Biochemical Pharmacology, Vol. 46, No. 10, pp. 1749–1757, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EFFECTS OF ANTI-FREE RADICAL INTERVENTIONS ON PHOSPHATIDYLCHOLINE HYDROPEROXIDE IN PLASMA AFTER ISCHEMIA–REPERFUSION IN THE LIVER OF RATS

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(*Received* 16 *June* 1993; *accepted* 4 *August* 1993)

Abstract—The present study set out to investigate whether plasma phosphatidylcholine hydroperoxide (PCOOH) levels could accurately reflect lipid peroxidation linking to liver damage due to ischemia–reperfusion. PCOOH is a primary peroxidative product of phosphatidylcholine (PC), which is the most important functional lipid in the hepatocellular membrane, and may mediate oxidative stress. We quantified PCOOH and PC in the plasma and liver of rats subjected to hepatic ischemia–reperfusion by chemiluminescence detecting HPLC (CL-HPLC) method. Plasma PCOOH levels showed no significant rise in either the ischemia only group or in the sham-operation group, compared to controls (0.7 nmol/mL plasma). At 60 min subsequent to reperfusion, the PCOOH levels in plasma and liver, as well as the levels of several serum markers of liver injury [lactic dehydrogenase (LDH), glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT)] increased in proportion to the duration of ischemia (up to 60 min). During periods of reperfusion following 30 min of ischemia, plasma PCOOH increased biphasically (2 nmol/mL; 12–24 hr duration of reperfusion), and generally ran parallel to that in the liver after more than 60 min of reperfusion. Dose-dependent protective effects against warm ischemia (30 min)–reperfusion (12 hr) injury were clearly demonstrated in the groups treated with allopurinol, diclofenac Na, ascorbic acid (V.C), α-tocopherol and coenzyme Q_{10}, but not in those treated with *r-h*-superoxide dismutase or betamethasone. The rises in plasma PCOOH and serum GOT, GPT and LDH of the ischemia–reperfused rats were ameliorated most in the group pretreated with diclofenac Na, and next most in the group pretreated with V.C. These results indicate that the plasma PCOOH levels are a useful index both for liver cell damage induced by oxygen free radicals generated during ischemia–reperfusion, and to investigate the efficacy of drugs against oxidative stress.

Free radical-mediated lipid peroxidation in biomembranes is believed to be critically involved in several disease states, including ischemic disease, drug-associated toxicity, complications with transplantation etc. [1–3]. Lipid peroxidation may cause alterations in biomembrane-associated functions and structure, and may disrupt the function of the cell or subcellular organelles [1, 2, 4]. The liver is highly susceptible to the chain reaction of lipid peroxidation under aerobic conditions. Tissue lesions associated with hepatic ischemia–reperfusion may occur spontaneously from vessel deterioration or during liver transplantation [2, 4–6]. The lipid peroxidation may be initiated by a chain reaction of superoxides, the major source of which is not fully understood [7–10].

Thiobarbituric acid reactants indicate lipid peroxidation associated with cellular damage caused by free radicals [11]. However, this assay also measures aldehydes from processes other than hydroperoxide degradation, and therefore lacks specificity [12]. Phosphatidylcholine hydroperoxide (PCOOH†) has recently gained attention as a primary peroxidative product of phosphatidylcholine (PC), which is the most important functional lipid in the hepatocellular membrane [13]. Moreover, the occurrence of PCOOH may reflect biomembrane damage. Therefore, we developed a sensitive and specific quantitative assay for PCOOH which improves upon the chemiluminescence-HPLC (CL-HPLC), the methods of Yamamoto *et al.* [13] and Miyazawa *et al.* [14], and requires only minor modifications of previous methods. This assay begins with the separation of lipid classes with a normal phase HPLC. Total PC, including PC and hydroxy or hydroperoxide derivatives, is then detected by absorption at UV 205 nm due to its diene structure. Chemiluminescence (CL) from luminol oxidation, caused by the reaction of hydroperoxides with heme compounds, was also measured.

We previously used this method to determine the level of PCOOH generated in the liver of rats subjected to hepatic ischemia–reperfusion [15]. In that study, we also evaluated liver injury using microscopy to detect morphological changes and by noting the activity of serum lactic dehydrogenase

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† Abbreviations: CL, chemiluminescence; CL-HPLC, HPLC coupling with detection of chemiluminescence for hydroperoxide assay; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; LDH, lactic dehydrogenase; GOT, glutamic-oxalacetic transaminase; GPT, glutamic-pyruvic transaminase; V.C, ascorbic acid; CoQ_{10}, coenzyme Q_{10}; SOD, superoxide dismutase; NSAID, non-steroidal anti-inflammatory drug; PLA_2, phospholipase A_2; O_2^-, superoxide anion; $OH^·$, hydroxyl radical; α-toc, α-tocopherol; BHT, butylated hydroxytoluene; K.U., Karmen units; W.U., Wroblewski units.

