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OXIDATIVE STRESS IN ISOLATED RAT HEPATOCYTES DURING NAPROXEN METABOLISM

HIROYUKI YOKOYAMA, TOSHIHARU HORIE*† and SHOJI AWAZU

Department of Biopharmaceutics, Tokyo College of Pharmacy, Tokyo 192-03; and *Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan

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Abstract—Naproxen, a non-steroidal anti-inflammatory drug, induced lipid peroxidation in isolated hepatocytes of rats. The viability of the hepatocytes decreased upon lipid peroxidation, and this effect was accompanied by the formation of high molecular weight protein aggregates in the hepatocytes. Protein aggregation occurred slowly compared with the formation of thiobarbituric acid reactive substances (TBARS). The increase of TBARS was strongly correlated with the decrease of intracellular glutathione. Chemiluminescence was produced from the hepatocyte suspension during naproxen metabolism, and was correlated with the formation of TBARS. These results indicate that lipid peroxidation in the hepatocytes was provoked by reactive oxygens produced in the process of naproxen metabolism.

Key words: naproxen; oxidative stress; chemiluminescence; hepatocytes; glutathione; lipid peroxidation

Non-steroidal anti-inflammatory drugs are used over long-term periods in the treatment of patients with rheumatoid arthritis. Chronic treatment with these drugs sometimes induces various side-effects. Naproxen, (*S*)-6-methoxy-α-methyl-2-naphthaleneacetic acid, a non-steroidal anti-inflammatory drug widely used in cases of rheumatoid arthritis, is known to be effective and safe [1–3], but some side-effects have been reported: gastrointestinal toxicity [4], nephrotoxicity [5, 6], jaundice [7–9], and hepatotoxicity [10].

It has been suggested that the hypersensitive response to the drug is involved in the hepatic injury associated with naproxen therapy [6, 9]. However, there has been little clarification concerning the mechanism of the side-effects other than the hypersensitive response. We recently reported that naproxen induces lipid peroxidation in rat liver microsomes [11]. This lipid peroxidation is induced not by naproxen or its oxidative metabolite but by the reactive oxygens generated during naproxen oxidative metabolism. However, liver cells possess enzymatic and chemical defense systems that protect them from oxidative stress [12]. For example, SOD‡, catalase and glutathione peroxidase enzymatically dispose of superoxide anion, hydrogen peroxide and lipid hydroperoxides. Glutathione in the cytosol and mitochondria can depress lipid peroxidation by oxidizing its thiol group. α-Tocopherol in the membranes is well known as a chain-breaking (peroxyl radical-trapping) antioxidant. Is it possible that liver cells possessing such crucial defenses against oxidative stress undergo lipid peroxidation during naproxen metabolism, as shown in liver microsomes [11]? This point is very important in elucidating the mechanism of the *in vivo* toxicity induced by naproxen. Thus, in the present report, we intend to elucidate whether naproxen gives rise to oxidative stress in isolated rat hepatocytes.

MATERIALS AND METHODS

Materials. Naproxen (sodium salt) was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase (type I) and LDH-UV-Test Wako were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest purity available.

Preparation of isolated rat hepatocytes. Isolated rat hepatocytes were prepared from male Wistar rats (8–12 weeks of age) by collagenase perfusion, as described elsewhere [13]. Hepatocyte viability was determined by trypan blue exclusion. More than 85% of the hepatocytes were viable. The isolated hepatocytes were suspended in a standard buffer consisting of: 137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 $CaCl_2$, 5 mM glucose, 3 mM Na_2HPO_4, and 15 mM HEPES, pH 7.4. This buffer was used in all experiments unless otherwise stated.

Metabolism. The isolated hepatocyte suspension (4×10^6 cells/mL) in the standard buffer, which was put into a brown Erlenmeyer's flask, was preincubated at 37° for 10 min. The reaction was then initiated by adding naproxen dissolved in the standard buffer to the hepatocyte suspension and was terminated by 15% TCA.

