THE MULTIPLE HYDROPEROXIDES OF CHOLINE PHOSPHOLIPIDS OCCURRING IN PLASMA AFTER ISCHEMIA-REPERFUSION IN RAT LIVER

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ABSTRACT— Since phosphatidylcholine is a primary membrane component and its peroxidation may result in disruption and dysfunction of the biomembrane, we used phosphatidylcholine hydroperoxide (PCOOH) as an index of oxidative-stress-related injury. We detected the occurrence of PCOOH in both liver and plasma from rats subjected to hepatic ischemia-reperfusion, and demonstrated that liver and plasma PCOOH levels reflect the extent of liver injury. However, the accuracy in measuring PCOOH levels, particularly in blood samples, is still in question since PCOOH may be rapidly eliminated by phospholipase A₂ enzymes, which are widespread, easily activated, and have a preference for oxidized glycerophospholipids. When PCOOH levels were determined, using high-performance liquid chromatography to detect chemiluminescence (CL), the plasma samples showed a broad CL peak that began to elute at the same time as in the liver samples, but lasted longer. The peak appeared to contain hydroperoxides from multiple lipid classes. In addition to PCOOH, the hydroperoxides of sphingomyelin and lysophosphatidylcholine (SPHOOH and LPCOOH) were identified in the plasma of rats subjected to ischemia-reperfusion using thin layer chromatography separation and subsequent visualization, infrared spectroscopy, and hydrolysis under mild alkaline conditions. These results indicate that the occurrence of SPHOOH and LPCOOH, as well as PCOOH, may reflect liver damage related to ischemia-reperfusion.

KEY WORDS : Hepatic ischemia-reperfusion injury, HPLC coupled with the detection of chemiluminescence, Lipid peroxidation, Phosphatidylcholine hydroperoxide, Lysophosphatidylcholine hydroperoxide, Sphingomyelin hydroperoxide.

INTRODUCTION

Peroxidation of membrane lipids is proposed to occur through a free radical mechanism and can result in alterations in biomembrane-associated functions and structure, and in the disruption of function in cells or subcellular

organelles (Marubayashi *et al.*, 1984). In the pathogenesis of hepatic ischemic disease and liver transplantation complications (Horton *et al.*, 1987 ; Jaeschke *et al.*, 1991 ; Chien *et al.*, 1987), superoxides may overwhelm the strong antioxidant defense mechanisms found in liver tissue, and may cause peroxidation of biomembrane. Products commonly used to characterize this process include hydroperoxides and malondialdehyde. Many of these products also participate in prop-

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agation reactions, and may yield compounds which possess bioactive or cytotoxic properties (Horton *et al.*, 1987). Phosphatidylcholine (PC) is the most important functional lipid in the hepatocellular membrane and is susceptible to oxidation, primarily degenerating to PC hydroperoxide (PCOOH) (Chien *et al.*, 1987; Yamamoto *et al.*, 1985). Therefore, we previously used PCOOH as a marker of damage by oxidative stress, by determining PCOOH levels in liver and plasma with the chemiluminescence-detecting high performance liquid chromatography (CL-HPLC). Our results demonstrated that PCOOH is present in liver tissue and plasma from rats subjected to hepatic ischemia-reperfusion, and that increased concentrations of PCOOH in liver and plasma reflect liver cell damage (Takayama *et al.*, 1992; Egashira *et al.*, 1991; Egashira *et al.*, 1992), and that plasma PCOOH levels can be used to assess tissue injury following oxidative stress and for the investigation of drug efficacy (Takayama *et al.*, 1993). Despite this success, the possibility of a steady elevation in PCOOH, particularly in plasma, remains controversial (Miyazawa, 1989; Yamamoto *et al.*, 1992) due to the fact that phospholipid peroxides can be rapidly eliminated by biological defense mechanisms, such as phospholipase A₂ enzymes (PLA₂) (Sevanian *et al.*, 1983).

