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Plasma Phosphatidylcholine Hydroperoxide-reducing Activity in Pregnant Women

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Plasma glutathione peroxidase (PGPx) and apolipoproteins A-I, A-II, and B-100 reduce phosphatidylcholine hydroperoxide (PC-OOH) to its hydroxide (PC-OH). To elucidate the relative importance of the reduction pathways we developed a simple assay for measuring total PC-OOH-reducing activity. Human plasma was incubated with 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide and the time-dependent reduction was confirmed by its hydroxide formation, measured by reversed-phase high performance liquid chromatography. We determined the PC-OOH reducing activity in blood plasma of healthy men and women as 119 ± 7 (n = 13, aged 27 to 45) and 101 ± 4 μM/h (n = 5, aged 24 to 30), respectively. In addition, we also measured PC-OOH-reducing activity in the plasma of 53 pregnant women since they usually show hyperlipidemia or hyper-apolipoproteinemia. The average rate of PC-OOH reduction was 101 ± 34 μM/hr. The PC-OOH-reducing activity was not affected by the addition of iodoacetamide, an inhibitor of PGPx, suggesting that the activity is due to apolipoproteins. A significant correlation between plasma reducing activity with apolipoprotein B-100 was observed (r = 0.290), but not with apolipoprotein A-I (r = 0.118). In pre-eclamptic patients, about an 8% decrease in plasma PC-OOH-reducing activity was observed as compared to the normal pregnancy group, although the decrease was not statistically significant.

Key words : Phosphatidylcholine hydroperoxide, Phosphatidylcholine hydroperoxide-reducing activity, Plasma glutathione peroxidase, Apolipoprotein B-100, Pregnancy

INTRODUCTION

Lipid peroxidation has been proposed to play a critical role in the aging process and in degenerative diseases such as cancer, rheumatoid arthritis, heart attack, and stroke [1]. Lipid hydroperoxides are the primary stable products of lipid peroxidation. For example, free radical chain oxidation of lipoprotein yields phosphatidylcholine hydroperoxide (PC-OOH) and cholesterol ester hydroperoxide (CE-OOH) as major products. Lipid hydroperoxides need to be eliminated since they are toxic to cells *in vitro* and their metal-catalyzed decomposition gives rise to oxygen radicals which may further initiate the chain oxidation of biomolecules [2]. Circulating lipid hydroperoxides are damaging to endothelial cells *in vitro* [3], and therefore may be present in diseases related to endothelial cell dysfunction, such as atherosclerosis, and hence coronary disease. Pre-eclampsia is a pregnancy-specific condition characterized by hypertension, edema and proteinurea that resolves after delivery. Because these symptoms are thought to be secondary to endothelial cell dysfunction, plasma lipid hydroperoxide has been proposed as a candidate factor causing the endothelial cell dysfunction in pre-eclampsia [4, 5]. It has been shown that plasma glutathione peroxidase (PGPx) reduces PC-OOH to its hydroxide (PC-OH) [6, 7]. However, this enzyme can not reduce CE-OOH [6]. Glutathione transferase [8] and thioredoxin reductase [9] are also suggested

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Table 1 Patient characteristics

	Subjects (n)	age (Years)	Gestational age (weeks)	Systolic Blood pressure (mmHg)	Birth weight (g)
Normal pregnancy	53	32 ± 5	25 ± 9	109 ± 14	3066 ± 339
High-risk group for pre-eclampsia	11	29 ± 3	23 ± 9	108 ± 9	2718 ± 469
Pre-stage for pre-eclampsia	10	31 ± 6	21 ± 7	109 ± 12	2768 ± 383
Pre-eclampsia	11	29 ± 5	26 ± 10	131 ± 23	2367 ± 1098

Values are expressed as mean ± SD

as capable of reducing hydroperoxides. In addition to the enzymatic reduction of lipid hydroperoxides, it was found that apolipoproteins A-I, A-II, and B-100 reduce lipid hydroperoxides such as PC-OOH and CE-OOH [10, 11]. This reduction is methionine dependent and methionine sulfoxide is produced as a product [12, 13].

To evaluate the relative importance of the enzymatic and methionine dependent reductive pathways in removing lipid peroxide, we first developed a simple assay method to measure PC-OOH-reducing activity in human plasma using high performance liquid chromatography (HPLC). With this method, we measured the correlation of plasma PC-OOH-reducing activity with levels of apolipoprotein A-I, or apolipoprotein B-100 in plasmas from hyperlipidemic pregnant women. In addition, to estimate the patho-physiological roles of PC-OOH-reducing activity in the development of pre-eclampsia, activity was also measured in pre-eclamptic patients.

