Effect of Diclofenac, a Non-Steroidal Anti-Inflammatory Drug, on Lipid Peroxidation Caused by Ischemia-Reperfusion in Rat Liver

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ABSTRACT—The present study investigated the effects of diclofenac sodium (Dic Na) on lipid peroxidation (LPO) and liver injury in ischemia-reperfused rats. LPO was estimated from the levels of phosphatidylcholine hydroperoxide (PCOOH), a primary peroxidative product of phosphatidylcholine. Hepatic ischemia-reperfusion induced significant elevation of plasma PCOOH and caused liver injury in rats. Rats were treated daily with Dic Na or α-tocopherol (α-toc.), p.o., for 5 days and once at 1 hr prior to induction of ischemia. Both substances prevented LPO from decreasing the plasma PCOOH level, and they significantly suppressed the elevation of serum GOT and LDH, in a dose-dependent manner. Dic Na was able to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), but did not show radical-trapping ability for superoxide anion (O_2^-) or hydroxyl radicals (·OH), nor a suppressive ability for the NADPH-dependent LPO of microsomes. In contrast, α-toc. trapped both DPPH and O_2^−, but not ·OH, and it inhibited the NADPH dependent LPO in vitro. These results suggest that Dic Na may suppress liver injury caused by ischemia-reperfusion through stable radical scavenging and the inhibition of superoxide production in activated phagocytes, both of which may restrain the induction and progression of oxidative stress.

Keywords: Ischemia-reperfusion injury, Lipid peroxidation, Non-steroidal anti-inflammatory drug, Oxygen-derived free radical, Phosphatidylcholine hydroperoxide
MATERIALS AND METHODS

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 hr), with food and water available ad libitum. Dic Na (3 or 10 mg/kg), and α-toc. (30 or 100 mg/kg) were administered, p.o., daily for 5 days and once at 1 hr before the induction of ischemia. Each drug was administered to five rats per group, which were subjected to hepatic ischemia for 30 min and subsequent reperfusion for 12 hr (30 min-12 hr).

Chemicals
Dic Na (Ciba Geigy Japan, Ltd., Takarazuka) and α-toc. (Eisai Co., Ltd., Tokyo) were supplied as gifts from the respective companies. HPLC-grade reagents were used for the mobile phase of the CL-HPLC assay. We also used 3-aminophthaloyl hydrazine (luminol), xanthine oxidase, butylated hydroxytoluene (BHT), diethylentriamine pentaacetic acid (DETAPAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA) (Wako Pure Chemical Industries, Ltd., Osaka); cytochrome c (from horse heart type VI), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), xanthine (Sigma Chemical Co., St. Louis, MO, USA); and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) (Labotec Co., Ltd., Tokyo). All other reagents were of analytical grade.

Preparation of liver ischemia-reperfusion rats
We investigated drug efficacies on LPO and the liver injury in a hepatic, warm ischemia-reperfusion model with rats (14). The rats were first 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 chemiluminescence detecting high performance liquid chromatography (CL-HPLC) assay (13). Control rats were subjected to blood and liver collection, but not to ischemia-reperfusion.

Plasma preparation
Plasma was prepared from fresh heparinized blood by centrifugation at 3,000 r.p.m., for 10 min at 5°C, and BHT was added to 0.005%. Plasma was stored at −80°C until used in the lipid extraction procedure, to determine PCOOH levels with the CL-HPLC assay.

Biochemical assays
GOT, GPT, and LDH activities in the serum were determined by a Shimadzu CL20 Auto Analyzer (Shimadzu Co., Kyoto).

Equipment and chromatography conditions for CL-HPLC
The CL-HPLC system consisted of an injector (Rheodyne 7161; Rheodyne Inc., Cotati, CA, USA), a normal-phase column (Jasco Fine Pack SIL, 5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Tokyo), two pumps (Jasco 880-PU and Shimadzu LC10AS, Shimadzu Co.), 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 post-column reagent was prepared by dissolving 1 pg/ml of luminol and 10 pg/ml of cytochrome c in 20 mM borate buffer, pH 10.5, which was saturated with N₂ 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 the absorption at UV 205 nm, which was due to the diene structure. We then measured CL from luminol oxidation caused by the reaction of hydroperoxides with cytochrome c in the post-column CL reagent. The qualitative and quantitative detection limits of PCOOH with this assay are 0.5 and 2 pmole, respectively, based on active oxygen from hydroperoxide.