Assay of TBARS. TBARS formed in the hepatocyte suspension during the metabolism of

† Corresponding author: Toshiharu Horie, Ph.D., Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba-shi, Chiba 263, Japan. Tel. 043-290-2934; FAX 043-255-1574.

‡ Abbreviations: SOD, superoxide dismutase; LDH, lactate dehydrogenase; TCA, trichloroacetic acid; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; GSH, reduced glutathione; and GSSG, oxidized glutathione.

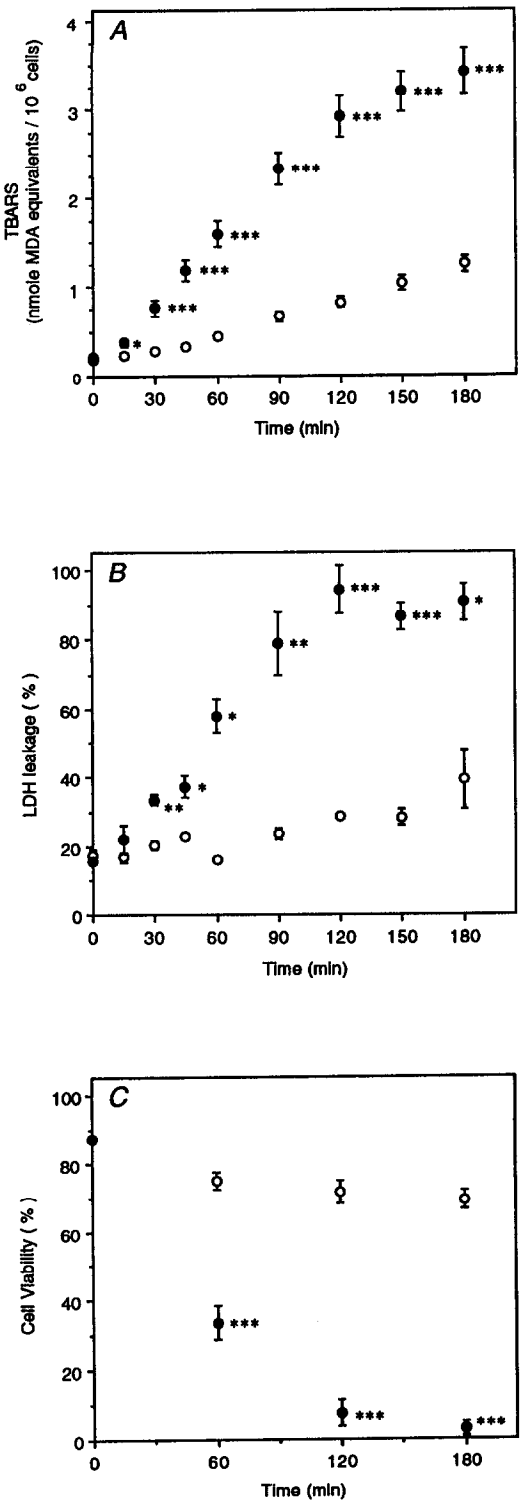

Fig. 1. Time courses of TBARS formation (A), LDH leakage (B) and change of cell viability (C) in isolated rat hepatocytes. The isolated rat hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° in the presence (●) and absence (○) of 10 mM naproxen. Data are the means ± SEM of 3–10 different experiments. Where absent, the SEMs were smaller than the symbols. Key: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$, significantly different from hepatocytes without naproxen at the same designated time.

naproxen were assayed according to Buege and Aust [14] and expressed as nanomoles of MDA equivalents per 10^6 cells. Briefly, a part of the hepatocyte suspension (0.5 mL) was added to 0.5 mL of 15% TCA. Thiobarbituric acid stock reagent (2 mL) containing 15% TCA, 0.375% thiobarbituric acid and 0.25 N HCl was added to the mixture, followed by boiling for 15 min. The mixture was then cooled and centrifuged at 1000 *g* for 15 min. The absorbance of the supernatant at 535 nm was determined with 1,1,3,3-tetraethoxypropane as the standard.