Utilizing the occurrence of CL generated from luminol oxidation in the presence of hydroperoxides and heme compounds, PCOOH was specifically detected after separating lipid classes by normal-phase HPLC. However, the CL peak of plasma PCOOH was asymmetrical and broad compared to the peak generated from liver samples. There are two possible explanations for the difference between plasma and liver peaks: either the peaks are affected by the fatty acid composition of PC or the plasma PCOOH peak contains other phospholipid hydroperoxides. To identify the plasma PCOOH increase following ischemia-reperfusion, we analyzed the lipid extracts of rat plasma using thin layer chromatography (TLC) and subsequent qualitative methods of visualization, and by hydrolysis in methanol under mild alkaline conditions. The substances recovered from the TLC plate and the hydrolyzed plasma lipids were then further examined by infrared spectroscopy and the CL-HPLC assay.

MATERIALS AND METHODS

1. *Experimental animals.*

Male Wistar rats, weighing 200–250 g, were kept in an environmentally controlled room (20–23°C, 50–60% humidity, illuminated from 7:00 to 19:00 h) with food and water available *ad libitum*.

2. *Chemicals.*

We used 3-aminophthaloyl hydrazine (luminol), butylated hydroxytoluene (BHT), and PC (egg lecithin) (Wako Pure Chemical Industries, Ltd., Osaka, Japan); cytochrome *c* from horse heart type VI and lysophosphatidylethanolamine oleoyl (LPE) (Sigma Chemical Co., St. Louis, U.S.A.); lysophosphatidylcholine oleoyl (LPC), and sphingomyelin from porcine brain (SPH) (Funakoshi, Japan). HPLC-grade reagents were used for the mobile phase of the CL-HPLC assay. All other reagents were of analytical grade.

3. *Preparation of liver ischemia-reperfusion rats.*

The rats were subjected to light ether anesthesia, then the abdomen was opened through a midline incision, and the left portal vein and hepatic artery were occluded with a microvessel clip. The abdomen was then closed and the rat was allowed to awaken. After liver ischemia, the vascular clip was released and the right lateral and caudate lobes were removed, leaving only the ischemic left lateral and median lobes intact. After liver reperfusion, a blood sample and the left lateral lobe were obtained for assessment of liver injury, and for the CL-HPLC assay (Nagai *et al.*, 1990). Blood samples and liver tissue were taken from control rats in a similar manner, but they were not subjected to ischemia-reperfusion.

4. *Tissue preparation.*

Immediately after dissection, liver tissue was placed in ice cold saline and all further preparative procedures were carried out on ice. Specimens were rinsed with chilled saline, cut into small pieces with scissors, and saline and BHT in methanol were added to achieve a final concentration of 200 mg wet liver tissue/ml and 0.005% BHT. Plasma was prepared from fresh heparinized blood centrifuged at 3,000 r.p.m., for 10 min at 10°C, and BHT was added to a concentration of 0.005%. Liver and plasma preparations were then stored at –80°C.

5. Auto-oxidation of phospholipid standards.

PC, LPC, LPE, and SPH were chosen as phospholipid standards, since previous studies suggested the simultaneous elution of these phospholipids, or elution following the PC peak, when subjected to separation on a HPLC normal phase column (Jungalwala *et al.*, 1976; Nakayama *et al.*, 1987). In addition, the tailing portion of the broad PCOOH peak of plasma lipid may contain choline or amine together with phosphoric acid. The phospholipid standards were left in a sunny place in the laboratory for one week to allow auto-oxidation to occur, then applied to the CL-HPLC assay to determine retention times.

6. CL-HPLC equipment and chromatography conditions.

The CL-HPLC system consisted of an injector (RHEODYNE 7161; RHEODYNE Inc., U.S.A.), a normal-phase column (JASCO Fine pack SIL, 5 μ m, 250 $\times$ 4.6 mm; Japan Spectroscopic Co., Tokyo, Japan), two pumps (JASCO 880-PU and SHIMADZU LC10AS; SHIMADZU Co., Kyoto, Japan), a UV detector (JASCO 875UV), a CL detector (JASCO 825), and two integrators (SHIMADZU CHROMATOPACK C-R6A). The mobile phase of acetonitrile (MeCN)/methanol (MeOH)/water (H₂O) (55:30:15, or 67:20:13, v/v) was delivered at 1 ml/min. The CL post-column reagent was prepared by dissolving 1 μ g/ml of luminol and 10 μ g/ml of cytochrome *c* in 20 mM borate buffer, pH 10.5, which was saturated with N₂ gas and contained 1% methanol. The flow rate was 1 ml/min (Takayama *et al.*, 1993).

