Determination of the Serum Unbound Fraction of Tadalafil in Children with Protein-Losing Enteropathy and Its Specific Binding to Human Serum Proteins in Vitro

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The purpose of this study was to determine the serum protein binding of tadalafil in children with protein-losing enteropathy (PLE) and to evaluate the specific binding of the drug to human serum-derived proteins in vitro. Seventeen serum samples from two PLE patients used after biochemical tests were collected, and the unbound fraction of tadalafil was determined by an ultrafiltration method. The serum albumin concentrations observed in patients #1 and #2 were 2.4–4.2 and 2.9–3.5 g/dL, respectively. The ranges of unbound fraction of tadalafil in patients #1 and #2 were 3.9–13 and 5.0–7.0%, respectively. This suggested that serum albumin was at least a binding carrier for tadalafil because the unbound fraction of tadalafil and serum albumin were slightly correlated. The unbound fraction of tadalafil at the total concentration of 300 ng/mL was negatively dependent on the serum albumin concentration (range: 1.0–5.0 g/dL) in vitro. In the presence of albumin, the additive effect of γ-globulin on the unbound fraction of tadalafil was marginal, but the addition of α1-acid glycoprotein to test samples decreased the unbound fraction of the drug. The decrease in the unbound fraction of tadalafil was greater at low albumin levels (2 g/dL). The addition of lipoprotein to test samples also decreased the unbound fraction of tadalafil, suggesting that lipoprotein was also a binding carrier of the drug. These results suggested that the disposition and/or response to tadalafil in PLE patients was altered by the change in protein bindings of the drug.

Key words: tadalafil; child; protein-losing enteropathy; unbound fraction

INTRODUCTION

Protein-losing enteropathy (PLE) is a life-threatening complication, and PLE patients have a poor prognosis with a 5-year survival of 46–59% and a total mortality rate of 50%.1,2) PLE has been reported in 5 to 13% of patients after Fontan operation, which subsequently to Glenn operation has been applied for the treatment of patients with congenital heart disease such as tricuspid atresia and complex heart malformations.1,3) It is speculated that the elevated systemic venous pressures associated with the Fontan circulation cause intestinal protein loss by increased pressures in the enteric lymphatic system.3) Excessive loss of protein to the gastrointestinal tract leads to hypoalbuminemia, and the serum albumin concentration decreases to 1.8 g/dL (range: 0.8–3.4 g/dL).1,2,4) Hypoalbuminemia is known to change the pharmacokinetics of drugs.5,6) In patients with nephrotic syndrome having albumin concentrations of less than 3 g/dL, for example, the unbound fraction of diphenylhydantoin (DPH) in plasma was significantly increased from a mean of 10.1 to 19.2% with 300 mg DPH, and the plasma clearance of DPH was 0.048 ± 0.019 and 0.022 ± 0.006 L/kg/h in the nephrotic patients and controls, respectively.5) These observations suggest that the maintenance dose of DPH in nephrotic patients should be increased to keep drug concentrations within the therapeutic range. At the same time, however, the risk of incidence of side effect increases with increasing dose, and the Boston Collaborative Drug Surveillance Program reported an increase in neurologic complications, hematologic reactions and rashes by DPH in patients with hypoalbuminemia.5) These studies refocused our attention on the optimal dosage regimen for the patients with hypoalbuminemia caused by PLE.

Tadalafil is a selective and potent phosphodiesterase-5 inhibitor used for the treatment of pulmonary arterial hypertension (PAH). We previously reported that the protein binding rate of tadalafil showed a large interindividual variability (84.6–99.4%) in children with PAH, those who have no hypoalbuminemia.7) In addition, the unbound tadalafil concentrations of the post-dose samples ranged from 2.3 to 56.9 ng/mL in PAH children receiving a mean dose of 0.97 mg/kg/d. Both serum albumin (ALB) and α1-acid glycoprotein (AGP) are known to be carrier proteins that bind to tadalafil.7) In the previous study, however, no effect of ALB and only a marginal effect of AGP on the unbound fraction of tadalafil were observed, probably because the ALB level was in the normal range. In contrast, it has been reported that serum components other than ALB and AGP, such as γ-globulin (GLB) and lipoprotein (LPP), are involved in the serum protein bindings of several drugs.8–11) We hypothesized that GLB and/or LPP are involved in the serum protein binding of tadalafil.

