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Chemiluminescence-HPLC assay of phosphatidylcholine hydroperoxide generated by ischemia–reperfusion in the liver of rats

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Abstract—To determine cellular damage due to "oxidative stress", we developed a sensitive and specific quantitative assay for phosphatidylcholine hydroperoxide (PCOOH) by coupling HPLC with detection of chemiluminescence (CL). The qualitative and quantitative detection limits of PCOOH by this assay were 0.5 and 2 pmol (based on active oxygen from hydroperoxide). Using this CL-HPLC method, we determined PCOOH levels caused by ischemia–reperfusion in rat livers. The PCOOH levels in livers of control, sham-operated and operated rats with only ischemic treatment were approximately 2 nmol/g wet liver weight. The PCOOH level and several serum parameters of liver injury increased with an increase in the duration of ischemia, and also increased in proportion to the duration of reperfusion. The determination of PCOOH in liver caused by ischemia–reperfusion could be a useful method for investigating liver damage induced by free radicals.

The functional and structural damage to liver cells caused by ischemia–reperfusion is believed to be the result of a free radical reaction process, involving lipid peroxidation in biomembranes [1], caused by "oxidative stress" [2, 3]. Usually, the thiobarbituric acid reactants are measured to indicate lipid peroxidation associated with cellular damage caused by free radicals [4]. However, this assay may also measure aldehydes and therefore lacks specificity [5]. Phosphatidylcholine hydroperoxide (PCOOH*) has recently gained attention as a primary peroxidative product of phosphatidylchloline (PC), which is the most important functional lipid in the cell membrane [6]. We have developed a sensitive and specific quantitative assay for PCOOH which improves the CL-HPLC methods of Yamamoto *et al.* [5] and Miyazawa *et al.* [7] with minor modifications. This assay begins with the separation of lipid classes with a normal phase HPLC. Total PC, including PC and hydroxy or hydroperoxide derivatives, are then detected by absorption at UV 205 nm due to its diene structure, and the chemiluminescence (CL) from luminol oxidation caused by the reaction of hydroperoxides with heme compounds is measured.

In the present study, to examine liver injuries caused by ischemia–reperfusion, we determined the levels of PCOOH and PC in lipid extracts from the liver tissues of our models with time course using the CL-HPLC assay. We also demonstrated the elevation of liver PCOOH levels induced by ischemia–reperfusion.

Materials and Methods

Preparation of standard PC and PCOOH. Standard PC was prepared by purification of PC (from egg yolk, Wako Pure Chemical Co.) by passage through a reversed phase column (Lichroprep RP-8, Merck). PCOOH was prepared by photosensitized oxidation of PC [7] and subsequently purified in the same manner as PC.

Confirmation of PCOOH. The purity of PCOOH was confirmed by CL-HPLC assay (the integrated CL area at PC retention time was greater than 95% of total) and TLC [8] and also detected qualitatively by (a) dimethyl-*p*-phenylenediamine reagent for peroxide derivative, (b) Dragendorf reagent for substances containing choline [9] and (c) UV irradiation for structure of conjugated diene structures.

* Abbreviations: CL, chemiluminescence; CL-HPLC, HPLC coupling with detection of chemiluminescence for hydroperoxide assay; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; BHT, butylated hydroxytoluene.

Determination of PCOOH concentration. Concentration of PCOOH was determined by the iodometric method of KI oxidation using $AlCl_3$ as a catalyst and was expressed as moles of active oxygen from hydroperoxide [10].

Equipment and chromatography conditions of CL-HPLC. The CL-HPLC system consisted of a Rheodyne 7161 injector, column (JASCO Fine pack SIL, 5 μm, 250 × 4.6 mm), pump (JASCO 880-PU and Shimazu LC10AS pump), UV detector (JASCO 875UV), CL detector (JASCO 825) and integrator (Shimazu Chromatopack C-R6A). The mobile phase for HPLC was acetonitrile/methanol/water (5.5:3:1.5, by vol.). The CL reagent was prepared by dissolving 1 μg/mL of luminol (3-aminophthaloyl hydrazine, Wako Pure Chemical Co.) and 10 μg/mL of cytochrome *c* (from horse heart type VI, Sigma Chemical Co.) in 20 mM of borate buffer, pH 10, which was saturated with N_2 gas and contained 1% methanol. The flow rates were 1 mL/min.

Preparation of liver ischemia–reperfusion models [11]. To prepare the rats (male Wistar rats weighing 200–250 g), the abdomen was opened through a midline incision under light ether anesthesia, 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 resected as controls, leaving only the ischemic left lateral and median lobes intact. After liver reperfusion, the left lateral lobe was obtained at the ischemic tissue. Sham-operated rats were treated in the same manner, except for clamping.