The CL-HPLC assay began with the separation of lipid classes using a normal phase HPLC. Total PC, including PC and hydroxyl or hydroperoxide derivatives, could then be detected by absorption at UV 205 nm, due to the diene structure. We also measured the CL generated from luminol oxidation caused by the reaction of hydroperoxides with cytochrome *c* in the CL post-column reagent. Individual lipid classes were eluted in increasing order of polarity by using the CL-HPLC assay with a normal-phase column. After plasma lipid extracts were classified on TLC plates or hydrolyzed under mild alkaline conditions, they were also analyzed by the CL-HPLC assay.

7. Extraction of liver and plasma lipids.

The extraction procedure was performed

under a nitrogen stream. Liver lipids were extracted from each 1 ml aliquot of homogenate by adding 5 ml of chloroform/methanol (2 : 1, v/v), containing 0.005% BHT. Plasma lipids were extracted using 4 ml of chloroform/methanol (3 : 1, v/v), containing 0.005% BHT, for each 0.5 ml of plasma (Takayama *et al.*, 1993). The mixture was shaken vigorously for 1 min and centrifuged at 3,000 r.p.m. for 10 min at 10°C. The lower layer was then collected, dried under a nitrogen stream at 30°C, and reconstituted in a chloroform/methanol (1 : 1, v/v) solution. A portion of the final solution, corresponding to lipids extracted from 10 mg of liver or 50 μ l of plasma, was then injected into the CL-HPLC equipment. Plasma lipid extracts were further classified on a TLC plate or hydrolyzed under mild alkaline conditions.

8. TLC separation of plasma lipids.

Plasma lipid extracts and PC, SPH, and LPC standards were spotted onto Merck TLC plates coated with silica gel 60 F₂₅₄ for visualization, and the lipid extract was also applied as a narrow band to a preparative plate. The plates were developed to a distance of 15 cm in chloroform/MeOH/1 M ammonia water (65:25:4, v/v) (Creer *et al.*, 1985). After being developed and dried, the plates for visualization were sprayed with (a) Dragendorff reagent to visualize substances containing choline (Beiss, 1964), (b) Ninhydrin reagent to visualize substances containing amine (Allen and Good, 1971), and (c) Ryu-MacCoss reagent to visualize substances containing phosphoric acid (Ryu and MacCoss, 1979), and subjected to (d) UV irradiation to visualize the conjugated diene structure in peroxidant. Identification of individual lipid classes in the spots was performed by comparing the R_f values in the present study with those in the Creer *et al.* study (Creer *et al.*, 1985), and by visualization. Bands corresponding to respective spots on the visualized plates were scrapped from the preparative TLC plate and then re-extracted separately for subsequent infrared spectra and CL-HPLC analyses.

9. Hydrolysis of plasma lipids under mild alkaline conditions in methanol.

Plasma lipid extracts from rats were dissolved in a methanolic solution of 0.1 N NaOH at room temperature for 12 hr. Under these condi-

tions, the ester bonds of acyl-glycerophospholipids between fatty acids and glycerin were cleaved, while the acid amide bond of sphingomyelin between fatty acids and the sphingosine base remained. Thus, sphingomyelin was distinguished from acyl-glycerophospholipids (Sweeley, 1963). The medium was neutralized and re-extracted for subsequent analysis on the CL-HPLC.

10. Infrared analysis.

Infrared experiments were carried out after the crude separation of plasma lipid extracts. Samples (PC and SPH standards, and re-extracted substances from TLC 1, 2, and 3) were dissolved in dehydrated chloroform. Each solution was transferred to an infrared transmission cell. The transmittance of infrared spectra with wave numbers in the region of $4000\text{--}640\text{ cm}^{-1}$ was recorded with a JIR-100 Fourier transform infrared spectrometer (JOEL Ltd., Tokyo, Japan), operating Globar light source, single beam, with a resolution of 4 cm^{-1} , and a scan speed of 2 mm/sec . We were able to distinguish

acyl-glycerophospholipids from sphingomyelin since acyl-glycerophospholipids exhibited a band at 1740 cm^{-1} assigned to the carbonyl (C=O) stretching mode, while sphingomyelin exhibited two bands at 1550 cm^{-1} and 1640 cm^{-1} assigned to the C=O stretching and amide (NH) bending vibration mode, respectively (Desormeaux *et al.*, 1992).

