FEBS Letters 490 (2001) 28–31 FEBS 24581

p-Hydroxyphenylacetaldehyde, the major product of tyrosine oxidation by the activated myeloperoxidase system can act as an antioxidant in LDL

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Received 20 December 2000; revised 25 December 2000; accepted 25 December 2000

First published online 22 January 2001

Edited by Shozo Yamamoto

Abstract The oxidative modification of low density lipoprotein (LDL) may play a significant role in atherogenesis. HOCl generated by the myeloperoxidase/H_2O_2/Cl^- system of activated neutrophils may be operative in vivo making LDL atherogenic. Tyrosine has been found to be oxidized by HOCl to *p*-hydroxyphenylacetaldehyde (*p*-HA) capable of modifying phospholipid amino groups in LDL. As an amphiphatic phenolic compound, *p*-HA may have the potential to act as an antioxidant in the lipid phase of LDL. The present results show that (a) tyrosine exerts a protective effect on LDL modification by HOCl, (b) *p*-HA could act as antioxidant associated with the lipoprotein preventing cell- and transition metal ion-mediated LDL oxidation and (c) *p*-HA was able to scavenge free radicals.

Key words: *p*-Hydroxyphenylacetaldehyde; Tyrosine; LDL oxidation; Myeloperoxidase; Antioxidant

1. Introduction

The oxidative modification of low density lipoprotein (LDL) may play an important role in the early events of atherogenesis [1–4]. The pathophysiological relevant pathway(s) of atherogenic LDL alterations, however, have not been elucidated at present. There is some evidence that the myeloperoxidase/hydrogen peroxide/chloride system (MPO/H_2O_2/Cl^-) of activated neutrophils may be operative in vivo modifying LDL [5–9]. However, a pivotal role of MPO in neutrophil-induced oxidation of LDL has been questioned by Noguchi et al. using MPO knockout mice [10]. In addition, the MPO/H_2O_2/NO_2^- system by producing reactive nitrogen species [11] has been identified as a further candidate for in vivo LDL atherogenic modification by MPO [12,13]. The reaction product HOCl of the MPO/H_2O_2/Cl^- system has been shown to induce the formation of protein chloramines in LDL and secondary derived radicals initiate lipid oxidation [14–16]. Actually, HOCl-modified proteins have been detected in atherosclerotic lesions [9,17]. Free amino acids react with HOCl by forming chloramines which decompose by deamination and decarboxylation to the respective aldehyde. These aldehydes are capable of modifying protein amino groups via the formation of Schiff bases [18–25]. Recently, Heller et al. [26] reported that tyrosine in presence of reagent HOCl, MPO/H_2O_2/Cl^- or activated neutrophils which employ the MPO system was converted to the lipid-soluble aldehyde, *p*-hydroxyphenylacetaldehyde (*p*-HA). *p*-HA was found to modify phospholipid amino groups (phosphoethanolamine) in LDL [26]. Such *p*-HA-modified LDL particles were actually isolated from atherosclerotic plaques indicating that this reaction may also occur in vivo [26]. However, the pathophysiological role of this particular LDL modification is uncertain at present. One may assume that *p*-HA, due to its phenolic structure and amphiphatic nature, may have the property to act as an antioxidant in the lipid phase of LDL. Therefore, in the present paper we have evaluated (a) the influence of tyrosine on HOCl-initiated LDL modification and (b) the sensitivity of *p*-HA pretreated LDL to lipid oxidating processes. The present results show that pretreatment of LDL with HOCl/tyrosine by generating *p*-HA protected LDL from further oxidative attacks mediated by endothelial cells or transition metal ions.

2. Materials and methods

Sodium hypochlorite (NaOCl) solution was from Aldrich Chemical Company, Inc. Tyrosine (sodium salt), phenylalanine, tyrosine [ring-3,5-^{3}H] specific activity 45.5 Ci/mmol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and galvinoxyl were purchased from Sigma-Aldrich Chemical Corp. All other chemicals used were of analytical grade.

2.1. LDL isolation

LDL preparations were isolated by ultracentrifugation as reported

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Abbreviations: CHOD, cholesterol oxidase; DTPA, diethylene triamine-pentaacetate; HOCl, hypochlorous acid; NaOCl, sodium hypochlorite; HUVEC, human umbilical vein endothelial cell; LDL, low density lipoprotein; PBS, phosphate buffered saline; *p*-HA, *p*-hydroxyphenylacetaldehyde; REM, relative electrophoretic mobility; TBARS, thiobarbituric acid reactive substance

PII: S0014-5793(01)02131-7

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 commercial test kit (Bio-Rad Laboratories) using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml.