(LDH), glutamic-oxalacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) as indices of leakage of hepatic enzymes into the circulation [16]. Our results showed that increased levels of PCOOH in the liver can reflect hepatic ischemia–reperfusion injury, and confirmed that these levels are a useful index of hepatocellular damage caused by oxidative stress. However, the tissue samples required for this analysis are difficult to collect clinically.

It has recently been suggested that the uptake of circulating lipid hydroperoxides into peripheral tissue may play a pivotal role in some diseases, such as atherosclerosis [17, 18]. Since lipid hydroperoxides can generate alkoxyl or peroxyl radicals in the presence of transition metals, they may mediate oxidative stress [19]. High steady-state levels of these radicals in tissue may be a source of oxidative stress [18]. If PCOOH generated by ischemia–reperfusion leaks into circulation then the plasma PCOOH level can be expected to increase, and the entire body may experience oxidative stress.

In the present study, we examined whether plasma PCOOH levels can reflect liver injury caused by ischemia–reperfusion. We determined serum markers (LDH, GOT and GPT activities) of liver injury and quantified the levels of PCOOH and PC in lipid extracts from plasma samples over time, using a CL-HPLC assay. We also evaluated the effects of the drugs which intervene in free radical chain reactions at the levels of plasma PCOOH and the serum markers of liver injury.

MATERIALS AND METHODS

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

Chemicals. Allopurinol was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and diclofenac Na (Ciba Geigy, Ltd, Japan), α-tocopherol (α-toc) and coenzyme Q_{10} (CoQ_{10}) (Neuquinon®) (Eisai Co., Ltd, Tokyo, Japan), ascorbic acid (V.C) (Vitacimin® inj.), *r-h*-superoxide dismutase (*r-h*-SOD) (Takeda Pharmaceutical Industries, Osaka, Japan) and betamethasone (Rinderon® inj.) (Shionogi Co., Ltd, Osaka, Japan) were supplied as gifts of the respective companies. HPLC-grade reagents were used for the mobile phase of the CL-HPLC assay, and all other reagents were of analytical grade.

Liver ischemia–reperfusion [20]. The abdomen of the rat was opened through a midline incision under ether anesthesia, and the left portal vein and hepatic artery were occluded with a microvessel clip. Later the abdomen was 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 CL-HPLC assay. Sham-operated rats were treated in the same manner, but were not clamped. Control rats only underwent blood and liver collection, without ischemia–reperfusion.

Dose–response studies for hepatic ischemia–reperfusion injury. Allopurinol 10, 40 mg/kg, diclofenac Na 3, 10 mg/kg, α-toc 30, 100 mg/kg, and CoQ_{10} 6, 20 mg/kg were administered p.o. daily for 5 days and once at 1 hr prior to the induction of ischemia. V.C 100, 400 mg/kg and *r-h*-SOD 4, 10 mg/kg were given i.v. 5 min prior to the onset of reperfusion. The dosage: 4, 15 mg/kg of betamethasone, was divided in half and given i.v., twice. One dose was given at 5 min prior to the induction of ischemia and the other was given at the onset of reperfusion. Each drug was administered to five rats per group, which were subjected to hepatic ischemia for 30 min and subsequent reperfusion for 12 hr (0.5–12 hr).

Biochemical assay. GOT, GPT and LDH activities in serum were determined using a Shimadzu CL20 Auto Analyzer (Shimadzu Co., Kyoto, Japan).

Tissue preparation. Immediately after resection, the liver tissue was placed in saline at 0–5° and all further preparative procedures were carried out at this temperature. After rinsing, the specimens were cut into small pieces and mixed with saline and butylated hydroxytoluene (BHT, Wako Pure Chemical Industries, Ltd) in methanol, to achieve a concentration of liver homogenate containing 200 mg wet liver tissue/mL and 0.005% of BHT. Plasma was prepared from fresh heparinized blood by centrifugation of 3000 rpm for 10 min under cooling, and BHT was added to 0.005%. Both preparations were stored at −80° until use in the lipid extraction procedure for the CL-HPLC assay.

CL-HPLC assay. (1) Preparation of standard PC and PCOOH. Standard PC was made by passing PC (from egg yolk, Wako Pure Chemical Industries, Ltd) through a reversed-phase column (Lichroprep RP-8, Merck and Co., Rahway, NJ, U.S.A.) with 1.8 mL/min of eluant (chloroform–methanol–water, 10:1:0.5, v/v). PCOOH was prepared by photosensitized oxidation of PC with methylene blue in methanol [14] and was subsequently purified in the same manner as PC, following removal of methylene blue by silica gel PF_{254} (Merck and Co.). Each fraction with absorption at a wavelength of 235 nm for conjugated diene structure, was injected into the CL-HPLC equipment. The fractions were collected as the purified product, if their CL chromatograms showed strong peaks near the retention time for PC, of which the integrated areas occupied greater than 95% of the total.

