Plasma lipid oxidation induced by peroxynitrite, hypochlorite, lipoxygenase and peroxy 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 peroxy radicals with a microplate reader. A clear lag phase was observed in the plasma oxidation mediated by aqueous peroxy 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 peroxy 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.

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Keywords:
Antioxidant
Diphenyl-1-pyrenylphosphine (DPPP)
Hypochlorite
Lipid oxidation
Lipoxygenase
Peroxy nitrite

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, peroxy 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.

Peroxy nitrite 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...
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 chloramines, 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 hydroperoxy radicals, and 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 hydroperoxy radicals, which undergo decomposition to give carbon- and nitrogen-centered radicals.

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]. Given 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 various 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 lipooxygenase 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-aminopropane) dihydrochloride (AAPH) and 2,2’-azobis-(4-methoxy-2,4-dimethylvaleronitrile) (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-hydroxycromin (Troxlox) were purchased from Cayman Chemical Company (Michigan, USA). Sodium hypochlorite, nordihydroguaiaretic acid (NDGA), and baicalin 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...
vitamin C and uric acid inhibited plasma oxidation initiated by free radicals produced in the aqueous phase, whereas the oxidation induced by lipoxygenase and hypochlorite was not inhibited by endogenous antioxidants in plasma. Hydroperoxides formed by the peroxyl radicals derived from MeO-AMVN may account for some of FI. Concentration of DPPP was changed from 0 to 150 μM. The FI increased with an increase in DPPP concentration, but reached almost plateau at 100 μM (data not shown). Therefore, the oxidation was performed with 100 μM DPPP.

AAPH and MeO-AMVN decompose thermally to produce carbon-centered radicals, which react rapidly with oxygen to give peroxyl radicals [42,43]. The peroxyl radicals thus formed attack plasma lipids to induce chain reaction of lipid peroxidation yielding cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides as major products [44,45]. The effects of AAPH and plasma concentrations were studied (Fig. 2). The lag phase was directly proportional to 1/[AAPH] and plasma concentration (Fig. 2B and D). Similarly, MeO-AMVN, a lipid soluble azo initiator which produces peroxyl radicals in lipophilic domain [42], induced plasma lipid peroxidation giving rise to an increase in FI in a concentration dependent manner (Fig. 1B). It may be noted that the reaction of peroxyl radicals derived from MeO-AMVN with antioxidant or polyunsaturated lipids produce hydroperoxides, which react with DPPP to give DPPP oxide.

Furthermore, SIN-1 induced lipid oxidation and increased FI in a concentration dependent manner (Fig. 1C). SIN-1 decomposes thermally to give superoxide and nitric oxide, which react rapidly to yield peroxynitrite, ONOO-, the rate constant being $10^{10} M^{-1}s^{-1}$. It may be present also as peroxynitrous acid, ONOOH, the pKa being 6.8 [6]. At the physiological pH of 7.4, peroxynitrite anion will be present in proportion of 80%. Peroxynitrous acid decomposes homolytically to hydroxyl radical and nitrogen dioxide radical. Peroxynitrite reacts with carbon dioxide, the rate constant being $4.6 \times 10^{4} M^{-1}s^{-1}$, to give a nitrosoperoxocarboxylate adduct (ONOOCO$_2^-$) that undergoes a fast homolysis to $^1$NO$_2$ radical and carbonate radical anion ($CO_3^{2-}$) in ~34% yields, with the remaining yielding carbon dioxide and nitrate, NO$_3$ [5,6,46]. Thus, peroxynitrite may give several reactive oxidants and induce lipid oxidation.

15-Lipoxygenase also induced oxidation of plasma lipids without lag phase and a concentration dependent increase in FI was observed (Fig. 1D). It has been reported that rabbit reticulocytes 15-lipoxygenase oxidizes plasma to give phosphatidylcholine and cholesterol ester hydroperoxides regio-, stereo-, and enantio-specifically [14,15].

Hypochlorite similarly induced lipid oxidation in plasma to produce lipid hydroperoxides (Fig. 1A). It was reported that proteins were major target of hypochlorite in plasma [47], but it also
induces lipid oxidation by both non-radical and radical mechanisms [16,17]. The free radical derived from chloramines may induce lipid peroxidation.

The plasma oxidation induced by AAPH was studied in some more detail (Fig. 2A and B). The lag phase decreased with increasing AAPH concentration, as expected in proportion to 1/[AAPH], since lag phase is inversely proportional to the rate of free radical flux which is proportional to AAPH concentration. The effects of plasma concentration on the increase in FI are shown in Fig. 2C. A clear lag phase was observed, which increased proportionally with increasing plasma concentration (Fig. 2D). The slope of the plot of lag phase against plasma concentration was obtained as 29.5 s/%.

In Fig. 3 are shown the effects of plasma concentration on the lipid oxidation induced by SIN-1. The lag phase increased with increasing plasma concentration and the plot of lag phase as a function of plasma concentration gave a straight line with a slope 48 s/% and an intercept on Y-axis 300 s.