2.2. Endothelial cell culture

Endothelial cells (HUVECs) were isolated from human umbilical veins and maintained in culture as reported previously [28]. For experiments cells were passaged into 35 mm culture dishes. All incubations were done in RPMI-1640 medium.

2.3. LDL oxidation

2.3.1. HOCl-mediated LDL oxidation. Prior to LDL oxidation, the lipoprotein was equilibrated in 0.1 mol/l phosphate buffer pH 7.4 containing 100 μmol/l DTPA using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (2 mg/ml) was incubated with reagent HOCl up to 1 mmol/l at 37°C for 18 h. Stock HOCl concentration was estimated spectrophotometrically in 0.01 mol/l NaOH using ε_{292} as 350 M^{-1} cm^{-1} [15]. For LDL treatment, stock HOCl was diluted with phosphate buffer. At the end of incubations LDL samples were applied to small Sephadex columns (Nick column, Pharmacia) equilibrated in PBS pH 7.4 to separate low molecular weight compounds prior to analytical procedures or further treatment.

2.3.2. Cell-mediated LDL oxidation. When cell-mediated LDL oxidation was performed, HUVEC confluent monolayers were washed with RPMI-1640 medium and subsequently incubated with the respective LDL preparation (100 μg/ml) in RPMI-1640 for 24 h. Results were obtained with four different HUVEC preparations.

2.3.3. Transition metal ion-mediated LDL oxidation. LDL preparations obtained after gel chromatography on Nick columns (see above) were diluted to 200 μg/ml protein and lipid oxidation was initiated by addition of Cu^{2+} (10 μmol/l). Samples were incubated at 37°C.

2.4. Estimation of LDL oxidation

2.4.1. Lipid hydroperoxides. Total lipid hydroperoxides were measured with the CHOD iodide color reagent ($\varepsilon_{365} = 1.73 \times 10^4$ M^{-1} cm^{-1}; E. Merck) as described by El-Saadani et al. [29] and validated for LDL by Gebicki et al. [30] under the conditions of Wallin and Camejo [31]. To avoid interference of any remaining chloramines with the CHOD reagent, methionine (2 mmol/l) was added at the end of the treatment and the samples were further incubated for 60 min at 37°C. Subsequently 0.4 ml sample was mixed with 0.4 ml CHOD iodide reagent and after incubation for 60 min at 37°C the absorbance was estimated at $A_{365\,nm}$. All values are given as μmol/l in the assay.

2.4.2. Conjugated dienes. Conjugated diene formation was monitored as the increase in absorbance at $A_{234\,nm}$ [32] using a Hitachi U-2000 spectrophotometer. 'Lag time' of LDL oxidation was calculated as defined by [32].

2.4.3. Thiobarbituric acid assay. LDL oxidation products in cell culture supernatants were assayed as TBARS as described [27].

2.5. Lipid electrophoresis

Aliquots (10 μl) of treated or untreated LDL were applied to agarose gels (1% in veronal buffer), run for 90 min and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Immuno-Baxter AG). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [33], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as 1.

2.6. Preparation of p-hydroxyphenylacetaldehyde

p-HA was prepared by mixing equimolar amounts of tyrosine and HOCl in phosphate buffer at 4°C and subsequently the mixture was incubated for 60 min at 37°C following the protocol of [24]. Samples were supplemented with LDL (2 mg/ml) and further incubated for 2 h at 37°C. Low molecular weight compounds were separated by gel chromatography as described above.

2.7. Binding of p-HA to LDL

p-HA (500 μmol/l) was prepared as described above using [^{3}H]tyrosine as a tracer (6.25 μCi/ml). Samples were supplemented with LDL (2 mg/ml) and further incubated for 2 h at 37°C. Low molecular weight compounds were separated by gel chromatography and bound *p*-HA was estimated by liquid scintillation counting. Parallel incubations were run as background controls using radioactive tyrosine only (no HOCl added).