RESULTS

Figure 1 shows typical chromatograms recording simultaneously ultraviolet (UV) 205 nm absorption and CL intensity (UV and CL chromatograms) following injections of liver and plasma lipid samples from a 30 min ischemia-12 hr reperused rat. The CL-HPLC assay was run with a mobile phase of MeCN/MeOH/ H_2O (55:30:15, v/v). The peaks (about 10 min) in UV chromatogram were total PC (PCs), including hydroperoxide and hydroxyl derivatives, and the peaks (about 10 min) in the CL chromatogram were appeared to be PCOOH, which in-

Fig. 1. Typical chromatograms of liver and plasma samples. Chromatograms were obtained by simultaneous detection of ultraviolet (UV) 205 nm absorption and chemiluminescence (CL) intensity, when a mobile phase of acetonitrile/methanol/water (55:30:15, v/v) was delivered at 1 ml/min in the CL-HPLC assay. Samples were lipid extracts corresponding to 10 mg of liver or $50\text{ }\mu\text{l}$ of plasma from a rat subjected to 30 min of hepatic ischemia followed by 12 hr of reperfusion.

creased after ischemia-reperfusion. The CL chromatogram shows plasma PCOOH beginning to be eluted at 10 min, and continuing to 20 min, with a shoulder at the front of the peak. The liver PCOOH peak was eluted at the same retention time as the front shoulder of the plasma PCOOH peak. Moreover, in simultaneously recorded UV and CL chromatograms from a plasma sample, the asymmetrical and broad PCOOH peak covered PCs peak and the following two minor peaks (Fig. 1).

When MeCN/MeOH/H₂O (67:20:13, v/v) was used for the mobile phase, the CL peak of PCOOH in plasma became broader, but still ranged from the latter half of total PC peak to the end of the second minor peak on the simultaneously detected UV chromatogram (Fig. 2, left). After hydrolysis in a methanolic solution

of 0.1 N NaOH, the first minor UV peak observed in the pre-hydrolyzed plasma sample still remained and the CL peak became narrower (Fig. 2, right). Comparing UV chromatograms of the plasma sample (Fig. 2, left) and the auto-oxidized phospholipid standards (Fig. 3, left), revealed that the PC, SPH, and LPC standards had a similar retention time to the PCs of plasma, the first and second minor peaks were eluted subsequently, and PE and LPE were eluted before PC. The CL peaks of PCOOH, SPHOOH, and LPCOOH (Fig. 3, right), obtained from analysis of the auto-oxidized phospholipid standards, were not eluted separately, but were co-eluted with the broad CL peak of plasma PCOOH (Fig. 2, left), and PEOOH and LPEOOH were eluted before PCOOH.

On the visualized TLC plate, the plasma

Fig. 2. Chromatograms of plasma samples. Chromatograms were obtained by simultaneous detection of UV absorption at 205 nm and chemiluminescence (CL) intensity, when a mobile phase of acetonitrile/methanol/water (67:20:13, v/v) was delivered at 1 ml/min in the CL-HPLC assay. *plasma lipid*: lipid extract corresponding to 50 μ l of plasma from a rat subjected to 30 min of hepatic ischemia followed by 12 hr of reperfusion. *hydrolyzed plasma lipids*: lipids re-extracted after hydrolyzing plasma lipid extracts from ischemia-reperfused rats, under mild alkaline conditions.

Fig. 3. Separation of several phospholipid standards by the CL-HPLC assay. Chromatograms of auto-oxidized phospholipid standards were obtained by simultaneous detection of UV absorption at 205 nm and chemiluminescence (CL) intensity, when a mobile phase of acetonitrile/methanol/water (67:20:13, v/v) was delivered at 1 ml/min in the CL-HPLC assay. Peaks: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanol-amine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SPH, sphingomyelin; LPCOOH, LPEOOH, PCOOH, SPHOOH, the respective hydroperoxides.

