P-Glycoprotein-Mediated Drug Secretion in Mouse Proximal Tubule Perfused In Vitro

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Abstract. To examine the functional significance of drug-transporting P-glycoprotein (P-gp), studies were conducted in the isolated and perfused proximal tubule S2 segment from mice disrupting both mdr1a and mdr1b genes [mdr1a/1b(−)(−)] and their wild-type mice. Efflux of the intracellular fluorescence of rhodamine 123, a fluorescence substrate of P-gp, into the lumen was measured, and the decay half-time of the intracellular fluorescence of rhodamine 123, a fluorescence substrate of P-gp, the present study was increased to 434 ± 41 s by the addition of luminal verapamil, a P-gp inhibitor. In the mdr1a/1b(−)(−) mice, the T1/2 was 407 ± 16 s (n = 10) at the basal period and was no longer affected by the luminal addition of verapamil. The digoxin content in the kidney after a repeated administration of the drug was markedly elevated in the mdr1a/1b(−)(−) mice. In conclusion, P-gp-mediated drug efflux capacity indeed exists in the apical membrane of proximal tubule cells from the wild-type mice, whereas it is absent in that of the mdr1a/1b(−)(−) mice.

P-glycoprotein (P-gp) was initially identified through its ability to confer multidrug resistance (MDR) in mammalian tumor cells (1). P-gp is a member of the ATP-binding cassette superfamily of transporters (1) and uses ATP to pump hydrophobic drugs out of the cells, decreasing their intracellular concentrations and hence their toxicity. Humans have one drug-transporting P-gp (MDR1), whereas mice have two genes encoding drug-transporting P-gp, mdr1a (also called mdr3) and mdr1b (also called mdr1) (1–3). The mouse mdr1a gene is predominantly expressed in intestine, liver, and blood capillaries of brain and testis, whereas the mdr1b gene is predominantly expressed in adrenal glands, placenta, and ovaries (1). Similar levels of mdr1a and mdr1b expression are observed in the kidney (4,5). The mdr1a and mdr1b in the mouse together fulfill the same function as MDR1 in humans.

The expression of P-gp in the kidney is thought to occur exclusively in the apical membrane of the proximal tubule epithelium, where P-gp was expected to participate in the excretion of xenobiotics (6,7). Consistent with a role for P-gp as an excretory transporter, cell lines that express many properties of proximal tubule cells, including LLC-PK1 cells (8–10) and opossum kidney (OK) cells (11), primary cultures of flounder proximal tubule epithelial cells (12), cultured mouse proximal tubule cells (13), and intact killifish proximal tubules (12), exhibit net secretion of several MDR substrates, including vinblastine, verapamil, cyclosporin A, digoxin, and daunomycin. However, it is not known whether drug-transporting P-gp is functionally expressed in intact mammalian proximal tubule cells.

Drug metabolism in mammals is a complex process that involves parallel pathways that overlap with drug specificities. Although these can often be distinguished by the use of inhibitors, few inhibitors act on a single target. To resolve the problems, Schinkel et al. (3,5,14–16) recently generated mice with a genetic disruption of the genes, including the mdr1a and mdr1a/1b, and examined the physiologic and pharmacologic roles of these genes. However, no essential function of P-gp in the excretion of xenobiotics has been demonstrated in the kidney. The mice that lack the mdr1a gene [mdr1a(−) mice] have a normal lifespan when they are kept in the laboratory environment and are not challenged with P-gp–transported drugs (3). The mice that lack both mdr1a and mdr1b genes [mdr1a/1b(−)(−) mice] also have the same phenotype as their wild-type counterparts (16). However, the drug accumulation in the kidney of mdr1a(−) mice was identical to that of the wild-type mice (14,16), and mdr1b mRNA was expressed with a greater extent than that of their wild-type mice (5), whereas drug accumulation in the kidney of mdr1a/1b(−)(−) mice was greater than that of the wild-type mice (3). These findings suggest the possibility that the mdr1b gene but not the mdr1a gene may be important for P-gp–mediated drug excretion in the kidney. However, there is no direct evidence of this possibility. Therefore, the present study used mdr1a/1b(−)(−) mice. In combination with in vitro microperfusion technique and measurements of intracellular fluorescence of rhodamine 123 (R-123), a fluorescence substrate of P-gp, the present study was
therefore designed to examine whether drug-transporting P-gp is functionally expressed in the isolated perfused proximal tubule S2 segment from the wild-type and mdr1a/1b(−)(−) mice.

