Mechanism Responsible for the Decreased Hepatic NADPH Generation Rate in Rats with Bilateral Ureter Ligation-Induced Renal Failure

Takashi HIGASHI, Misato URAI, Masato TAGUCHI, and Yukiya HASHIMOTO*

Graduate School of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930-0194, Japan. Received March 24, 2005; accepted June 1, 2005

We previously reported that a decrease in hepatic NADPH generation results in reduced hepatic first-pass clearance of propranolol in rats with bilateral ureteral ligation (BUL)-induced renal failure. The aim of the present study was to evaluate the mechanisms responsible for the reduced NADPH generation in the supernatant of liver homogenates (SUP) obtained from rats with BUL. The NADPH generation in the SUP in the presence of NADP⁺ was decreased in BUL rats as compared with control rats. After the addition of glucose-6-phosphate or 6-phosphogluconic acid, the increase in NADPH in the SUP of BUL rats was similar to that in control rats. The NADPH generation in the SUP after the addition of the ultrafiltrate of BUL rat SUP was smaller than that after the addition of the ultrafiltrate of control rat SUP. These findings suggest that the enzymatic activities in the pentose phosphate pathway were not decreased significantly in BUL rats, and that the decrease in the generation of NADPH in BUL rats was mainly caused by the decreased concentration of endogenous substrate(s) and/or the increased concentration of endogenous inhibitor(s) for the pentose phosphate pathway.

Key words NADPH generation; bilateral ureter-ligated rat; bile duct-ligated rat; pentose phosphate pathway

The intestinal absorption of orally administered propranolol is essentially complete, and no metabolism of this drug occurs in the gut.^{1,2)} After the oral administration of propranolol, the liver is the principal site of extensive pre-systemic and systemic metabolism, and less than 1% of the intact drug is found in the urine.^{1,3)} However, Bianchetti *et al.* showed that the area under the concentration–time curve for orally administered propranolol in renal failure patients not on hemodialysis is 7- to 8-fold higher than that in healthy volunteers.⁴⁾ The pharmacokinetics of propranolol has been extensively investigated using uranyl nitrate-induced renal failure model rats.^{5,6)} These studies showed increased bioavailability and reduced hepatic first-pass extraction of propranolol in rats with renal failure, although the precise biochemical and/or physiological mechanism for the decreased presystemic clearance is unclear.^{7,8)}

Because changes in government regulations regarding the production of radioactive substances have made uranyl nitrate less available, we investigated the mechanisms responsible for the increased bioavailability of propranolol in rats with cisplatin-induced renal failure.⁹⁾ The hepatic intrinsic clearance of propranolol was not significantly altered in rats with renal failure as compared with control rats. However, hepatic first-pass extraction of propranolol was dose-dependent and saturable in both renal failure and control rats, and the initial absorption rate of the drug from the intestine in rats with renal failure was significantly greater than that in control rats. Accordingly, the increased bioavailability of propranolol in rats with cisplatin-induced renal dysfunction is mainly a result of the increased initial absorption rate in the intestine followed by the partial saturation of hepatic first-pass metabolism.⁹⁾

The mechanism responsible for the increased bioavailability of propranolol in bilateral ureter-ligated (BUL) rats is different from that in the rats with cisplatin-induced renal failure.^{2,10)} That is, Laganière and Shen investigated the pharmacokinetics of intravenously and orally administered propranolol in BUL rats, and showed that the bioavailability is increased in BUL rats as compared with control rats.²⁾ They re-

ported that the gastrointestinal absorption of propranolol is not altered in BUL rats as compared with control rats. We also investigated the pharmacokinetics of propranolol in BUL rats, and confirmed that the intestinal absorption rate of propranolol in BUL rats was similar to that in control rats.¹⁰⁾ Therefore, the absorption rate-dependent decrease in hepatic first-pass clearance of the drug due to saturation kinetics was marginal in BUL rats. On the other hand, we found that the blood concentrations of propranolol following intra-portal infusion in BUL rats were significantly higher than those in control rats.¹⁰⁾ Therefore, the increased bioavailability of propranolol in BUL rats was attributed to diminished hepatic first-pass metabolism. The activity of CYP2D2, which is responsible for the metabolism of propranolol in the rat liver, was not altered by BUL, whereas the NADPH generation rate in the liver cytosolic fraction of BUL rats was lower than that of control rats.^{10,11)} Accordingly, we thought that the decrease in the hepatic metabolic activity and extraction of propranolol in BUL rats is mainly a result of the reduced generation of NADPH in the liver.¹⁰⁾