Fig. 4. Thin layer chromatograms of plasma lipid extracts of rats. ○ ; contains choline and phosphoric acid, ▨ ; contains amine and phosphoric acid. R_f values are shown on the left of the plates. Right panel represents the preparative plate and the left panel summarizes the data from two visualized plates; one sprayed with Dragendorff reagent followed by Ryu-McCoss reagent, the other sprayed with Ninhydrin reagent followed by Ryu-McCoss reagent.

lipid extract separated in several spots. Three of the spots contained choline and phosphoric acid and developed at almost the same distance as the PC, SPH, and LPC standards (Fig. 4, left). The bands (TLC 1, 2, and 3) on the preparative plate (Fig. 4, right) corresponded to the three spots on the visualized plate, and were re-extracted and further analyzed. TLC 1 and 2 contained amine phospholipids impurities.

The recovered phospholipids from TLC 1 and 3 had CL peaks and UV peaks that were identical to the second minor peak (about 21 min) (TLC 1) and the PCs peak (about 12 min) (TLC 3) obtained from the plasma sample in Fig. 2 (Fig. 5). The peaks obtained from the CL-HPLC assay of TLC 1 and 3 (Fig. 5, left and right) were also similar to those of auto-oxidized LPC and PC (Fig. 3), respectively, when MeCN/MeOH/H₂O (67:20:13, v/v) was used as the mobile phase. In contrast, the recovered phospholipids from TLC 2 (Fig. 5, middle), and from plasma hydrolyzed under mild alkaline conditions (Fig. 2, right), each had a peak identical to the first minor UV peak from pre-hydrolyzed plasma (Fig. 2, left) which eluted near auto-oxidized SPH, but with a lightly shift (Fig. 3).

The infrared spectra of TLC 1 and 3 showed

a band at 1740 cm^{-1} which was assigned to the carbonyl ($\text{C}=\text{O}$) stretching mode in acylglycerophospholipids (Fig. 6). On the other hand, the infrared spectrum of TLC 2 showed bands at 1550 cm^{-1} and 1640 cm^{-1} , both assigned to $\text{C}=\text{O}$ stretching and NH bending vibration mode, respectively, in acid amide residues of sphingolipids.

These results suggest that TLC 1, 2, and 3 contain LPC, SPH, and PC, respectively, and more specifically, that (A) TLC 1, 2, and 3 contain choline phospholipids and may also contain amine phospholipid impurities; (B) the recovered phospholipids from TLC 3, 2, and 1 were co-eluted with PCs (TLC 3) and the subsequent first (TLC 2) and second (TLC 1) minor peaks of plasma lipid, and were eluted at the same time as PC (TLC 3), near SPH (TLC 2), and at the same time as LPC (TLC 1), while other phospholipid standards (LPE and PE) were eluted before PC under the same CL-HPLC conditions; and finally, (C) TLC 2 contains a phospholipid resistant to hydrolysis by mild alkaline in methanol, and appears to contain sphingophospholipids, unlike

TLC 1 and 3, which were hydrolyzed under mild alkaline conditions, and contain glycerophospholipids. Therefore, the asymmetrical and broad PCOOH peak from plasma lipid extracts consists of PC, SPH, and LPC hydroperoxides (PCOOH, SPHOOH, and LPCOOH).

