Effect of Carrageenan-induced Acute Peripheral Inflammation on the Pharmacokinetics and Hepatic Metabolism of Midazolam in Rats

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Summary: The effect of carrageenan-induced acute peripheral inflammation (API) on the pharmacokinetics of the heptically metabolizing compound midazolam (MDZ) was investigated in rats. Rats were subcutaneously treated with \( \lambda \)-carrageenan in the hind paw to induce API. When MDZ was intravenously administered in male rats, it was demonstrated that the plasma concentration profile of MDZ slightly alters in API rats compared with that in normal rats, while the plasma concentrations of its metabolites, 4-hydroxy and 1\(^\prime\)-hydroxy MDZ, are markedly reduced with delayed appearances in API rats. In the incubation study with rat liver microsomes, it was clearly indicated that the generation rates of the two metabolites decrease in API rats. Western blot analysis revealed that hepatic CYP3A1 expression increases, while CYP3A2 expression decreases in API rats. In female rats, in which CYP3A2 is barely expressed in the liver, MDZ metabolism is little affected by API. These findings indicate that the hepatic handling of a therapeutic compound varies with API, largely due to altered hepatic expression of the drug-metabolizing enzyme.

Keywords: acute inflammation; carrageenan; CYP3A2; hepatic metabolism; midazolam; sex difference

Introduction

It has been recognized that diseases, infections, and injuries sometimes influence remote and unrelated organs, modulating their functions beyond the areas affected. Periodontal disease has been reported to be associated with cardiovascular disease and arteriosclerosis.\(^1\)\(^-\)\(^4\) Rheumatoid arthritis and renal failure have both been reported to increase the arteriosclerosis risk in patients.\(^5\)\(^-\)\(^6\) The mechanism responsible for the influence is not fully understood at present, but the inflammatory reaction accompanying the primary symptom appears to be involved, since it is generally observed that the plasma level of the inflammation marker protein, C-reactive protein, concurrently increases.\(^3\)\(^-\)\(^6\) As is well recognized, inflammation occurs as a physiological defensive reaction in response to external stimuli, such as tissue injury and infection, and it occasionally inflames to cause the overproduction of reactive oxygen species and excessive microvessel permeability, along with the over-expression of interleukin-6, tumor necrosis factor-\( \alpha \), and other cytokines.\(^7\)\(^-\)\(^8\) These cytokines probably reach remote organs via the circulation, affecting the organs’ functions.

In conjunction with these considerations and the fact that the hepatic production of an acute-phase protein, \( \alpha_1 \)-acid glycoprotein, increases in response to peripheral inflammation,\(^9\)\(^-\)\(^10\) part of the influence is suggested to reach the liver, leading to modulation and/or stimulation of the hepatic functions. Since the liver is well known to play an important role in regulating the pharmacokinetics of therapeutic compounds, its functional alteration probably has a non-negligible impact on the blood concentration profiles of the therapeutic compounds, causing variation in their pharmacological effects. Altered pharmacokinetics with hepatitis is frequently reported for various compounds, while little is known about the effects of peripheral inflammation on the hepatic drug handling. Therefore, the effects of peripheral inflammation on the pharmacokinetics and hepatic metabolism of therapeutic compounds were examined in rats with experimentally induced inflammation. For the induction of inflammation, the use of a method employing \( \lambda \)-carrageenan, an anionic polysaccharide with a repetitious structure of 1,3-linked \( \beta \)-D- and 1,4-linked \( \alpha \)-D-galactophyranoses, seems to be appropriate, as it has been frequently used in various experiments examining the acute phase of inflammation itself and inflammation-related physiological reactions in rodents.\(^11\)\(^-\)\(^13\) It has been reported that \( \lambda \)-carrageenan induces inflammation in two steps: histamine, serotonin, and bradykinin are first released from the site of carrageenan injection, and then neutrophil infiltration and
prostaglandin generation occur with the release of reactive oxygen species, free radicals, nitric oxide, and cytokines such as tumor necrosis factor and interleukin-1β. Additionally, to examine whether the peripheral inflammation influences the hepatic function in a remote and indirect manner, it is advantageous for carrageenan to be subcutaneously administered into their limbs for induction, as it scarcely reaches the liver via the circulation, having little influence on the hepatic functions in a direct manner.

In this study, we examined whether the plasma concentration profile of therapeutic compounds alters with inflammation in rats, employing λ-carrageen for the induction of acute peripheral inflammation (API). Their altered plasma concentration profiles probably lead to a change in their therapeutic potency. As a representative therapeutic compound, midazolam (MDZ) was utilized, since it is mainly metabolized in the liver.17,18 The effect of acute inflammation on the hepatic drug-metabolizing activity was also investigated, focusing on the cytochrome P450 (CYP) 3A subfamily in rats, aiming to understand the mechanism underlying the alteration of hepatic drug metabolism in the presence of peripheral inflammation.