(2) Confirmation of PCOOH. The purified product was concentrated by evaporation at room temperature, and was then applied as a narrow band to Merck TLC plates coated with silica gel $60F_{254}$. The plates were developed to a distance of 15 cm in chloroform–methanol–1 M NH_4OH (65:25:4, v/v) [21]. After development and drying, the plates underwent visualizing procedures by (a) dimethyl-*p*-phenylenediamine reagent for peroxide derivative, (b) Dragendorf reagent for substances containing choline [22], (c) Ryu-MacCoss reagent for substances containing phosphoric acid [23] and (d) UV irradiation for the structure of conjugated diene structures. Identification of purified product to

PCOOH was performed by comparing the R_f value in the present study with that in the Creer *et al.* study [21], and by visualizing procedures (data not shown). Similar confirmation procedures were done with lipid extracts from the plasma and liver.

(3) Determination of PC and PCOOH concentrations. The concentration of standard PC was determined by a colorimetric method measuring phosphorus and was expressed as moles. The 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 [24].

(4) Equipment and chromatography conditions of CL-HPLC. The CL-HPLC system consisted of an injector (Rheodyne 7161; Rheodyne Inc., U.S.A.), a normal-phase column (Jasco Fine pack SIL, 5 μm, 250 × 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). Acetonitrile–methanol–water (5.5:3:1.5, v/v) was used for the mobile phase of HPLC. The CL reagent sent to post-column was prepared by dissolving 1 μg/mL of luminol (3-aminophthaloyl hydrazine, Wako Pure Chemical Industries, Ltd) and 10 μg/mL of cytochrome *c* (from horse heart type VI, Sigma Chemical Co., St Louis, MO, U.S.A.) in 20 mM borate buffer, pH 10.5, which was saturated with N_2 gas and contained 1% methanol. The flow rates were 1 mL/min.

The CL-HPLC assay began with the separation of lipid classes using a normal phase HPLC. Total PC, including PC and hydroxy or hydroperoxide derivatives, was then detected by absorption at UV 205 nm due to diene structure. We then measured the CL from luminol oxidation caused by the reaction of hydroperoxides with cytochrome *c* in the CL reagent sent to the post-column. The qualitative and quantitative detection limits of PCOOH by this assay are 0.5 and 2 pmol, based on active oxygen from hydroperoxide.

Lipid extraction procedure for CL-HPLC assay. The extraction procedure was performed under a nitrogen stream. The lipids in liver homogenate were extracted with 5 mL of chloroform–methanol (2:1, v/v) containing 0.005% BHT to each 1 mL of homogenate [25]. The lipids in plasma were extracted with 4 mL of chloroform–methanol (3:1, v/v) containing 0.005% BHT to each 0.5 mL of plasma. The mixture was shaken vigorously for 1 min and centrifuged at 3000 rpm for 10 min at 10°. The lower layer was then collected and dried under a nitrogen stream at 30° and reconstituted in chloroform–methanol (1:1, v/v) solution. The portion corresponding to lipid extracts from 5 to 20 mg of liver or 50 μL of plasma was then injected into the CL-HPLC equipment.

Statistics. All values are expressed as means or means ± SE for four or five rats. The significance of mean differences was analysed by the Student's *t*-test for unpaired data.

RESULTS

Determination of PCOOH by CL-HPLC

The typical calibration curve for this CL-HPLC assay of PCOOH is shown in Fig. 1. The CL counts integrated for the peak area were proportional to the amount of PCOOH from 2 to 1000 pmol according to active oxygen from hydroperoxide. The minimum amount of PCOOH detectable by the present CL-HPLC was 0.5 pmol. An intra-assay variation of 7% and inter-assay variation of 10% were obtained at 100 pmol. The use of chloroform–methanol (3:1) 4 mL, instead of chloroform–methanol (2:1) 3 mL, as an extracting solvent to 0.5 mL of plasma, improves the recovery rate of PCOOH by 4-fold (from 11 to 44.9%). The coefficient variation for recovery rates of additional PCOOH in plasma was within 4%.

