

Plasma phosphatidylcholine hydroperoxide as a new marker of oxidative stress in alcoholic patients

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Abstract Quantitative analysis of plasma phosphatidylcholine hydroperoxide (PCOOH) is an important step in evaluating the biochemical processes leading to oxidative injury. However, secondary products of lipid peroxidation are now used as indices. One hundred nine alcoholic patients, aged 22–81 years (mean \pm SEM, 52.0 ± 1.3 years), and 21 healthy volunteers, aged 41–79 years (51.2 ± 2.2 years), participated in this study. Plasma PCOOH was measured by HPLC with chemiluminescence detection. Plasma PCOOH concentration was significantly higher in alcoholic patients (46.1 ± 4.1 pmol/ml) than in controls (15.6 ± 1.8 pmol/ml). It was significantly higher in patients with blood alcohol (88.0 ± 10.5 pmol/ml) than in those without alcohol (32.6 ± 3.1 pmol/ml). The patients with high levels of aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transpeptidase (γ -GTP), and triglyceride (TG) showed significantly higher PCOOH concentrations than did patients with normal levels. The PCOOH level was positively correlated with levels of γ -GTP, HDL, blood alcohol concentration, and TG. Plasma PCOOH levels in 29 alcoholic patients after a 6 week abstinence were decreased significantly (22.8 ± 11.1 pmol/ml), which was associated with improvement on liver function tests. This is the first measurement of plasma PCOOH in alcoholic patients. **These results suggest the involvement of lipid peroxidation in alcohol-induced liver damage and confirm that the PCOOH plasma concentration is a new marker of alcohol consumption as well as oxidative stress in alcoholic patients.**—Adachi, J., S. Matsushita, N. Yoshioka, R. Funae, T. Fujita, S. Higuchi, and Y. Ueno. **Plasma phosphatidylcholine hydroperoxide as a new marker of oxidative stress in alcoholic patients.** *J. Lipid Res.* 2004. 45: 967–971.

Supplementary key words high-performance liquid chromatography • liver disorder • lipid peroxidation

The induction of CYP2E1 in hepatocytes (1, 2) as well as in Kupffer cells (3) by chronic alcohol has been shown

to generate free radicals and cause liver disease attributable to the peroxidation of membrane lipids.

Previously, we developed methods for quantifying cholesterol hydroperoxides by HPLC with chemiluminescence detection (HPLC-CL) (4) and oxysterols by HPLC with UV detection (5). Subsequently, we demonstrated increased membrane cholesterol peroxidation by analyzing 7-hydroperoxycholesterols as well as oxysterols in skeletal muscle (6), liver (7), and heart (5) of rats with chronic administration of alcohol. However, direct demonstration of increased lipid peroxidation in patients with alcoholic liver disease (ALD) has been difficult because plasma concentrations of 7-hydroperoxycholesterol and oxysterols are too low to be detected. Recently, lipid peroxidation was assessed by analyses of 4-hydroxynonenal (8), serum malondialdehyde (9, 10), and F₂-isoprostane excretion (11), secondary products of phospholipid peroxidation. Plasma phosphatidylcholine hydroperoxide (PCOOH), a direct peroxidation product, may be a sensitive and specific index of lipid peroxidation in vivo.

To assess oxidative stress in alcoholic patients with ALD, PCOOH plasma concentration was measured in 109 alcoholic patients and 21 healthy controls. Additionally, factors relevant to lipid peroxidation among various clinical tests were investigated in alcoholic patients.

MATERIALS AND METHODS

Materials

3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), luminol (3-aminophthaloylhydrazine), and cytochrome C (from horse heart, type

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; CL, chemiluminescence detection; γ -GTP, γ -glutamyl transpeptidase; PCOOH, phosphatidylcholine hydroperoxide; TC, total cholesterol; TG, triglyceride.

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VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 1-Palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (C16:0/C18:2-OOH) was synthesized by reaction with methylene blue under tungsten lamp irradiation at 15°C for 8 h, monitoring by HPLC and TLC. The whole reaction mixture was subjected to HPLC (Daiso-gel, SP-120-40/60-ODS-B, CHCl₃-methanol, UV 235 nm, at 50 ml/min). The product obtained was checked by mass spectrometry, TLC, and HPLC. Additionally, phospholipid amount was quantitatively analyzed as 1.95 mg/ml [mass spectrometry: 790.6 (M+H, monoperoxide) and 812.5 (M+Na, monoperoxide)].