The purpose of this study was to determine the serum protein binding of tadalafil in routinely-treated children with PLE. In addition, the binding property of tadalafil to carrier proteins was evaluated in vitro.

MATERIALS AND METHODS

Materials Tadalafil was purchased from Toronto Research Institute, Toronto, Canada, and the serum samples were collected from two children with protein-losing enteropathy. Four healthy children were selected as controls. Tadalafil was added to the test samples at a total concentration of 300 ng/mL, and the unbound fraction of tadalafil was determined by an ultrafiltration method. The serum albumin, AGP, γ-globulin (GLB), and LPP were determined using the immunonephelometric method. The serum proteins were also determined using an immunofixation method. The serum albumin and lipoprotein concentrations were slightly correlated. The unbound fraction of tadalafil at the total concentration of 300 ng/mL was negatively dependent on the serum albumin concentration (range: 1.0–5.0 g/dL) in vitro. In the presence of albumin, the additive effect of γ-globulin on the unbound fraction of tadalafil was marginal, but the addition of α1-acid glycoprotein to test samples decreased the unbound fraction of the drug. The decrease in the unbound fraction of tadalafil was greater at low albumin levels (2 g/dL). The addition of lipoprotein to test samples also decreased the unbound fraction of tadalafil, suggesting that lipoprotein was also a binding carrier of the drug. These results suggested that the disposition and/or response to tadalafil in PLE patients was altered by the change in protein bindings of the drug.

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Chemicals (North York, ON, Canada). Human serum albumin was purchased from Nacalai Tesque (Kyoto, Japan). α1-Acid glycoprotein from human plasma and human γ-globulin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipoprotein was isolated from normal pooled human serum by ultracentrifugation, as described by Haberbosch et al. with minor modification as follows: 5 mL of human serum (BJ7001A, Cosmobio KOJ, Tokyo, Japan) was mixed with 1.5 mL of 0.15 M NaCl and centrifuged for 18 h at 50000 rpm using a Beckman 70.1 Ti rotor. After removing the upper fraction, the residual solution was adjusted to a density of 1.210 g/mL with KBr and centrifuged for 48 h at 65000 rpm. The upper fraction including lipoproteins was then collected. After dialysis against 0.9% NaCl including 0.01% Na2 ethylenediaminetetraacetic acid (EDTA), 10⁻³ M NaN3 at pH 7.4 at 4°C, the samples were stored at −30°C until use. The fractions were analyzed by LipoSEARCH (Skylight Biotech Inc., Akita, Japan), and the total lipoprotein concentration was 102.10 mg/dL (CM: 1.23, very low density lipoprotein (VLDL): 18.64, low density lipoprotein (LDL): 39.73, high density lipoprotein (HDL): 42.50 mg/Lipoprotein/dL). All other chemicals and solvents were of the highest purity available.

**Serum Samples** We recruited patients with PLE who were hospitalized at the University of Toyama Hospital between October 2016 and February 2017. The patients were routinely treated with oral administration of tadalafil (Adcirca tablet; Nippon-shinyaku Co., Ltd., Kyoto, Japan), and the dose was then evaporated to dryness with a SpeedVac system (Savant, Farmingdale, NY, U.S.A.). The residue was dissolved in 200 µL mobile phase, and 50 µL was injected onto the column. The flow rate of the mobile phase was 1.0 mL/min, and the column temperature was 40°C. Peaks were monitored at an excitation wavelength of 275 nm and an emission wavelength of 335 nm, and the retention time was 8.0 min for tadalafil. The quantitation limit for tadalafil was 10 ng/mL.

**Data Analysis** Values are expressed as the mean ± standard error (S.E.). The statistical significance of the differences between two groups was evaluated using the Student’s t-test if the variances of the groups were similar. If this was not the case, the Mann–Whitney U-test was applied. p < 0.05 was considered to be statistically significant.

