Changes of Hepatic Microsomal Oxidative Drug Metabolizing Enzymes in Chronic Renal Failure (CRF) Rats by Partial Nephrectomy

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ABSTRACT—Male SD rats, 7-weeks-old, were used to investigate the changes in the hepatic drug metabolizing system of chronic renal failure (CRF) model rats. Partial nephrectomy (5/6) was performed in a two-stage surgical procedure. After nephrectomy, the rats were housed under regular conditions at least 21 days. After confirming the CRF states, trimethadione (TMO, 100 mg/kg, i.p.) was administered for evaluation of the hepatic drug metabolizing capacity; the ratio of dimethadione (DMO: the only metabolite of TMO) to TMO (DMO/TMO) in the serum and the dialysate from the blood microdialysis method were ascertained. The hepatic drug metabolizing enzyme contents and activities were also determined. In the CRF rats, the DMO/TMO ratios decreased significantly; total cytochrome P450 (CYP) contents, aminopyrine N-demethylase activity and δ-aminolevulinic acid synthetase activity also decreased significantly in the CRF rats. The extent of the alterations of these enzyme contents and activities correlated well with the severity of the CRF states evaluated by the serum blood urea nitrogen and creatinine concentrations. With Western blot analysis, the levels of CYP2C6, CYP2C11 and CYP3A2 decreased considerably in the CRF rats. These results suggest that CRF states induce not only a reduction of renal function but also an alteration of hepatic metabolism.

Keywords: Chronic renal failure, Partial nephrectomy, Drug metabolism, Trimethadione, Cytochrome P450

Drug metabolism and excretion are closely associated with liver and renal functions. It is a well-known phenomenon that several unexpected adverse effects occur when these functions are impaired (1). Despite the adjustment of dosage to compensate for reduced renal clearance, the frequency and severity of adverse drug effects are significantly greater in patients with renal failure than in patients who do not suffer from renal dysfunction (2, 3). We have reported that the changes in pharmacokinetic parameters of rilmazafone, a benzodiazepine derivative, in patients with renal failure were due not only to decreased renal excretion but also to changes in the activity of hepatic drug metabolizing enzymes and/or the drug metabolic pathway (4); similar findings were also reported by Bianchetti et al. (5) and Lowenthal et al. (6). There are several reports on the changes of hepatic drug metabolism in experimental renal failure rats (7–13). However, most of these studies have been performed by in vitro experimental procedures, and very few reports exist about experiments under in vivo conditions in chronic renal failure (CRF) model rats.

In the present study, to clarify the mechanism of drug metabolism alteration in CRF states, in vivo and ex vivo experiments were conducted using CRF model rats to evaluate the changes in hepatic drug metabolizing enzyme systems. Changes of in vivo hepatic drug metabolizing capacity in CRF rats were evaluated by employing trimethadione (TMO) as a model probe. TMO is converted into the only metabolite, dimethadione (DMO), through N-demethylation by microsomal cytochrome P450s. Tanaka et al. reported that the ratio of serum concentration of DMO to TMO (DMO/TMO) is a useful parameter to assess the hepatic oxidative drug metabolizing enzyme capacity under several conditions in experimental animals and also in humans (14–18). For the in vivo experiment, the microdialysis method is a useful tool since it is capable of continuous measurement of drug levels in the plasma with no body fluid (including
blood) loss. In this study for the determination of pharmacokinetic parameters of TMO, we applied a new technique of blood microdialysis that enabled us to measure plasma levels of the drug continuously in freely moving rats (19). Changes in hepatic drug metabolizing enzymes in CRF rats were also determined using the microsomal fraction and Western blot analysis.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Japan Medical Animals Co., Ltd., Tokyo), 7-weeks-old, weighing approximately 250 g were used in this study.

Surgical procedure for partial nephrectomy

Partial nephrectomy (five-sixths) was performed in two stages according to Hayslett et al. (20) under pentobarbital anesthesia (30 mg/kg). In the first surgical procedure, 2/3 of the left kidney was removed by cutting off both poles. Before cutting off the poles, the capsule of the kidney had been removed, and to minimize blood loss the renal artery had been clamped. Seven days after the first surgical procedure, the right kidney was completely removed. The nephrectomy was carried out in the same sequence as that of the pole resection. Throughout the experiment, 6 rats were housed per cage and fed a commercial diet (Oriental Yeast Co., Ltd., Tokyo) ad libitum for at least 21 days in order to achieve a CRF condition. Sham-operated (sham) animals were subjected to laparotomy only on the same days as the CRF group. The control animals were untreated.