From the four control rats, the right lateral and caudate lobes were obtained as controls for untreated tissue in each model and the left lateral lobes were obtained as controls for the ischemic tissue of each model.

Liver homogenate. After resection of the liver, homogenate (200 mg wet liver weight/mL saline) containing 0.002% butylated hydroxytoluene (BHT, Wako Pure Chemical Co.) was prepared with a teflon–glass homogenizer under ice cooling and stored at −80° until the lipid extraction procedure for CL-HPLC assay.

Lipid extraction procedure from homogenate. In a test tube filled with N_2 gas, lipids were extracted by the method of Folch *et al.* [12]. Lipids were extracted with 5 mL of chloroform/methanol (2:1) solution containing 0.002% BHT for each 1 mL of homogenate. The lower layer was collected and dried under N_2 gas at 30° and redissolved in 200 μL of chloroform/methanol (1:1) solution. The 5–20 μL samples, which contained the lipids extracted from 5–20 mg of liver, were injected into the CL-HPLC equipment for hydroperoxide assay.

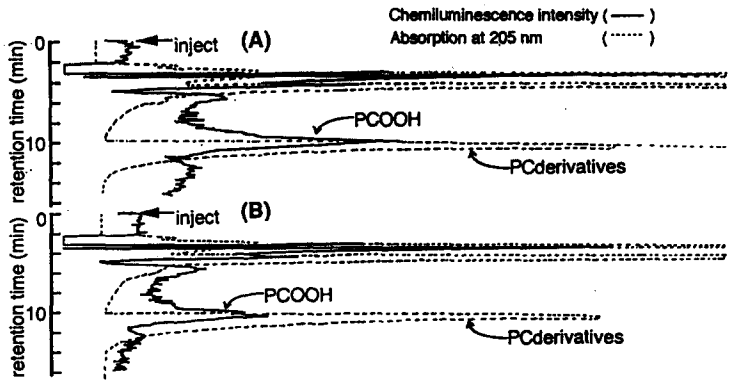

Fig. 1. Chromatograms of lipid extracts from the liver homogenates (based on 5 mg liver extracts) of a rat subjected to hepatic ischemia–reperfusion. (A) Ischemia–reperfused liver, (B) control liver. Both samples were obtained from a rat subjected to 30 min of hepatic ischemia followed by 6 hr of reperfusion. The solid line represents chemiluminescence detection and the dashed line represents UV detection at 205 nm following HPLC separation.

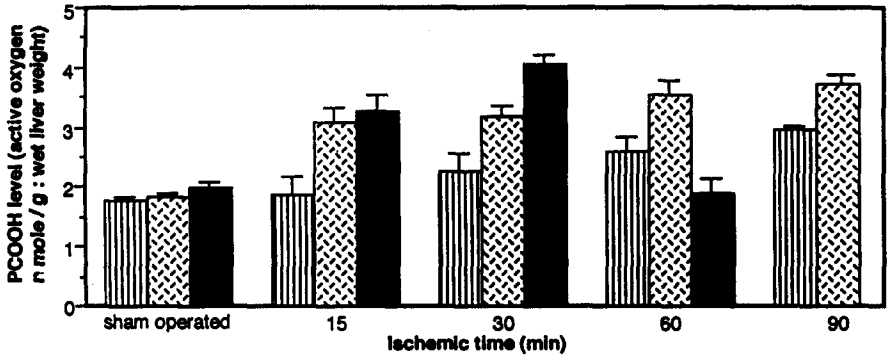

Fig. 2. PCOOH levels in rat livers after various durations of hepatic ischemia–reperfusion. Rats were subjected to hepatic ischemia for the durations shown on the horizontal axis. Vertical columns represent the duration of subsequent reperfusion [(▥) 1 hr reperfusion, (▨) 6 hr reperfusion, (■) 24 hr reperfusion]. Bars on the columns represent the mean ± SD of 3 rats.