It is reported that the activity of glucose-6-phosphate dehydrogenase (G-6-PD), which is one of the enzymes generating NADPH in the pentose phosphate pathway, is increased in rats with bile duct ligation (BDL)-induced liver impairment.¹²⁾ However, the mechanism responsible for the decreased NADPH generation in BUL rats is still unclear.¹⁰⁾ In the present study, we compared the activity of enzymes involved in the pentose phosphate pathway in BUL rats with that in BDL rats. In addition, we also investigated the concentration of endogenous substrate(s) for NADPH-generating enzymes in BUL rats.

MATERIALS AND METHODS

Materials Glucose, glucose-6-phosphate (G-6-P), 6-phosphogluconic acid (6-PG), NADP⁺, and NADPH were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade available.

BUL and BDL Rats The animal experiments were per-

* To whom correspondence should be addressed. e-mail: yukiya@ms.toyama-mpu.ac.jp

formed in accordance with the Guidelines for Animal Experiments of Toyama Medical and Pharmaceutical University. Male Wistar rats (230–260 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan), and the rats were housed in a temperature- and humidity-controlled room with free access to water and standard rat chow. BUL was performed according to the method of Giacomini *et al.*,¹³⁾ and the rats were used for experiments 28 h after the operation. Briefly, the abdominal cavity was opened under pentobarbital anesthesia, and a double ligature was placed on each ureter isolated. Similarly, BDL was performed under pentobarbital anesthesia, and the rat was used for experiments 7 d after the operation. Sham-operated rats served as respective controls. In this paper, SHAM (28 h) and SHAM (7 d) represent the control rats for BUL and BDL, respectively.

Preparation of the Hepatic Cytosol Fraction Hepatic cytosol fractions were prepared as previously reported.¹⁰⁾ Briefly, the liver was removed from the rat after 24-h fasting, and homogenized with 4 volumes of ice-cold 1.15% potassium chloride dissolved in 50 μ M phosphate buffer (pH 7.4) using a Potted-Elvehjem homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min, and supernatant was transferred and centrifuged again at 105000 *g* for 60 min. The supernatant after the 105000 *g* centrifugation (SUP) was collected as the hepatic cytosol fraction, and stored at -85°C until used.

Generation of NADPH in the Hepatic Cytosol Fraction The incubation buffer, which consisted of 300 μ l of SUP and 100 μ l of 1.15% potassium chloride dissolved in 50 mM phosphate buffer (pH 7.4), was preincubated for 10 min at 37°C . The reaction was started by adding 50 μ l of an NADP⁺ solution (final concentration, 3 mM) and 50 μ l of a substrate (glucose, G-6-P, or 6-PG) solution, and was allowed to run for 30 min at 37°C . At the end of the reaction, an equal amount of acetonitrile was added to precipitate the protein. The concentration of NADPH generated was assayed spectrophotometrically based on the absorbance at 340 nm.¹⁴⁾

Effect of Endogenous Substrate(s) on NADPH Generation To evaluate the concentration of endogenous substrate(s) in the cytosol fraction of BUL rats, we prepared an ultrafiltrate of SUP (SUP filtrate) by centrifugal ultrafiltration (ULTRAFREE[®]-MC 10000 NMWL filter unit, Millipore Co., MA, U.S.A.). The incubation buffer consisting of 100 μ l of control rat SUP and 300 μ l of SUP filtrate was preincubated for 10 min at 37°C , and the reaction was started by adding 100 μ l of an NADP⁺ solution (final concentration, 3 mM). The reaction was allowed to run for 30 min at 37°C .