LDH assay. The hepatocyte suspension incubated with and without naproxen was centrifuged at 50 *g* for 2 min at the designated times. LDH activity in the supernatant was assayed using LDH-UV-Test Wako, a kit for the measurement of LDH activity [15], and is expressed as a percentage of the total LDH activity of the cells treated with 0.5% Triton X-100.

Assay of high molecular weight protein aggregates. SDS–PAGE (7.5%) was performed according to Weber and Osborn [16], as reported elsewhere [17]. The samples were mixed with 0.2 M sodium phosphate buffer (pH 7.4) containing 5% SDS, 20% glycerol, 2 mM 2-mercaptoethanol and 0.06% phenol red and were applied to the gels. The gel bands stained with 0.1% Coomassie Brilliant Blue were analyzed with a Shimadzu chromatoscanner CS-9000 by dual wavelengths (550 nm as a sample and 490 nm as a reference).

Chemiluminescence. Chemiluminescence was measured using a single photoelectron counting system, CLD-100 and CLC-10 (Tohoku Electronic Industries Co., Ltd., Sendai, Japan), connected to a personal computer, PC-9801 NS (NEC Corp., Tokyo, Japan), for integration. One half-milliliter of the hepatocyte suspension (4×10^6 cells/mL) incubated with and without naproxen was taken from the reaction mixture at the designated times and placed in a stainless steel dish (diameter: 50 mm; height: 10 mm) containing 1.5 mL of the standard buffer; the temperature was maintained at 37°. The chemiluminescence emitted from the hepatocyte suspension (10^6 cells/mL) was measured by counting the number of photons per minute.

Assay of GSH. GSH was determined according to Ellman [18]. Briefly, 1 mL of the hepatocyte suspension incubated with naproxen was taken from the reaction mixture at the designated times and was mixed with 1 mL of 10% metaphosphorus. The mixture was centrifuged at 1000 *g* for 10 min. One milliliter of the supernatant was mixed with 4 mL of 0.1 M sodium phosphate buffer, pH 8.0. Forty microliters of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in 0.1 M sodium phosphate buffer, pH 7.0, was added to 2 mL of the sample, and the absorption at 412 nm was measured to determine the concentration of GSH.

Statistical analysis. Statistical analysis was performed by Student's *t*-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

TBARS formation, LDH leakage, and cell viability.

The isolated rat hepatocyte suspension was incubated at 37° with and without naproxen. TBARS formation in the hepatocyte suspension during the incubation was determined at the designated times (Fig. 1A). The hepatocyte suspension with 10 mM naproxen showed a marked increase of TBARS, but TBARS formation was low in the hepatocyte suspension without naproxen. LDH leakage from the hepatocytes was observed during the incubation (Fig. 1B), and it was almost complete after 120 min. LDH leakage from the hepatocytes without naproxen was low. The viability of the hepatocytes with 10 mM naproxen, determined by trypan blue exclusion, decreased as the incubation proceeded, and almost all of the hepatocytes were dead after 120 min (Fig. 1C). On the other hand, the viability of hepatocytes without naproxen was affected only slightly. We also examined TBARS formation, LDH leakage, and cell viability in the hepatocyte suspension with 0.1, 1 and 5 mM naproxen. In the hepatocyte suspension with 0.1 and 1 mM naproxen, each value was similar to that without naproxen. The presence of 5 mM naproxen affected the respective values, although not as much as 10 mM naproxen (data not shown).