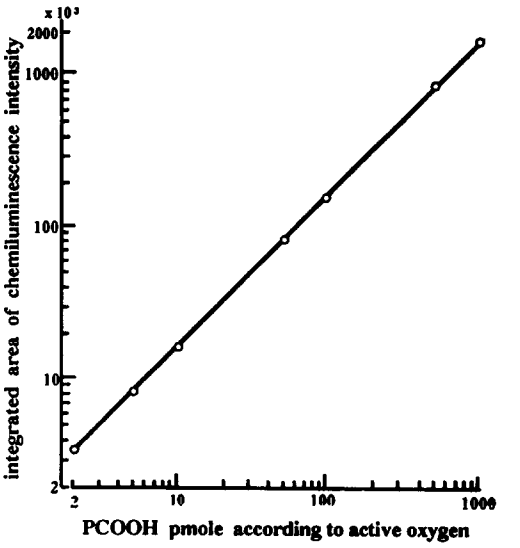

Fig. 1. Calibration line of PCOOH with the CL-HPLC assay. Open circles represent the mean integrated area from three assays of authentic PCOOH. Horizontal axis represents moles. Each coefficient variance of three assays is within 10%.

With this extraction procedure and CL-HPLC assay, we were able to measure PCOOH and PC in rat plasma. Figure 2 shows typical chromatograms of PCOOH obtained from plasma. In the control and ischemia–reperfusion plasma samples, the CL peak (about 11 min) was identified as PCOOH and became larger compared with that of rats subjected to 30 min of ischemia and 12 hr of reperfusion (0.5–12 hr). This increase was suppressed by pretreatment with α-toc.

The PCOOH level in control rat plasma was 0.689 ± 0.227 (mean ± SE of five rats).

Influence of length of ischemia on levels of serum GOT, GPT, LDH and plasma PCOOH

The serum markers (GOT, GPT and LDH) on liver injury slightly increased in the rats subjected to only sham-operation and ischemia; however, after 60 min of reperfusion increased depending on the duration of ischemia (Fig. 3). The values for 30 min of ischemia in Fig. 3 were not different from those

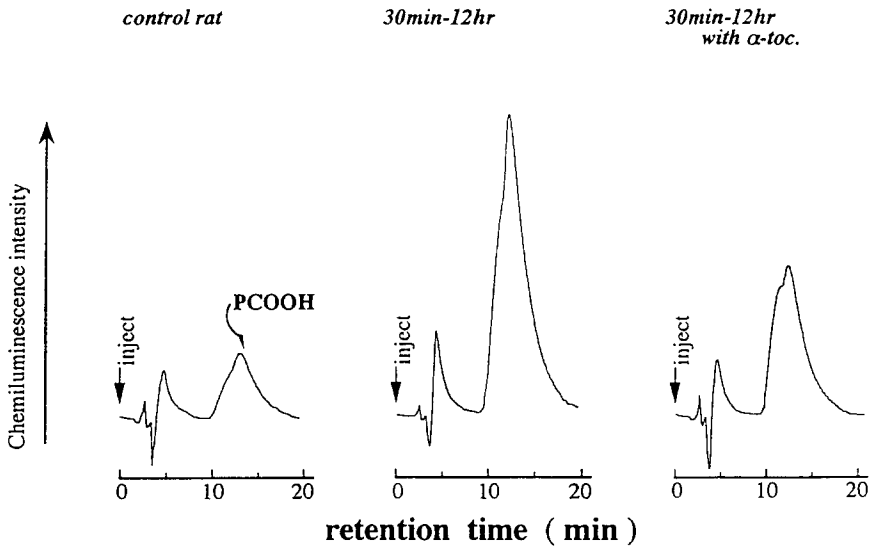

Fig. 2. Chromatograms obtained from detection of the intensity of chemiluminescence. Each was recorded after an injection of lipid extract from 50 μL of rat plasma into CL-HPLC equipment for PCOOH assay. Control rat data are for a blood sample only, 0.5–12 hr data are for a rat subjected to 30 min of hepatic ischemia followed by 12 hr of reperfusion, 0.5–12 hr with α-toc data are for a 0.5–12 hr rat with pre-ischemic administration of α-toc 100 mg/kg/day, p.o., for 5 days, and an additional dose.

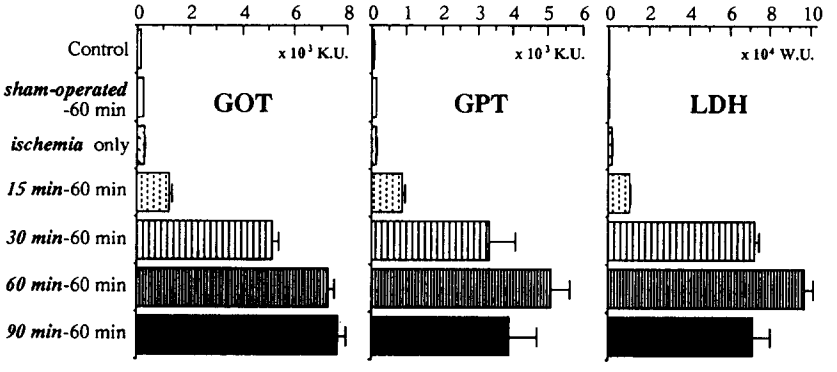

Fig. 3. Changes in serum GOT, GPT and LDH levels after 60 min of reperfusion following various durations of hepatic ischemia. Rats were subjected to ischemia lasting for the period shown on the vertical axis. Each column represents a mean ± SE of four or five rats.

of the 15, 60 and 90 min ischemia only groups (data not shown).