Lipid extraction procedure for CL-HPLC assay
The extraction procedure (13) was performed under a nitrogen stream. Plasma lipids were extracted with 4 ml of chloroform-methanol (3:1, v/v), containing 0.005% BHT, for each 0.5 ml of plasma. The mixture was shaken vigorously for 1 min and centrifuged at 3,000 r.p.m. for 10 min at 5°C. The lower layer was then collected and dried under a nitrogen stream at 30°C and reconstituted in a chloroform-methanol (1:1, v/v) solution. The portion corresponding to lipids extracted from 50 μl of plasma was then injected into the CL-HPLC equipment.

Radical trapping studies
Radical-scavenging abilities of Dic Na for O₂−, hydroxyl radical (·OH), and stable radical were estimated with an electron spin resonance (ESR) spectrometer (JES-RE1X) (Jeol Ltd., Tokyo).

The radical-trapping ability, for a stable free radical, was estimated using DPPH. DPPH was dissolved in ethanol to a concentration of 100 μM. Dic Na and α-toc.
were dissolved in dimethyl sulfoxide (DMSO) to concentrations of 20 \( \mu \)M, 0.2 mM, and 2 mM. Each test drug, in DMSO, was mixed with an equal volume of DPPH solution in a cuvette. The blank assay was performed in the same manner using only DMSO instead of the test drug solution. At 40 sec after mixing, the ESR spectra were recorded at room temperature in a quartz flat cell (Labotec Co., Ltd.) with a JES-RE1X spectrometer operating at the X band (9.415 GHz) and a modulation frequency of 100 kHz, microwave power of 8 mW, receiver gain of 1.6 x 10, field modulation width of 0.79 x 0.1, sweep time of 2 min, and time constant of 0.1 sec. The central field ± sweep width was set at 335 ± 10 mT. Then the relative DPPH radical intensity height was calculated to divide the signal height of DPPH by that of simultaneously recorded Mn²⁺. The scavenging ability of a drug for a DPPH radical was estimated by the comparison of the relative intensity height between a test drug assay and a blank assay.

The radical-trapping abilities of Dic Na and a-toc. solutions (25 \( \mu \)M, 0.25 mM, and 2.50 mM, final concentrations) for \( O_2^- \) and \( \cdot \)OH were determined using DMPO as a radical-trapping reagent. Unless otherwise indicated, determinations for \( O_2^- \) and \( \cdot \)OH were performed under the previously described ESR-spectrometer settings. To examine the scavenging ability for \( O_2^- \), 15 \( \mu \)l of 3.6 M DMPO was mixed with 50 \( \mu \)l of 2 mM hypoxanthine in a 0.1 M phosphate buffer solution (PBS), pH 7.8, and 35 \( \mu \)l of 5.5 mM DETAPAC in PBS, 50 \( \mu \)l of DMSO (the blank) or 50 \( \mu \)l of a test drug (Dic Na or a-toc.) dissolved in DMSO, and finally 50 \( \mu \)l of 0.4 U/ml xanthine oxidase. We began to record the ESR spectra of DMPO-OOH, the spin-trapped adduct of superoxide anion, 40 sec after xanthine oxidase was added to the sample. The central field ± sweep width was set at 334.8 ± 5 mT and the receiver gain, at 5 x 10.

Effects on NADPH-dependent LPO of liver microsomes

To investigate the effects of Dic Na and a-toc. on the NADPH-dependent LPO of liver microsomes, 3 mg microsomal protein/ml (50 \( \mu \)l) and 0.1 M PBS, pH 7.4, (200 \( \mu \)l) were preincubated with 50 \( \mu \)l of Dic Na or a-toc. (1 \( \mu \)M to 10 mM) in DMSO-0.1 M PBS, pH 7.4, (2 : 3, v/v) at room temperature for 20 min. The mixture was then combined with 4 mM NADPH (100 \( \mu \)l) and 1 mM ferric pyrophosphate (100 \( \mu \)l) and incubated at 37°C for 15, 20, and 30 min. The blank assay was performed in the same manner, using only DMSO-0.1 M PBS, pH 7.4, instead of the test drug solutions. To stop the reactions, 2.5 ml of 20% trichloro-acetic acid was added to each, followed by mixing for 10 sec. The LPO index of the TBA reactants (TBARS) was then measured by a modification of the method of Talcott et al. (15). After adding 1.0 ml of 0.67% TBA, the tubes were capped and put in boiling water for 15 min and then cooled. The samples were then extracted with 4.0 ml of n-butanol. The samples were centrifuged (3,000 r.p.m., 10 min), and the optical density of the butanol layer was read at 532 nm.