**RESULTS**

In the present study, two pediatric patients and their parents gave written informed consent to use the serum samples remaining after biochemical tests. Patient #1 was a 13-year-old girl who underwent Glenn operation at the age of 7 months, and then underwent Fontan operation at the age of 2 for tricuspidal atresia. Patient #1 was first diagnosed as PLE at the age of 10, and was receiving tadalafil twice a day (10 mg BID). Nine serum samples were available for analysis from her frequent hospitalizations due to recurrent PLE (days 13, 22, 27, 30, 51, 132, 135, 142 and 149) (Fig. 1). The ranges of serum albumin and the unbound fraction of tadalafil were 2.4–4.2 g/dL and 3.9–13%, respectively, revealing that both serum albumin and the unbound fraction of the drug had greatly fluctuated (Fig. 1A). The patient #1 had no significant hepatic and renal impairment, and no inflammatory symptoms were noted, although some part of clinical laboratory data was missing (Fig. 1B). In addition, the unbound concentration of tadalafil was determined between 12.9 and 42.4 ng/mL (Fig. 1B). Patient #2 was an 8-year-old girl who underwent Glenn operation at the age of 2 for single ventricular disorder complicated with complex cardiac malformation. Patient #2 was first diagnosed as PLE at the age of 7, and was receiving tadalafil once a day (15 mg SID). Eight serum samples were available for analysis from hospitalization due to acute
Fig. 1. (A) Concomitant Drugs and the Course of Serum Albumin Concentration and Unbound Fraction of Tadalafil in Patient #1
Open and closed circles represent serum albumin and unbound fraction, respectively. (B) The course of unbound concentration of tadalafil and the corresponding clinical laboratory data in patient #1. a) time after dose of tadalafil (h), b) ALT (U/L), c) AST (U/L), d) BUN (mg/dL), e) CRP (mg/dL), f) AGP (mg/dL), and g) total cholesterol (mg/dL). NA; not available.

Fig. 2. (A) Concomitant Drugs and the Course of Serum Albumin Concentration and Unbound Fraction of Tadalafil in Patient #2
Open and closed circles represent serum albumin and unbound fraction, respectively. (B) The course of unbound concentration of tadalafil and the corresponding clinical laboratory data in patient #1. a) time after dose of tadalafil (h), b) ALT (U/L), c) AST (U/L), d) BUN (mg/dL), e) CRP (mg/dL), f) AGP (mg/dL), and g) total cholesterol (mg/dL). NA; not available.
gastroenteritis (days 2, 9, 11, 14, 23, 25 and 28) (Fig. 2). The observed ranges of serum albumin and the unbound fraction of tadalafil were 2.9–3.5 g/dL and 5.0–7.0%, respectively, indicating that serum albumin was lower than normal, but the unbound fraction of the drug had been relatively stable compared to patient #1 (Fig. 2A). The patient #2 had no significant hepatic and renal impairment, but persistent inflammation was identified throughout the period of our observation (Fig. 2B). In addition, the unbound concentration of tadalafil was determined between 16.3 and 79.1 ng/mL (Fig. 2B).

Figure 3 shows the relationship between unbound fraction of tadalafil and serum albumin observed in the present study and our previous study. The unbound fraction of tadalafil was slightly correlated with serum albumin in the two PLE patients (closed symbols). In order to understand the effect of hypoproteinemia on the unbound fraction of tadalafil, our previous data from 22 pediatric patients with normal serum albumin (open symbols) were added to Fig. 3. The correlation between the unbound fraction of tadalafil and serum albumin appeared more clearly, when three outlying patients (open triangles) were omitted. The correlation coefficient \( r \) was improved from 0.365 (closed symbols) to 0.551 (closed symbols and open circles). It is impressive to note that the open circle indicated by the arrow is the data of patient #2 at the age of three (5 years ago).