Formation of high molecular weight protein aggregates. The hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° for 3 hr with and without 10 mM naproxen. The protein molecular distribution of hepatocytes was examined by SDS–PAGE. The electrophoretic pattern of the hepatocytes incubated without naproxen is shown in Fig. 2A. Similar patterns were obtained from the hepatocytes incubated for 0 hr with and without 10 mM naproxen (data not shown). In the hepatocytes incubated for 3 hr with 10 mM naproxen, the high molecular weight proteins appeared at the top of the gel rod, while the proteins around the middle of the gel rod decreased (Fig. 2B). The time courses of the high molecular weight protein aggregates formed in the hepatocytes incubated at 37° with and without 10 mM naproxen also were examined. The protein aggregation was expressed as described elsewhere [17], i.e. the high molecular weight proteins that appeared at the top of the gel rods were expressed as a percentage of the amount of total hepatocyte proteins (Fig. 3). The formation of high molecular weight protein aggregates in the hepatocytes with 10 mM naproxen was facilitated markedly after a

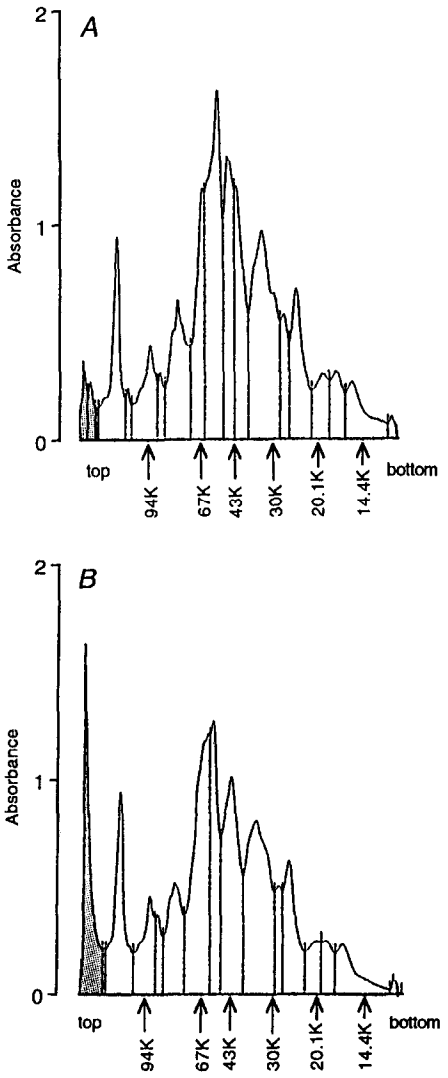

Fig. 2. SDS–PAGE of hepatocytes. The isolated rat hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° for 3 hr without naproxen (A) and with 10 mM naproxen (B). The proteins of the hepatocytes were separated, and the gels were stained with Coomassie Brilliant Blue. The stain intensity in the gels was measured by a chromatoscanner, and absorbance is expressed on the ordinate of the figure. Standard proteins: phosphorylase *b* (*Mr* 94K); bovine serum albumin (*Mr* 67K); ovalbumin (*Mr* 43K); carbonic anhydrase (*Mr* 30K); soybean trypsin inhibitor (*Mr* 20.1K); and α-lactoalbumin (*Mr* 14.4K).

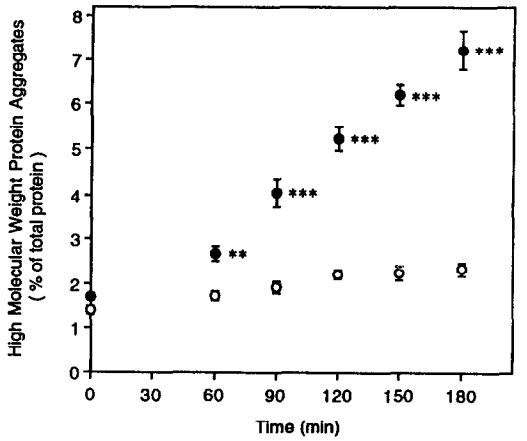

Fig. 3. Formation of high molecular weight protein aggregates in hepatocytes. The isolated rat hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° in the presence (●) and absence (○) of 10 mM naproxen. The gel bands of the separated hepatocyte proteins were stained with Coomassie Brilliant Blue. High molecular weight proteins that appeared at the top of the gels are expressed as a percentage of the total proteins. Data points are means ± SEM of 4 different experiments. Where absent, the SEMs were smaller than the symbols. Key: (**) $P < 0.01$ and (***) $P < 0.001$, significantly different from the hepatocytes without naproxen at the same designated time.