MATERIALS AND METHODS

Patients: 92 pregnant patients were included in this study. Of these, 53 were normal pregnancies, 11 were mild pre-eclampsia, 18 were pre-stage of pre-eclampsia, (these patients developed pre-eclampsia after blood sampling), and 11 were high-risk group for pre-eclampsia (6 had nephritis, 2 asthma, 2 had pre-eclampsia during a previous pregnancy, and 1 Behcet's disease; none developed pre-eclampsia until delivery). Detailed clinical features are shown in table 1. Pre-eclampsia was defined as: the onset of hypertension, blood pressure over 140/90 mmHg; protein-urea over 30mg/dl; and edema with body weight gain over 500g/week. The patients with diagnosed pre-eclampsia were given drugs to lower blood pressure and improve blood supply. After obtaining informed consent, 5 ml of blood was drawn by venipuncture for routine laboratory investigations from each patient. After centrifugation at 2000g for 20 min at 4℃, the plasma was removed and aliquoted into 1.5ml Ependorf tubes, then stored at − 80℃ until analysis.

Assay method of PC-OOH-reducing activity: 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) and soybean lipoxidase (type 1B) were purchased from Sigma Japan (Tokyo). PLPC-hydroperoxide (PLPC-OOH) was prepared as described previously [14]. Briefly, PLPC was oxidized with soybean lipoxygenase under aerobic conditions and PLPC-hydroperoxide (PLPC-OOH) was purified by HPLC (Superiorex ODS column, 20 × 250 mm, Shiseido, Tokyo) using methanol containing 0.02% triethylamine as the mobile phase. The PC-OOH-reducing activity was measured as follows: 2 μl of 0.5 mM PLPC-OOH in methanol (final 50 μM) was mixed with 4 μl of 1 M Tris buffer (pH 7.2, final 0.2 M), 1 μl of 0.1 M EDTA (final 5 mM), 11 μl of water and 2 μl of human plasma. The reaction mixture was then incubated at 37℃ for 1 hr under aerobic conditions. PLPC-OOH and PLPC-OH were extracted by adding 80 μl of methanol, and centrifuged at 12,000g for 10 min at 4℃. The supernatant (10 μl) was injected onto a CAPCELL PAK ODS column (5 μm, 4.6 × 250 mm; Shiseido, Tokyo) with UV detection at 234 nm. The mobile phase used was acetonitrile-methanol-water 100:99:1 (v/v/v) containing 10 mM chorine chloride and the flow rate was 1 ml/min [15].

Other biochemical methods: Plasma lipid

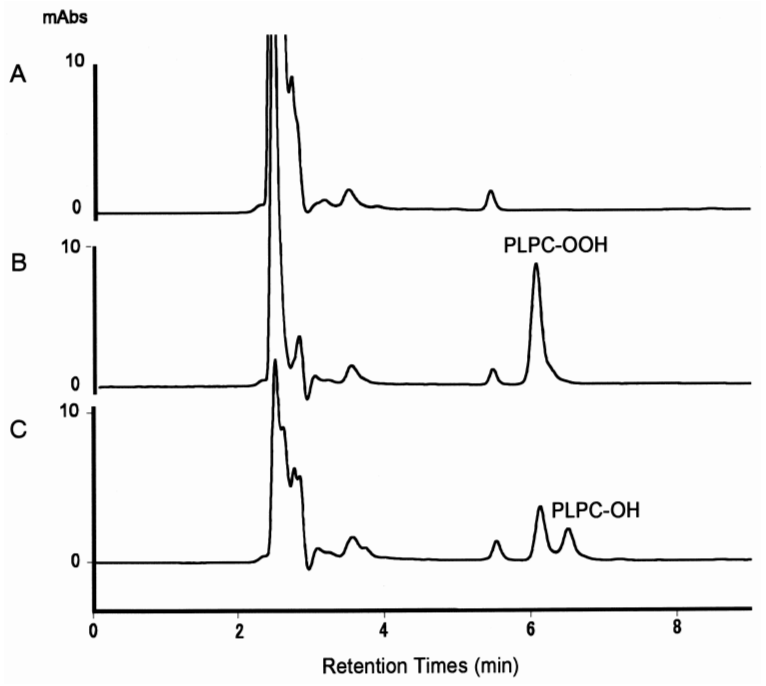

Fig. 1 Reversed-phase HPLC chromatograms of methanol extracts of (A) human plasma, (B) human plasma treated with 50 μM PLPC-OOH for 0 min, and (C) that incubated at 37℃ for 60 min.

hydroperoxide level was measured by using a Lipid Hydroperoxide (LPO) Assay Kit (CAYMAN CHEMICALS, MI USA) according to the manufacturer's instruction. PGPx activity in the plasma was determined spectrophotometrically at 340 nm, as previously described using cumene-hydroperoxide (0.23 mM) as a substrate [16]. ApoA1 and apoB levels were determined by immunonephelometry (Behring diagnostic, Germany).