DISCUSSIONS

PC constitutes the largest class of biomembrane phospholipids, and polyunsaturated fatty acyl residues at *sn*-2-positions in PC are most susceptible to oxidation degenerating to PCOOH (Chien *et al.*, 1987; Yamamoto *et al.*, 1985). We previously had determined the levels of PCOOH generated in the liver and plasma of rats subjected to hepatic ischemia-reperfusion to be used as an indicator of lipid peroxidation (Takayama *et al.*, 1992; Takayama *et al.*, 1993). Our previous results demonstrated that increased PCOOH levels in plasma and liver can reflect liver cell damage (Takayama *et al.*, 1992), and that plasma PCOOH levels can be a reliable indicator for assessing tissue injury (Takayama *et*

Fig. 5. Chromatograms of plasma samples separated on thin layer chromatography (TLC). After a crude separation of plasma lipid extracts on a preparative TLC plate, the recovered samples from bands (TLC 1, 2, and 3) were injected into the CL-HPLC equipment. Chromatograms were obtained by simultaneous detection of UV absorption at 205 nm (A 205 nm) and chemiluminescence intensity (CL), when a mobile phase of acetonitrile/methanol/water (67:20:13, v/v) was delivered at 1 ml/min in the CL-HPLC assay.

Fig. 6. Infrared spectra of separated lipid extracts from rat plasma and choline phospholipid standards. The spectra show the transmittance of each substance indicated in the figure. PC; phosphatidylcholine, SPH; sphingomyelin. TLC 1, 2, and 3 were recovered from a preparative TLC plate (shown in Fig. 4) after the crude separation of plasma lipid extracts from ischemia-reperfused rats. $\uparrow$; center of the band assigned to the C=O stretching mode in the ester bond, or the centers of two bands assigned to the C=O stretching and NH bending vibration mode in the acid-amide bond.

al., 1993).

However, as seen in Fig. 1, the CL chromatogram of plasma PCOOH showed a broad peak compared to that of liver. An examination of the simultaneously generated UV and CL chromatograms suggests that the broad plasma peak results from the presence of multiple hydroperoxides.

PC and SPH are the major lipid components of biomembranes and serum lipoprotein in mammals, and are located in the external surface of membranes (Suyama *et al.*, 1979; Barenholz and Thompson, 1980). The fatty acid composition of SPH consists predominately of saturated fatty acids (Kuksis, 1992), and PC consists primarily of saturated fatty acids located in the *sn*-1-position and unsaturated fatty acids in the *sn*-2-position

(Suyama *et al.*, 1979).

Since phospholipids are important membrane constituents, their peroxidation can result in membrane disruption and alter the function of cells or subcellular organelles (Marubayashi *et al.*, 1984). Biological systems have mechanisms for the elimination of modified lipids. Oxidized glycerophospholipids (GPL) can be immediately hydrolyzed by PLA₂ (Chang *et al.*, 1987). PLA₂ is widespread and easily activated by even minor stimulation (Chang *et al.*, 1987). Recently, PLA₂ has been shown to function as an antioxidant enzyme, since it hydrolyzes oxidized glycerophospholipid and cleaves the *sn*-2-acyl bond to produce lyso-GPC and a free fatty acid peroxide that can then be disposed of by glutathione peroxidase (Sevanian *et al.*, 1983). The

degeneration of PC to LPC has been shown to occur during the oxidative modification of membranes and low density lipoprotein (Sevanian *et al.*, 1983). In the present study, SPHOOH and LPCOOH were identified as the hydroperoxides occurring in plasma during hepatic ischemia-reperfusion, as well as in PCOOH from rat plasma lipid extracts using TLC separation, hydrolysis under mild alkaline conditions, and subsequent qualitative analyses.

Although PC is rich in unsaturated fatty acids, when compared with SPH and LPC, the CL chromatogram of plasma lipid extract revealed that the main CL peak eluted simultaneously with SPH and LPC (Fig. 2). SPHOOH and LPCOOH, rather than PCOOH, appeared to increase in plasma during ischemia-reperfusion injury. The present study suggests that the occurrences of SPHOOH and LPCOOH, in addition to PCOOH, in plasma may reflect lipid peroxidation in membranes and lipoproteins, resulting in tissue injury.

Since SPH, a sphingolipid, and LPC can not be cleaved by PLA₂, the biological defense system may have more difficulty eliminating the oxidative products, SPHOOH and LPCOOH, than PCOOH. In addition, hydroperoxides may participate in further propagation reactions to yield peroxy and alkoxyl lipid radicals with bioactive or cytotoxic properties (Gonzalez Flecha *et al.*, 1991). Thus, SPHOOH and LPCOOH may play extremely important roles as mediators and propagators of oxidative stress.

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