Assessment of CRF states

After 5/6 nephrectomy, a 0.8-ml blood sample was collected from the jugular vein every 7 days. The extent of renal failure was assessed by the elevation of serum blood urea nitrogen (BUN) and creatinine concentrations.

In vivo microdialysis study

The microdialysis probe was newly developed in our department with Eicom Co., Ltd. (Kyoto). The blood dialysis probe was implanted in the right jugular vein under ether anesthesia; the guide tube was taken out of the neck and connected to the microinjection pump (EP-60, Eicom) and the fraction collector (EF-80, Eicom). Microdialysis sampling was done using the microinjection pump; a perfusion solution (5% glucose solution) was pumped to the blood microdialysis probe at a flow rate of 3 pl/min in all experiments. Dialysate was collected every hour for 24 hr with a fraction collector. TMO was injected (100 mg/kg, i.p.) more than 3 hr after implantation of the probe. Concentrations of TMO and DMO in the dialysate were analyzed by gas chromatography according to the method of Tanaka et al. (21) with minor modifications. For determination of DMO/TMO ratios, serum samples were taken 2 hr after TMO administration. The dialysate DMO/TMO ratio was calculated using the mean value of the TMO or DMO concentration of the dialysate sample between 1 to 2 hr and 2 to 3 hr.

Ex vivo study

Enzyme source preparation: Rats were sacrificed 3 weeks after 5/6 nephrectomy. The liver was resected and divided into two portions. One portion of the liver was perfused with an ice cold 0.9% NaCl solution and homogenized with 4 vol. of a 1.15% KCl solution. The homogenate was centrifuged at 9,000 × g for 20 min. The resulting supernatant fraction was centrifuged again at 105,000 × g for 90 min to prepare the microsomal fraction. The resulting pellets, microsomal fractions, were washed once and resuspended in 0.1 M Na-K phosphate buffer (pH 7.4) and used as the microsomal enzyme source for the determination of enzyme contents and activities. The other portion of the liver was homogenized with 3 vol. of 0.9% NaCl containing 10 mM Tris-HCl buffer (pH 7.4) and 0.5 mM EDTA and used for the determination of δ-aminolevulinic acid synthetase (ALAS) activity.

Enzyme assay: The contents of microsomal cytochrome P450, cytochrome b5 and protoheme were determined according to the method of Omura and Sato (22). NADPH-cytochrome c reductase activity was analyzed by the method of Williams and Kamin (23). Aminopyrine N-demethylase activity was measured by the method of Imai et al. (25). Aniline hydroxylase activity was measured by the method of Lowry et al. (28).

Statistical analyses

Western blot analysis

Western blot analysis was performed by the method of Isogai et al. (29). Antibodies for rat hepatic microsomal P450s were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo).
serum BUN and creatinine, overall group differences between the control, the sham and the CRF groups were determined by the Kruskal-Wallis one-way analysis of variance. Statistical analysis was then carried out by Dunn’s test. For the DMO/TMO ratio, differences of the control and the CRF group were assessed by Student’s t-test.

RESULTS

Production of chronic renal failure by 5/6 nephrectomy

The development of renal failure was assessed by the elevation of serum BUN and creatinine concentrations (Table 1). A significant decrease in body weight was observed in the CRF rats compared with the sham and the control rats. The average serum BUN concentration was significantly elevated in the CRF rats as compared with both the sham and control rats. Serum creatinine concentrations also increased significantly in the CRF rats. Thus, 5/6 nephrectomy produced chronic renal failure; the rats, 21 days after the 5/6 nephrectomy, were used in this study as the CRF model.