Iodoacetamide treatment of human plasma: In order to estimate the contribution of PGPx to PC-OOH-reducing activity, human plasma was pretreated with iodoacetamide to inactivate PGPx. Human plasma was incubated in the presence or absence of iodoacetamide at 4℃ for 1 hour, followed by measurement of PC-OOH-reducing activities as described above.

Statistical Analysis: Statistical analysis was evaluated by Student's t-test, and data expressed as mean ± SD. Differences were considered significant at $p > 0.05$.

RESULTS AND DISCUSSION

Assay method of PC-OOH-reducing activity: We developed an HPLC method for the measurement of total PC-OOH-reducing activity in human plasma using PLPC-OOH as a substrate. Figure 1 demonstrates that PLPC-OOH and its reduction product (PLPC-OH) can be quantitated simultaneously and the methanol extract of plasma does not interfere with measurement. The standard assay procedure is described in Methods section. Briefly, 50 μM PLPC-OOH was incubated in 20 μl of buffer solution containing 2 μl of human plasma. Figure 2 shows that the rate of reduction was constant for at least 60 min. The amounts that PLPC-OOH decreased and PLPC-OH formed were equivalent, suggesting that reduction is the major reaction. The rate of PLPC-OOH reduction increased with increasing content of plasma in the reaction mixture (Fig. 2).

PC-OOH-reducing activity in healthy human plasma: By using the assay method, we initially determined the PC-OOH-reducing activity in the blood plasma of healthy males and females. This was estimated as 119 ± 7 (n = 13, aged 27 to 45) and 101 ± 4 μM/h (n = 5, aged 24 to 30), respectively.

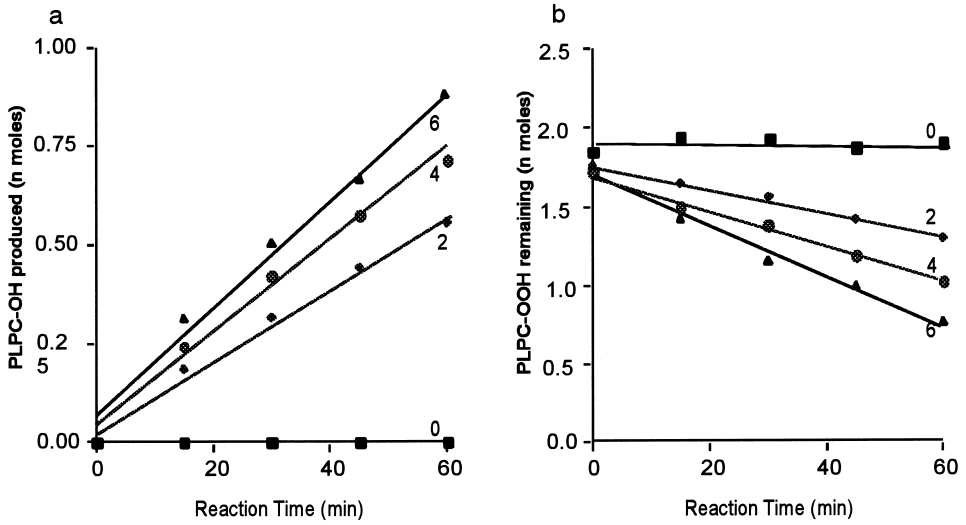

Fig. 2 Decrease of (A) PLPC-OOH and the formation of (B) PLPC-OH during the incubation of human plasma with 50 μM PLPC-OOH under aerobic conditions at 37 ℃. Numbers show volume (μl) of human plasma used.