The PCOOH level in the plasma of rats at 60 min after sham-operation was 0.592 ± 0.258 nmol/mL. The plasma PCOOH levels after 60 min of reperfusion increased in proportion to the duration of ischemia and the mean ± SE of the PCOOH level in cases of ischemia–reperfusion at 15–60 min, 30–60 min, 60–60 min and 90–60 min was 0.579 ± 0.141, 1.126 ± 0.273, 2.283 ± 0.317 and 3.017 ± 0.574 nmol/mL, respectively (Fig. 4). Similar results were obtained for liver PCOOH levels.

Effect of reperfusion period on levels of serum GOT, GPT, LDH and plasma PCOOH

In our previous study, serum GOT and GPT activities after 30 min of ischemia increased with reperfusion, and reached maximum levels (30-fold and 20-fold over control levels) at 12 hr of reperfusion. LDH activity increased immediately and reached a peak (150-fold over control levels) within 1 hr of reperfusion, and then decreased to a level that was 15-fold over that of controls at 12 hr of reperfusion [26].

The plasma PCOOH levels increased biphasically

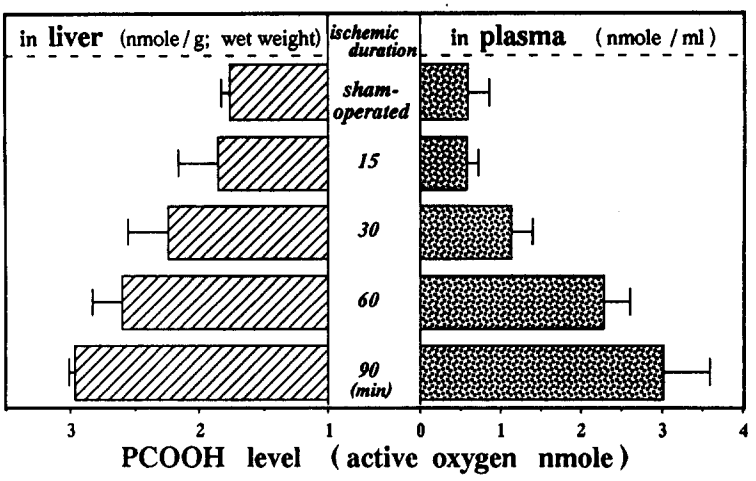

Fig. 4. Relationship between hepatic ischemia duration and increased PCOOH levels in liver and plasma of rats after 60 min of reperfusion. Rats were subjected to 60 min reperfusion following hepatic ischemia for the duration shown on the vertical axis. Sham-operated rats were treated in the same manner, but were not clamped. The PCOOH values are means ± SE for four or five rats.

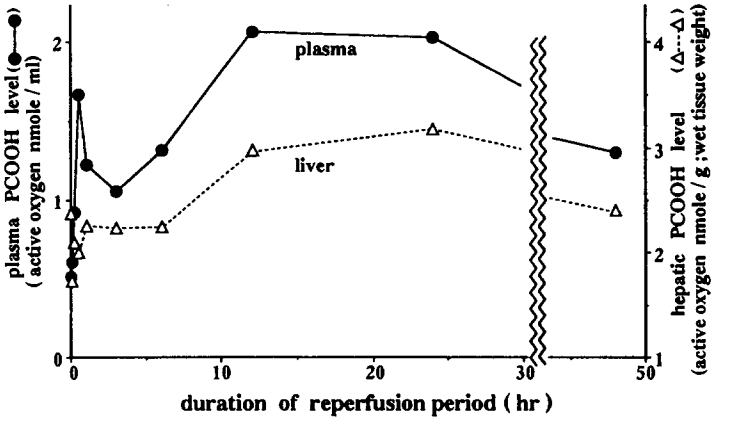

Fig. 5. Fluctuation of PCOOH levels in liver and plasma of rats with different durations of reperfusion following 30 min of ischemia. Rats were subjected to 30 min of hepatic ischemia and subsequent reperfusion of varied duration (reperfusion began at the end of the ischemic period). (●) Represents PCOOH levels in plasma, and (△) represents PCOOH levels in liver. Values are the mean for four or five rats. The PCOOH levels in plasma and liver of control rats were 0.689 ± 0.227 nmol (according to active oxygen)/mL and 2.007 ± 0.019 nmol/g; wet tissue weight, respectively.