Analytical Methods The plasma concentrations of glutamic pyruvic transaminase (GPT), direct bilirubin, and creatinine were measured using a kit purchased from Wako Pure Chemical Industries (Osaka, Japan). The protein levels in the SUP were determined using Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany). The ATP concentration was measured using an ATP assay kit (LL-100-1, Toyo B-Net Co., Tokyo). Briefly, SUP was diluted with a 99-fold volume of ice-cold Krebs-Henseleit bicarbonate buffer before use, and mixed with an identical volume of resolution buffer. After 100 μ l of luciferin–luciferase mixture was added to 10 μ l of the sample mixture, the luminescence was immediately measured for 10 s by using a luminometer (CT-9000D; Dia-Iatron, Tokyo, Japan). ATP levels were calcu-

lated as the value per protein weight in the SUP (nmol/mg protein).

Data Analysis Values are expressed as means $\pm$ S.E. for *n* animals. The statistical difference between mean values was evaluated using a non-paired *t*-test provided that the variances were similar. If this was not the case, the Mann-Whitney *U*-test was applied. *p* values less than 0.05 (two-tailed) were considered to be statistically significant.

RESULTS

Biochemical Parameters in BDL and BUL Rats Table 1 shows the biochemical parameters in BDL and BUL rats. The plasma GPT and direct bilirubin concentrations were significantly increased in BDL rats as compared with SHAM (7 d) rats. The plasma creatinine concentration was significantly higher in BUL rats than SHAM (28 h) rats.

NADPH Generation In the Hepatic Cytosol Fraction The protein concentration in the SUP of BDL rats (14.9 ± 0.3 mg protein/ml) was significantly ($p < 0.01$) lower than that of SHAM (7 d) rats (19.8 ± 0.4 mg protein/ml). On the other hand, the protein concentration in the SUP of BUL rats (18.4 ± 0.1 mg protein/ml) was similar to that of SHAM (28 h) rats (17.6 ± 0.4 mg protein/ml). Table 2 shows the rate at which NADPH was generated in the SUP of BDL and BUL rats at 30 min after the addition of 10 mM glucose and 3 mM NADP⁺. The rate for BDL rats was about 2-fold higher than that for SHAM (7 d) rats. In contrast, the rate of generation in the SUP of BUL rats was significantly lower than that of SHAM (28 h) rats, which was consistent with our previous finding.¹⁰⁾ We had hypothesized that the reduced rate in BUL rats is due to the decreased activity of glucokinase, which requires ATP and NADP⁺ to generate NADPH.¹⁰⁾ In the present study, we therefore examined the concentration of ATP in the SUP of BUL rats. The ATP concentration in the SUP of BUL and SHAM (28 h) rats was 25.7 ± 0.3 and 6.4 ± 0.3 pmol/mg protein, respectively. Since the amount of ATP in the SUP was much smaller than the concentration of

Table 1. Biochemical Parameters of Plasma Obtained from BDL and BUL Rats

	SHAM (7 d)	BDL	SHAM (28 h)	BUL
GPT (IU/l)	8.27 ± 0.45	$25.1 \pm 3.4^{**}$	13.4 ± 0.6	14.3 ± 2.3
Direct bilirubin (mg/dl)	0.029 ± 0.014	$5.04 \pm 1.14^{**}$	0.046 ± 0.022	0.094 ± 0.043
Creatinine (mg/dl)	0.655 ± 0.029	0.774 ± 0.046	0.734 ± 0.021	$4.14 \pm 0.12^{**}$

Values are expressed as the mean $\pm$ S.E. ($n=5-6$). $^{**}p < 0.01$ compared with SHAM rats.

Table 2. NADPH Generation in SUP of BDL and BUL Rats after the Addition of Glucose and/or NADP⁺

Addition	NADPH generation (nmol/30 min/mg protein)			
	SHAM (7 d)	BDL	SHAM (28 h)	BUL
Glucose and NADP ⁺	54.3 ± 1.4	$102 \pm 1^{**}$	55.8 ± 1.0	$31.7 \pm 1.2^{**}$
NADP ⁺ alone	45.1 ± 0.4	$88.8 \pm 0.9^{**}$	46.3 ± 1.0	$25.0 \pm 1.0^{**}$

Values are expressed as the mean $\pm$ S.E. ($n=6$). $^{**}p < 0.01$ compared with SHAM rats.