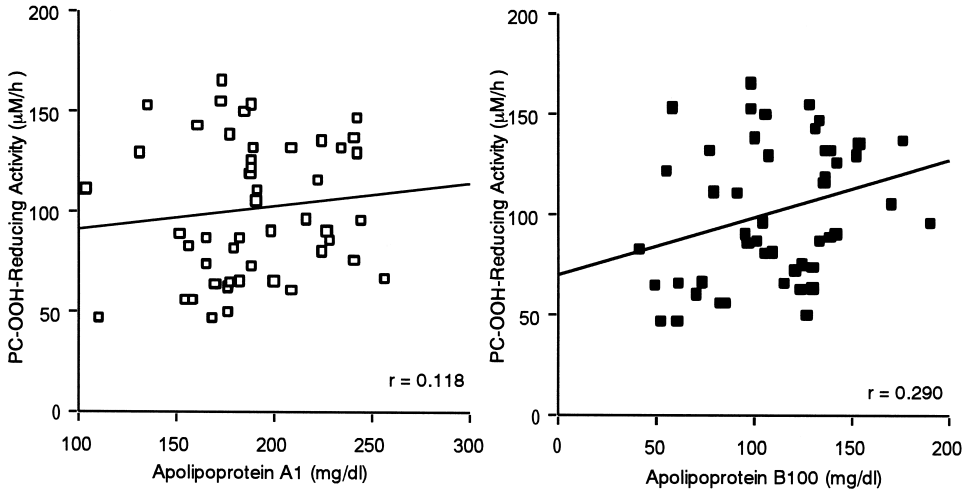

Fig. 3 PC-OOH-reducing activity against apolipoprotein A-I and apolipoproteins B-100 levels observed in plasmas from 53 pregnant women.

These data indicated that human plasma could reduce more than 100 μM PC-OOH to PC-OH within 1 hour. This value is much higher than previously obtained. Frei et al reported that human plasma could reduce over 10 μM PC-OOH and linoleic acid-OOH within 2 hours [17]. Additionally, it was reported that human and rat plasma could reduce 35 μM PC-OOH within 2 hours [10]. In these reports, lipid hydroperoxide added to plasma was completely reduced, suggesting that plasma could reduce more PC-OOH than had been reported. In our experiments, we used small amounts of plasma samples in the reaction system and relatively large amounts of substrate. Therefore, the large PC-OOH-reducing activity was obtained in the present study.

Relative importance of PGPx in PC-OOH-reducing activity: It has been reported that PGPx and apolipoproteins A-I, A-II, and B-100 can reduce PC-OOH to PC-OH [8–12].

Table 2 Effects of iodoacetamide pretreatment and addition of GSH on PC-OOH-reducing activity in blood plasma.

Treatment	PLPC-OOH	PLPC-OH	Total
No	0.500		
Plasma	0.390	0.127	0.517
Plasma + Iodoacetamide (1mM)	0.386	0.120	0.506
Plasma + Iodoacetamide (5mM)	0.339	0.103	0.441
Plasma + GSH (1mM)	0.210	0.248	0.458
Plasma + GSH (2mM)	0.099	0.422	0.521

Values (n moles) are the means of 3 experiments

Table 3 Lipid peroxide, PGPx and PC-OOH-reducing activity in Pre-eclampsia patients.

	Subjects (n)	Lipid peroxide (n moles)	PGPx activity (units/ml)	PC-OOH reducing activity (μM/h)
Normal pregnancy	53	0.89 ± 0.68	0.266 ± 0.051	100.9 ± 33.3
High-risk group for pre-eclampsia	11	0.82 ± 0.43	0.271 ± 0.060	116.7 ± 28.0
Pre-stage for pre-eclampsia	10	0.85 ± 0.23	0.268 ± 0.054	89.6 ± 29.1
Pre-eclampsia	11	1.15 ± 0.86*	0.266 ± 0.048	92.6 ± 34.2

Values are expressed as mean ± SD
*Statistically significant compared with normal pregnant group $P < 0.05$.

To elucidate the relative importance of these reduction pathways, we first measured PC-OOH-reducing activity before and after the iodoacetamide treatments, since it inactivates PGPx by binding with selenocysteine residue. As shown in Table 2, the iodoacetamide treatment resulted in little change in PC-OOH-reducing activity. PGPx requires 2 molecules of reduced glutathione (GSH) for the reduction of 1 molecule of PC-OOH, and human plasma contains about 5 μM GSH [18] suggesting that PGPx can reduce only about 2.5 μM PC-OOH in the assay condition. Since plasma PC-OOH was reported at submicromolar levels, the GSH concentration was enough to reduce plasma PC-OOH by PGPx. Addition of 2 mM GSH enhanced the PC-OOH-reducing activity by 3-fold (Table 1). It was reported that PGPx accounts for essentially all of the plasma GSH-dependent lipid-hydroperoxide reducing activity [19], and PLPC-OOH could not be reduced by incubation with only GSH (data not shown). Thus, it is likely that the enhanced PC-OOH-reducing activity with GSH would be due to the PGPx in the plasma. The present data indicates that PGPx can reduce large amounts of PC-OOH when GSH is supplied, and also suggests that the PC-OOH-reducing activity measured in this study is not due to PGPx.