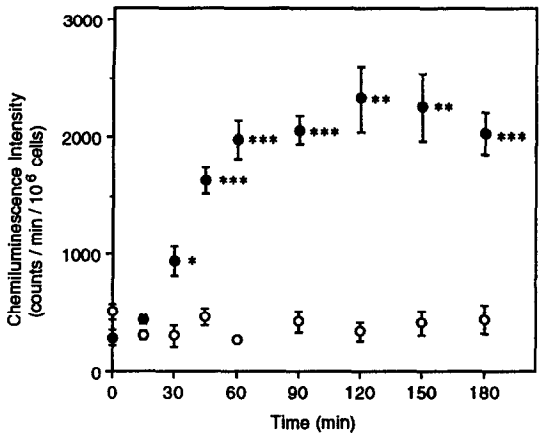

Fig. 4. Chemiluminescence production from the hepatocyte suspension. The chemiluminescence from the hepatocyte suspension was detected at 37° in the presence (●) and absence (○) of 10 mM naproxen. Chemiluminescence intensity is expressed in counts of photons per minute per 10^6 cells. Data are the means ± SEM of 3 experiments. Key: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$, significantly different from the hepatocytes without naproxen at the same designated time.

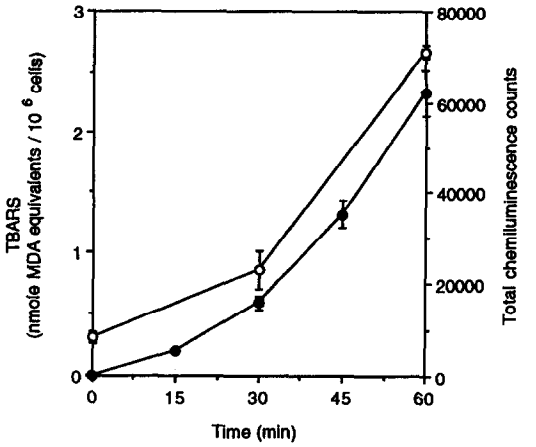

Fig. 5. Relationship between chemiluminescence production and TBARS formation in the hepatocyte suspension. The chemiluminescence (●) from the hepatocyte suspension (4×10^6 cells/mL) with 10 mM naproxen was measured over 1 hr at 37°. Chemiluminescence is expressed in the total counts of photons obtained by integrating the chemiluminescence as shown in Fig. 4 from 0 min to the designated time. TBARS formation (○) was measured in the same hepatocyte suspension in which the chemiluminescence was determined. Data points are the means ± SEM of 3 experiments. Where absent, the SEMs were smaller than the symbols.

1-hr incubation. Little increase in the protein aggregates in the hepatocytes without naproxen was observed throughout the incubation.

Chemiluminescence. The chemiluminescence from the hepatocyte suspension with and without 10 mM naproxen was measured directly at 37°. The time courses of chemiluminescence production are shown in Fig. 4, where chemiluminescence intensity is expressed as photoelectron counts per minute per 10^6 cells. The chemiluminescence from the hepatocyte suspension without naproxen was almost unchanged throughout the incubation. On the other hand, the hepatocyte suspension with 10 mM naproxen produced a marked chemiluminescence. The chemiluminescence increased with the length of the incubation and reached a maximum intensity around 1 hr. The chemiluminescence from the hepatocyte suspension with 0.1 and 1 mM naproxen was as low as that without naproxen (data not shown). The time course of total chemiluminescence integrated from 0 to designated times and that of the TBARS produced in the same sample in which chemiluminescence was measured are shown in Fig. 5. Both indicated a similar behavior of production.