Materials and Methods

Animals

The mdr1a/1b(−)(−) mice used in this study were originally described by Schinkel et al. (3). Male mdr1a/1b(−)(−) mice and FVB (wild-type) mice served as background (body weight, 25 to 40 g) and were purchased from Taconic Engineering (Germantown, NY). These animals were maintained under a controlled environment and had free access to a standard rodent chow and tap water ad libitum until the experiments. Ages of the knockout animals were matched with their wild-type controls.

In Vitro Microperfusion

The mice from both groups were anesthetized with intraperitoneal injection of pentobarbital sodium (4 mg/100 g body wt), and both kidneys were removed. Slices of 1 to 2 mm were taken from the coronal section of each kidney and transferred to a chilled dish containing modified Collins’ solution having the following composition: 14 mM KH$_2$PO$_4$, 14 mM K$_2$HPO$_4$, 15 mM KCl, 9 mM NaHCO$_3$, and 160 mM sucrose (pH 7.4). Proximal tubule S2 segments were then dissected by fine forceps under a stereomicroscope and transferred to a bath chamber mounted on an inverted microscope (IMT-2, Olympus, Tokyo, Japan). Each tubule was perfused in vitro according to the method of Burg et al. (17) with slight modifications in our laboratory (18–20). The details of the technique have been published previously (18–20); accordingly, they will be presented here only briefly. After suspending the tubules between the two pipettes, the lumen was perfused at 10 to 20 nl/min by changing the height of the reservoir connected to the back end of the perfusion pipette. A system of the flowing-through bath was used to permit rapid exchange of the bathing fluid. The flow rate of the bathing fluid was in a range of 5 to 10 ml/min. A water jacket was used to maintain the bath temperature at 37°C. The composition of the control luminal and bathing solution used in the study was as follows: 110 mM NaCl, 5 mM KCl, 25 mM NaHCO$_3$, 0.8 mM Na$_2$HPO$_4$, 0.2 mM NaH$_2$PO$_4$, 10 mM Na-acetate, 1.6 mM CaCl$_2$, 1.0 mM MgCl$_2$, 8.3 mM glucose, and 5 mM alanine. The control solution had an osmolality between 285 and 295 mOsm/kg H$_2$O and was equilibrated with 95% O$_2$/5% CO$_2$ and 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 8.3 mM glucose, and 5 mM alanine.

Measurement of Efflux of P-gp Substrate

P-gp–mediated drug transport was assessed by measuring the efflux of R-123, (13,21). After the tubules were hooked up to the perfusion system, they were perfused with the control solution. After a 30-min equilibration period, the tubules were perfused with the control solution containing 10 μM R-123 at 37°C for 15 to 20 min, and the measurement of intracellular R-123 fluorescence was started. Luminal perfusate was then rapidly changed to the control solution without R-123. In the preliminary study, no reduction in the intracellular R-123 fluorescence signal was detected until R-123 was removed from the lumen. Intracellular fluorescence signal of the dye from a focused single cell was monitored by microscopic fluorescence photometry system (OSP-3, Olympus, Tokyo, Japan) 100 times per minute. Perfused tubules were positioned at the bottom of the bath chamber as closely as possible and were viewed with a 40× fluorescence objective. The diameter of the beam of light focused on the single tubule cell was 8.5 μm. The light source was a 75 W xenon lamp (18). The R-123 fluorescence was excited at 490 nm, and fluorescence emission was measured at 530 nm. To minimize dye bleaching and cell damage, protocols were made as short as possible. The decay of the intracellular R-123 fluorescence upon rapid removal of the dye was followed as described previously (13,21). Because there is R-123 mitochondrial accumulation and quenching at high concentrations, decay of the dye was determined at the linear range of the fluorescence-concentration relationship, approximately 50% of the maximal intracellular fluorescence (13,21). The apparent rate constants for R-123 decay (k) were obtained by using PH/EDISIM® (Mediware, Amsterdam, the Netherlands), a computer program originating from pharmacokinetic analysis, to fit the data to the following equation (13,21)

\[ F_{R-123} = F_0 + F_{max}e^{-kt} \]

where $F_{R-123}$ is the intracellular fluorescence, $F_0$ is the background fluorescence, and $F_{max}$ is the R-123 intracellular fluorescence at $t = 0$.

