Effect of Carrageenan-Induced Inflammation on the Induction of Hepatic Microsomal Enzymes by Phenobarbital and Benzo[*a*]pyrene in Male Rats

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The inhibitory effect of carrageenan-induced inflammation was examined by utilizing rats treated with inducers of this drug metabolizing system. Animals were given sodium phenobarbital (PB, 80 mg/kg, i.p., 24 h prior to death), or benzo[*a*]pyrene (BP, 40 mg/kg, i.p., 24 h prior to death) as inducers. Some animals were also given carrageenan 24 h prior to death. Non-induced male rats exhibited significant decreases in hepatic 9000 × *g* supernatant (S-9) cytochrome P-450 and aminopyrine (AM) *N*-demethylase, benzphetamine (BenzP) *N*-demethylase and meperidine (MP) *N*-demethylase activity following carrageenan treatment. Carrageenan also depressed the induction of hepatic S-9 cytochrome P-450 content caused by PB treatment, and suppressed the induction of AM, BenzP, MP, arylhydrocarbon and 7-ethoxycoumarine metabolism by PB treatment. Cytochrome P-450 levels and related biotransformation activity which are elevated by BP treatment were not decreased by the injection of BP and carrageenan simultaneously to male rats. Non-induced, PB-treated and BP-treated female rats did not show inhibited carrageenan-induced reduction in hemoprotein content or inhibition of AM-*N*-demethylase, BenzP *N*-demethylase and aniline hydroxylase activities. These results demonstrate the selective nature of the inhibitory effects of carrageenan-induced inflammation upon drug metabolism in the rats.

Keywords — carrageenan-induced inflammation; hepatic drug-metabolizing enzyme activity; phenobarbital; benzo[*a*]pyrene; rat

Introduction

Cawthorne *et al.* demonstrated that carrageenan-induced inflammation was effective in prolonging hexobarbital sleeping time, decreasing hepatic cytochrome P-450* content, and inhibiting *in vitro* hydroxylase and demethylase activities 24 h after carrageenan injection in the rats.¹⁾ This inhibitory effect of carrageenan-induced inflammation modifies the response of the animal to various drugs *in vivo* and decreases the efficiency of the monooxygenase system of hepatic microsomes isolated from the animals. Accompanying these alterations in biotransformation are the inflammation-induced reductions in hepatic microsomal cytochrome P-450 content are observed. In the rat, carrageenan-induced inhibition in drug metabolism appears to be predominant in the males; treatment of female rats with carrageenan has not been reported to

alter the duration of hexobarbital hypnosis, the rate of hepatic microsomal oxidation of hexobarbital or ethylmorphine, or cytochrome P-450 content.²⁻⁶⁾

The finding that carrageenan-induced inflammation produces inhibitory effects in male rats, but not in female rats may be related to the sex difference in drug metabolism in the rats known to exist for some time.

Since the inhibitory effects of carrageenan-induced inflammation in the rats occur predominantly in males, the inflammation may interact differently with these two qualitatively different enzyme systems. Cytochrome P-450 mediated drug-metabolism can be broadly categorized into two major groups depending on whether they are induced by a class of chemicals whose prototype is phenobarbital or chemicals exemplified by polycyclic aromatic hydrocarbons. Recent advances in protein separation

* Cytochromes P-450 and P-448 as used in the present studies refer to the two broad categories of the hemoprotein which, on reduction and combination with CO, exhibit absorption maxima at 450 and 448 nm, respectively; no distinction is made between the several hitherto identified species of the hemoprotein which differ with respect to substrate specificity, immunochemical properties, *etc.*

techniques have further shown that a family of differing cytochrome P-450 molecules exists in mammalian hepatic microsomes and that different inducers of drug metabolism cause different forms of the enzyme to predominate in the induced microsomal fraction.⁷⁾ The various species of the hemoprotein differ in substrate specificity as well.⁷⁾

We examined further the degree of selectivity exhibited by carrageenan-induced inflammation with regard to the inhibition of drug metabolism exerted by the inflammation. To accomplish this goal, the interaction of carrageenan-induced inflammation with the hepatic microsomal (S-9 fraction) monooxygenase system of rats undergoing enzyme induction was examined.