NADPH after the addition of glucose and NADP^+ (Table 2), we thought that glucose added to SUP was used little in the generation of NADPH, and that the endogenous substrate(s) was utilized to generate NADPH in the experimental conditions used. We, therefore, examined the generation of NADPH in the SUP of BDL and BUL rats after the addition of NADP^+ alone (Table 2). The NADPH concentration in both BDL and BUL rats was similar to that after the addition of glucose and NADP^+ (Table 2). These results indicated that the rate at which NADPH was generated from endogenous substrate(s) and added NADP^+ was decreased in BUL rats, as compared with SHAM (28 h) rats.

Enzymatic Activities in Pentose Phosphate Pathway It is reported that the activity of G-6-PD, which is one of the enzymes generating NADPH in the pentose phosphate pathway, is increased in rats with BDL-induced liver impairment.¹²⁾ To examine the mechanism of the altered NADPH generation in BUL rats, we compared the activity of enzymes involved in the pentose phosphate pathway in BUL rats with that in BDL rats. Figure 1 shows the increase in NADPH generation in the SUP of BDL and BUL rats after the addition of 0.05–1 mM G-6-P and 3 mM NADP^+ . The increase was greater in the BDL rats than SHAM (7 d) rats. The increases in NADPH generation in BUL rats at the G-6-P concentrations of 0.5 and 1 mM were statistically different from those in SHAM (28 h) rats, because the variances of the measurement were small. However, there was only a marginal difference in the mean increases in the NADPH generation between BUL and SHAM (28 h) rats (Fig. 1). In addition, the increase in NADPH generation in the SUP of BDL rats after the addition of 0.05–1 mM 6-PG and 3 mM NADP^+ was also greater than that in the SUP of SHAM (7 d) rats, whereas that in the SUP of BUL rats was similar to that in the SUP of SHAM (28 h) rats (data not shown). These findings indicated that the enzymatic activity of the pentose phosphate pathway was not altered markedly in BUL rats, as compared with SHAM (28 h) rats.

Concentration of Endogenous Substrate(s) for Pentose Phosphate Pathway To elucidate the concentration of endogenous substrate(s) in the BUL rats, we evaluated the effect of the SUP filtrate on the generation of NADPH in the SUP. As shown in Fig. 2, the addition of the SUP filtrate of BUL rats to the reaction mixture resulted in an increase in the generation, but its effect in BUL rats was significantly less than that in SHAM (28 h) rats. These results indicated that concentration of the endogenous substrate(s) and/or inhibitor(s) for the pentose phosphate pathway was altered in BUL rats, as compared with SHAM (28 h) rats.

DISCUSSION

The aim of the present study was to evaluate the mechanisms responsible for the reduced generation of NADPH in the supernatant of liver homogenates (SUP) obtained from rats with BUL. The rate at which NADPH was generated in the SUP after the addition of NADP^+ alone was increased in BDL rats, while it was decreased in BUL rats as compared with SHAM rats (Table 2). After the addition of G-6-P or 6-PG, the increase in NADPH in BDL rats was greater than that in SHAM (7 d) rats, whereas the increase in BUL rats was similar to that in SHAM (28 h) rats (Fig. 1). The increase

Fig. 1. Increase in NADPH Generation in SUP of BDL (A) or BUL (B) Rats after the Addition of G-6-P and NADP^+

Each symbol and bar represents the mean $\pm$ S.E. ($n=3$) BDL/BUL (●) and SHAM (○) rats. * $p<0.05$, ** $p<0.01$ compared with SHAM rats.

Fig. 2. NADPH Generation in SUP of SHAM Rats after the Addition of BUL and SHAM (28 h) SUP Filtrate

Each column and bar represents the mean $\pm$ S.E. ($n=6$) ** $p<0.01$ compared with SHAM SUP filtrate.

in the NADPH generation after the addition of the SUP filtrate of BUL rats was smaller than that after the addition of the SUP filtrate of SHAM (28 h) rats (Fig. 2). These findings suggest that the increased NADPH generation in BDL rats was due to the increased enzymatic activities in the pentose phosphate pathway, whereas the decrease in the NADPH generation in BUL rats was mainly caused by the decreased concentration of endogenous substrate(s) and/or the increased concentration of endogenous inhibitor(s) for the pentose phosphate pathway.