In vivo microdialysis study

Changes in the concentrations of TMO and DMO in the dialysate over time are shown in Fig. 1. In the sham rats (Fig. 1A), the peak dialysate concentration of TMO (43.3 ± 4.3 μg/ml) was observed within 1 hr after TMO administration and then decreased with first order elimination kinetics until 17 hr. DMO appeared within 1 hr after TMO administration and increased gradually until 9 hr, reaching plateau levels of 44.1 ± 4.9 μg/ml for up to 24 hr. On the other hand, the peak dialysate concentration of TMO (66.4 ± 4.7 μg/ml) was observed 2 hr after TMO administration and decreased until 17 hr in the CRF rats (Fig. 1B). The DMO concentration reached plateau levels of 62.5 ± 7.8 μg/ml at 10 hr after TMO administration. The calculated elimination half-life of TMO in the dialysate was slightly prolonged in the CRF rats (2.9 ± 0.2 hr) compared with the sham rats (2.7 ± 0.4 hr: P > 0.05). Table 2 shows DMO/TMO ratios in the serum and the dialysate. DMO/TMO ratios decreased significantly in the CRF rats both in the serum (P < 0.05) and in the dialysate (P < 0.01).

Ex vivo study

No significant differences were observed in any of the microsomal drug metabolizing enzyme contents and activities between the sham and control rats (Table 3). Microsomal cytochrome P450 contents decreased significantly by 30% and 20% in the CRF rats as compared with the sham and control rats. The cytochrome b5 contents and NADPH cyt. c reductase activities were not altered in the CRF rats. In the CRF rats, the microsomal aminopyrine N-demethylase activities decreased significantly by 30% and 35% compared to the sham and control, respectively. No significant change was seen for aniline hydroxylase activities. ALAS activities decreased significantly in the CRF rats, by 50% and 45% compared to the sham and control, respectively. Heme oxygenase activities tended to increase and protoheme contents decreased in the CRF rats.

Figure 2 shows the relationship between BUN concentrations and P450 contents, and aminopyrine N-demethylase, aniline hydroxylase and ALAS activities. A significant correlation with serum BUN was found for P450 contents (P = 0.0007), aminopyrine N-demethylase activities (P = 0.002) and ALAS activities (P = 0.0001). A similar tendency of correlation was observed for serum creatinine concentrations (data not shown).

| Table 1. Characteristic observations in control, sham operation (sham) and chronic renal failure (CRF) rats |
|---------------------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                                                                      | Control (n=9) | Sham (n=12) | CRF (n=21) |
| Initial body weight (g)                                                              | 254.4 ± 2.4   | 247.1 ± 3.8 | 251.8 ± 1.8 |
| Duration of renal failure (day)                                                      | 21            | 21          | 21          |
| Final body weight (g)                                                                | 379.7 ± 14.1  | 353.8 ± 9.6 | 313.1 ± 10.3 *f |
| Serum BUN (mg/dl)                                                                   | 22.7 ± 1.1    | 22.0 ± 1.1  | 80.3 ± 6.2 44 |
| Serum creatinine (mg/dl)                                                             | 0.70 ± 0.03   | 0.68 ± 0.03 | 1.45 ± 0.09 44 |

CRF rats were given a partial nephrectomy (5/6) in a two-stage surgical procedure. Sham-operated rats were subjected to laparotomy only on the same days as the CRF group. The control rats were untreated. Final body weight, serum blood urea nitrogen (BUN) and serum creatinine concentrations were measured on the experimental day at 21 days after 5/6 nephrectomy. Values are means ± S.E. *P < 0.05 vs control group and *P < 0.05 vs sham group by Tukey’s multiple range test. †P < 0.05 vs control group and †P < 0.05 vs sham group by Dunn’s test.
Fig. 1. Changes in blood dialysate concentrations of trimethadione (TMO, ■) and dimethadione (DMO, □) after TMO administration (100 mg/kg, i.p.) in the sham (A: n=8) and chronic renal failure (CRF) rats (B: n=10). Microdialysis sampling was done using a microinjection pump; a perfusion solution (5% glucose solution) was pumped to the blood microdialysis probe at a flow rate of 3 μl/min in all experiments. The dialysate was collected every hour for 24 hr with a fraction collector. CRF rats were given a partial nephrectomy (5/6) in a two-stage surgical procedure. Sham-operated rats were subjected to laparotomy only on the same days as the CRF group. Values are means ± S.E.
Table 2. Effect of chronic renal failure (CRF) on trimethadione metabolism

|                          | Control (n = 3) | CRF (n = 3) |
|--------------------------|----------------|-------------|
| Serum DMO/TMO ratio      | 0.503±0.053    | 0.374±0.011*|
| Sham (n = 8)             | 0.583±0.126    | 0.390±0.043**|