Materials and Methods

Animals — Male Wistar strain rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) at 6 weeks of age. Rats were housed in stainless steel cages, and they were allowed free access to a solid diet (CE-2, Nihon Clea, Tokyo, Japan) and tap water. The breeding room was controlled so that the light–dark schedule (lighting time: 7:00–19:00) and room temperature ($25 \pm 1^\circ\text{C}$) were constant. Animals were housed in facilities accredited by the Japan Association of Laboratory Animals Care and the research protocols were approved by the Guideline for Animal Experimentation for Tohoku College of Pharmacy.

Chemicals — Carrageenan (PICNIN-A, Lot No. P-9) was obtained from Zushi Chemical Laboratory Inc., (Zushi, Japan). Food and water were given *ad libitum*. Hind paw edema was induced by carrageenan as previously described.²⁾ Briefly, one tenth ml of a 1.0% solution of carrageenan dissolved in sterile saline solution was injected subcutaneously into the plantar surface of the left hind paw of rats 24 h prior to measurement of the hepatic drug-biotransformation. The assay of carrageenan-induced edema has been described previously.²⁾ Sodium phenobarbital (PB, Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in saline and injected intraperitoneally at a dose of 80 mg/kg. Benzo[*a*]pyrene (BP,

Sigma Chemical Co., St. Louis, MO) was dissolved in corn oil and administered intraperitoneally at a dose of 40 mg/kg. Control animals received normal saline (5 ml/kg, i.p.). Corn oil (5 ml/kg, i.p.) had no effect on the hepatic mixed-function oxidase system.

Enzyme Assay — To obtain the $9\,000 \times g$ supernatant (S-9) of liver preparations rats were killed by cervical dislocation. The liver was rapidly excised and then perfused with 1.15% KCl solution. The minced liver was homogenized in 3 volumes of cold 0.1 M phosphate buffer (pH 7.4) using a Potter Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 10 000 rpm ($9\,000 \times g$) in a Hitachi model 65P-7 ultracentrifuge (Model Rp-50 rotor) at 4°C for 20 min.

Activities of aminopyrine (AM) *N*-demethylase, benzphetamine (BenzP) *N*-demethylase, meperidine (MP) *N*-demethylase, aniline (AN) hydroxylase, arylhydrocarbon (AH) hydroxylase, and 7-ethoxycoumarin (EC) *O*-deethylase were determined in a cofactor mixture with final concentrations of KCl 50 μmol , MgCl_2 2.5 μmol , glucose-6-phosphate 3 μmol , glucose-6-phosphate dehydrogenase 10 units, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) 0.5 μmol . In addition, an enzyme preparation with (S-9 protein about 0.5 mg for AH hydroxylase, and EC *O*-deethylase, and 1–2 mg for AM *N*-demethylase, BenzP *N*-demethylase, MP *N*-demethylase and AN hydroxylase). Substrates: AM 2.5 μmol , BenzP 3.0 μmol and AN 2.5 μmol in 0.1 M phosphate buffer; AH 80 nmol/50 μl in acetone and EC 500 nmol/25 μl in 50% ethanol and 50 mM sodium phosphate buffer, pH 7.4, added to a final volume of 1 ml. Incubation was started with an enzyme preparation (AM *N*-demethylase, BenzP *N*-demethylase, MP *N*-demethylase and AN hydroxylase) or the substrate (AH and EC) after a 2-min preincubation period in a metabolic shaker under atmospheric air at 37°C . Incubation lasted for 10 min (AH), 15 min (EC) or 60 min (AM, BenzP, MP and AN), linearity being maintained for this time.