Change in intracellular glutathione. The hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° with and without 10 mM naproxen, and intracellular glutathione was determined at designated times. As the incubation proceeded, the intracellular GSH in the hepatocytes with 10 mM naproxen decreased markedly up to 90 min and then decreased slowly (Fig. 6). It almost disappeared after incubating for 3 hr. On the other hand, GSH in the hepatocytes without naproxen decreased slowly and reached about one-half of the intracellular glutathione content at 0 min after incubating for 3 hr.

Relationship between intracellular glutathione and TBARS. The intracellular glutathione in the hepatocytes incubated with 10 mM naproxen was compared with the TBARS formation (Fig. 7). The TBARS formation was well correlated with the decrease of intracellular glutathione, indicating a negative correlation between them (correlation coefficient: $r = 0.963$).

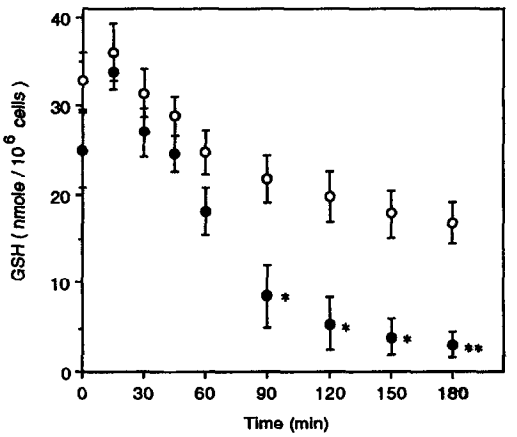

Fig. 6. Effect of naproxen on hepatocellular glutathione. The hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° in the presence (●) and absence (○) of 10 mM naproxen. Data points are the means ± SEM of 4 different experiments. Key: (*) $P < 0.05$ and (**) $P < 0.01$, significantly different from hepatocytes without naproxen at the same designated time.

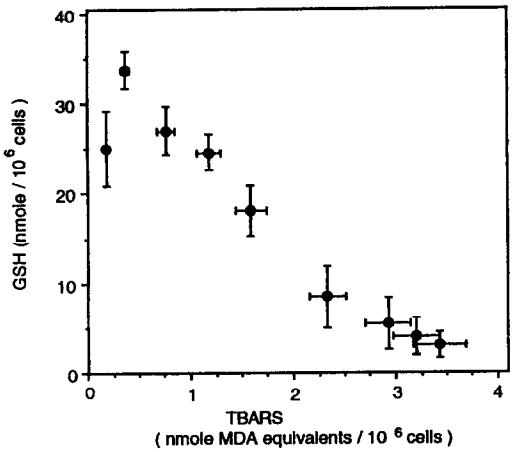

Fig. 7. Relationship between hepatocellular glutathione and TBARS formation. The hepatocyte suspension (4×10^6 cells/mL) was incubated at 37° for 3 hr with 10 mM naproxen. Data points are the means ± SEM of 4 different experiments. The correlation coefficient was 0.963.

DISCUSSION

Lipid peroxidation occurred in rat liver microsomes during naproxen oxidative metabolism, although neither naproxen nor its oxidative metabolite, 6-demethylnaproxen (6-hydroxy-α-methyl-2-naphthaleneacetic acid), induced it [11]. This suggested that the lipid peroxidation was caused by reactive oxygens generated from the cytochrome P450 monooxygenase system. On the other hand, hepatocytes have various protective devices against oxidative stress [12]. If naproxen induces lipid peroxidation in hepatocytes with such defense systems, it could be a possible mechanism of the naproxen-induced hepatotoxicity.