Correlations of PC-OOH-reducing activity with apolipoproteins: We correlated PC-OOH-reducing activity with apolipoprotein levels. For this purpose, we used plasmas from pregnant women since they usually show hyperlipidemia [20]. The average rate of PC-OOH reduction was 101 ± 34 μM/hr (n = 53). Figure 3 shows that the correlation with the apolipoprotein B-100 level was moderate ($r = 0.290$), but that with the apolipoprotein A-I level was poor ($r = 0.118$). The reduction of PC-OOH by apolipoprotein is methionine dependent, and 78 methionines are present in one molecule of apolipoprotein B-100 and 3 methionines in apolipoprotein A-I. A poor correlation between reducing activity and the

apolipoprotein A-I level may indicate some of methionine residues are oxidized to methionine sulfoxide. This idea is hypothetical and needs further study.

Pre-eclampsia and PC-OOH-reducing activity: Pre-eclampsia is a pregnancy-specific condition characterized by hypertension, edema and proteinurea. These symptoms are thought to be secondary to endothelial cell dysfunction. Therefore, plasma lipid hydroperoxide has been proposed as a factor causing endothelial cell dysfunction in pre-eclampsia [4, 5, 21]. Consistent with previous reports, we also noted a significant increase in plasma lipid peroxide levels in pre-eclamptic patients, but not in the pre-stage and high-risk groups for pre-eclampsia, as compared to the normal pregnancy group (Table 3). PGPx is one of the primary antioxidants present in blood plasma that limits the concentration of lipid peroxide [22]. In severe pre-eclamptic patients, Uotila et al reported that PGPx activity was elevated over that in women experiencing a normal pregnancy, and suggested that this increase might be caused by an adaptive response to the elevated lipid peroxide [5]. However, we found no PGPx changes in the 4 groups we studied (Table 3), although the lipid peroxide level was increased significantly in the pre-eclamptic group. Uotila et al measured activity in patients with severe pre-eclampsia, while we measured activity in patients with mild pre-eclampsia. This difference might explain why PGPx activity was unchanged in our study. PC-OOH- reducing activity, measured in the 4 groups studied, revealed an 8% decrease in PC-OOH-reducing activity in the pre-eclampsia group compared to the normal pregnancy group (Table 3). However, the decrease was not statistically significant. Among various speculations regarding the etiology of elevated lipid peroxide in pre-eclampsia, one line of thought has considered a possible deficiency in antioxidants. Although the decrease of PC-OOH-reducing activity was not statistically significant, it is likely that the decrease may cause an accumulation of lipid peroxides in blood plasma. Interestingly, a decreased PC-OOH-reducing activity was also observed in the pre-stage pre-eclamptics. At the time of blood sampling, these patients did not develop pre-eclampsia and their lipid peroxide levels did not change. This data suggests that the pre-stage group might be susceptible to lipid peroxidative stress, which may lead to pre-eclampsia. In contrast, the high-risk group showed higher levels of activity than the normal pregnancy group. This group did not develop pre-eclampsia until delivery, and consisted of pregnant patients with nephritis, asthma, Behcet's disease and a history of pre-eclampsia in a previous pregnancy. The increased PC-OOH-reducing activity, which might have developed in response to increased oxidative stress caused by the basal diseases, may have facilitated the recovery of plasma lipid hydroperoxide to the normal range.

In summary, we described an HPLC method for the measurement of total PC-OOH-reducing activity in human plasma. With this assay method, the PC-OOH-reducing activity in the blood plasma of healthy males and females was estimated as 119 ± 7 (n=13, aged 27 to 45) and 101 ± 4 μM/h (n=5, aged 24 to 30), respectively. A significant correlation between the reducing activity with apolipoprotein B-100 was observed ($r = 0.290$), but not with apolipoprotein A-I ($r = 0.118$). In pre-eclamptic patients, about an 8% decrease in plasma PC-OOH reducing activity was observed compared to the normal pregnancy group, but the decrease was not statistically significant.

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