Determination of Digoxin Concentration in Serum and Kidney from Wild-Type and mdr1a/1b(−)(−) Mice

For this purpose, the mice from both groups at 14 to 20 wk old were used. A 2.0-mg/ml stock solution of digoxin was prepared with a 0.1-M glucose solution containing 2.6 M propylene glycol and 8.7 M ethanol. It was then diluted with a 0.3-M glucose solution to make a final concentration of 0.05 mg/ml for intraperitoneal injection. Digoxin (20 μg/100 g body wt) was intraperitoneally injected once a day into the mice for 7 d. Twenty-four h after the final dosing, the animals were anesthetized with intraperitoneal pentobarbital sodium (5 mg/100 g body wt). Blood samples were then collected from the inferior vena cava. Both kidneys were removed after blood had been flushed out with saline and were weighed and homogenized with distilled water for assay of digoxin content. The digoxin concentrations in serum and tissue homogenate were measured by an automatic fluorescence polarization immunoassay (TDx autoanalyzer, Dinabot Co., Ltd., Tokyo, Japan) (22).

Chemicals

R-123 was purchased from Molecular Probe (Eugene, OR). Others were obtained from Sigma (St. Louis, MO) with highest grade. Verapamil was dissolved in DMSO at 1 mg/ml final concentration. Tetraethylammonium (TEA) was dissolved in distilled water at 1 mg/ml final concentration. Equivalent concentrations of vehicle were added as a control for individual protocols.

Statistical Analyses

All of the data are expressed as the mean ± SEM. Comparisons were performed by $t$ test, one-way ANOVA in combination with Fisher’s protected least significant difference, and Mann-Whitney $U$ test, as needed. $P < 0.05$ was regarded as significant.

Results

Efflux of the Intracellular R-123 Fluorescence in the Proximal Tubule S2 Segment from the Wild-Type Mice

First, we measured intracellular fluorescence signal of R-123 as a function of time in proximal tubule cells from the wild-type mice. A representative record of its intracellular fluorescence is shown in Figure 1A. Results from the efflux experiments are summarized in Figure 2. There was no change in intracellular fluorescence signal before the dye was washed out from the lumen. However, upon rapidly removing the dye
from the lumen, the decay of the intracellular fluorescence was observed and followed a single exponential function of time, as described previously (13,21). The calculated T1/2 was 34 ± 6 s (n = 36), a value compatible with that previously reported in mouse proximal tubule cell line expressing P-gp (13). The T1/2 was significantly (P < 0.001) prolonged to 434 ± 64 s (n = 11) by the addition of luminal verapamil (5 μM), an inhibitor of P-gp, and was completely reversible after removal of the drug. Also, the apparent rate constants were significantly (P < 0.001) decreased from 1.22 ± 0.25 to 0.09 ± 0.03 min⁻¹ (n = 11) after the addition of verapamil. Conversely, the T1/2 was not altered at all by the addition of luminal TEA (1 mM), a model substrate for renal organic cation transport system (Figures 1B and 2). The longer exposure to TEA also caused no effect on T1/2 (data not shown). These findings indicate that R-123 is indeed extruded from the cell into the lumen via P-gp.

**Efflux of the Intracellular R-123 Fluorescence in the Proximal Tubule S2 Segment from the mdr1a/1b(-/-) Mice**

Next, we examined whether the P-gp–mediated drug efflux is present in the proximal tubule cell from mdr1a/1b(-/-) mice. A representative record of intracellular R-123 fluorescence is shown in Figure 3A. Results from the efflux experiments are summarized in Figure 3B. The T1/2 at the basal period was 407 ± 6 s (n = 10), a value significantly (P < 0.001) greater than that of the wild-type mice. The apparent rate constants (0.11 ± 0.03 min⁻¹, n = 10) were significantly (P < 0.001) smaller than those of the wild-type mice (1.22 ± 0.03 min⁻¹, n = 11). In sharp contrast to the wild-type mice, the T1/2 was no longer affected by the addition of luminal verapamil (5 μM; Figure 3). Also, in the knockout mice, the apparent rate constants (from 0.11 ± 0.03 to 0.09 ± 0.03 min⁻¹, n = 10) were unchanged after the addition of verapamil. The values of T1/2 and the apparent rate constants seen after the addition of verapamil in the wild-type mice were similar to those of the knockout mice, indicating that the mdr1a/1b(-/-) mice never possess drug-transporting P-gp activity at the apical membrane of proximal tubule cells.