2.8. Free radical scavenging

Radical scavenging ability of *p*-HA was estimated using the stable free radical compounds DPPH and galvinoxyl [34–36]. To methanolic solutions of DPPH (50 μmol/l) or galvinoxyl (10 μmol/l) *p*-HA was added (50 μmol/l) and the absorption was read at 517 nm (DPPH) or 429 nm (galvinoxyl), respectively. 2,6-di-*tert*-butyl-4-methylphenol (BHT, 50 μmol/l) was run as a positive control. The decrease in absorption was taken as an indicator of radical scavenging ability [34–36].

3. Results and discussion

HOCl, the product of the activated myeloperoxidase system ($MPO/H_2O_2/Cl^-$) is a potent bio-reagent [37] known to modify LDL [14–16,38]. HOCl (1 mmol/l) treatment of LDL resulted in modification of the apoprotein as indicated by increased REM of the lipoprotein (Fig. 1). When tyrosine was present in the incubations (molar ratio 1:1) the amino acid suppressed REM from 1.88 (HOCl) to 1.55 (see Fig. 1) presumably by scavenging HOCl. Estimation of lipid hydroperoxides revealed that tyrosine (0.250–1 mmol/l) effectively inhibited lipid oxidation in LDL initiated by HOCl (Fig. 1). It should be noted that the concentration of 1 mM HOCl which was used in this experiment is about 10 times higher than the concentration of HOCl generated by 1×10^6 neutrophils/ml. Tyrosine in presence of reagent HOCl (or $MPO/H_2O_2/Cl^-$) is converted in quantitative yield (mol/mol) to the phenolic aldehyde, *p*-HA [24–26]. In general, phenolics have antioxidative properties. To evaluate the effect of *p*-HA on LDL oxidation, LDL was preincubated for 18 h at 37°C with tyrosine/HOCl (500 μmol/l each) to generate *p*-HA and after gel separation LDL samples were subjected to cell-mediated oxidation using HUVECs as an LDL oxidating system [39]. Lipid oxidation products (TBARS) were estimated in culture media of HUVECs treated with the respective LDL preparation. As seen in Fig. 2, LDL pretreated with HOCl/tyrosine was less prone to lipid oxidation compared to native LDL. Under these conditions, about 50% lower TBARS formation was observed in tyrosine/HOCl pretreated LDL. This is obviously due to *p*-HA associated with the lipoprotein as all LDL prep-

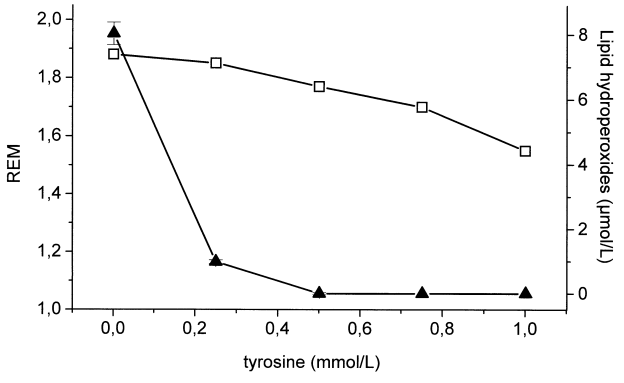

Fig. 1. Influence of tyrosine on protein and lipid modification in LDL initiated by HOCl. LDL (2 mg/ml) was incubated in 0.1 mol/l phosphate buffer, pH 7.4, 100 μmol/l DTPA in the absence or presence of 1 mmol/l HOCl and tyrosine (0–1 mmol/l) for 18 h at 37°C. At the end of incubation, REM (□) and lipid hydroperoxides (▲) (after gel chromatography) were estimated as given in Section 2.