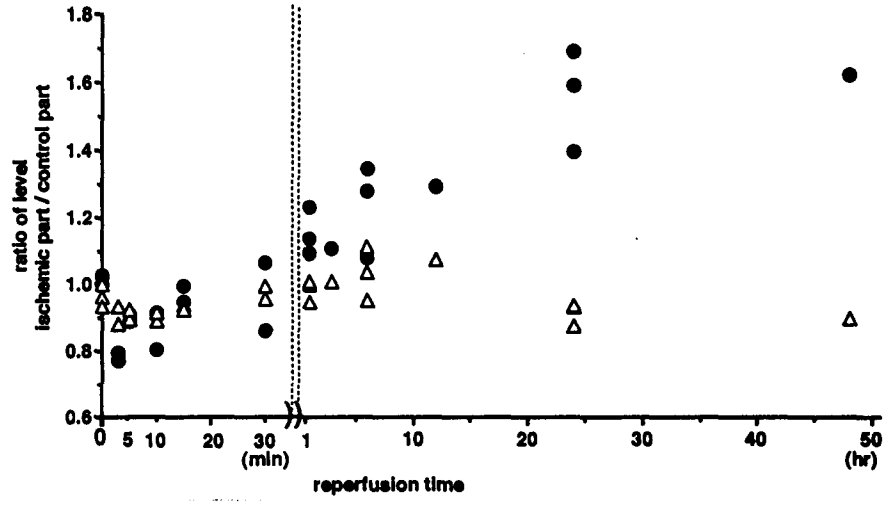

Fig. 3. The ratios of PCOOH and PC levels in ischemic tissue to those in control liver tissue (I/C ratio) from the same rat. (●) Represents the I/C ratio of PCOOH and (△) represents that of PC after 30 min ischemia followed by a period of reperfusion indicated on the horizontal axis.

Results and Discussion

Miyazawa *et al.* [7] have previously reported a CL-HPLC method for the assay of PCOOH at a detection limit of 10 pmol of hydroperoxide. In the present CL-HPLC assay, which incorporates changes to both the pH of the CL reagent and the composition of the mobile phase, we were able to detect PCOOH at concentrations of about 0.5 pmol and to obtain quantitative determinations at levels of 2 pmol (based on active oxygen from hydroperoxide). We were also able to determine PC levels. Using this assay, we determined PCOOH and PC concentrations in rat liver following ischemia–reperfusion. Figure 1 shows typical chromatograms of PCOOH and PC obtained by this CL-HPLC method. In the control and ischemia–reperfusion rat liver samples, a sharp chemiluminescence peak (10 min), which represented major hydroperoxide compounds under these chromatographic conditions, was identified as PCOOH (Fig. 1A and B).

The PCOOH levels in the livers of control, sham-operated and operated rats with only ischemic treatment were approximately 2 nmol/g wet liver weight. The effects of changing the duration of ischemia or reperfusion on PCOOH levels were also determined. As can be seen in Fig. 2, PCOOH levels increased with an increase in the duration of ischemia, except in the group in which 24 hr of reperfusion following 60 min ischemia (60 min–24 hr). The PCOOH level of the 90 min–24 hr group could not be determined because the rats died within 24 hr of 90 min hepatic ischemia. After 15 or 30 min ischemia, the PCOOH level increased during periods of reperfusion (up to 24 hr).

We compared the PCOOH and PC levels in ischemic tissue with those in control tissue from the same rat (I/C ratio). The I/C ratio of PCOOH was less than 1 during the 30 min of reperfusion following 30 min of ischemia. The I/C ratio of PC was also lowered (lowest value 0.88). However, the I/C ratio of PCOOH increased during reperfusion lasting more than 1 hr, while that of PC was near or below 1 (Fig. 3). These results suggest that PC is hydrolysed by the release of free fatty acids [13] in the early phases of reperfusion, and PC turn-over would be expected to increase. However, lipid peroxidation caused by chain reactions with the reactive oxygens may involve phospholipids in biomembranes, resulting in the accumulation of PCOOH. Therefore, ischemia–reperfusion may cause damage which is beyond the ability of PC turn-over to correct, resulting in the degeneration of hepatic tissue. This was also suggested in our models, from 10-, 12- and 30-fold increases in the levels of lactic dehydrogenase, glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase, the increased level of lipid peroxide and the microscopy of the livers, which revealed various morphological changes [14]. Moreover, administration of α-tocopherol 100 mg p.o. for 5 days showed the suppressive action on the lipid peroxidation caused by the hepatic ischemia–reperfusion [15].

Ischemia and subsequent reperfusion cause functional and structural damage to liver cells, possibly by free radical reaction processes caused by "oxidative stress" [2, 3]. High steady-state levels of oxyradicals and of alcoxyl and peroxyl radicals in tissue have been considered as sources of oxidative stress due to the destructive potential of these species [16]. In this study, we examined PCOOH levels in rat liver, since PCOOH is a primary peroxidative product from PC, the most important functional lipid in hepatocellular membranes. We first demonstrated the generation of PCOOH in rat liver following ischemia-reperfusion, which might produce oxygen free radicals linked to further chain reactions.

These results suggest that the simultaneous determination of PCOOH and PC levels by this CL-HPLC assay could be useful for determining the extent of cellular damage of various organs induced by free radicals generated during surgery or by drugs or disease, and for aiding in the development of drugs for diseases involving free radicals.

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