3.2. Effects of antioxidants against plasma lipid oxidation induced by multiple oxidants

The effect of Trolox on the plasma oxidation induced by AAPH is shown in Fig. 4A and B. The plot of lag phase produced by Trolox against Trolox concentration gave a straight line: lag phase = \( n[\text{Trolox}]/Ri \) [423x362] The stoichiometric number, \( n \), for Trolox is 2. The rate of free radical flux from AAPH, \( Ri \), is calculated from the slope of the plot shown in Fig. 4B: \( Ri = 2/\text{slope} \). The average \( Ri \) with 100 mM AAPH was obtained from the slope of three independent experiments as \( 4.7 \times 10^{-8} \text{ M/s} \). The concentration of endogenous antioxidants which produced lag phase in the oxidation of plasma is then calculated from the lag phase obtained from the results in Fig. 2D, 29.5 s/%, and the above \( Ri \) as \( n[\text{antioxidant}] = \text{lag phase} \times Ri = 29.5 \times 10^2 \times 4.7 \times 10^{-8} = 1.4 \times 10^{-4} \text{ M} \), assuming \( n \) is 1.

The total concentration of antioxidants contained in plasma, termed total antioxidant capacity (TAC), may be estimated from the lag phase produced by plasma in the consumption of a probe such as fluorescein and pyranine induced by AAPH. It was estimated in the previous study that the mouse plasma contained \( 9.74 \times 10^{-4} \text{ M} \) antioxidants, more strictly speaking the plasma could scavenge \( 9.74 \times 10^{-4} \text{ M} \) peroxyl radicals [41]. This is about 7 times larger than the above value, \( 1.4 \times 10^{-4} \text{ M} \), estimated from the lag phase observed in the oxidation plasma lipids. This difference is attributed to the fact that TAC estimated from the lag phase produced by unreactive probe such as fluorescein and pyranoine counts weak antioxidants as well which are not reactive enough to scavenge peroxyl radicals in competition with biological substrates to suppress plasma oxidation.

The effects of Trolox on the SIN-1 induced plasma oxidation were also studied (Fig. 4C, D). Trolox increased lag phase and the plot of lag phase against Trolox concentration gave a straight line with a slope and Y-axis intercept 67.3 s/\( \mu \)M and 800 s, respectively. The rate of free radical flux from 0.5 mM SIN-1 under the present reaction conditions is calculated as \( Ri = n/\text{slope} = 2/67.3 \mu \text{M}/s = 3.0 \times 10^{-8} \text{ M/s} \). With these \( Ri \) and Y-axis intercept, the
apparent endogenous antioxidant concentration which produced lag phase in the SIN-1 induced plasma lipid oxidation is calculated as $n[H]=800 \times 3.0 \times 10^{-8}=2.4 \times 10^{-5}$ M, which is much smaller than the above value $1.4 \times 10^{-4}$ M obtained from the lag phase observed for oxidation induced by AAPH. This difference reflects the different reactivities in the initiating species derived from AAPH and SIN-1.

The antioxidant effects against lipid peroxidation induced by azo-initiators have previously been studied in detail [44, 45]. In the present study, the effects of several antioxidants on the plasma lipid oxidation induced by peroxynitrite, hypochlorite, and 15-lipoxygenase were assessed.

The effects of $\alpha$-tocopherol, $\gamma$-tocopherol, ascorbic acid, uric acid, and Trolox on the plasma lipid oxidation induced by SIN-1 are shown in Fig. 5A. $Fl$ increased after a lag phase of around 1000 s independent of the presence or absence of added antioxidant, which was produced by the endogenous antioxidant in the plasma. The $Fl$ did not increase linearly with time in the oxidation induced by SIN-1, which may be ascribed to complex initiation mechanisms of lipid oxidation mediated by multiple oxidants derived from SIN-1. Hydrophilic antioxidant such as ascorbic acid, Trolox, and uric acid suppressed oxidation more significantly than lipophilic $\alpha$-tocopherol and $\gamma$-tocopherol. As observed previously [12], $\gamma$-tocopherol exhibited more potent antioxidant effect than $\alpha$-tocopherol against SIN-1 induced lipid oxidation (Fig. 5C), which implies the contribution of $^{\bullet}$NO$_2$ radical. However, considering much higher bioavailability and physiological concentration of $\alpha$-tocopherol than $\gamma$-tocopherol and the difference between the two tocopherols shown in Fig. 5C, it may be surmised that $\alpha$-tocopherol exerts more potent effect than $\gamma$-tocopherol in vivo against SIN-1 mediated plasma oxidation.

The effects of glutathione, bilirubin, baicalein, and fucoxanthin...
Fig. 4. Effect of Trolox on the lipid oxidation of plasma induced by AAPH (A, B) and by SIN-1 (C, D). Plot of lag phase against Trolox concentration in the oxidation by AAPH (B) and SIN-1 (D). Plasma (10%) was oxidized with 100 mM AAPH or 0.5 mM SIN-1 in the presence of 100 μM DPPP and added Trolox at 37 °C and an increase in fluorescence intensity (FI) was measured as described in the Section 2. The numbers in the Figures A and C are Trolox concentration in μM.