Subjects

One hundred nine alcoholic patients, 93 men and 16 women, 22–81 years old (mean \pm SEM, 52.0 \pm 1.3 years), were used, and 21 healthy volunteers, 20 men and 1 woman, 41–79 years old (51.2 \pm 2.2 years), were recruited after obtaining their informed consent. This experiment had the approval of the ethical committee of the National Institute on Alcoholism, Kurihama National Hospital. The investigation conforms to the principles outlined in the Declaration of Helsinki. Blood samples were collected from alcoholic patients at the first medical examination and after a 6 week abstinence.

Serum transaminase [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] activities, γ -glutamyl transpeptidase (γ -GTP) activity, and the levels of serum albumin, cholinesterase, total bilirubin, total cholesterol (TC), triglyceride (TG), HDL, and creatinine were examined using routine laboratory techniques.

Six weeks after hospitalization, the levels of type 4 collagen and anti-hepatitis C virus antibody of 29 patients were determined. Of these, we diagnosed 9 patients with alcoholic hepatitis and 11 patients with liver cirrhosis using ultrasonography and laboratory data.

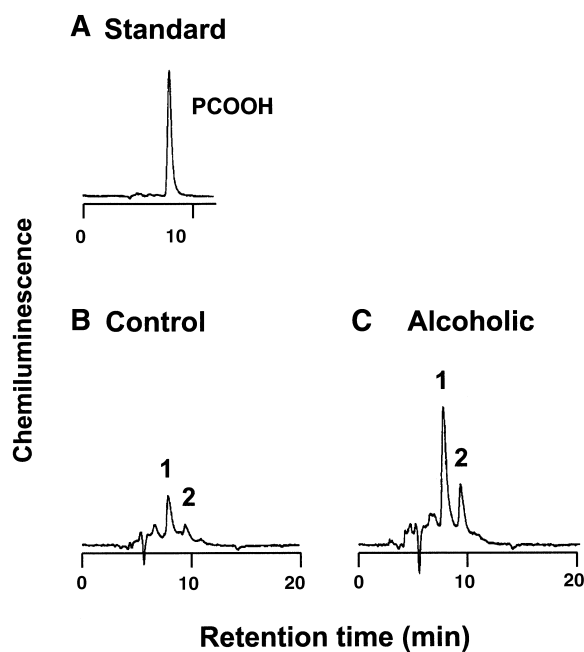


Fig. 1. HPLC chromatograms of phosphatidylcholine hydroperoxide (PCOOH). HPLC analyses of standard PCOOH and plasma samples of a control subject and an alcoholic patient by chemiluminescence detection. A: PC C16:0/C18:2-OOH. B: Control subject. C: Alcoholic patient.

Extraction

Three milliliters of blood was collected in a test tube containing 0.3 mg of EDTA.2Na and centrifuged at 4°C and 800 *g* for 10 min, and the plasma was fractionated. Total lipid was extracted by adding 0.5 ml of distilled water and 8 ml of ice-cold chloroform-methanol (3:1, v/v) containing 0.005% (v/v) BHT (as antioxidant) to 0.5 ml of plasma. The mixture was spun vigorously for 1 min and then centrifuged at 800 *g* for 20 min. The chloroform layer was aspirated off and concentrated in a rotary evaporator, then dried under a nitrogen stream. The phospholipid fraction then was isolated from the total lipid by solid-phase extraction. A silica column (Sep-Pak; Waters Co., Milford, MA) of 3 ml capacity packed with aminopropyl-derivatized silica (-NH₂) initially was conditioned by washing with 5 ml of acetone and 10 ml of *n*-hexane. The total lipid sample, dissolved in a small amount of chloroform, was layered on the column, which then was flushed with a mixture of 2 ml of chloroform and 1 ml of isopropanol, giving an eluate consisting mainly of cholesterol. The column next was flushed with methanol containing 0.005% BHT, giving an eluate consisting mainly of phospholipid. This was concentrated in a rotary evaporator, dried under a nitrogen stream, and dissolved in 150 μ l of methanol, and a 10 μ l portion was injected into the HPLC column.