during periods of reperfusion following 30 min of ischemia (Fig. 5). These levels did not show increases at the end of ischemia (0.516 ± 0.166 nmol/mL) and were nearly identical to those of the control group. The levels increased to 1.667 ± 0.351 nmol/mL at 30 min of reperfusion and then decreased and again increased to approximately 2 nmol/mL at 12–24 hr of reperfusion. Finally, the plasma PCOOH levels decreased again at 48 hr (1.300 ± 0.197 nmol/mL). The plasma PCOOH levels generally ran parallel to those in the liver, except for the elevation in early phase of reperfusion seen in the plasma (Fig. 5).

Effects of anti-free radical interventions on levels of serum GOT, GPT, LDH and plasma PCOOH with 0.5–12 hr ischemia–reperfusion

The levels of serum markers were significantly raised by ischemia–reperfusion of 0.5–12 hr [GOT: 4583 ± 509 (0.5–12 hr) from 143 ± 5 K.U. (control), GPT: 1400 ± 112 from 64 ± 11 K.U., and LDH: 4603 ± 2093 from 287 ± 53 W.U.]. These increases were suppressed by treatments with drugs that inhibited production of active oxygen during ischemia–reperfusion or that scavenge radical species (Table 1).

Figure 6 shows the effects of these drugs on plasma PCOOH levels. The increased plasma PCOOH levels (2.073 ± 0.302) in rats after 0.5–12 hr of ischemia–reperfusion was lowered to 1.019 ± 0.142 by allopurinol 40 mg/kg, to 0.589 ± 0.064 by diclofenac Na 10 mg/kg, to 0.810 ± 0.036 by V.C 400 mg/kg, to 1.188 ± 0.129 by α-toc 100 mg/kg, and to 1.514 ± 0.088 nmol/mL by CoQ_{10} 20 mg/kg. When *r-h*-SOD was used, the suppressive effect on

Table 1. Several serum parameters for liver injury caused by ischemia–reperfusion in rats.

	GOT (K.U.)	GPT (K.U.)	LDH (W.U.)
Control	143 ± 5§	64 ± 11§	287 ± 53§
0.5–12 hr	4583 ± 509	1400 ± 112	4603 ± 2093
Allopurinol (10 mg)	1974 ± 327†	900 ± 77†	845 ± 288
Allopurinol (40 mg)	1880 ± 271†	928 ± 237	930 ± 127
Diclofenac Na (3 mg)	625 ± 82‡	1555 ± 224	517 ± 82*
Diclofenac Na (10 mg)	487 ± 128‡	1328 ± 96	496 ± 110*
V.C (100 dmg)	2128 ± 289†	774 ± 111†	754 ± 89*
V.C (400 mg)	2062 ± 132‡	700 ± 55‡	754 ± 93*
α-toc (30 mg)	2573 ± 511*	1589 ± 35	1061 ± 298
α-toc (100 mg)	1681 ± 209†	1295 ± 99	843 ± 96
CoQ_{10} (6 mg)	2620 ± 224†	1490 ± 462	780 ± 152
CoQ_{10} (20 mg)	1593 ± 80†	935 ± 305	810 ± 258
SOD (4 mg)	3155 ± 449*	1883 ± 401	1216 ± 629
SOD (10 mg)	2778 ± 515*	1609 ± 333	989 ± 242
Betamethasone (4 mg)	2983 ± 210*	870 ± 151*	1829 ± 164
Betamethasone (15 mg)	3600 ± 850	888 ± 409*	3645 ± 1242

*$P < 0.10$, †$P < 0.05$, ‡P and §$P < 0.01$ (Student's *t*-test for unpaired data).
*, †, ‡ Means a comparison of ischemia–reperfusion rats with a drug to ischemia–reperfusion rats without drug. §Means a comparison of control rats to ischemia–reperfusion rats without a drug.