Determination of protein concentrations

Protein concentrations were measured according to the method of Lowry et al. (16), using bovine serum albumin as a standard.

Statistics

Results are expressed as the means or means ± S.E. for four or five rats. The significance of the mean difference was determined by Student’s t-test for unpaired data or one way analysis of variance (ANOVA) followed by Scheffé’s test.

RESULTS

Effects of Dic Na and a-toc. on liver injury, and plasma PCOOH levels in ischemia-reperfusion rats

Figure 1 shows typical chromatograms of PCOOH obtained from plasma. The chemiluminescence peak (at about 11 min) was identified as PCOOH, and this increased in rats subjected to 30 min of ischemia-12 hr of reperfusion (30 min-12 hr), but did not differ between rats treated with 30 min of ischemia only and control rats. The increase seen in 30 min-12 hr rats was suppressed by pretreatment with 3 mg/kg of Dic Na.

The 30 min-12 hr rats showed a 3-fold increase in plasma PCOOH levels, from 0.689 ± 0.227 (mean ± S.E. for five control rats) to 2.073 ± 0.302 nmol/ml (mean ± S.E. for four or five rats) (Table 1); a 30-fold increase in serum GOT, to 4583 ± 509 K.U.; a 20-fold increase in serum GPT, to 1400 ± 112 K.U.; and a 15-fold increase in serum LDH, to 4603 ± 2093 W.U., when compared to control rats (Fig. 2). Dose-response studies clearly demonstrate the protective effects of both Dic Na and a-toc. against warm ischemia-reperfusion injury. Daily pretreatments of 3 or 10 mg of Dic Na significantly suppressed the elevation of serum GOT, LDH, and plasma PCOOH levels to 625 ± 82, 487 ± 128 K.U., 517 ± 82, 496 ± 110 W.U., and
1.069 ± 0.120, 0.589 ± 0.064 nmol/ml, respectively. In addition, daily pretreatments of 30 or 100 mg/kg of a-toc. suppressed the elevation of serum GOT, LDH, and plasma PCOOH levels to 2573 ± 511, 1681 ± 209 K.U., 1061 ± 298, 843 ± 96 W.U., and 1.449 ± 0.053, 1.188 ± 0.142 nmol/ml, respectively (Fig. 2, Table 1). However, neither Dic Na nor a-toc. had any effect on the elevation of GPT.

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### Table 1. Effect of diclofenac sodium (Dic Na) and α-tocopherol (α-toc.) on PCOOH elevation in rat plasma after ischemia-reperfusion

| Treatment to rats | PCOOH level in plasma (nmol/ml) |
|-------------------|----------------------------------|
| No treatment      | 0.689 ± 0.227                   |
| 30 min ischemia only | 0.516 ± 0.166                  |
| 30 min-12 hr      | 2.073 ± 0.302                   |
| pretreated with Dic Na (3 mg/kg) | 1.069 ± 0.120*                 |
| pretreated with Dic Na (10 mg/kg) | 0.589 ± 0.064*                 |
| pretreated with α-toc. (30 mg/kg) | 1.449 ± 0.053*                 |
| pretreated with α-toc. (100 mg/kg) | 1.118 ± 0.129*                 |

No treatment means untreated rats. The 30 min ischemia only and 30 min-12 hr groups are rats subjected to 30 min of hepatic ischemia followed by 0 and 12 hr of reperfusion, respectively. Dic Na: 3, 10 mg/kg and α-toc.: 30, 100 mg/kg data are for 30 min-12 hr rats treated with pretreatment of 3 or 10 mg/kg/day of Dic Na or with 30 or 100 mg/kg/day of α-toc., respectively, p.o. Each value represents the mean ± S.E. for four or five rats. *P < 0.01, vs. no treatment rats; **P < 0.01, vs. 30 min-12 hr rats. One way ANOVA followed by Scheffe's test.

