Biochemical Pharmacology, Vol. 45, No. 8, pp. 1721–1724, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Lipid peroxidation in rat liver microsomes during naproxen metabolism

(*Received* 8 *September* 1992; *accepted* 22 *January* 1993)

Abstract—Naproxen, a non-steroidal anti-inflammatory drug, is known to be highly effective and relatively safe, but some side-effects in the liver have been reported. In the present study, the effect of naproxen metabolism on rat liver microsomes was studied by determining lipid peroxidation in terms of thiobarbituric acid reactive substances (TBA-RS), high molecular weight protein aggregates and fluorescent substances formed in the microsomal suspension containing naproxen, NADPH and $MgCl_2$. Lipid peroxidation was found to occur at 10 mM naproxen. Production of chemiluminescence from the microsomal suspension was observed during naproxen metabolism. The time course of 6-demethyl-naproxen formation by O-demethylation of naproxen appeared to be comparable to that of the chemiluminescence production in their initial periods of production. These results suggest that the lipid peroxidation was provoked through the reactive oxygen species generated during the oxidative metabolism of naproxen.

Naproxen (*S*-6-methoxy-α-methyl-2-naphthaleneacetic acid), a non-steroidal anti-inflammatory drug, is currently widely used in the treatment of rheumatoid arthritis. This drug is known to be a highly effective and relatively safe drug [1, 2]. However, some side-effects induced by naproxen have been reported: gastrointestinal toxicity [3], nephrotoxicity [4], jaundice [5–7] and hepatotoxicity [8].

Naproxen is metabolized and excreted in the urine of humans, rats, guinea pigs, rhesus monkeys and mini pigs, and in the feces of dogs [9]. Naproxen metabolites, i.e. 6-demethyl-naproxen (6-hydroxy-α-methyl-2-naphthaleneacetic acid; 6-DMN*), naproxen glucuronide, 6-DMN glucuronide and 6-DMN sulfate, were found in the urines [10]. No metabolites have been reported to induce the side-effects mentioned above [3–8]. Little has been revealed as to the above side-effects, besides the suggestion that the hypersensitive response to the drug contributes to the hepatic injury associated with naproxen therapy [7]. Thus far, the suggestion of hypersensitivity has not been further substantiated. In this study, we investigated the effect of the oxidative metabolism of naproxen on liver microsomes.

Materials and Methods

Materials. Naproxen sodium salt was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest purity available.

Liver microsomes. Liver microsomes were prepared from male Wistar rats (8–12 weeks of age), without pretreatment with phenobarbital, as described elsewhere [11]. The protein concentration of the microsomes was determined by the method of Lowry *et al.* [12] with bovine serum albumin as the standard.

Metabolism. Liver microsomes (1 mg protein/mL) were incubated at 37° with and without naproxen in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.2 mM NADPH and 5 mM $MgCl_2$, and the reaction was terminated by 5% trichloroacetic acid or ice-cold methanol.

Assay of thiobarbituric acid-reactive substances (TBA-RS). TBA-RS formed in the microsomal suspension were assayed according to Buege and Aust [13] and expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein.

Assay of high molecular weight protein aggregates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) of the microsomes was performed as we described elsewhere [14], which was based on the method of Weber and Osborn [15].

Assay of fluorescent substances. Fluorescent substances were determined by a slight modification of the method we reported previously [11].

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. Two milliliters of the microsomal suspension (1 mg protein/mL) containing 0.2 mM NADPH and 5 mM $MgCl_2$ with and without naproxen was placed in a stainless steel dish (diameter: 50 mm; height: 10 mm) where the temperature was maintained at 37°. The reaction mixture without naproxen and NADPH was preliminarily incubated at 37° for 5 min in the dish and the reaction was started by adding naproxen and NADPH solution to the reaction mixture. Chemiluminescence was measured by counting the amount of photons per minute.

6-DMN synthesis. 6-DMN was synthesized according to Abdel-Moety *et al.* [16].

Determination of 6-DMN. 6-DMN was determined by HPLC according to the method of Van Loenhout *et al.* [17] with a slight modification: column, μBondapak C18; mobile phase, 0.1 M sodium citrate buffer, pH 6.5: methanol (6:4, v/v); internal standard, 2-naphthylacetic acid; detection, fluorescence (excitation wavelength, 280 nm and emission wavelength, 355 nm).