HPLC-CL analysis

PCOOH was analyzed by HPLC-CL. The apparatus consisted of two LC-10AD vp pumps (Shimadzu, Kyoto, Japan), a CLD-10A chemiluminescence detector (Shimadzu), and a Chromatopac C-R8A integrator (Shimadzu).

An LC-18-DB column (250 \times 4.6 mm internal diameter; Supelco) with methanol containing 0.01% triethylamine as the mobile phase was used. Both the mobile phase and the chemiluminescent reagent were delivered at 0.7 ml/min. The reagent consisted of cytochrome C and luminol (10 and 2 μ g/ml, respectively) in alkaline borate buffer, pH 10. After the column eluent was passed through a UV detector set at 234 nm, it was mixed with the luminescent reagent in the postcolumn mixing joint of the chemiluminescence detector.

Individual peak areas were calculated with an integrator (Chromatopac C-R8A; Shimadzu). Standard PCOOH was injected at least three times per day to calculate the concentration, because chemiluminescent intensity was sometime unstable, particularly during the early period of analysis. The recoveries from the plasma extracts were determined by comparison of the peak area obtained after injection of a plasma extract spiked with a known concentration. The recoveries were \sim 70%.

Statistical analysis

All data are presented as means \pm SEM. Differences between two groups were assessed using Student's *t*-test. Step-wise multiple regression analysis was performed to provide a simultaneous model of prediction.

TABLE 1. BAC (mg/ml) at the first medical examinations

Group	n
BAC = 0	81
0 < BAC \leq 0.1	3
0.1 < BAC \leq 1	14
1 < BAC \leq 2	7
2 < BAC	3

BAC, blood alcohol concentration.

TABLE 2. Plasma PCOOH concentrations in alcoholic and control subjects

Group	n	Age	PCOOH	P
		years	pmol/ml	
Alcoholic	109	52.0 ± 1.3	46.1 ± 4.1	0.0001
Control	21	51.2 ± 2.2	15.6 ± 1.8	

PCOOH, phosphatidylcholine hydroperoxide. Values are means ± SEM.

RESULTS

Typical HPLC-CL chromatograms for PCOOH are shown in **Fig. 1**, which illustrates the chromatographic separation of PCOOH in a standard solution and in plasma samples from control and alcoholic subjects. Standard PCOOH gave a single peak at 7.82 min. The plasma extract had peak 1 at 7.81 min and peak 2 at 9.36 min. Accordingly, the retention time of peak 1 corresponded to that of standard PCOOH.

Blood alcohol was detected in 27 alcoholic patients at the first medical examination. Of these, half of the patients had blood alcohol concentration between 0.1 and 1 mg/ml (**Table 1**).

Comparison of the PCOOH concentration in alcoholic and control subjects showed that the PCOOH concentration was significantly higher in alcoholics (**Table 2**). The mean concentration in alcoholics was 46.1 ± 4.1 pmol/ml, whereas in controls it was 15.6 ± 1.8 pmol/ml. PCOOH concentrations in alcoholic patients for blood alcohol, liver function, and lipid metabolism are shown in Tables 3–5. The PCOOH concentration was significantly higher in patients with blood alcohol (88.0 ± 10.5 pmol/ml) than in patients without blood alcohol (32.6 ± 3.1 pmol/ml) at the first medical examination (**Table 3**). Thus, blood alcohol evidently affected the accumulation of PCOOH in plasma. Comparison of the PCOOH concentration in alcoholic patients showed that the PCOOH concentrations were significantly higher in patients with high levels of AST, ALT, and γ -GTP (**Table 4**). Thus, the accumulation of plasma PCOOH was affected by clinical tests related to liver function. Moreover, the PCOOH concentration was significantly higher in patients with high levels of TG, whereas it was not higher in patients with high levels of TC (**Table 5**). Statistical Analysis System (SAS) revealed that γ -GTP, HDL, blood alcohol concentration, and TG correlated positively and independently with plasma PCOOH concentration (**Table 6**). Thus,

TABLE 3. Blood alcohol and plasma PCOOH concentrations at the first medical examination

Group	n	PCOOH	P
		pmol/ml	
BAC > 0	27	88.0 ± 10.5	0.0001
BAC = 0	81	32.6 ± 3.1	

Values are means ± SEM.