The formation of formaldehyde from AM, BenzP and MP were determined by the method of Nash.⁸⁾ The formation of *p*-aminophenol

from AN was determined by the method of Kato and Gillette.⁹⁾ The fluorescence of EC was measured according to Greenlee and Poland.¹⁰⁾ The formation of fluorescent phenolic AH metabolites was determined according to Nebert and Gelboin.¹¹⁾

The hepatic S-9 cytochrome P-450 or P-448* content was determined from the carbon monoxide (CO) difference spectrum of dithionite-treated microsomes, assuming values of 91 mM⁻¹cm⁻¹, for molar extinction coefficients between 450 or 448 and 490 nm.¹²⁾

Protein content of the prepared samples was measured according to the method of Bradford,¹³⁾ using bovine serum albumin as the standard.

Statistical Analysis — Student's *t*-test was used to test for statistical significance, taking *p* = 0.05 as the limit of significance.

Results

Interaction between PB or BP and Carrageenan-Induced Inflammation on Cytochrome P-450 or

Fig. 1. Time Course for the Development of Carrageenan-Induced Hind Paw Edema in the Rat

Groups (*N* = 6) of male (○) or female (●) rats received carrageenan, and increase in edema volume of the injected hind paws were measured by volume meter (MK-550, Muromachi Instruments Co., Ltd., Tokyo, Japan) immediately before, hourly for 8 h and again 10, 12 and 24 h after carrageenan injection, comparison with the volume of the carrageenan-untreated paw. Each value represents the mean of increase swelling percent of control.

448 Content in Male Rats

The development of carrageenan-induced hind paw edema in the rats is shown in Fig. 1. Rapid swelling develops within the first 7 hours after injection of the carrageenan to a peak edema of about 130%, thereafter rebounded gradually to the control level in male and female rats. But, swelling of hind paw edema in both rats shows a significant elevation at 24 h as compared to control. Area under the increase in edema volume of paw treated by carrageenan was determined by cutting out on graph paper and weighing. The ratio of area for edema volume in male rats compared to female rats was 0.95. In the previous reports,⁵⁾ carrageenan-induced inflammation was shown to most effectively depress cytochrome P-450 24 h after administration in male rats. Therefore, in subsequent studies carrageenan was given 24 h prior to assay.

Male rats received a single subcutaneous in-

Fig. 2. Hepatic 9 000 × *g* Supernatant Cytochrome P-450 or P-448 Content after Administration of Carrageenan, PB, and/or BP in Male Rats

Male rats were administered carrageenan (CAR), PB (80 mg/kg, i.p.), BP (40 mg/kg, i.p.), carrageenan plus PB or carrageenan plus BP, and 24 h later cytochrome P-450 or P-448 contents were measured, as described in Materials and Methods. Control rats (*N*) received saline. Dotted and shaded bars display data from control or carrageenan-treated animals, respectively. Each value represents the means ± S.E. of 4–6 animals. Data were analyzed by Student's *t*-test; a) *p* < 0.05 with respect to control and b) *p* < 0.05 with respect to corresponding control.

jection of carrageenan and intraperitoneal injection PB (80 mg/kg, i.p.) or BP (40 mg/kg, i.p.) simultaneously, 24 h before they were killed. Figure 2 shows the effects of these treatments upon the cytochrome P-450 or P-448 content. In animals not receiving PB or BP, carrageenan administration reduced the content of hepatic S-9 cytochrome P-450 to 65.5%, as compared to the controls.

PB significantly raised the content, and concomitant carrageenan treatment inhibited the induction of cytochrome P-450 content caused by PB treatment.

The microsomal drug-metabolizing enzyme

system which arises following aromatic hydrocarbon administration is qualitatively different from that occurring in untreated or PB-treated animals. The absorption peak of the CO-induced difference spectrum in using reduced microsomes isolated from aromatic hydrocarbon-treated rats appears at 448 nm, compared to 450 nm in untreated or PB-treated animals.¹⁴⁾ In addition, different forms of the hemoprotein predominate in the microsomes prepared from aromatic hydrocarbon-treated animals, as compared to the control or PB-treated animals.⁷⁾ Thus, we examined the effects of carrageenan-induced inflammation in

Fig. 3. Hepatic Drug-Metabolizing Enzymes Activities after Administration of Carrageenan, and/or PB in Male Rats. Male rats were administered carrageenan (CAR), and/or PB (80 mg/kg, i.p.) at 24 h prior to sacrifice and subsequent measurement of (A) AM *N*-demethylase, (B) BenzP *N*-demethylase, (C) MP *N*-demethylase, (D) AH hydroxylase and (E) EC *O*-deethylase.