Results and Discussion

The effect of naproxen metabolism on rat liver microsomes was studied by determining three different types of indicators of lipid peroxidation, using the same procedures that we used previously [18]. TBA-RS do not remain in the microsomes after they are formed, but are released into the medium [11]. The protein aggregates and fluorescent substances are retained in the microsomes [14, 18]. The liver microsomal suspension containing NADPH and $MgCl_2$ was incubated at 37° for 30 min with and without naproxen. The amount of TBA-RS formed at 10 mM naproxen was significantly larger than that without naproxen (Fig. 1A). The pattern of microsomal proteins

* Abbreviations: 6-DMN, 6-demethyl-naproxen (6-hydroxy-α-methyl-2-naphthaleneacetic acid); TBA-RS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

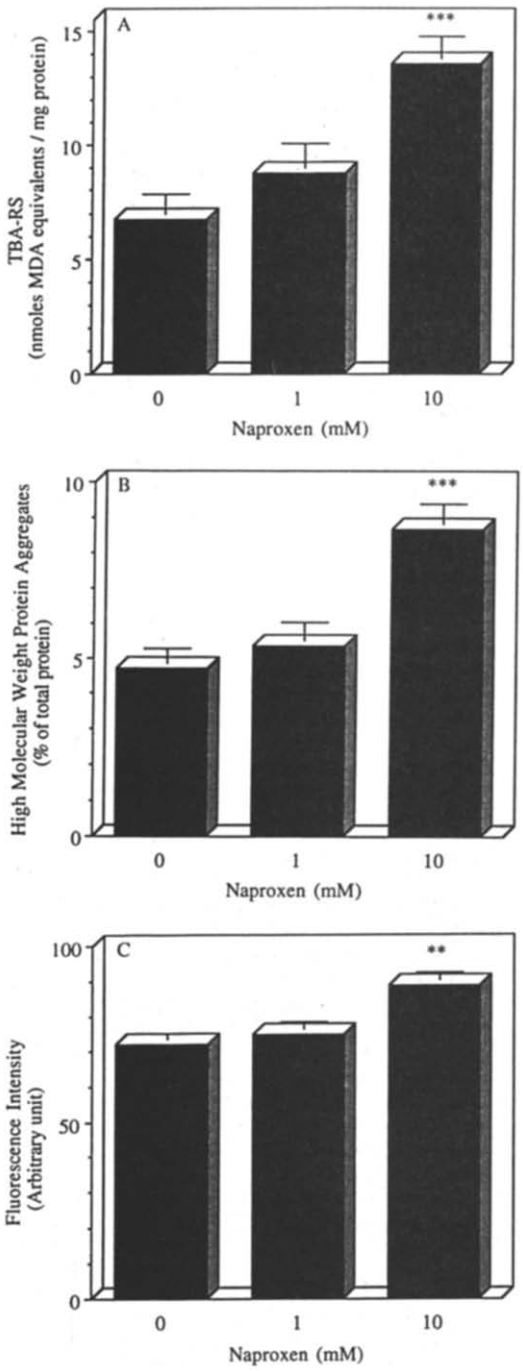

Fig. 1. Evaluation of lipid peroxidation in rat liver microsomes by the presence of naproxen. Rat liver microsomes were incubated at 37° for 30 min in the presence of 0, 1 and 10 mM naproxen with NADPH and $MgCl_2$. (A) TBA-RS formation in rat liver microsomal suspension. Values are means ±SEM of 8–15 experiments. (B) High molecular weight protein aggregates formed in liver microsomal suspension. The reaction mixtures were separated by SDS–PAGE. The high molecular weight proteins which appeared at the top of the gels are expressed as a percentage of the amount of total proteins. Values are means ±SEM of 4–7 experiments. (C) Fluorescent substances formed in liver microsomal suspension. Values are means ±SEM of 3 experiments. Key: (**) $P < 0.01$ and (***) $P < 0.001$, significantly different from the data without naproxen.

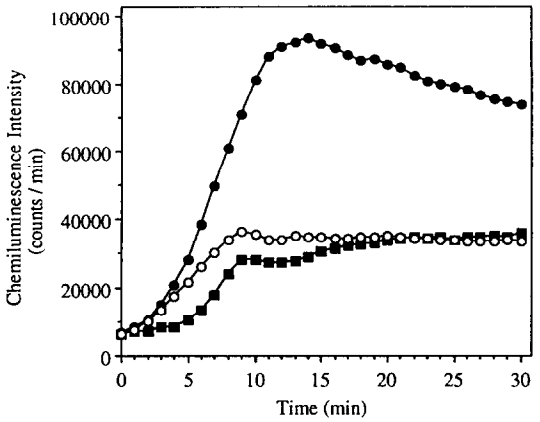

Fig. 2. Chemiluminescence produced from the liver microsomal suspension. Chemiluminescence was detected at 37° in the presence of 0, 1 and 10 mM naproxen with NADPH and $MgCl_2$. The chemiluminescence intensity is expressed in counts of photons per minute. Key: (■) 0 mM naproxen; (○) 1 mM naproxen; and (●) 10 mM naproxen. Data from one typical experiment (out of 4–15 experiments) are shown.

was determined by SDS–PAGE. The high molecular weight proteins which appeared at the top of the gel rods were expressed as a percentage of the amount of total proteins of the microsomes [14, 18]. The amount of high molecular weight proteins increased significantly at 10 mM naproxen (Fig. 1B). Fluorescent substances were determined by measuring the fluorescence emitted from the microsomes (Fig. 1C). The fluorescent substances also increased significantly at 10 mM naproxen. Thus, lipid peroxidation was found to occur in the microsomal suspension and was stimulated by 10 mM naproxen.