TABLE 4. PCOOH concentrations for biochemical variables related to liver function

Variable	Range	n	PCOOH	P	
			pmol/ml		
AST	High	≥40 IU/l	65	59.3 ± 6.2	0.0001
	Normal	<40 IU/l	44	26.6 ± 2.7	
ALT	High	≥42 IU/l	37	66.3 ± 8.8	0.0027
	Normal	<42 IU/l	72	35.7 ± 3.9	
γ -GTP	High	≥72 IU/l	77	53.2 ± 5.5	0.0003
	Normal	<72 IU/l	32	28.9 ± 3.2	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ -GTP, γ -glutamyl transpeptidase. Values are means ± SEM.

these four biochemical factors have significant influences on increased PCOOH. In contrast, time between blood collection and the final drink did not correlate with plasma PCOOH concentration by SAS.

Figure 2 shows plasma PCOOH concentrations in 29 patients on admission and after the 6 week abstinence. The data of the exceptional case are shown by open circles. The mean concentration (41.8 ± 6.7 pmol/ml) decreased almost to the control value (22.8 ± 2.0 pmol/ml) except in one patient. The biochemical profiles of this patient were practically normal on admission, whereas the fasting blood sugar after the 6 week abstinence increased to 150 mg/dl.

Table 7 shows the PCOOH concentrations in patients with ALD. The patients with liver cirrhosis had significantly higher type 4 collagen and lower cholinesterase activity but significantly lower PCOOH concentrations than patients with alcoholic hepatitis.

DISCUSSION

The major findings of this study are as follows: 1) the plasma PCOOH concentration was significantly higher in alcoholic patients than in controls; 2) the PCOOH level positively correlated with levels of γ -GTP, HDL, blood alcohol concentration, and TG; and 3) the increased PCOOH concentration returned to normal after the 6 week abstinence.

Phosphatidylcholine is more easily peroxidized to produce PCOOH than cholesterol is to cholesterol hydroperoxide, so plasma PCOOH is easily detectable, whereas cholesterol hydroperoxide is not. Accordingly, plasma PCOOH may be used as a general indicator of lipid perox-

TABLE 5. PCOOH concentrations and lipid metabolism

Variable	Range	n	PCOOH	P	
			pmol/ml		
TG	High	≥151 mg/dl	36	67.5 ± 9.3	0.002
	Normal	<151 mg/dl	73	35.6 ± 3.6	
TC	High	>220 mg/dl	37	51.8 ± 7.8	0.331
	Normal	≤220 mg/dl	72	43.2 ± 4.8	

TC, total cholesterol; TG, triglyceride. Values are means ± SEM.

TABLE 6. Correlation between PCOOH concentration and clinical tests

Variable	Regression Coefficient	P
	% (95% confidence interval)	
γ -GTP	0.02966 (0.0136–0.046)	0.0005
HDL	0.26045 (0.081–0.440)	0.0054
BAC	8.58888 (0.183–16.994)	0.0478
TG	0.06516 (0.041–0.089)	<0.0001

The multiple adjusted regression coefficient was $R = 0.613$.

itation. Indeed, the accumulation of plasma PCOOH was observed not only in dialysis patients with diabetic nephropathy (12) but in patients with hyperlipidemia (13); this may be related to the development and progression of atherosclerosis. In addition, the increase of serum PCOOH was dependent on glycemic control in type 2 diabetic patients (14). The effects of hemodialysis in patients with uremia (15) and tea catechin supplementation in healthy volunteers on antioxidant activity (16) were estimated by plasma PCOOH.

Because the main component (peak 1 in Fig. 1) of plasma PCOOH is PC C16:0/C18:2-OOH, we used it as a standard compound. We were able to identify it and quantify its concentration accurately in the present study. The remainder shown as peak 2 in Fig. 1 may be PC C18:0/C18:2-OOH. We estimate the concentration of peak 2 by calculating peak area based on PC C16:0/C18:2-OOH. The mean area ratio of PC C18:0/C18:2-OOH to PC C16:0/C18:2-OOH was 0.22, almost constant; accordingly, total PCOOH concentration may be 1.22 times higher than that of PC C16:0/C18:2-OOH alone.