To clarify the carriers involved in the serum protein binding of tadalafil, we performed a protein binding assay of the drug in vitro. First, we confirmed the effect of ALB on the unbound fraction of tadalafil at the total concentration of 300 ng/mL (Fig. 4). The unbound fraction of tadalafil decreased with increasing concentration of ALB; however, the unbound fraction levels observed in vitro were higher than those in serum samples (Figs. 3, 4). These results suggested that other binding carriers were involved in the serum protein binding of tadalafil. We then evaluated the effects of AGP and GLB on the unbound fraction of tadalafil with 5 and 2 g/dL of ALB, which mimic normal conditions and hypoalbinemia, respectively. Considering the quantification limit for tadalafil by HPLC, the tadalafil concentration was set at 500 ng/mL in the following experiments. Each point represents the mean ± standard error (S.E.) for 6 experiments. *\( p < 0.05 \) compared with controls.
of AGP (Figs. 5A, B), indicating that AGP is also involved in the serum protein binding of tadalafil. It is noteworthy that the additive effect of AGP on the unbound fraction of tadalafil was greater in the samples with 2 g/dL of ALB than in those with 5 g/dL of ALB (Figs. 5A, B). In contrast, no effect of GLB was observed (Figs. 5C, D), suggesting that the contribution of GLB to the serum protein binding of tadalafil was negligible. Finally, we evaluated the effects of LPP on the unbound fraction of tadalafil (Fig. 6). The unbound fraction of tadalafil was significantly decreased when LPP was added to the test samples, suggesting that LPP also contributed to the serum protein binding of the drug. Moreover, the additive effect of LPP on the unbound fraction of tadalafil was greater in the samples with 2 g/dL of ALB than in those with 5 g/dL of ALB (Figs. 6A, B). These results suggested that the serum protein binding of tadalafil is susceptible to fluctuation in patients with hypoalbuminemia.

DISCUSSION

The purpose of this study was to determine the serum protein binding of tadalafil in children with PLE and to evaluate the specific binding of the drug to major human serum derived proteins (ALB, AGP, GLB, and LPP). Seventeen serum samples from two PLE patients used after biochemical tests were collected, and the unbound fraction of tadalafil was determined by an ultrafiltration method. The serum albumin concentrations and ranges of the unbound fraction of tadalafil were 2.4–4.2 g/dL and 3.9–13% in patient #1, and 2.9–3.5 g/dL and 5.0–7.0% in patient #2 (Figs. 1, 2). An in vitro study confirmed that the unbound fraction of tadalafil was negatively dependent on the ALB concentration (Fig. 4). The addition of AGP or LPP decreased the unbound fraction of tadalafil more with 2 g/dL of ALB than with 5 g/dL, but there was little effect of GLB on the unbound fraction of the drug (Figs. 5, 6). These results suggested that the disposition and/or response to tadalafil in PLE patients fluctuates with changes in the protein bindings of the drug.

Clinical study on the two PLE patients suggested that not only serum albumin but also AGP could affect the unbound fraction of tadalafil. The plasma protein binding of tadalafil and serum albumin were fluctuated in patient #1, but no other clinically meaningful correlation was detected with her laboratory data (Fig. 1). As compared with the patient #1, the plasma protein binding was stable in patient #2 probably because the change of her serum albumin levels was small. In the case of patient #2, however, persistent inflammatory status associated with an elevated AGP levels (Fig. 2) may also contribute to the stable unbound fraction of the drug. This is supported by an in vitro observation, which unbound fraction of tadalafil was minimized in the presence of 200 mg/dL of AGP (Fig. 5B). On the other hand, concomitant drugs may compete for protein binding sites with tadalafil, because the binding rate of macitentan, beraprost, spironolactone, furosemide, tolvaptan and aspirin to human plasma proteins are reported to be >99,<sup>14</sup> 89,<sup>15</sup> >89,<sup>16</sup> 93,<sup>17</sup> 99,<sup>18</sup> and 80–90%<sup>19</sup> respectively. Further in vitro study determining physicochemical factors susceptible to affect the protein bindings of tadalafil should be warranted to assess the competitive displacement of a drug from serum proteins.

We found LPP to be a binding carrier for tadalafil in vitro at the total tadalafil concentration of 500 ng/mL. (A) ALB conc. was 5 g/dL, (B) ALB conc. was 2 g/dL. Total lipoprotein conc. was 81.68 mg/dL. Each point represents the mean ± standard error (S.E.) for 6 experiments. * p < 0.05 compared with controls.

![Fig. 6. Effect of LPP on the Unbound Fraction of Tadalafil](image)

Total tadalafil concentration was 500 ng/mL. (A) ALB conc. was 5 g/dL, (B) ALB conc. was 2 g/dL. Total lipoprotein conc. was 81.68 mg/dL. Each point represents the mean ± standard error (S.E.) for 6 experiments. * p < 0.05 compared with controls.