TBARS formation is a generally used indicator of lipid peroxidation, and high molecular weight protein aggregates are an indicator that remains in the membranes after being formed [17, 19, 20]. The two indicators showed that naproxen provoked lipid peroxidation in the isolated rat hepatocytes (Figs. 1A, 2 and 3). Hepatocyte injury proceeded in parallel with the lipid peroxidation (Fig. 1B and C). These results suggested that the occurrence of lipid peroxidation inflicts damage on the hepatocytes, resulting in their death.

Chemiluminescence is a useful technique for detecting reactive oxygen species, since it is considered to be associated with lipid peroxidation [21–24]. The singlet oxygens and/or excited carbonyls are reported to emit chemiluminescence [22, 23, 25]. We reported that rat liver microsomes produced a striking chemiluminescence during naproxen demethylation [11,26]. This production of chemiluminescence was well correlated with that of TBARS, and the origin of the chemiluminescence was shown to be the singlet oxygen [26]. In the present study, chemiluminescence was detected in the hepatocyte suspension with 10 mM naproxen, in parallel with the progress of lipid peroxidation (Figs. 4 and 5). Thus, the naproxen-induced lipid peroxidation in the isolated rat hepatocytes was indicated to be provoked through the reactive oxygens.

Intracellular glutathione decreased markedly in hepatocytes incubated with naproxen (Fig. 6). This is not due to the consumption of glutathione by the formation of conjugates, since glutathione conjugates of naproxen have not been observed thus far [27, 28]. GSH, under oxidative stress, is changed to GSSG and reacts with protein thiols and radicals [12, 29–32], resulting in a decrease of intracellular glutathione. The close relationship between the decrease of hepatocellular glutathione and the formation of TBARS, as shown in Fig. 7, indicated the occurrence of oxidative stress in the hepatocytes during naproxen metabolism.

When naproxen is administered to the rat, it is excreted into the urine as naproxen, 6-demethylnaproxen, naproxen sulfate, naproxen glucuronide, 6-demethylnaproxen sulfate and 6-demethylnaproxen glucuronide [27, 28]. However, the formation of reactive metabolites has not been reported thus far. We reported that lipid peroxidation during the oxidative metabolism of naproxen in rat liver microsomes was induced by the reactive oxygens generated from the cytochrome P450 monooxygenase system [11, 26]. Thus, the lipid peroxidation in the isolated hepatocytes was suggested to be induced not by the reactive metabolites of naproxen but by the reactive oxygens produced from the cytochrome monooxygenase system in the cellular organelles (endoplasmic reticulum), as was proved by the chemiluminescence study in Fig. 4. As is well known, the oxidative metabolism of drugs in liver microsomes is by monooxygenation via cytochrome P450 [33]. Isolated liver microsomes are suggested to produce superoxide anion (O_2^-) and hydrogen peroxide during mixed-function oxidation. These two reactive oxygen intermediates are believed to be produced primarily from the decay of oxycytochrome P450 complex [34–36]. The reactive oxygens broken away from the cytochrome monooxygenase system could provoke lipid peroxidation, resulting in cell death [37].

Naproxen pharmacokinetics suggests that it is a relatively safe drug, sharply in contrast with aspirin, when used in large doses [38, 39]. However, there are a few reports on its side-effects [4–7]. The present study has shown that lipid peroxidation occurs in isolated rat hepatocytes during the metabolism of naproxen. Naproxen is administered over long-term periods to patients with rheumatoid arthritis. Such drugs used for chronic treatments, even if the drugs themselves are safe, may have side-effects through lipid peroxidation, because the potency of the patient's defense system against oxidative stress may decrease during the chronic treatments; this, in turn, may make the patient susceptible to oxidative stress due to reactive oxygen species generated from the cytochrome monooxygenase system during the metabolism of naproxen and/or other coadministered drugs.

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