Kurose reported that the activity of G-6-PD is increased in rats with BDL-induced liver impairment.¹²⁾ Sanz *et al.* reported that an induction of mRNA expression and increase in G-6-PD activity were observed in rats with cirrhosis induced by long-term thioacetamide administration.¹⁵⁾ In addition, G-6-PD activity was increased in rats with liver injury produced by dimethylnitrosamine.¹⁶⁾ G-6-P is converted to 6-phosphoglucono- δ -lactone by G-6-PD, whereas 6-PG is converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (6-PGD). These reactions accompany the generation of NADPH from NADP^+ . In the present study, we found that the increase in the generation of NADPH in the SUP after the addition of 6-PG as well as G-6-P was accentuated in BDL rats as compared with SHAM (7 d) rats. The present finding suggested that the rate at which NADPH was generated from endogenous substrate(s) in the BDL rat SUP was much higher than that in the SHAM (7 d) rat SUP (Table 2), since the activity of not only G-6-PD but also 6-PGD was increased in BDL rats as compared with SHAM (7 d) rats.

Shafir *et al.* reported that the activity of G-6-PD was decreased in aminonucleoside-induced nephrotic rats.¹⁷⁾ In contrast, Szolkiewicz *et al.* reported increased enzymatic activity of G-6-PD in 5/6-nephrectomized rats.¹⁸⁾ Previously, we reported that the activity of G-6-PD was not altered significantly in BUL rats.¹⁰⁾ The present finding that the increase in NADPH generation in the BUL rat SUP after the addition G-6-P and NADP⁺ was similar to that in SHAM (28 h) rat SUP (Fig. 1), is consistent with our previous findings. In the present study, the increase in NADPH generation in the BUL rat SUP after the addition of 6-PG and NADP⁺ was similar to that in SHAM (28 h) rat SUP, suggesting that the activity of 6-PGD in BUL rat SUP was not decreased markedly. In addition, although the drug-metabolizing activity of CYP2D2 in the presence of NADPH in liver microsomes of BUL rats is not significantly different from that of normal rats, the CYP2D2 activity is highly dependent on the concentration of NADPH.^{10,11)} Therefore, although the precise biochemical mechanism responsible for the decreased concentration of endogenous substrate(s) for the pentose phosphate pathway (or the chemical structure of the endogenous inhibitor) in BUL rat SUP is still unclear, the decreased rate of generation of NADPH in the liver seemed to be one of the most plausible explanations for the reduced hepatic extraction of propranolol in BUL rats.¹⁰⁾

Another possible explanation for the diminished hepatic extraction of drugs is the presence of uremic toxins during renal failure.¹⁹⁾ Several reports have suggested possible effects of endogenous uremic substance(s) on hepatic drug metabolizing activity. Yoshitani *et al.* reported that the unbound clearance of losartan in rat hepatic microsomes in the presence of 10% uremic serum obtained from uranyl nitrate-treated and BUL rats is significantly lower than that in the presence of control serum.²⁰⁾ Terao and Shen also reported that some endogenous substance(s) in the blood of the uranyl nitrate-induced uremic rat is capable of inhibiting the hepatic extraction of propranolol in perfused liver, although it is not clear whether endogenous uremic substances inhibit P450 activity directly.⁶⁾ Further systematic studies may be necessary to determine the inhibitory effect of endogenous uremic substances on drug metabolizing activity.¹⁰⁾

In conclusion, the decreased concentration of endogenous substrate(s) and/or the increased concentration of endogenous inhibitor(s) for the pentose phosphate pathway in BUL

rats leads to a decrease in the generation of NADPH in the liver, which may be at least partly responsible for the decreased hepatic drug metabolism. This may provide new insight into the altered bioavailability of drugs during renal failure.

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