Naproxen in the rat liver microsomes is preferentially metabolized into 6-DMN by O-demethylation [10]. Neither naproxen nor 6-DMN alone induced lipid peroxidation in the rat liver microsomal suspension incubated without NADPH and $MgCl_2$ (data not shown).

Howes and Steele [19] originally reported the production of chemiluminescence from rat liver microsomal suspension with NADPH and oxygen. The production of chemiluminescence by the microsomes is considered to be associated with lipid peroxidation, since the chemiluminescence can come from the reactive oxygens which can peroxidize lipids [19–23]. Therefore, chemiluminescence is a useful method to detect the presence of reactive oxygen species. However, little has been reported concerning the production of chemiluminescence from microsomes by clinically used drugs. Interestingly, chemiluminescence was found to be produced at 10 mM naproxen in the microsomal suspension containing NADPH (Fig. 2). This indicates the generation of reactive oxygen species through the oxidation of naproxen by the microsomes. Though the origin of chemiluminescence was not identified here, the generation of electronically excited species, predominantly singlet oxygen and/or excited carbonyls, was suggested, since they are understood to emit light [22, 24].

As shown in Fig. 3, the production of 6-DMN at 10 mM naproxen was fast and almost reached a plateau at 15 min. This fast production of 6-DMN was comparable to that of chemiluminescence in Fig. 2, indicating that the production of chemiluminescence is closely related to the demethylation of naproxen. As is well known, the oxidative metabolism of drugs in liver microsomes is monooxygenation via

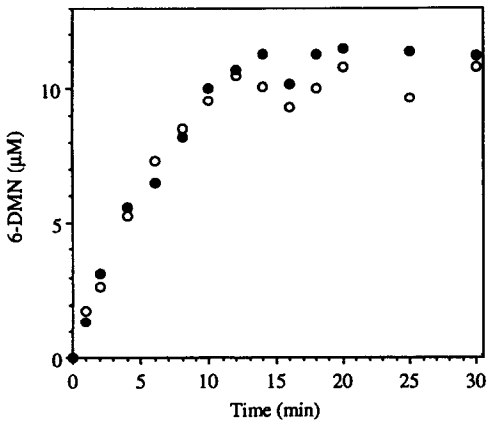

Fig. 3. Time course of naproxen demethylation. Microsomes (1 mg protein/mL) were incubated at 37° in the presence of 1 mM (○) and 10 mM (●) naproxen with 0.2 mM NADPH and 5 mM $MgCl_2$, and the reaction was terminated at designated times by adding ice-cold methanol. 6-DMN produced by the demethylation of naproxen was plotted against time. Data obtained from one typical experiment are shown. Similar results were obtained from 6 different experiments.

cytochrome P450 [25]. In this monooxygenase system, superoxide anion (O_2^-) radicals are suggested to originate from a dissociation of the oxycomplex [26]. They can be a source of reactive oxygen species such as hydrogen peroxide. Thus, the reactive oxygen species which break away from the monooxygenase system could produce the chemiluminescence and induce lipid peroxidation in the liver microsomes. To support this possibility, the effect of carbon monoxide on TBA-RS formation in liver microsomes with 10 mM naproxen was studied according to a slight modification of the method of Kuthan *et al.* [26]; cytochrome P450 inhibitors, SKF-525A and piperonyl butoxide, were also used for this purpose. SKF-525A and piperonyl butoxide completely inhibited TBA-RS formation and carbon monoxide inhibited it to some extent. These results support the present mechanism of the naproxen-stimulated lipid peroxidation. On the other hand, the time course of naproxen demethylation at 1 mM naproxen was similar to that at 10 mM naproxen, suggesting the saturation of demethylation. This was unlike the chemiluminescence data in Fig. 2. Although a definite explanation for this phenomenon cannot be given at present, the binding of naproxen to microsomes may induce a change in membrane structure which stimulates the uncoupling phenomenon.

Naproxen is known to be relatively safe [1]. However, there are a few reports on side-effects [3–8]. Little is known as to why the drug induces such side-effects. The hypersensitivity response to the drug is only a suggestion presented to explain the side-effects [7], but little has been further specified for the hypersensitivity. The present study has shown that lipid peroxidation occurs in rat liver microsomes during the oxidative metabolism of naproxen. This may contribute to the side-effects, since lipid peroxidation damages the membranes in various ways [27, 28]. This may also contribute to the hypersensitivity, since the production of reactive oxygens can vary among individuals depending on the amount of endogenous radical scavengers.

Acknowledgements—The authors are grateful to Misses Sumie Oki and Hiroko Uchida for their technical assistance.

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