**Radical-trapping abilities of Dic Na and α-toc.**

Dic Na had a concentration-dependent radical-trapping ability for the stable free radical DPPH. The radical intensity height observed with 50 μM DPPH was diminished by approximately 10%, 22%, and 70% with the addition of Dic Na at concentrations of 10 μM, 0.1 mM, and 1 mM, respectively, while inhibition by α-toc. was 28%, 92% and almost 100% at the same concentrations (Fig. 3). Dic Na was unable to trap DMPO-OOH and DMPO-OH adducts up to concentrations of 2.50 mM, while α-toc. could trap DMPO-OOH adducts by about 20% and 50% at concentrations of 0.25 and 2.50 mM, respectively, but also was unable to trap DMPO-OH at concentrations up to 2.50 mM (Figs. 4 and 5).

**Effects of Dic Na and α-toc. on NADPH-dependent LPO of liver microsomes**

Additions of Dic Na up to 10 mM had no suppressive effect against the formation of TBA reactants (TBARS) induced by the NADPH-dependent LPO of liver microsomes. In contrast, the addition of 1 μM of α-toc. produced 50% inhibition, and the suppressive effect was dependent on α-toc. concentration. Figure 6 shows the effects of incubation time on the NADPH-dependent LPO of liver microsomes in the presence of Dic Na and α-toc.
DISCUSSION

The subjection of rats to 30 min of hepatic ischemia followed by 12 hr of reperfusion resulted in LPO and liver injury, which were directly expressed by significant increases in plasma PCOOH levels (Table 1) and by the severe leakage of hepatic enzymes (GOT, GPT and LDH) into the circulation system (Fig. 2). Such abnormal leakages of hepatic enzymes suggest that structural and functional disorders in the hepatic microsomes also occurred, which were shown in our previous studies and by Frederiks et al. (14, 17 -19). Therefore we examined the effects of Dic Na and α-toc. on the NADPH-dependent LPO of liver microsomes, but the former was demonstrated to have no effect (Fig. 6). However, the protective effects of Dic Na and α-toc. against the hepatic warm ischemia-reperfusion injury were clearly demonstrated by the lowered levels of plasma PCOOH and serum GOT and LDH, but not GPT of hepatic ischemia-reperfused rats (Table 1 and Fig. 2). We do not know the reason why both agents had no effect on the elevation of serum GPT levels.

Recently, oxygen-derived free radicals and phagocytes (Kupffer cells and neutrophils) have been implicated in the pathogenesis of ischemia-reperfusion injury (4);
**Fig. 4.** Effect of diclofenac sodium (Dic Na) and α-tocopherol (α-toc.) on the formation of the DMPO spin-trapped adduct DMPO-OOH. Dic Na and α-toc. were dissolved in DMSO. The top ESR spectrum was recorded as a control after the addition of DMSO to the \( \text{O}_2^- \) generation system. Other spectra were recorded after addition of the test drug solution, shown at the left of each spectrum.

**DMPO-OOH**

- **Dic Na**
  - 25 µM
  - 0.25 mM
  - 2.5 mM

- **α-toc.**
  - 25 µM
  - 0.25 mM
  - 2.5 mM

**Fig. 5.** Effect of diclofenac sodium (Dic Na) and α-tocopherol (α-toc.) on the formation of the DMPO spin-trapped adduct DMPO-OH. Dic Na and α-toc. were suspended in 1% methylcellulose. The top ESR spectrum was recorded as a control after addition of 1% methylcellulose to the \( \cdot \text{OH} \) generation system. Other spectra were recorded after addition of the test drug solution, shown at the left of each spectrum.

**DMPO-OH**

- **Dic Na**
  - 25 µM
  - 0.25 mM
  - 2.50 mM

- **α-toc.**
  - 25 µM
  - 0.25 mM
  - 2.50 mM
however, these implications are controversial. Nordstrom et al. (5) had previously demonstrated the beneficial effect of allopurinol on liver ischemia-reperfusion; thus, the hypoxanthine-xanthine oxidase system has been proposed to be one of the primary sources of active oxygen species (6). Moreover, it is well-known that superoxides in a biological system are generated by myeloperoxidase in activated phagocytes, leakages of reactive oxygen from the mitochondrial respiratory chain, the arachidonic acid cascade, the oxidation of NAD(P)H in the microsomes, etc. (10, 20–22).