CRF rats were given a partial nephrectomy (5/6) in a two-stage surgical procedure. Sham-operated rats were subjected to laparotomy only on the same days as the CRF group. The control rats were untreated. The serum DMO/TMO ratio was calculated using the serum concentrations of trimethadione (TMO) and dimethadione (DMO) at 2 hr after TMO administration (100 mg/kg, i.p.). Microdialysis sampling was done using a microinjection pump; a perfusion solution (5% glucose solution) was pumped to the blood microdialysis probe at a flow rate of 3 μl/min in all experiments. The dialysate was collected every hour for 24 hr with a fraction collector. The blood dialysate DMO/TMO ratio was calculated using the mean value of TMO or DMO concentrations of the dialysate sample between 1 to 2 hr and 2 to 3 hr. Values are means±S.E. *P<0.05 vs control group and **P<0.01 vs sham group by Student’s t-test.

Western blot analysis

Western blot analysis of hepatic microsomal P450s in the control, sham and CRF rats are shown in Fig. 3. Western blot analysis for microsomal P450s was performed with the microsomal fraction obtained from the ex vivo study described above. Cytochrome P450 isoforms that were reactive towards the antibody against CYP3A2 were decreased considerably, and CYP2C6 and CYP2C11 also decreased slightly in the CRF rats compared with the control and sham rats. CYP1A2 increased slightly in the CRF rats compared with the control and sham rats.

DISCUSSION

There are several methods for producing CRF model rats (7–13). The use of drugs for producing the CRF model, for example, cisplatin, may cause damage to not only the renal function but also the liver function including hepatic drug metabolizing enzyme systems depending on the dosage used. Therefore, we selected 5/6 partial nephrectomy for producing CRF model rats in this study to avoid direct effects of a drug to the liver.

Changes of hepatic drug metabolism in renal failure model rats have been reported with in vitro experimental procedures (7–13). There are, however, very few reports on the changes in the hepatic drug metabolizing system under in vivo conditions in CRF rats. Kurata et al. (19) have recently reported that freely moving blood microdialysis was capable of continuous measurement of blood drug levels in conscious, unrestrained rats with no body fluid loss, including blood.

In the present study, the changes in TMO metabolism under CRF conditions were determined by the microdialysis method. DMO/TMO ratios decreased in the CRF rats in both the serum and the dialysate. These results indicated that TMO N-demethylase capacity was reduced by some of the factors with CRF conditions. Theuson et al. reported that there was little binding of TMO and DMO to serum albumin. Only a small amount of unchanged TMO, less than 3%, is eliminated by the extrahepatic pathway in the rat. Of the administered dose of TMO, 8% was recovered as DMO in the 24-hr urine in male rats (30). Although the concentration of TMO was higher in the CRF rats than the sham rats, as shown in Fig. 1, it might be that this result was not due to decreased renal excretion of TMO and DMO by nephrectomy but due to inhibition of TMO metabolism by the CRF condition. Nakayama et al. (31) reported that TMO
Fig. 2. Correlation between serum blood urea nitrogen (BUN) concentrations and cytochrome P450 contents (A), δ-aminolevulinic acid synthetase (ALAS) activity (B), aminopyrine N-demethylase activity (C) and aniline hydroxylase activity (D). Each plot is derived from the data obtained in the ex vivo experiment (n = 18) shown in Table 3. The regression lines were calculated by the least squares method.
is metabolized by several species of P450s, especially the CYP2C subfamilies. Terao and Shen (8) reported a decrease in the metabolism of propranolol in the uremic rat and suggested that an inhibitory factor in uremic blood inhibits the metabolizing enzymes involved in propranolol metabolism. An addition of serum from CRF rats to the in vitro reaction mixture inhibited aminopyrine N-demethylase activity (unpublished data, N. Uchida et al.). Therefore, some inhibitory substance of P450 isoforms may exist in the blood of CRF rats. The decrease of DMO/TMO ratio suggested that P450 isoforms, which participate in TMO metabolism, were inhibited by CRF conditions.