Each value represents the mean $\pm$ S.E. of 4–6 animals. Data were analyzed by Student's *t*-test; a) $p < 0.05$ with respect to control and b) $p < 0.05$ with respect to corresponding control.

Fig. 4. Hepatic Drug-Metabolizing Enzyme Activities after Administration of Carrageenan, and/or BP in Male Rats. Male rats were administered carrageenan (CAR), and/or BP (40 mg/kg, i.p.) 24 h prior to sacrifice and subsequent measurement of (A) AH hydroxylase and (B) EC O-deethylase.

Each value represents the means $\pm$ S.E. of 4–6 animals. Data were analyzed by Student's *t*-test; *a)* $p < 0.05$ with respect to control.

animals undergoing aromatic hydrocarbon induction as well.

BP administered concomitantly with carrageenan significantly raised the hemoprotein content in rats. However, the cytochrome P-448 level which is elevated following BP treatment was not decreased by the injection of BP and carrageenan simultaneously to male rats. These findings indicated that the hepatic drug-metabolizing activity was disrupted.

Interaction between PB and Carrageenan-Induced Inflammation on Drug Metabolism in Male Rats

The effect of carrageenan on drug metabolism was measured using AM, BenzP, MP, AH and EC as substrates (Fig. 3). In the male rats injected with carrageenan, the rates of biotransformation of the sex-dependent compounds, AM, BenzP and MP were decreased significantly. By contrast, the rate of biotransformation of the sex-independent compounds, AH and EC were not reduced. PB treatment produced a 1.5- to 3-fold increase in all of the parameters measured, and carrageenan inhibited the induction of all drug-metabolizing enzyme activities caused by PB treatment.

TABLE I. Effect of Carrageenan, PB, and/or BP on Hepatic 9 000 $\times$ g Supernatant Cytochrome P-450 or P-448 Content and Hepatic Drug-Metabolizing Enzymes Activities in Female Rats

Treatment	Cyt. P-450 or P-448 (nmol/mg protein)	AM <i>N</i> -demethylase (nmol/60 min/mg protein)	BenzP <i>N</i> -demethylase (nmol/60 min/mg protein)	AN hydroxylase (nmol/60 min/mg protein)	AH hydroxylase (nmol/10 min/mg protein)	EC <i>O</i> -deethylase (nmol/15 min/mg protein)
Control	0.042 $\pm$ 0.0037	0.062 $\pm$ 0.007	0.14 $\pm$ 0.027	0.023 $\pm$ 0.0013	0.020 $\pm$ 0.0039	0.050 $\pm$ 0.0098
CAR	0.046 $\pm$ 0.0007	0.056 $\pm$ 0.007	0.12 $\pm$ 0.021	0.022 $\pm$ 0.0009	0.013 $\pm$ 0.0011	0.013 $\pm$ 0.0028 ^{a)}
PB	0.061 $\pm$ 0.058 ^{a)}	0.081 $\pm$ 0.098 ^{a)}	0.19 $\pm$ 0.021 ^{a)}	0.030 $\pm$ 0.0030 ^{a)}	0.041 $\pm$ 0.0046 ^{a)}	0.111 $\pm$ 0.0104 ^{a)}
PB + CAR	0.058 $\pm$ 0.0048	0.075 $\pm$ 0.008 ^{a)}	0.18 $\pm$ 0.019 ^{a)}	0.029 $\pm$ 0.0028 ^{a)}	0.028 $\pm$ 0.0028 ^{a,b)}	0.070 $\pm$ 0.0069 ^{a,b)}
BP	0.053 $\pm$ 0.0046 ^{a)}	0.071 $\pm$ 0.068	N.D.	0.029 $\pm$ 0.0026 ^{a)}	0.023 $\pm$ 0.0028 ^{a)}	0.212 $\pm$ 0.0193 ^{a)}
BP + CAR	0.056 $\pm$ 0.0038	0.057 $\pm$ 0.061 ^{c)}	N.D.	0.026 $\pm$ 0.0023	0.018 $\pm$ 0.0016 ^{a,c)}	0.179 $\pm$ 0.0161 ^{a)}