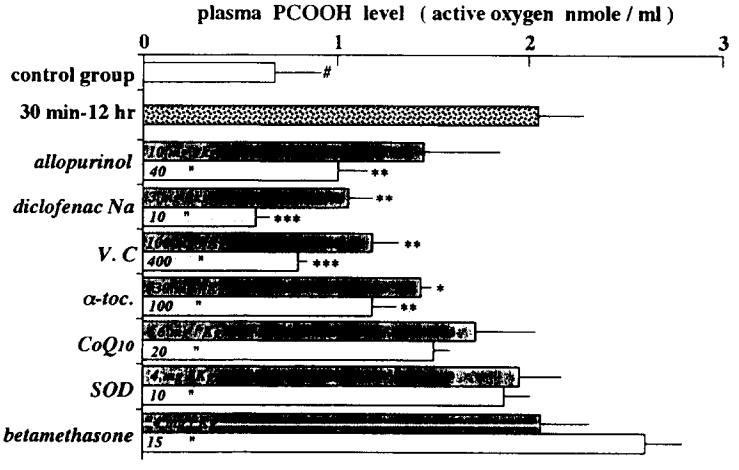

Fig. 6. Effect of drugs on increased PCOOH levels in rat plasma caused by hepatic ischemia–reperfusion. Only blood samples were collected for the control group. All others were subjected to 30 min of hepatic ischemia followed by 12 hr of reperfusion with or without pretreatment with a drug (vertical axis). Each value is the mean ± SE for four or five rats, and comparisons are made with rats that received no drugs. *$P < 0.10$, **$P < 0.05$, ***P and #$P < 0.01$ (Student's *t*-test for unpaired data). * Means a comparison of ischemia–reperfusion rats with a drug to ischemia–reperfusion rats without drug, and # means a comparison of control rats to ischemia–reperfusion rats without a drug.

the elevation of PCOOH level and the protective effect on liver injury were weak or absent. By contrast, the high dose administration (15 mg/kg) of betamethasone i.v., prior to ischemia and reperfusion, raised the plasma PCOOH levels to 2.632 ± 0.186 nmol/mL, while 4 mg/kg betamethasone i.v. had no effect on PCOOH levels but depressed GOT, GPT and LDH.

DISCUSSION

It has been suggested that many disease states arise from tissue injuries caused by alterations to cellular membranes as a result of free radical chain reactions [2, 3, 27]. Liver cells are protected from oxygen-derived radical injury by naturally occurring free radical scavengers and antioxidant pathways.

When these protective mechanisms are overwhelmed, liver tissues become susceptible to damage by oxygen radicals [28].

The liver injury following ischemia and reperfusion in rats, directly expressed by the leakage of hepatic enzymes into the circulation, had previously [16] been defined by pathohistological changes in liver preparations, hepatic microsomal enzyme activity and survival rates. We had also demonstrated the occurrence of PCOOH in liver following ischemia–reperfusion [15]. PCOOH is the primary peroxidative product of PC, which is the most important functional lipid in the hepatocellular membrane. PCOOH may generate its alkoxyl or peroxyl radicals in the presence of transition metals, and may therefore mediate oxidative stress [17–19]. We monitored hepatocellular damage by the occurrence of lipid peroxidation as indicated by PCOOH in the plasma, and examined the relationship between PCOOH levels in liver and those in plasma.

We found an increase in the levels of PCOOH in liver and plasma, and in serum GOT, GPT and LDH following 60 min of reperfusion, and that this increase was proportional to the duration of ischemia up to at least 60 min (Figs 3 and 4). We also found that serum GOT, GPT and LDH levels in ischemia–reperfused rats at 0.5–12 hr increased to 30-, 20- and 15-fold of those in controls (Table 1). Such high levels are rare, and reflect hepatic dysfunction and considerable tissue injury. This abnormal leakage of enzymes suggests structural and functional disorders in the hepatic microsomes [16]. After 30 min of hepatic ischemia in rats, the plasma PCOOH levels increased biphasically and paralleled those in the liver during subsequent reperfusion.

Superoxides in biological system can be produced by xanthine oxidase in endothelial cells, NAD(P)H oxidase and myeloperoxidase in activated phagocytes (neutrophils, monocytes and Kupffer cells), leakage of reactive oxygen from the mitochondrial respiratory chain, the arachidonic acid cascade, the oxidation of NAD(P)H at the microsomes etc. [7–10, 29]. However, the major source of these superoxides during hepatic ischemia–reperfusion is still unclear. It is clinically and pathophysiologically important to clarify the mechanism of the cellular damage caused by ischemia–reperfusion in order to prevent and treat organ dysfunction and to enhance viability of the organ for transplantation.

We tried various compounds that interfered with free radical chain reactions at different points in order to examine more precisely the role of active oxygen species in hepatric ischemia–reperfusion injury. These compounds included allopurinol [5] and diclofenac Na [8, 30] to inhibit the production of superoxides; V.C [31–33], α-toc [34] and SOD [5] to scavenge active oxygen species; α-toc and CoQ_{10} to suppress lipid peroxidation in biomembrane [35]; and betamethasone to inhibit phospholipase A_2 (PLA_2) [36].