There was a significant decrease in the total P450 contents and activities of aminopyrine N-demethylase and ALAS in the CRF rats. However, cytochrome b\textsubscript{5} contents and activities of NADPH cyt. c reductase, aniline hydroxylase and heme oxygenase were not significantly different among the control, sham and CRF rats. There are several studies, using models of CRF or acute renal failure (ARF), on the changes in drug metabolizing activities (9–13). Leber and Georg (11) reported that aminopyrine N-demethylase, p-nitroanisole O-demethylase and acetanilide hydroxylase activities and cytochrome P450 contents decreased 21 days after nephrectomy. However, cytochrome b\textsubscript{5} contents and activities of NADPH cyt. c reductase and 6-methylmercapturine S-demethylase were not altered. Van Peer and Belpaire (9) reported a decrease in aminopyrine N-demethylase activities without changes in aniline hydroxylase activities in ARF rabbits produced with uranyl nitrate. Katayama et al. (13) reported that hepatic clearance of propranolol was significantly reduced in uranyl nitrate induced ARF rats. All of these reports support our results on patterns of changes in the microsomal drug metabolizing enzyme system.

In addition, Leber and Georg (11) reported that P450 contents in rats with renal failure could be normalized by treatment of with ß-aminolevulinic acid during the last 48 hr before the animals were sacrificed. They concluded that the decreased P450 contents were due to a disturbance in heme synthesis caused by a deficiency of ß-aminolevulinic acid in liver mitochondria in rats with renal failure. In our study, the alteration of the drug metabolizing enzyme contents and activities correlated significantly and negatively with serum BUN and creatinine concentrations. These results suggest that the decrease in total P450 contents in the CRF rats was caused by the decrease in ALAS activities, the rate-limiting enzyme for heme synthesis, which correlated with the CRF states evaluated by serum BUN and creatinine concentrations. However, aniline hydroxylase activities and other microsomal oxidative drug metabolizing
enzyme systems were not significantly altered in the CRF rats. Although heme oxygenase activity increased slightly and protoporphyrin content tended to decrease, these changes did not seem to affect the P450 content. These results, therefore, suggested that the changes of hepatic drug metabolizing systems in CRF rats are not generalized by biosynthesis of the heme but by some specific regulation of biosynthesis of the cytochrome P450 protein. This phenomenon is supported by the Western blot analysis data, where the density of some P450s isozymes such as CYP3A2, CYP2C6 and CYP2C11 decreased, and CYP1A2 increased but others did not change. Known substrates for CYP1A2 such as caffeine and theophylline may not be used for the microdialysis method, since they have moderate plasma protein binding that makes data evaluation more complicated. Further experiments with another method will be needed to clarify the effect of the CRF condition on CYP1A2.

In the present study, a significant decrease in body weight was observed in the CRF rats compared with both the control rats and the sham rats on day 3 weeks after 5/6 nephrectomy. However, the increase ratio of body weight was almost the same between the CRF rats and the sham rats for 1 to 3 weeks after 5/6 nephrectomy (data not shown). These results indicated that the CRF rats recovered from operation stress within 1 week, and the delayed body weight gain may be caused by operation stress during the first week after 5/6 nephrectomy. Imaoka et al. (32) and Ma et al. (33) reported that caloric restriction decreased hepatic expression of CYP2C11 and increased CYP3A2 expression. In our study, the level of CYP2C11 decreased in the CRF rats. However, the level of CYP3A2 also decreased in the CRF rats in spite of the low body weight. Furthermore, Leber and Georg used a group of sham rats that were given a restricted amount of food so that they lost an equal amount of body weight during the same time interval as nephrectomized rats. That report suggested that the metabolic changes in the liver of uremic rats were not the same as those in the liver of sham rats with caloric restriction (11). Therefore, we concluded that the decrease of CYP2C11 and CYP3A2 in our study was not due to caloric restriction by stress of the CRF condition, but due to the effects of the CRF condition on hepatic drug metabolizing enzymes.

Nakayama et al. (31) reported that the CYP2C subfamily partly participated in TMO metabolism. Tanaka et al. suggested that CYP3A2 was also involved in TMO metabolism (personal communication, E. Tanaka et al.). In our Western blot analysis, both P450s decreased in the CRF rats. Taking these results into consideration, the observed reduction of in vivo TMO metabolism in the CRF rats may be caused by the decreased contents of the CYP2C subfamily and/or the CYP3A2 by the CRF condition. Thus, CRF affects the pharmacokinetics of certain drugs not only by reducing the renal excretion but also by altering the hepatic metabolism. CYP2C11 and 3A2 were reported to be male specific enzymes (34). The CRF condition may directly affect those male specific isozymes through sex and/or growth hormones. Further experiments will be needed to clarify the effect of these hormones and specific inhibitor(s) on each P450 isozyme.

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