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Research paper

Plasma lipid oxidation induced by peroxynitrite, hypochlorite, lipoxygenase and peroxyl radicals and its inhibition by antioxidants as assessed by diphenyl-1-pyrenylphosphine

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ABSTRACT

Lipid oxidation has been implicated in the pathogenesis of many diseases. Lipids are oxidized in vivo by several different oxidants to give diverse products, in general lipid hydroperoxides as the major primary product. In the present study, the production of lipid hydroperoxides in the oxidation of mouse plasma induced by multiple oxidants was measured using diphenyl-1-pyrenylphosphine (DPPP) as a probe. DPPP itself is not fluorescent, but it reacts with lipid hydroperoxides stochiometrically to give highly fluorescent DPPP oxide and lipid hydroxides. The production of lipid hydroperoxides could be followed continuously in the oxidation of plasma induced by peroxynitrite, hypochlorite, 15-lipoxygenase, and peroxyl radicals with a microplate reader. A clear lag phase was observed in the plasma oxidation mediated by aqueous peroxyl radicals and peroxynitrite, but not in the oxidation induced by hypochlorite and lipoxygenase. The effects of several antioxidants against lipid oxidation induced by the above oxidants were assessed. The efficacy of antioxidants was dependent markedly on the type of oxidants. α-Tocopherol exerted potent antioxidant effects against peroxyl radical-mediated lipid peroxidation, but it did not inhibit lipid oxidation induced by peroxynitrite, hypochlorite, and 15-lipoxygenase efficiently, suggesting that multiple antioxidants with different selectivities are required for the inhibition of plasma lipid oxidation in vivo. This is a novel, simple and most high throughput method to follow plasma lipid oxidation induced by different oxidants and also to assess the antioxidant effects in biologically relevant settings.

1. Introduction

Lipid oxidation proceeding inevitably in vivo gives rise to deleterious effects such as functional loss of biological membranes, protein modification, enzyme deactivation, and nucleic acid damage, which has been implicated in the pathogenesis of various diseases [1,2]. Lipids are oxidized in vivo by multiple oxidants. Among them, peroxynitrite, hypochlorite, lipoxygenases, cyclooxygenase, cytochrome P450, and singlet oxygen have been shown to induce lipid oxidation to give diverse products [3,4]. In the free radical mediated lipid peroxidation, peroxyl radicals act as chain carrying species independent of the type of initiating radical species to produce lipid hydroperoxides as primary major products, while non-radical oxidants oxidize lipids to give other specific products.

Peroxynitrite is one of the major reactive oxidants and nitrating species [5,6]. Neither superoxide nor nitric oxide is reactive enough per se to induce lipid oxidation, but they react with each other rapidly to give peroxynitrite. It was reported that peroxynitrite or simultaneous production of nitric oxide and superoxide induces plasma oxidation to produce malonaldehyde and conjugated diene [7], cholesteryl ester hydroperoxide [8,9] and F2-isoprostanes [10]. It has been reported also that peroxynitrite induces lipid peroxidation of LDL [11,12]. The formation of phosphatidylcholine and phosphatidylethanolamine hydroperoxides was observed in the oxidation of erythrocytes by peroxynitrite [13].

Lipoxygenases oxidize polyunsaturated fatty acids to produce regio-, stereo-, and enantio-specific hydroperoxides by non-radical mechanisms [14]. Rabbit reticulocyte 15-lipoxygenase was found

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DPPP, diphenyl-1-pyrenylphosphine; FI, fluorescence intensity; LOX, lipoxygenase; MeO-AMVN, 2,2′-azobis-(4-methoxy-2,4-dimethyllvaleronitrile); NDGA, nordihydroguaiaretic acid; SIN-1, 3-morpholinosydnonimine

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to oxidize plasma directly to give cholesteryl ester and phosphatidylcholine hydroperoxides specifically [15].

Hypochlorous acid (HOCl) produced in stimulated neutrophils via the myeloperoxidase (MPO) catalyzed reaction of hydrogen peroxide with Cl^- is a reactive oxidant [16,17] and it oxidizes unsaturated fatty acids and cholesterol to produce chlorohydrins [18]. Hypochlorite reacts with amines to produce choloramines, which undergo decomposition to give carbon- and nitrogen-centered radicals. It also reacts with hydrogen peroxide to produce singlet oxygen, while it reacts with lipid hydroperoxides to give alkoxyl/peroxyl radicals [19]. Singlet oxygen also gives hydroperoxides from unsaturated fatty acids, cholesterol, and their esters as primary products. On the other hand, cyclooxygenase, cytochrome P450, and ozone do not produce lipid hydroperoxides.

With increasing evidence showing the involvement of oxidative modification of biological molecules in the pathogenesis of various diseases, the role and effects of antioxidants have received much attention, but recent large scale human intervention studies gave disappointing and inconsistent results [20]. Giving large doses of dietary antioxidant supplements to human subjects has, in most studies, demonstrated little or no preventive or therapeutic effect. Such “antioxidant paradox” has been the subject of extensive arguments [21].