The experimental conditions, parameters and abbreviations are as in Figs. 2 and 3. *a)*, *b)* and *c)* indicate significant difference ($p < 0.05$) from control, PB alone and BP alone, respectively. N.D., not done.

Interaction between BP and Carrageenan-Induced Inflammation on Drug Metabolism in Male Rats

Figure 4 presents the results of an experiment using male rats treated with BP with or without injection of carrageenan 24 h prior to death. As in the previous experiments, carrageenan treatment of non-induced male rats did not produce significant inhibition in the rate of metabolism of AH and EC. The rate of biotransformation of the AH and EC was elevated by BP treatment. But or moreover, the increase was not altered by concurrent carrageenan administration.

Interaction of PB or BP and Carrageenan-Induced Inflammation on Cytochrome P-450 or P-448 Content and Drug Metabolism in Female Rats

The effect of carrageenan-induced inflammation upon animals undergoing microsomal enzyme induction was also examined in female rats. Female rats received injection of PB or BP with or without injection of carrageenan 24 h prior to death. As seen in Table I, the administration of carrageenan to non-induced female rats did not alter either cytochrome P-450 content or the rate of biotransformation of AM, BenzP, AN and AH, as compared to control animals a finding consistently with previous findings.²⁻⁶⁾ However, EC *O*-deethylase activity was decreased.

PB treatment stimulated the rates of oxidation of all five substrates and increased the cytochrome P-450 content. These elevations were not affected with the exception of AH hydroxylase and EC-*O*-deethylase by carrageenan-induced inflammation.

BP treatment produced a 1.2- to 4-fold increase in all of the parameters measured, and carrageenan inhibited the induction of AH hydroxylase caused by BP. However, cytochrome P448 content, AN hydroxylase or EC-hydroxylase activity which is elevated following BP treatment were not decreased by the injection of BP and carrageenan simultaneously to female rats.

Discussion

The monooxygenase enzyme system of the male rat has been characterized as being very la-

bile following a wide diversity of experimental manipulations. Kato and Gillette, for example, have shown that the hepatic microsomal metabolism of sex-dependent compounds is inhibited by abnormal physiological states, such as starvation, adrenalectomy, corticotropin (ACTH) administration, hypoxia, alloxan-induced diabetes, and castration.^{15,16)} In female rats, these treatments either had no effect upon, or slightly enhanced, the biotransformation of these same sex-dependent substrates. None of these physiological manipulations impair the metabolism of sex-independent substrates in male or female rats.^{15,16)} Similarly, the administration of aromatic hydrocarbons markedly reduces the biotransformation¹⁷⁾ or spectral binding¹⁸⁾ of androgen sensitive substrates in male rats. In contrast, aromatic hydrocarbon treatment of female rats does not alter the hepatic oxidation of sex-dependent compounds.¹⁷⁾ By contrast, following PB induction, a stimulation of the metabolism of a wide variety of foreign compounds occurs, regardless of the sex of the animal or the sex dependency of the substrate examined.¹⁷⁾

In male rats undergoing PB induction carrageenan treatment decreased cytochrome P-450 content and hepatic drug metabolism. By contrast, neither PB-treated nor non-induced female rats were subject to alterations in drug metabolism with the exception of AH and EC metabolism or cytochrome P-450 content following carrageenan administration. The specificity of inflammation-induced inhibition in drug metabolism was further elucidated in experiments employing aromatic hydrocarbon inducers. The data indicated that neither the synthesis of cytochrome P-448, nor cytochrome P-448-mediated reactions (in the case AN hydroxylation, AH hydroxylation and EC deethylation), were subject to interference by carrageenan-induced inflammation, regardless of the sex of the animal. However, in female rats, the effects of the administration of both carrageenan and BP upon the metabolism of a sex-dependent compound, AM were puzzling. The inhibition of biotransformation produced by carrageenan-induced inflammation and BP have always been shown to be sex-related in the past.