Fig. 6. Effect of diclofenac sodium (Dic Na) and α-tocopherol (α-toc.) concentration and the incubation period on NADPH-dependent lipid peroxidation of rat liver microsomes. The vertical axis represents the formation of TBA reactants (TBARS). Dic Na and α-toc. were dissolved in DMSO. Hepatic microsomes were incubated with: 1) Vehicle (C), DMSO only as a control, 2) 10 mM Dic Na (△), or 3) α-toc. with concentrations of 1 μM (○), 10 μM (△) or 10 mM (□).

Dic Na, which is an NSAID (1), an agent that interferes with arachidonate metabolites by inhibiting cyclooxygenase, has recently been shown to suppress the production of $O_2^-$ by disrupting the activation of NAD(P)H oxidase in neutrophils and monocytes (2, 3). NSAIDs have also been shown to reduce the infarct size in the heart by preventing the accumulation of neutrophils in ischemic tissue (4). Therefore, Dic Na may be able to help prevent LPO and subsequent liver dysfunction caused by hepatic ischemia-reperfusion.

Using the ESR method, we demonstrated that Dic Na could scavenge a stable free radical (Fig. 3). However, Dic Na did not have radical-trapping ability for $O_2^-$ (Fig. 4) or ·OH (Fig. 5), and was unable to inhibit the NADPH-dependent LPO of microsomes (Fig. 6), while α-toc. was able to scavenge a stable radical and $O_2^-$, but not ·OH, and could inhibit the NADPH-dependent LPO of microsomes. The in vitro study showed that the antioxidant properties of α-toc. were clearly superior to those of Dic Na. α-Toc., a lipid-soluble, strong antioxidant, exists in biomembranes and functions as a chain-breaking agent to protect membranes from free radical injury by scavenging peroxyl and alkoxyl lipid radicals (23) and superoxides (24), and also acts as a free radical scavenger (25). Since stable radicals have a relatively long half-life compared to ·OH and $O_2^-$ (26), they may act as mediators in the propagation of oxidative stress. The scavenging ability of Dic Na for a stable radical was weaker than that of α-toc., but much stronger than that of coenzyme Q$_{10}$, which also has scavenging abilities (10). These results suggest that Dic Na may act directly, as a chain-breaking agent in a free radical chain-reaction, scavenging peroxyl and alkoxyl lipid radicals to inhibit the progression of peroxidation. This suggestion is further supported by the fact that Dic Na is a relatively lipophilic compound that may approach biomembranes. Treatment with α-toc. had previously been demonstrated to protect against reperfusion injury mediated by oxygen free radicals (22, 27). However, daily administration of 10 mg/kg of Dic Na improved liver injury more effectively than the same treatment with 100 mg/kg of α-toc., a strong antioxidant (Table 1). These results suggest that mechanisms in addition to the stable radical scavenging ability may participate in the protective effect of Dic Na on ischemia-reperfusion injury in liver.

Recently, a comprehensive hypothesis has been presented that focuses on the extracellular generation of reactive oxygen in the hepatic sinusoids, where large Kupffer cell-derived, reactive oxygen species seem to be involved in initial vascular and parenchymal cell injury, and indirectly in neutrophil recruitment into the liver (4). As Kupffer cells are derived from monocytes and exist in the liver, and Dic Na has been shown to disrupt the activation of NAD(P)H oxidase in phagocytes (2, 3), it may suppress the production of $O_2^-$ at initial and subsequent phases of reperfusion.

Current results suggest that Dic Na acts to protect against LPO and liver injury caused by ischemia-reperfusion by scavenging stable radicals, inhibiting superoxide production in activated phagocytes (4), and through the production of active oxygen species from arachidonates (1). We used Dic Na as a pharmacological tool to inhibit the production of active oxygen species in activated phagocytes and from arachidonates, and first demonstrated the extreme protection of Dic Na against LPO and liver injury caused by ischemia-reperfusion, despite the inferiority of Dic Na to α-toc. with respect to antioxidant properties. To prevent hepatic ischemic disease and liver transplantation complications, further studies must clarify whether activated phagocytes or arachidonates are implicated in hepatic ischemia-reperfusion injury.
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