Materials and Methods

Materials: MDZ that is commercially available for medical purposes from Sandoz (Tokyo, Japan) was used without further purification.19 The product is supplied as an injectable solution in a sterilized isotonic buffer (pH 2.8–3.8), in which MDZ is dissolved at a concentration of 5 mg/ml 4-Hydroxy midazolam (4-OH MDZ) and 1′-hydroxy midazolam (1′-OH MDZ) were purchased from BD Biosciences (San Jose, CA) and Cerrilliant (Round Rock, TX), respectively. λ-Carrageenan was obtained from Wako Pure Chemical Industries (Osaka, Japan). Purified rabbit anti-rat cytochrome P450 enzyme CYP3A1 and CYP3A2 polyclonal antibodies were from Millipore (Billerica, MA). As rat CYP3A1 and CYP3A2 standard proteins, cDNA-expressed CYP3A1 and CYP3A2 BD-Supersomes34 (BD Biosciences) were used, respectively. All other chemicals were of the finest grade available from local distributors.

Animals: Male and female Wistar rats at 7 weeks old were purchased from Japan Charles River (Yokohama, Japan). Body weights were 230–300 and 150–165 g for male and female rats, respectively. They were housed in stainless steel cages placed in an air-conditioned room, in which the temperature and relative humidity were controlled at 20–25°C and 40–50%, respectively. A 12-h light and dark cycle was maintained. All animal experiments were performed in accordance with the guidelines for animal experimentation of Okayama University, after being approved by the institutional animal ethics committee (OKU-2012382).

Induction of API in rats: API was induced in rats with λ-carrageenan treatment, as previously reported.21 Briefly, after being anesthetized with pentobarbital (50 mg/kg, i.p.), rats were treated with λ-carrageenan solution prepared at a concentration of 40 mg/ml with phosphate-buffered saline (PBS). The pH of the PBS was adjusted to 7.4. The carrageenan solution was intramuscularly injected into the soles of left and right hind paws at a dose of 64 mg/ml by employing a 1-ml syringe with a 26-gauge needle. For a comparison, normal rats were treated with the PBS instead of the carrageenan solution. The rats were then subjected to experiments following a 24-h rest period.21

Evaluation of plasma concentration profiles of MDZ and its metabolites in rats: After being anesthetized with pentobarbital, a male rat was fixed on its back, and the right femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho, Tokyo, Japan) for blood collection. The polyethylene tubing was heparinized to prevent coagulation. The rat was then placed on a water jacket constantly maintained at 37°C, and the MDZ solution described above was gently administered into its left femoral vein at a dose of 20 mg/kg by employing a 1-ml syringe with a 27-gauge needle. A 200-µl sample of blood was drawn via the cannula at 3, 10, 15, 30, 60, 90, 120, and 150 min after the administration. Plasma specimens were obtained by centrifugation of the blood samples at 2,000 × g for 10 min at 4°C, and they were immediately supplied for the MDZ determination described later.

The plasma concentration profiles of MDZ and its two major metabolites, 4-OH and 1′-OH MDZ, were evaluated based on their AUC values. The AUC values were calculated using the trapezoidal rule until the last time-point in the experiment. For the calculation of the AUC value of MDZ, the extrapolation was additionally performed, in which four or five data points in the terminal phase of the plasma concentration profile of MDZ were utilized for extrapolation to infinity. The AUC value is used to calculate the total body clearance of MDZ, in which the MDZ dose administered is divided by the AUC value. The extrapolation was also utilized to calculate the elimination half-time of the plasma concentration of MDZ. Subsequently, the ratio between the AUC values of MDZ and its metabolites was calculated in each experiment by the AUC value of the metabolite being divided by the AUC value of MDZ.

Evaluation of hepatic MDZ metabolism in rats: The hepatic drug-metabolizing activity was evaluated using MDZ as a substrate in the incubation experiments with the pooled liver microsomes as previously reported.19 Four groups of liver microsomes were prepared from normal male and female rats and API rats, respectively, in a conventional manner with ultra-centrifugation. Five rats were used in each group. The prepared microsomes were stored at −80°C until use. Their protein contents were determined with the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA).