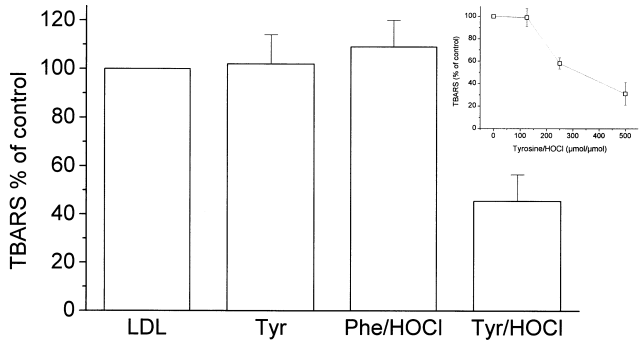

Fig. 2. LDL oxidation by human endothelial cells. LDL (2 mg/ml) in 0.1 mol/l phosphate buffer, pH 7.4, 100 μmol/l DTPA was pretreated with HOCl (500 μmol/l), tyrosine (500 μmol/l), HOCl/tyrosine (both 500 μmol/l) or HOCl/phenylalanine (both 500 μmol/l) for 18 h at 37°C. At the end of incubation samples were subjected to gel chromatography (see Section 2) and the respective LDL preparation was added to HUVEC cultures (100 μg/ml medium) and incubated for 24 h. TBARS were estimated in the cell culture supernatants. Untreated LDL processed as described above served as control. Inset: Concentration-dependent effect of HOCl/tyrosine/LDL pretreatment on cell-mediated LDL oxidation. LDL (2 mg/ml) in 0.1 mol/l phosphate buffer, pH 7.4, 100 μmol/l DTPA was pretreated with HOCl (125, 250, 500 μmol/l)/tyrosine (125, 250, 500 μmol/l) for 18 h at 37°C and processed as described above.

arations were subjected to gel chromatography to separate low molecular weight compounds prior to cell-mediated oxidation. Using [^{3}H]tyrosine/HOCl reaction mixtures to generate radioactively labelled *p*-HA about 6.85 ± 0.82 nmol of *p*-HA/mg of protein ($n = 3$) were found to be associated with the lipoprotein fraction after 18 h incubation at 37°C. LDL pretreatment with phenylalanine/HOCl (500 μmol/l each) had no antioxidative effect in this system. This is in accordance to the chemical nature of the compound formed by phenylalanine/HOCl, namely phenylacetaldehyde [22] – a lipophilic

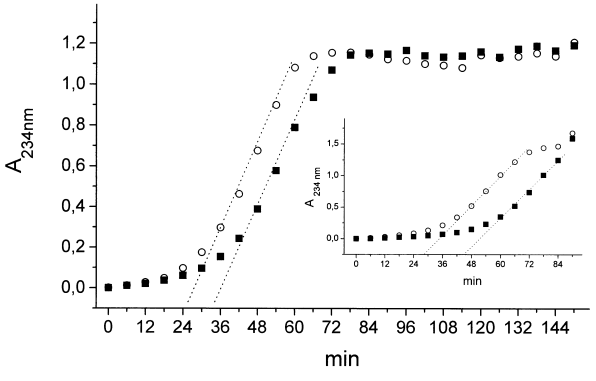

Fig. 3. Influence of tyrosine/HOCl pretreatment on LDL oxidizability by transition metal ions. LDL (2 mg/ml) in 0.1 mol/l phosphate buffer, pH 7.4, 100 μmol/l DTPA was treated with HOCl/tyrosine (500 μmol/l each) for 18 h at 37°C. After gel chromatography, LDL samples were diluted to 0.2 mg/ml and lipid oxidation was initiated by the addition of Cu^{2+} (10 μmol/l). Conjugated diene formation was monitored as the increase in absorbance at $A_{234\,nm}$ and lipid oxidation lag phase was calculated according to [31]. Untreated LDL processed as described above served as control. Untreated LDL (○), tyrosine/HOCl pretreatment (■). Inset: Influence of reagent *p*-HA treated LDL on oxidizability by Cu^{2+}. Reagent *p*-HA was prepared as given in Section 2. LDL (2 mg/ml) was treated with 2 mmol/l) *p*-HA for 2 h at 37°C. After separation of low molecular weight compounds, lipid oxidation was initiated by 10 μmol/l Cu^{2+} and conjugated dienes monitored. Untreated LDL (○), reagent *p*-HA pretreatment (■).

but non-phenolic compound unable to act as an antioxidant. Tyrosine (no HOCl present) pretreatment of LDL had also no protective effect on subsequent cell-mediated LDL oxidation (Fig. 2). The inset in Fig. 2 shows the concentration-dependent effect of tyrosine/HOCl mixtures (125–500 μmol/l each) on LDL oxidation by HUVECs. The maximal antioxidant effect on cell-mediated LDL oxidation was observed at 500 μmol/l tyrosine and HOCl (molar ratio 1:1). The (relative) potential of an antioxidant can be estimated by the kinetic assay of conjugated diene formation in LDL [32]. Native, HOCl or tyrosine/HOCl (generating *p*-HA) pretreated (18 h at 37°C) LDL was therefore subjected to copper ion-mediated lipid oxidation and conjugated dienes were monitored. As depicted in Fig. 3, tyrosine/HOCl pretreated LDL showed a longer resistance to Cu^{2+} (10 μmol/l)-initiated LDL oxidation compared to native LDL. This was indicated by a prolonged lag phase of lipid oxidation (24 min versus 33 min). The inset in Fig. 3 shows the effect of 2 mmol/l *p*-HA on Cu^{2+}-initiated lipid oxidation in LDL incubated for 2 h with already formed *p*-HA (see Section 2) using another LDL preparation. This 'short-time' treatment effectively protected LDL from lipid oxidation, too. As described above, *p*-HA suppressed the oxidation of LDL by endothelial cells as well as transition metal ions. It has been shown that potent antioxidants react with