Although various serum markers, such as γ -GTP (17), carbohydrate-deficient transferrin (17, 18), metalloproteinase-9 (19, 20), tissue polypeptide-specific antigen (21), type 4 collagen (22), laminin (20), hyaluronic acid (23), and CYP2E1 activity (24), have been introduced for the evaluation of alcohol consumption and ALD, it has remained unclear which is the best marker.

To understand the significance of increased plasma PCOOH concentration in this study, factors relevant to the PCOOH concentration were studied using data collected from alcoholic patients at the first medical examination and after a 6 week abstinence.

First, the relationship between blood alcohol and PCOOH concentration was examined. As expected, the PCOOH concentration was significantly higher in patients with blood

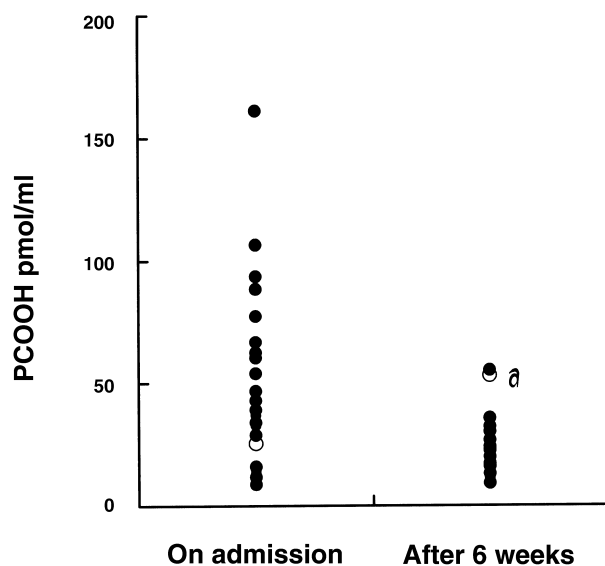


Fig. 2. Plasma PCOOH concentrations in 29 patients on admission and after a 6 week abstinence. The data of the exceptional case are shown by open circles.

alcohol than in patients without blood alcohol, indicating that increased oxidative stress is attributable to the direct action of alcohol.

Next, γ -GTP, AST, and ALT are hepatic enzymes that leak from hepatocytes into the blood when hepatocytes are damaged by various causes, including alcohol consumption. In these studies, the PCOOH plasma concentrations increased significantly in patients with higher activity of these enzymes, correlating with γ -GTP activity, indicative of increased oxidative stress in alcoholic liver injury.

The PCOOH concentration increased significantly in patients with high levels of TG, which may be caused by decreased activity of lipoprotein lipase, hepatic TG lipase (25), and long-term alcohol consumption.


The mean PCOOH concentrations decreased in 29 alcoholic patients after the 6 week abstinence, showing that PCOOH is a marker of alcohol consumption.

Finally, accumulation of 7-hydroperoxycholesterols was observed in alcoholic fatty liver but not in alcoholic cirrhotic liver (26). The present results, showing increased plasma PCOOH in patients with alcoholic hepatitis but low levels in patients with alcoholic liver cirrhosis, consistent with previous liver data, suggest increased lipid peroxidation in the early stage of alcoholic liver injury.

TABLE 7. PCOOH concentrations in patients with alcoholic liver disease

Condition	n	On Admission					
		PCOOH	AST	γ -GTP	ChE	TC	Type 4 Collagen
		pmol/ml	IU/l	IU/l	IU/l	mg/dl	ng/ml
Alcoholic hepatitis	9	66.0 \pm 13.7	104.2 \pm 30.6	433.6 \pm 137.2	160.7 \pm 14.1	234.4 \pm 26.1	8.0 \pm 1.0
Liver cirrhosis	10	21.6 \pm 5.8	84.2 \pm 21.1	346.5 \pm 136.6	100.3 \pm 13.6	152.2 \pm 19.1	16.1 \pm 1.8
P		0.006	0.591	0.659	0.006	0.019	0.001

ChE, cholinesterase. Values are means \pm SEM.

In conclusion, the present findings confirm that plasma PCOOH is a new marker of alcohol consumption as well as oxidative stress in alcoholic patients. 

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