The hepatic drug-metabolizing activity was evaluated in the incubation study.19 The incubation mixture was prepared with potassium phosphate buffer containing 200 µg of the microsomal protein. MDZ was dissolved in it at various concentrations. For each set of incubation experiments, 5 kinds of incubation mixture were prepared, in which MDZ was dissolved at concentrations of 1, 2.5, 5, 7.5, and 10 µM, respectively. Following a 5-min pre-incubation period, the incubation mixture was spiked with 50 µl of β-NADPH solution to initiate the metabolic reaction. The final concentration of β-NADPH in the mixture was 1 mM. The final volume of the incubation mixture was 1 ml. After the incubation had been carried out for 10–30 min at 37°C, the incubation mixture was quickly mixed and vigorously agitated with 6 ml of ice-cold diethyl ether to terminate the metabolic reaction. The incubation mixture was then processed for the determination of MDZ and its metabolites, as described later. The incubation experiments were also carried out without β-NADPH in the same manner as described above to assess non-specific MDZ reactions in the liver microsomes, in which potassium phosphate buffer was added to the incubation mixture instead of the β-NADPH solution.

In each set of incubation experiments, the hepatic drug-metabolizing activity was evaluated based on the β-NADPH-dependent disappearance of MDZ and the associated generation of
its two major metabolites, 4-OH and 1’-OH MDZ, in which the disappearance and generation rates of the compounds were characterized on the assumption that the rate is described with the Michaelis-Menten equation (Eq. 1):

\[ v = \frac{V_{\text{max}} \times C}{K_m + C} \]  

where \( v \) is the rate at which MDZ disappears or the metabolite generates, \( C \) is the MDZ concentration in the incubation mixture. The apparent Michaelis-Menten constant is designated as \( K_m \), and the maximum rate of MDZ disappearance or metabolite generation is indicated as \( V_{\text{max}} \). In each set of experiments, the values of \( K_m \) and \( V_{\text{max}} \) were estimated by performing non-linear least square calculation for the data given in the experiments. The intrinsic clearance \( CL_{\text{int}} \) was obtained by dividing \( V_{\text{max}} \) by \( K_m \).

**Evaluation of hepatic expressions of the CYP3A subfamily in rats:** The hepatic expressions of the CYP3A subfamily were examined with Western blotting, as previously reported. In brief, the aforementioned liver microsomes prepared for the incubation study were suspended with PBS at a microsomal protein concentration of 0.6 mg/ml. Following 5-min boiling with 2-mercapto-ethanol, the solution was applied to an SDS-polyacrylamide (10%) gel at 3 μg of microsomal protein/lane. The gel was subjected to electrophoresis and subsequent transfer to a nitrocellulose membrane. The membrane was then blocked with 4% skim milk containing 0.3% Tween 20 at 4°C, and, after that, it was incubated for 60 min with the anti-CYP3A1 or anti-CYP3A2 antibodies. The antibodies were diluted at 1:1,000 for use. The migration pattern consisting of 55% methanol and 45% sodium phosphate buffer (10 mM, pH 3.1). The mobile phase was used at a flow rate of 1 ml/min, and 4-OH MDZ, MDZ, 1’-OH MDZ, and the internal standard diazepam were eluted in this order at about 4.4, 5.7, 6.9, and 10.0 min, respectively. The detection was performed with spectrophotometry at a wavelength of 229 nm.

**Data analysis:** Data are shown as means ± S.E. The significance of differences between two values was evaluated by Student’s \( t \)-test, and \( p < 0.05 \) was considered significant.

**Results**

**Evaluation of plasma concentration profiles of MDZ and its metabolites in male API rats:** The plasma concentrations of MDZ and its two major metabolites, 4-OH and 1’-OH MDZ, were examined following the intravenous administration of MDZ to male rats. As shown in Figure 1A, the plasma concentration of MDZ decreases in a two-exponential manner in normal rats, in which the concentration rapidly decreases in an early phase, followed by a gradual reduction in the subsequent terminal phase. The elimination half-time of the terminal phase is 34.2 ± 0.2 min. The total body clearance of MDZ is 35.7 ± 4.8 ml/min/kg. Two metabolites of MDZ are detected in the plasma specimens, in which the concentrations of 4-OH MDZ are generally higher than those of 1’-OH MDZ (Fig. 1A). In API rats, the plasma concentration of MDZ tends to decrease at a slower rate, with the half-time being 40.4 ± 5.0 min, and the total body clearance of MDZ is calculated as 26.2 ± 3.8 ml/min/kg, but these values are not significantly different from those in normal rats (Fig. 1B). As for

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**Fig. 1. Plasma concentration profiles of MDZ and its two main metabolites following intravenous administration of MDZ at a dose of 20 mg/kg in male rats**

The concentration profiles observed in normal rats are indicated with open symbols in Panel A, and those in API rats are presented with closed symbols in Panel B. In both panels, the profiles of MDZ, 4-OH, and 1’-OH MDZ are shown with circles, squares, and triangles, respectively. Data are expressed as the mean ± S.E. of 3 experiments. *p < 0.05: significantly different from the corresponding value in normal rats shown in Panel A.