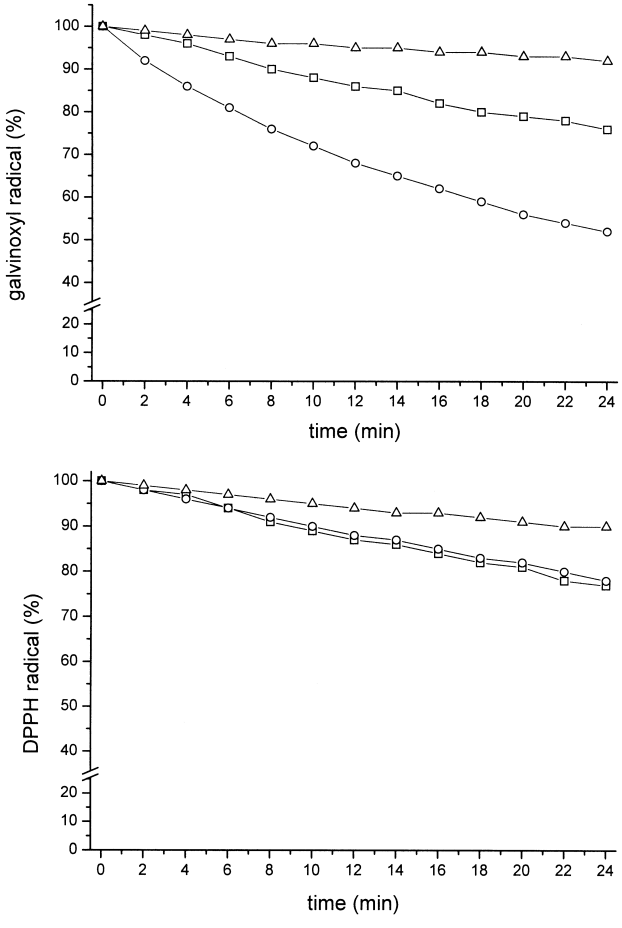

Fig. 4. Radical scavenging ability of *p*-HA. Reagent *p*-HA (50 μmol/l) was added to a methanolic solution of DPPH radical (50 μmol/l) or galvinoxyl radical (10 μmol/l) and the decay in absorption was monitored for DPPH at 517 nm and galvinoxyl at 429 nm, respectively. BHT (50 μmol/l) was run as a positive control. No addition (△), *p*-HA (□), BHT (○).

stable free radicals such as DPPH (*N*-centered radical) and galvinoxyl (*O*-centered radical) [34–36]. Thus, the reactivity of *p*-HA as a hydrogen donor was estimated by following a decay of visible absorption of DPPH and galvinoxyl using BHT as a positive control. The results show that *p*-HA reacts with both radical species (Fig. 4) further indicating its potential antioxidant properties.

The present results show that free tyrosine has a protective effect on LDL modification by HOCl and that *p*-HA, the reaction product of tyrosine and HOCl, could act as an antioxidant associated with the lipoprotein influencing its alterations induced by endothelial cells or transition metal ions. On the other hand beside its bare antioxidant ability, *p*-HA may bind to regions in the LDL molecule blocking copper binding sites in the lipoprotein [40–43], thus resulting in lower oxidizability of LDL by transition metal ions. In this respect it should be emphasized that a role – if any – of metal ion-initiated LDL oxidation in atherogenic transformation of LDL is not clear at present and is still a matter of debate [7,44]. Thus one may assume that in vivo multiple modifications may take place in parallel [7,45]. Although the described antioxidant effect of *p*-HA may be of benefit regarding the pivotal role of LDL oxidation in atherogenesis [1], the impact of *p*-HA-modified lipoproteins [46] in the pathophysiological process of atherosclerosis must be kept in mind.

Acknowledgements: We thank Claudia Müllner and Daniela Seidinger for expert technical assistance.

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