Study Limitations First, we did not evaluate each fraction of LPP, and the LPP used in vitro study was crude for simplicity, because LPP sample was unstable during further separation and/or storage. Second, it is difficult to evaluate pharmacokinetics in PLE because the drugs bound to serum proteins may leak into the intestinal tract. Third, failure to

**Bioavailability and Extent of Drug Metabolism by Metabolic Enzymes**

Classifies drugs into four groups according to their water solubility. More than 20% of the drugs, including ticlopidine and amiodarone, were detected mainly in the lipoprotein fraction. Next, they classified these drugs based on the Biopharmaceutics Drug Disposition Classification System (BDDCS), which classifies drugs into four groups according to their water solubility and extent of drug metabolism by metabolic enzymes such as cytochrome P-450. Most lipoprotein-associated drugs are categorized as class 2 drugs, which exhibit low water solubility and extensive metabolism.

**We found LPP to be a binding carrier for tadalafil in vitro at the total tadalafil concentration of 500 ng/mL.** Yamamoto et al.<sup>20</sup> examined whether drugs associate with lipoproteins in vivo. They randomly selected 42 drugs for oral administration to mice, and the lipoprotein-associated drug content was determined by quantifying the drug concentrations in serum lipoprotein fractions collected by size-exclusion chromatography. More than 20% of the drugs, including ticlopidine and amiodarone, were detected mainly in the lipoprotein fraction. Next, they classified these drugs based on the Biopharmaceutics Drug Disposition Classification System (BDDCS), which classifies drugs into four groups according to their water solubility and extent of drug metabolism by metabolic enzymes such as cytochrome P-450.<sup>21</sup> Most lipoprotein-associated drugs are categorized as class 2 drugs, which exhibit low water solubility and extensive metabolism.<sup>20</sup> Benet et al.<sup>21</sup> reported that tadalafil was also classified as class 2.

Lee et al.<sup>22</sup> reported the effects of hyperlipidemia on the pharmacokinetics of tadalafil in rats with experimental hyperlipidemia. They administered tadalafil to control rats and rats with poloxamer-407-induced hyperlipidemia. Hyperlipidemia dramatically increased tadalafil’s total area under the curve from time 0 to infinity after intravenous (2.09 fold) and oral (11.9 fold) administration, and decreased total body clearance (0.537 fold) and apparent volume of distribution at the steady state (0.438 fold) after intravenous administration of tadalafil. The plasma protein binding values of tadalafil were 91.1 and 98.4% for control rats and hyperlipidemia rats, respectively. They suggested that the alterations in the pharmacokinetics of tadalafil observed in hyperlipidemia rats may be attributable to a decrease in the unbound fraction of tadalafil in the plasma.<sup>22</sup> Kanno et al.<sup>23</sup> suggested that a decrease in serum albumin led to increased hepatic lipoprotein synthesis. The observed range of the total cholesterol concentrations was 115–232 mg/dL in patient #1, and 92–144 mg/dL in patient #2, which were generally in the normal range. A large and systematic study is needed to clarify the chronic effect of PLE status on the total cholesterol and each LPP fraction.

**Study Limitations** First, we did not evaluate each fraction of LPP, and the LPP used in vitro study was crude for simplicity, because LPP sample was unstable during further separation and/or storage. Second, it is difficult to evaluate pharmacokinetics in PLE because the drugs bound to serum proteins may leak into the intestinal tract. Third, failure to
unify the blood sampling time for the 17 serum samples may have affected the protein binding rate of tadalafil. Fourth, the number of PLE cases was limited.

In conclusion, we demonstrated variability in the serum albumin concentrations and the unbound fraction of tadalafil in two PLE patients, and found that not only ALB and AGP but also LPP is involved in the serum protein binding of tadalafil, and the additive effect was greater with 2 g/dL of ALB, which is a normal albumin level. As far as we know, this is the first report documenting the alteration of serum protein binding properties of tadalafil in pediatric patients with PLE.

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Conflict of Interest The authors declare no conflict of interest.

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