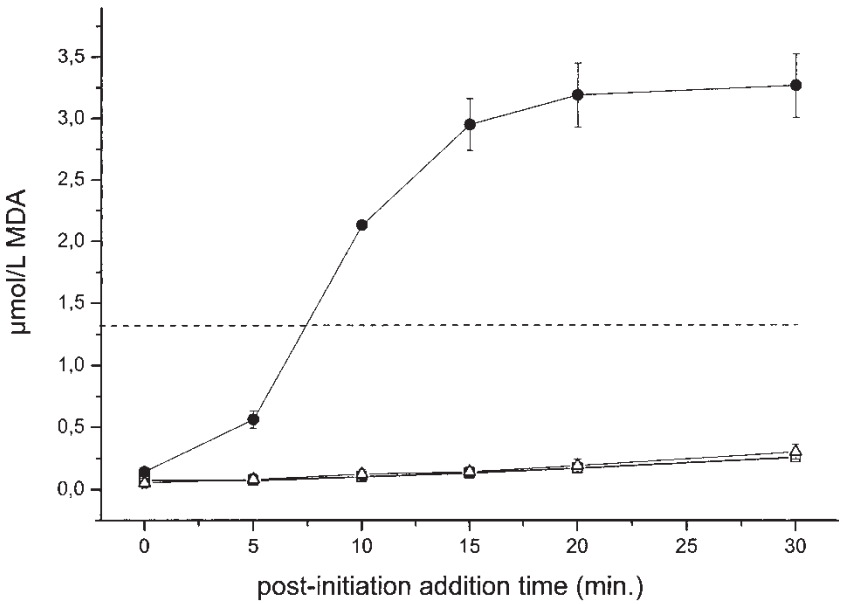

FIGURE 7 Influence of DOPA added at various time points after copper-initiated LDL oxidation. Oxidation of LDL (0.2 mg/ml) was initiated by Cu^{2+} (5 µmol/l). DOPA (50 µmol/l) was added immediately (0 min) or at the indicated time points. After a total incubation time of 60 min, MDA was measured as described in Methods. ● DOPA, □ EDTA, △ DOPA + EDTA. The dotted line represents MDA formation in LDL oxidized by Cu^{++} for a total time of 60 min at 37°C.

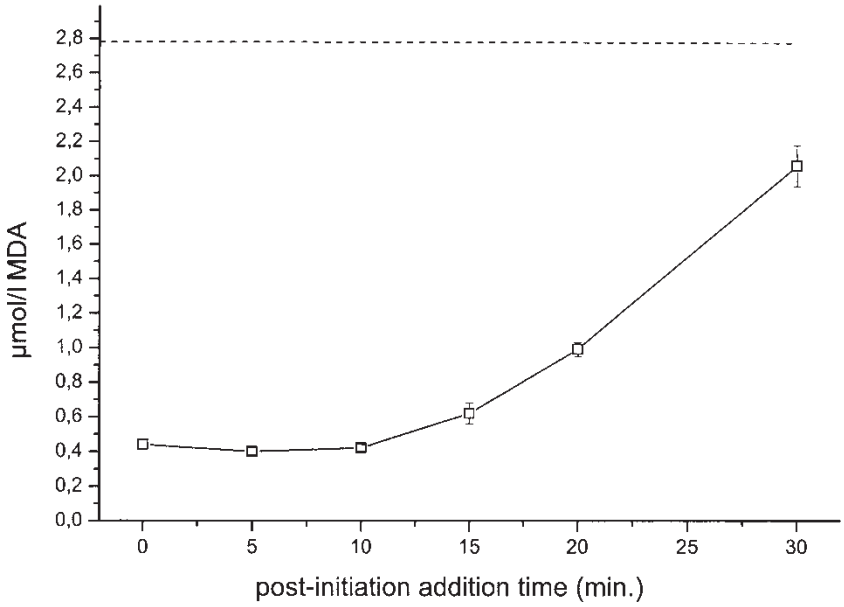

FIGURE 8 Influence of DOPA added at various time points after AAPH-initiated LDL oxidation. Oxidation of LDL (0.2 mg/ml) was initiated by AAPH (10 mmol/l). DOPA (50 µmol/l) was added immediately (0 min) or at the indicated time points. After a total incubation time of 60 min MDA was measured as described in Methods. □ DOPA. The dotted line represents MDA formation in LDL oxidized by AAPH for a total time of 60 min at 37°C.

For a recent review on copper-dependent LDL oxidation see Burkitt.[42] Free and PB-DOPA can reduce Cu^{++} to Cu^{+}[39] thus one may assume that DOPA may facilitate copper-mediated LDL oxidation. However, the present results indicate that DOPA (free or peptide-bound) has strong antioxidant properties when LDL oxidation was initiated by Cu^{++}. As a first crucial step of LDL oxidation by Cu^{++}, Cu^{++} has been found to bind to LDL histidyl residues.[42,43] As a possible mechanism of the observed antioxidant effects of free or PB-DOPA, DOPA may complex the copper ions thus preventing their binding to ApoB resulting in inhibition of LDL oxidation. On the other hand, the DOPA "oxychelate" formed which has been shown to promote DNA oxidative modifications may not be able to induce LDL oxidation. In contrast, Cu^{++} mediated LDL oxidation already in progress was stimulated in the presence of DOPA, an effect which was inhibited in presence of EDTA, pointing to the role of metal ions involved in this process. This may be due to facilitated breakdown of early lipid oxidation products (lipid hydroperoxides) to MDA by the DOPA "oxychelate".

DOPA did also inhibit the free radical initiated metal ion-independent lipid oxidation. In contrast to metal ion initiated LDL oxidation, AAPH mediated LDL oxidation already in progress was inhibited by DOPA. This may be due to the scavenging of the free radicals derived from the azo-compound leading to formation of DOPA oxidation products, e.g. DOPA-chrome (see results). DOPA-chrome formation was also observed in DOPA-Cu^{++} incubation mixtures, which could further explain the inhibitory action of DOPA on the copper-mediated LDL oxidation. One may speculate, that due to DOPA oxidation the concentration of DOPA measured in atherosclerotic plaques[25] could underestimate the total tyrosine oxidation.

That DOPA is a molecule with beneficial, as well as unfavorable effects in respect to its role in oxidative processes has been reported by Spencer *et al.* and Li *et al.*[44,45] who found pro- and antioxidant effects of DOPA examined in mouse brain homogenates and ox brain phospholipids vesicles, respectively.

In summary, the present results show that free or peptide-bound DOPA has the ability to act as an efficacious antioxidant of copper ion or free radical initiated LDL oxidation, but DOPA could stimulate further oxidative modification of already pre-oxidized LDL in presence of redox-active metal ions.

Acknowledgements

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