Treatment with allopurinol, α-toc, CoQ_{10} and SOD with catalase has previously been demonstrated to protect against reperfusion injury mediated by oxygen free radicals [5, 6]. Allopurinol was shown to prevent the disorder after myocardial ischemia–reperfusion [5] by inhibiting the production of superoxide anion (O_2^-) by acting as a competitive substrate in the xanthine–xanthine oxidase system. The compound α-toc exists in biomembrane and functions as a chain-breaking agent to protect polyunsaturated lipids from peroxidation by scavenging peroxyl and alkoxyl radicals of lipids [6] and superoxides [34], and also acts as a free radical scavenger. This compound has also been demonstrated to prevent ischemia–reperfusion injuries [4]. CoQ_{10} has been shown to protect against hepatic ischemia–reperfusion damage [37], and to scavenge peroxyl [38] and tocopheroxyl radicals [37], although it is not as effective as vitamin E. Thus, it is likely that CoQ_{10} may exert an antioxidant effect by recycling vitamin E.

In the present study, protective effects against warm ischemia–reperfusion injury were clearly demonstrated in the groups treated with allopurinol, diclofenac Na, V.C, α-toc and CoQ_{10}, but not in those treated with *r-h*-SOD. These protective effects were consistent with the finding of several previous studies. It has been reported that the combination of SOD and catalase may be more effective than treatment with SOD alone [11]. The use of SOD alone may be insufficient because the H_2O_2 generated may be broken down in the presence of transition metals into the extremely reactive hydroxyl radical ($OH^\cdot$).

The plasma PCOOH and serum GOT, GPT and LDH levels were significantly depressed by pretreatment with diclofenac Na, in a dose-dependent manner. Diclofenac Na is used as a non-steroidal anti-inflammatory drug (NSAID) and has been shown to suppress the production of O_2^- by disrupting the activation of NAD(P)H oxidase of phagocytes, mainly neutrophils and monocytes [30, 39]. Steroid and NSAID have been shown to reduce infarct size by preventing the accumulation of neutrophils in the ischemic tissue [8]. Moreover, NSAID may reduce the production of active oxygen species from the arachidonic cascade by inhibiting cyclooxygenase. These effects may enable diclofenac Na to prevent lipid peroxidation and the subsequent liver dysfunction.

In the present study, V.C was very effective in preventing hepatic injury following 0.5–12 hr of ischemia–reperfusion, and significantly suppressed both PCOOH levels in plasma and the leakage of GOT, GPT and LDH from the liver of 0.5–12 hr rats with a single i.v. dose prior to the reperfusion. V.C can generate damaging radical species including $OH^\cdot$ in the presence of iron or copper salts [40], and reacts quickly with O_2^-, $OH^\cdot$, hypochlorous acid (HOCl) [31] and carbon center radicals (peroxyl radicals, alkoxyl radicals and tocopheroxyl radicals). Although V.C is hydrophilic and cannot directly inhibit lipid peroxidation in biomembranes, it can rapidly and effectively scavenge radicals synergistically with vitamin E [32]. Since $OH^\cdot$ and HOCl are strong oxidants and are likely to be the oxygen-derived free radicals that interact with tissue components to initiate cellular damage [33], their removal may account for the protective effect of V.C against ischemia–reperfusion injury.

Administration of betamethasone 4 mg/kg suppressed liver injury without decreasing plasma

PCOOH levels, but a dose of 15 mg/kg had no effect on liver injury caused by 0.5–12 hr of ischemia–reperfusion. Betamethasone, a glucocorticoid, by inhibiting PLA_2 plays a role in the turnover of glycerophospholipids in biomembranes. PLA_2 during ischemia [41] promotes the arachidonic cascade, and the dysfunction and disruption of biomembranes but functions as an antioxidant, since it prefers an oxidized glycerophospholipid and hydrolyses and releases a free fatty acid peroxide that can be destroyed by glutathione peroxidase [36].

The present results suggested that the reagents that inhibit the production of O_2^- were the most effective in preventing hepatic ischemia–reperfusion injury. Comparison of the changes in plasma PCOOH levels and serum markers of liver injury between the diclofenac Na group and the allopurinol group suggests that NAD(P)H oxidase and the arachidonic cascade may play a more important role in production of O_2^- and oxygen-derived free radicals than xanthine oxidase during ischemia–reperfusion. Further, our results indicate that compounds that are able to scavenge the tocopheroxyl radicals OH^* and HOCl are very effective, and that OH^* and HOCl are likely to be involved in the cellular damage of warm ischemia–reperfusion injury in the liver. Plasma PCOOH levels appear to be a reliable indicator of hepatic injury caused by ischemia–reperfusion and other oxidative stress. Since blood samples are much easier to obtain than tissue samples, the quantification of plasma PCOOH is clinically and pathophysiologically useful, for the assessment of tissue injury following oxidative stress.

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