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Next, hepatic expression of the CYP3A subfamily was examined in normal and API rats, since the subfamily is known to be primarily involved in the hepatic metabolism of MDZ. As shown in Figure 2B, the expression level of CYP3A1 in API rats was revealed to increase by 29 ± 5% compared with that in normal rats, while the level of CYP3A2 in API rats decreased by 37 ± 4% from that in normal rats.

Evaluation of the hepatic activity of MDZ metabolism in female API rats: The effect of API on the hepatic drug-metabolizing activity was also examined in female rats, in which CYP3A2 is barely expressed in the liver. In normal female rats, hepatic MDZ metabolism occurs at a slower rate compared with that in normal male rats, as expected (Figs. 2A and 3A). In female rats, the metabolic profiles of MDZ elimination and metabolite generation in API rats are indistinguishable from those in normal rats (Fig. 3A), and, thereby, the kinetic parameters in API rats are comparable to those in normal rats (Table 2). As shown in Figure 3B, on examination of the hepatic expressions of the CYP3A subfamily, expressions in API rats are not different from those in normal rats.

Discussion

Inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, are known to influence the function of remote and seemingly unrelated organs, which sometimes leads to systemic disturbance. Such disturbance is likely to affect the liver and modulate the hepatic function, which may result in altered plasma concentration profiles of therapeutic compounds. Our current observations support this speculation. There have been reports that the hepatic drug-metabolizing enzymes and transporters are influenced by inflammation in vitro, but it is required to be demonstrated in vivo that the plasma concentration profiles of the compounds that are related to the therapeutic potency alter with inflammation. As shown in Figure 1, the plasma concentration of MDZ in API rats tends to decrease in an impaired manner compared with that in normal rats. The impairment of MDZ elimination

![Figure 2. Effects of API on hepatic MDZ metabolism and hepatic expression of drug-metabolizing enzymes in male rats](image)

In Panel A, the metabolic profiles of MDZ in liver microsomes are presented using the Michaelis-Menten plot. The values for normal and API rats are indicated with open and closed symbols, respectively. The elimination profiles of MDZ are shown with circles, and the generation profiles of 4-OH and 1'-OH MDZ are presented with squares and triangles, respectively. Data are shown as the mean ± S.E. of 4 sets of incubation experiments. Some error bars are behind the symbols. The solid and dotted lines are the best-fit lines for the corresponding data group. In Panel B, the hepatic expressions of CYP3A1 and CYP3A2 isozymes are presented using a bar graph with the representative results yielded in Western blotting analysis. The values for normal and API rats are indicated with open and closed columns, respectively, as the mean ± S.E. of 3 experiments. *p < 0.05: significantly different from the normal value.
seems to be reflected by the suppressed generation of metabolites. That is, the AUC ratio of metabolites is markedly lower in API rats compared with that in normal rats (Fig. 1 and Table 1), demonstrating that the plasma concentration of the therapeutic compound alters with API. It was previously reported that hepatic drug metabolism is suppressed when lipopolysaccharides are systemically administered to rats, which is consistent with our current findings. Although lipopolysaccharides may affect the hepatic function in a direct manner, it is plausible that lipopolysaccharide-induced inflammation is responsible for the suppression.

The suppression of metabolite generation observed in API rats is likely to be related to the suppression of hepatic MDZ metabolism. In the experiments with the rat liver microsomes, it was shown that the hepatic MDZ metabolism is influenced in API rats, in which metabolites are generated at a slower rate in API than normal rats (Fig. 2A). The CLint values for metabolite generation are reduced by 39.4 and 26.2% for 4-OH MDZ and 1’-OH MDZ, respectively, as compared with those in normal rats (Table 2). It was additionally demonstrated that hepatic expression of the CYP3A subfamily alters in API rats, in which the expression of CYP3A1 increased while that of CYP3A2 decreased (Fig. 2B). It is therefore plausible that the relative abundance of each of the hepatic CYP3A isoforms changes in API rats, resulting in the fact that CYP3A2 contributes to MDZ metabolism to a lesser degree in API than normal rats. Since metabolite generation is largely mediated by CYP3A2, the altered expression of the hepatic CYP3A subfamily is probably responsible for the suppressed metabolite generation in API rats. It should be noted that the sum of the CLint values for metabolite generations is disproportionally smaller than that expected from the CLint value for MDZ elimination in both normal and API rats (Table 2). The reason for this remains to be elucidated in this study. It may be partly involved in the fact that the kinetic profiles of the two metabolites are probably not well characterized, giving a disproportional CLint value for metabolite generation, as the MDZ metabolites, 4-OH and 1’-OH MDZ, are known to be further metabolized to 1,4-dihydroxy MDZ.