Fig. 5. Effect of antioxidants on the plasma oxidation induced by SIN-1. Plasma was oxidized with 0.5 mM SIN-1 in the presence of 100 μM DPPP and 50 μM (A) α-tocopherol, γ-tocopherol, ascorbic acid, uric acid, (B) glutathione, bilirubin, baicalein, and fucoxanthin. (C) Plot of FI(+IH)/FI(-IH) against α-tocopherol (black) and γ-tocopherol (red) concentration.
on the SIN-1 induced plasma oxidation are shown in Fig. 5B. They suppressed the oxidation partially, but kinetic analysis is difficult.

The results of antioxidant effects against plasma lipid oxidation induced by hypochlorite are shown in Fig. 6. Among the antioxidants tested, bilirubin and fucoxanthin inhibited lipid oxidation efficiently, while \( \alpha \)-tocopherol, ascorbic acid, uric acid, glutathione, and bilirubin (50 \( \mu \)M) and the production of lipid hydroperoxides was followed as described in the Section 2.

Neither \( \alpha \)-tocopherol nor Trolox exerted significant antioxidant effect against 15-LOX-induced plasma lipid oxidation, whereas nordihydroguaiaretic acid (NDGA), baicalein, and caffeic acid inhibited oxidation efficiently in a concentration dependent manner (Fig. 7), the IC50 being 2.8, 6.8, and 29 \( \mu \)M respectively.

### 4. Discussion

The above results show that peroxynitrite, hypochlorite, 15-LOX, as well as the peroxyl radicals produced by azo initiator all induced plasma lipid oxidation to give lipid hydroperoxides. This is a novel method using DPPP as a probe to follow the production of lipid hydroperoxides in the oxidation of plasma induced by different kinds of oxidants and also to assess the antioxidants effects of specific compounds and natural products.

Azo compound, although biologically irrelevant, generates peroxyl radicals in the presence of oxygen at a constant rate and controlled site, which is useful for kinetic study on the oxidation of biological molecules and its inhibition [43]. The peroxyl radicals derived from azo compounds and those formed in vivo physiologically and pathologically react with biological molecules similarly.

Peroxynitrite is one of the important oxidants produced in vivo [5,49,50], which has been reported to induce plasma lipid oxidation to produce cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides [8–12]. This study showed continuous accumulation of plasma lipid hydroperoxides mediated by peroxynitrite and other
oxidants derived from it including hydroxyl radical, nitrogen dioxide, and carbonate radical anion [5,6,49].

Hypochlorite is another important oxidant playing important role especially at the site of inflammation. It has been reported that proteins are major target in plasma for hypochlorite [47], but the relative importance of lipid oxidation and protein oxidation is not clear and the contribution of protein hydroperoxides in DPPP oxide formation cannot be ruled out. The characterization of lipid hydroperoxides produced by hypochlorite is a subject of future study.

The above results show that antioxidant effect against lipid oxidation induced by different oxidants is assessed easily by the present method using DPPP in biologically relevant system. It is important to understand that multiple oxidants with different reactivity and selectivity contribute to the oxidation of biological molecules in vivo and that the efficacy of antioxidants depends on the type of oxidants. It has been shown that vitamin E and vitamin C inhibit plasma lipid peroxidation quite efficiently [44,45], but the effects of antioxidants against plasma oxidation by other oxidants have not been studied as extensively.

Ascobic acid exerted more potent antioxidant effect against peroxy nitrite induced oxidation than uric acid and bilirubin. γ-Tocopherol suppressed lipid oxidation more than α-tocopherol (Fig. 5) as reported previously [12]. γ-Tocopherol which is contained in diets as much as α-tocopherol deserves more attention [51], but much lower bioavailability than α-tocopherol should be taken into consideration [33].

α-Tocopherol exerted poor antioxidant effect against 15-LOX mediated oxidation, while NDGA, baicalein, and caffeic acid inhibited it in a concentration dependent manner. NDGA used anciently in folk medicine for the treatment of multiple diseases is known as lipoxigenase inhibitor [52] and showed most potent inhibition among the antioxidant tested in this study. The inhibitory effect of baicalein against 12/15-LOX was reported previously [53].

Glutathione is one of the most important antioxidants in vivo, working as an essential reducing agent with glutathione peroxidases. In the present study, glutathione did not exert a potent antioxidant effect against plasma lipid oxidation induced by peroxynitrite and hypochlorite, although glutathione has been reported to scavenge hypochlorite rapidly with the rate constant 1.1 × 10^9 M^-1 s^-1 [54] and it reacts with peroxynitrous acid by a rate constant 7.3 × 10^8 M^-1 s^-1 [55]. Glutathione scavenges peroxyl radical slower than ascorbic acid [56] and further the resulting glutathione thyl radical is not stable, making glutathione less potent antioxidant against lipid oxidation.

Collectively, the method employed in the present study using DPPP as a probe may be useful to follow plasma lipid oxidation induced by multiple oxidants and also to assess the antioxidant capacity in biologically relevant settings.

Conflict of interest

None.

References