It must be noteworthy that, as stated above, lipids are oxidized in vivo by multiple oxidants and the effects of antioxidants depend on the oxidants. The inconsistent and conflicting results of human trials on vitamin E, the most widely studied biological antioxidant, may be, at least in part, due to the facts that multiple oxidants are involved in the oxidative damage, while vitamin E is effective against only free radical mediated mechanisms, but not against non-radical mechanisms [22]. It is therefore imperative to specify the reactive oxidants and measure the effects of antioxidants against different oxidants.

Many kinds of methods have been developed to measure lipid oxidation products. The recent advancement of mass spectrometric analysis enabled to identify and measure numerous kinds of oxidation products in their intact forms [4]. However, lipid oxidation in vivo gives diverse products with many positional and stereo isomers, making it practically quite difficult to measure all of them, while it is also necessary to measure total lipid oxidation for assessment of, for example, oxidative stress status or screening of effective antioxidants.

In the present study, the production of lipid hydroperoxides was measured in the oxidation of plasma induced by several oxidants using diphenyl-1-pyrenylphosphine (DPPP) as a probe. DPPP itself is not fluorescent, but it reacts with lipid hydroperoxides stoichiometrically to give the corresponding lipid hydroxides and DPPP oxide, which is strongly fluorescent. By virtue of this property, DPPP has been used in the measurement and analysis of lipid hydroperoxides in biological samples [23–28]. The formation of lipid hydroperoxides in the cultured cells under oxidative stress has been measured also by DPPP [29–33]. The uptake of oxidized LDL containing DPPP oxide into macrophages has been analyzed [34]. This was applied also for the assessment of antioxidant activity against lipid peroxidation [33,35–38]. It was reported that the DPPP method was amenable for high-throughput screening to the inhibitor of lipoxygenase reaction [39]. Recently, we reported the production of lipid hydroperoxides in the oxidation of plasma induced by singlet oxygen and its inhibition by antioxidants as assessed by DPPP [40]. In this study, peroxyl radicals, peroxynitrite, 15-lipoxygenase, and hypochlorite were chosen as biological oxidant which produces lipid hydroperoxides.

2. Materials and methods

2.1. Materials

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis-(4-methoxy-2,4-dimethyllvaleronitrile) (MeO-AMVN), used as water-soluble and lipid-soluble radical initiator respectively, were obtained from Wako Pure Chemical Industries Ltd., Japan. Peroxynitrite was generated by simultaneous formation of superoxide and nitric oxide from 3-morpholinosydnonimine (SIN-1) [11], which was obtained from DOJINDO Laboratories, Japan. DPPP and 2-carboxy-2,5,7,8-tetramethyl-6-hydroxychroman (Trolox) were purchased from Cayman Chemical Company (Michigan, USA). Sodium hypochlorite, nordihydroguaiaretic acid (NDGA), and baicalein were obtained from WAKO Pure Chemical Industries Ltd., Japan. Rabbit reticulocytes 15-lipoxygenase was purchased from Enzo Life Science Inc. (New York, USA). Caffeic acid was obtained from Nakalai Tesque Co., Japan. Other chemicals were those of the highest grade available commercially.

Wild type male C57BL/6J mice purchased from Shimizu Laboratory Supplies Co. Ltd., Japan were maintained under standardized conditions of 12-h/12-h light/dark schedule. Blood was collected in heparin-containing tubes from mice. Plasma was obtained by centrifugation at 3500 rpm for 15 min at 4 °C and frozen on ice immediately and stored until use as reported previously [41]. The animal experiments and care were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

2.2. Measurement of lipid hydroperoxides

Plasma (10 vol%, unless otherwise specified) was oxidized with multiple oxidants at 37 °C in PBS (pH 7.4) under air in the absence and presence of antioxidant. Lipid hydroperoxides was measured from the fluorescence intensity by DPPP oxide, the excitation and emission wavelength being 351 and 380 nm respectively, with a microplate reader, Spectra Max M2 (Molecular Devices, Sunnyvale, CA) equipped with a thermostatted cell maintained at 37 °C under air as reported previously [41]. The oxidation was started by the addition of the respective oxidant into the PBS solution of plasma in the presence of DPPP and additives. DPPP and antioxidants were added as DMSO solution. The concentration of DMSO was kept to or less than 2.5 vol%.

Since the plasma obtained from different mice contained different composition of lipids and antioxidants, the same plasma was used for a set of experiments. The experiments were repeated at least twice, in most cases more than three times, and the reproducibility was satisfactory.

3. Results

3.1. Plasma lipid oxidation induced by peroxyl radicals, peroxynitrite, hypochlorite, and 15-lipoxygenase

The production of lipid hydroperoxides was followed continuously in the oxidation of mouse plasma induced by multiple oxidants by an increase in fluorescence intensity (FI) due to DPPP oxide formed by the reaction of DPPP and lipid hydroperoxides. AAPH, MeO-AMVN, SIN-1, hypochlorite, and 15-lipoxygenase all induced lipid hydroperoxide production as shown in Fig. 1A. The concentration dependence on MeO-AMVN, SIN-1, and 15-lipoxygenase is shown in Fig. 1B–D, respectively. It may be noteworthy that a lag phase was observed before rapid increase in FI in the oxidation induced by AAPH and SIN-1, but not in the oxidation induced by MeO-AMVN, lipoxygenase, and hypochlorite. This may be because hydrophilic endogenous antioxidants in plasma such as