**Digoxin Content in the Kidney from Knockout and Their Wild-Type Mice**

Finally, we examined whether the digoxin content in the kidney from mdr1a/1b(-/-) mice is actually affected, be-
cause digoxin, a model of P-gp substrate drug, is excreted from kidneys. Experiments were performed in five wild-type and mdr1a/1b\((2)\)(2) mice. Average body and kidney weights, respectively, were 28.0 ± 0.7 g and 424 ± 31 mg in wild-type mice and 26.2 ± 0.8 g and 439 ± 29 mg in mdr1a/1b\((2)\)(2) mice, values not significantly different between the genotypes.

As shown in Figure 4, the kidney digoxin content was significantly higher in the mdr1a/1b\((2)\)(2) mice (12.3 ± 7.4 pg/mg kidney, \(n = 5\), \(P < 0.001\)) than in their wild-type mice (0.4 ± 0.3 pg/mg kidney, \(n = 5\)). These findings confirm a previous report (3). Conversely, serum digoxin concentrations in the knockout mice (4.52 ± 0.76 ng/ml, \(n = 5\), \(P = 0.136\)) were slightly higher but not significantly different from those of the wild-type mice (2.15 ± 1.21 ng/ml, \(n = 5\)).

**Discussion**

In combination with in vitro microperfusion techniques and measurements of intracellular R-123 fluorescence, the present study examined whether drug-transporting P-gp is actually expressed in the apical membrane of proximal tubule cells from wild-type and mdr1a/1b\((2)\)(2) mice. As shown in Figure 1, upon rapid removal of the dye from the lumen, the decay of the intracellular R-123 fluorescence was observed and followed a single exponential function of time. Conversely, the intracellular R-123 fluorescence was unchanged before the dye was washed out from the lumen. Also, when the dye was rapidly removed from the lumen, the decay of the intracellular fluorescence was observed in the wild-type mice (see Figure 1), whereas it was not found in the knockout mice (see Figure 3). Therefore, the decay of the intracellular R-123 fluorescence is not due to photobleaching but due to the drug-transporting P-gp activity. Our data demonstrate that the P-gp–mediated drug efflux mechanism indeed exists in the apical membrane of proximal tubule cells from the wild-type mice but lacks in that of the mdr1a/1b\((2)\)(2) mice. This is the first study to demonstrate that disruption of both mdr1a and mdr1b genes causes lack of drug-transporting P-gp activity in the proximal tubule cell.

In mdr1a\((2)\) mice, the cumulative urinary excretion of the P-gp substrate digoxin was increased compared with their wild-type mice (14). This could be due to the activity of other transporters, e.g., mdr1b P-gp, for which the mRNA levels were increased in the mice with disrupted mdr1a gene (5). In sharp contrast to the mdr1a\((2)\) mice, in the mdr1a/1b\((2)\)(2) mice, the kidney digoxin content was significantly increased compared with that of the wild-type mice (see Figure 4) (3). In the proximal tubule from the mdr1a/1b\((2)\)(2) mice, the decay half-time of intracellular R-123 was markedly increased and the apparent rate constants were markedly decreased, and the increased decay half-time or the decreased apparent rate constants were no longer affected by the addition of luminal verapamil. These results indicate that in the proximal tubule...
from the mdr1a/1b(−)(−) mice, the P-gp-mediated drug efflux capacity is completely lost and other transporters do not compensate for the absence of mdr1a/1b P-gp. Taken together with our findings and other reports, the mdr1b gene but not the mdr1a gene could play an important role in the maintenance of drug-transporting P-gp activity in the proximal tubule.

In conclusion, we clearly demonstrated that drug-transporting P-gp activity is actually expressed in the apical membrane of the proximal tubule from the wild-type mice, whereas it abolishes in that of the mdr1a/1b(−)(−) mice. These findings will be helpful to get a better understanding of the physiologic role of P-gp on the xenobiotic efflux in normal kidney epithelial cells.

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