As shown in Figure 3, the hepatic metabolism of MDZ was shown not to vary with inflammation in female API rats. It is known that CYP3A2 is little expressed in female rats, being contrasted with the fact that it is expressed 40–50% more than normal.

Table 2. The kinetic parameters of hepatic metabolism of MDZ and its two metabolites in liver microsomes prepared from male and female rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal rats</th>
<th>API rats</th>
</tr>
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<tbody>
<tr>
<td>MDZ</td>
<td>4-OH MDZ</td>
<td>1’-OH MDZ</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keq (µM)</td>
<td>4.13 ± 0.41</td>
<td>9.13 ± 1.08</td>
</tr>
<tr>
<td>Vmax (nmol/min)</td>
<td>3.29 ± 0.28</td>
<td>2.62 ± 0.19</td>
</tr>
<tr>
<td>CLint (ml/min)</td>
<td>0.81 ± 0.06</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keq (µM)</td>
<td>1.36 ± 0.27</td>
<td>2.82 ± 0.15</td>
</tr>
<tr>
<td>Vmax (nmol/min)</td>
<td>0.200 ± 0.016</td>
<td>0.033 ± 0.002</td>
</tr>
<tr>
<td>CLint (ml/min)</td>
<td>0.159 ± 0.021</td>
<td>0.014 ± 0.001</td>
</tr>
</tbody>
</table>

*p < 0.05: significantly different from the corresponding value in normal rats.

Values are normalized by the protein content (in mg) of microsomes. Values are indicated as the mean ± S.E. for 4 sets of incubation experiments. The solid and dotted lines are the best-fit lines for the corresponding data group. Some error bars and lines are behind the symbols. In Panel B, the hepatic expressions of CYP3A1 and CYP3A2 isoforms are presented using a bar graph with the representative results yielded in Western blotting analysis, in which no CYP3A2 expression is detected in either normal or API rats at the position where the standard CYP3A2 protein has migrated (indicated with arrow heads). The values for normal and API rats are indicated with open and closed columns, respectively, as the mean ± S.E. of 4 experiments. N.D.: not detected.

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CYP3A1 in male rats. Therefore, the observation in female rats suggests that the decreased hepatic expression of CYP3A2 primarily causes alterations in the pharmacokinetics and metabolism of MDZ in male API rats. It seems that the impact of inflammation on the pharmacokinetics of therapeutic compounds varies depending on the extent to which the compound is hepatically metabolized and on which CYP isozyme mainly contributes to the metabolism. That is, in the case of a compound that is markedly metabolized by hepatic drug-metabolizing enzymes and the enzymes' hepatic expressions vary with inflammation, the plasma concentration profile of the compound would alter under inflammatory conditions. It is considered that the drug-metabolizing enzyme CYP3A4 in humans corresponds to CYP3A2 in rats. Besides the correspondence, the hepatic expressions of CYP3A2 and CYP3A4 are both known to be readily influenced by various hepatic conditions and diseases. It is therefore probable in a clinical setting that the pharmacokinetics and disposition of CYP3A4 substrates alter under inflammatory conditions.

It was shown that various inflammation-related factors, such as tumor necrosis factor-α, are largely released into the circulating blood in an early phase of inflammation, but, thereafter, they are rapidly eliminated to an undetectable level. In this study, while we could not identify a factor mediating the phenomenon whereby peripherally occurring inflammation has a functional influence in remote and unrelated organs, it appears to modulate the hepatic expression of CYP3A2 in a different manner from that of CYP3A1 (Figs. 2B and 3B). Although further study is necessary to elucidate the factors responsible for the mediation, interleukins, such as interleukin-1β and -6, can be considered as candidates, since they were reported to suppress mRNA expression of CYP3A2, with little effect on that of CYP3A1.

In this study, we demonstrated that the plasma concentration profiles of MDZ and its metabolites alter with API in male rats. The change in hepatic expression of CYP3A2, which mainly mediates MDZ metabolism, is responsible for the alteration. These findings will provide helpful information to optimize a regimen, taking patients’ conditions into consideration.

References
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