Neither carrageenan nor BP when administered alone interfered with the oxidation of AM substrate. When given concurrently an unexpected result of slight but significant reduction in AM *N*-demethylation was observed in female rats. Perhaps a small amount of androgen-sensitive monooxygenase activity present in female hepatic microsomes and this amount is too low to be unmasked by normal doses of sex-related inhibitors of drug metabolism.

The reductions in drug metabolism occurring in male rats following carrageenan treatment are accompanied by a decrease in cytochrome P-450 content of hepatic microsomes.^{3-6,19)} In female rats, carrageenan administration does not alter the levels of hepatic microsomal cytochrome P-450.³⁻⁶⁾ Thus, the inhibitory actions of carrageenan with respect to drug metabolism are apparently related to the ability of the carrageenan-induced inflammation to influence the turnover of the terminal oxidase.^{19,20)}

The cytochrome P-450 fraction of mammalian hepatic microsomes is heterogeneous, consisting of a family of hemoproteins which differ in inducibility and substrate specificity.⁷⁾ The inhibition of the male monooxygenase system to inflammation-induced lowering of cytochrome P-450 content, and the lack of such an effect in female rats, might be accounted for on the basis of the heterogeneity of the hemoprotein fraction. Apparently, the expression of these male-specific species of the hemoproteins results from hormonally controlled neonatal imprinting, since alterations in the hormonal environment of neonatal male rats abolishes the subsequent sex differences in drug oxidations of adult animals, and also renders these animals insensitive to inflammation-induced inhibition of biotransformation.^{21,22)} The increased capacity for biotransformation of certain foreign compounds in males, compared to females, rat liver, might be accounted for by the presence of these male-specific forms of cytochrome P-450, which are particularly involved in the metabolism of compounds which have been termed sex-dependent, or androgen-sensitive, substrates. The sensitivity of the drug-metabolizing enzyme system of male rats to various physiological manipulations,^{14,15)} aromatic hydrocarbon treatment,^{17,18)}

and carrageenan administration could occur because turnover of these male-specific species of cytochrome P-450 is affected to a much greater degree relative to the other forms of the hemoprotein. The present findings support the view that carrageenan-induced inflammation can selectively influence different species of the hemoprotein since the induction and activity of cytochrome P-448 was not influenced by concurrent carrageenan administration. The antagonistic effect of the carrageenan-induced inflammation inhibition on this monooxygenase system induced by PB in male rats could occur if this inducer stimulates the synthesis of forms of the hemoprotein other than, or in addition to, the androgen sensitive species.

The mechanism of the sex-related depression of carrageenan-induced inflammation on hepatic drug-metabolizing enzyme activity is not clear.

During the acute phase response to inflammation, the synthesis of hepatic negative acute phase proteins (such as albumin and α_{2u} -globulin) is decreased.^{23,24)} Recently, Morgan demonstrated that the synthesis of male-specific cytochrome P-450 and female-specific cytochrome P-450 is also suppressed in an acute phase reaction to inflammation, but the suppressive effect of inflammation seems to be greater for male-specific cytochrome P-450 isozyme than for female-specific cytochrome P-450 isozyme.²⁵⁾ The negative acute phase protein α_{2u} -globulin is a male-specific hepatic gene product.²⁶⁾ Because carrageenan-induced inflammation produces α_{2u} -globulin suppression, the expression of male-specific cytochrome P-450 isozymes may be decreased in cytochrome P-450. Experiments are now in progress to test this hypothesis. Further studies are needed to clarify the mechanism of the inhibitory effect of the carrageenan-induced inflammation.

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