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Enhancing effect of β-Lactoglobulin on the rate of Cyclosporin absorption

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Abstract

The aim of this study was to determine the effects of β-lactoglobulin (β-LG) on the intestinal absorption of cyclosporin A (CsA) *in vivo* and *in vitro*. After intraduodenal co-administration of CsA (5 mg/kg) with 150 mg/kg β-LG, T_{max} was significantly lower than that of control rats which were given the same dose of CsA without β-LG. C_{max} and AUC were significantly increased as compared with the control while $T_{1/2}$ were not significant. Moreover, a deconvolution method revealed that the rate of CsA absorption was enhanced by β-LG (150 mg/kg) by about 12 times. These results indicate that β-LG significantly enhanced the rate of CsA absorption and the extent of CsA bioavailability in the gut. β-LG did not change the solubility of CsA *in vitro,* but β-LG increased the apical-to-basolateral permeability clearance of CsA (PS_{cell}), with pronounced increase in the permeability of unbound CsA ($PS_{u,cell}$), across the Caco-2 monolayers in a concentration-dependent manner *in vitro*. It was thus considered that β-LG-mediated transport of CsA might be a possible mechanism to enhance the intestinal absorption of CsA *in vivo*.

Keywords: β-lactoglobulin; cyclosporine; Caco-2; permeability; absorption enhancement

Introduction

β-Lactoglobulin (β-LG) is a major whey protein of bovine milk, and has been reported to help absorption of lipophilic vitamins in neonates whose bile acids secretion is not matured. [1] β-LG shows a resistance to acidic pH therefore it has been introduced as a carrier to increase the stability for several compounds and nutrients.[2,3] However, the effect of β-LG on the gastrointestinal absorption of lipophilic drugs has not been examined quantitatively so far. Cyclosporine (CsA) is an immunosuppressive drug, and its oral bioavailability is low due to its poor absorption which is related to water insolubility, P-glycoprotein (P-gp)-mediated efflux, and first-pass metabolism by CYP3A4 in the liver and gut.[4] Some strategies have been proposed to enhance the absorption of CsA by using absorption enhancers including surfactants, fatty acids, or chitosan derivatives to modify cell membrane permeability or modulate gut tight junctions.[5] However, local toxicities of these absorption enhancers on intestinal epithelial cells are the matter of concern, and the development of a safer and effective absorption enhancer for CsA has thus been paid much attention. The aim of this study, therefore, was to determine the effect of β-LG on the intestinal absorption of CsA *in vivo* and estimate the rate of absorption by a deconvolution method. Moreover, the effects of β-LG on the solubility of CsA and the permeability of CsA across the Caco-2 monolayer model were determined *in vitro*.

Materials and Methods

Animals Male Wistar rats weighing from 250 to 280 g were provided by Nihon Ikagaku Doubutsu, Saitama, Japan. The rats were housed under standard food and water *ad libitum* and acclimatized to environmental control (a temperature-controlled facility with a 12-h light/dark cycle) for at least one week before use. All the animal experiments were conducted according to the guidelines for Animal Experimentation in Showa University.

Reagents β-LG was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Caco-2 cells from American type culture collection (Manassas, VA, USA). CsA was supplied by Novartis (Tokyo, Japan), and [mebmt-β-^{3}H]-cyclosporin A ([^{3}H]-CsA) from Amersham Life Science (Buckinghamshire, England). All other reagents used were commercially available and of analytical grade.

Effect of β-LG on intestinal absorption of CsA

The rats were fasted for approximately 12 h with water given ad libitum and then anesthetized by pentobarbital (40 mg/kg). The femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho, Tokyo, Japan) filled with saline and EDTA to facilitate blood sampling. In order to exclude the variability in effect as a result of gastric emptying, β-LG was administered intraduodenally to rats. Polyethylene micro-tubing (PE50) was used for duodenal cannulation according to the method previously

reported.[6] The abdominal cavity of the femoral artery-cannulated rats was opened and the distal part of the duodenum was exposed and clamped gently. The clamped duodenum was punctured using an 18-gauge needle and a cannula was inserted at the punctured site of the duodenum and the purse sutures were tightened around the cannula. The clamp was removed, and the cannula was flushed by physiological saline. The cannulated rat was kept for 18-24 hr in a Bolman cage and the experiment was performed following recovery from anesthesia.

CsA was dissolved in corn oil and given intraduodenally at the dose of 5 mg/kg (4.16 µmol/kg) alone (i.e. control group) or together with β-LG 15 mg/kg (0.81 µmol/kg) or 150 mg/kg (8.13 µmol/kg). At 0.08, 0.25, 0.5, 1, 2, 4, 8, and 12 hr after administration of CsA, blood samples (120µl) were taken into plastic tubes containing EDTA and stored at 4°C until assay. CsA concentrations in whole blood samples were assayed by high-performance liquid chromatography (HPLC), as described later.

Pharmacokinetic Analysis

Pharmacokinetic parameters of CsA, i.e., the area under the concentration-time curve from 0 to infinity (AUC0- ∞), elimination half-life (T1/2), mean residence time (MRT), the maximum blood concentration (Cmax), volume of distribution (Vdss/F), total body clearance (CLtot/F), and time to reach Cmax (Tmax), were calculated by a non-compartmental analysis using WinNonlinTM (version 4.0.1, Pharsight Corporation, Mountain Veiw, CA ,USA).

The intestinal absorption rate of CsA after intraduodenal administration to rats was calculated by a deconvolution method. Blood concentration of CsA after oral CsA administration as a function of time, Cpo(t), is expressed as follows[7]:

$$C_{po}(t) = \int_0^t f(\theta) C_{iv}(t-\theta) d\theta \quad (1)$$

where f(t) and Civ(t) are the absorption rate and the blood concentration, respectively, at time t after intravenous injection of CsA. When the amount of f(θ)Δθ is rapidly injected to the systemic circulation at time θ, blood concentration associated with the pulse is f(θ)ΔθCiv(t-θ) at time t. Equation 1 holds since an absorption rate-time profile is composed of an infinite number of the input pulses. Absorption profiles of CsA were estimated by deconvoluting Cpo(t) with Civ(t) in Eq. 1.[8] Civ(t) used in this analysis was cited from a previous study[9], where the same dose of CsA (5mg/kg) was intravenously administered to rats.

Effects of β-LG on the solubility of CsA

The solubility of CsA was determined according to a method described by Jiko et al. [10] with some modifications. Briefly, CsA (10 mg) was added to 0.5% sodium carboxymethyl cellulose (CMC-Na) solution (5 ml) in the absence or presence of β-LG (30 mg). Samples were centrifuged at 15,000 x g for 20 min. Then, aliquots of supernatant were taken, diluted with saline and filtrated through a microfilter (pore size 0.45µm) before CsA was quantified by HPLC.

Effect of β-LG on permeability of CsA across the Caco-2 monolayer model

Caco-2 cells, seeded at a density of 6 x 104 cells/cm2 on the Transwell® permeable polycarbonate filters (12 mm diameter, 0.4 mm pore size, 1 cm2 growth area) (Coring Costar Corp., Cambridge, MA, USA) were cultured as previously described. [10] The Caco-2 monolayer cultured on a polycarbonate filter was then mounted into the diffusion apparatus (Coring Costar Corp.). The cells and PC membrane faced the donor and receiver chambers nominated as the apical (A) and basolateral (B) sides, respectively. Then, 5 ml of HBSS was pipetted into the A and B sides of the diffusion cells maintained at 37 C, where 95%O2/5%CO2 was bubbled continuously. Transport study was initiated by adding [3H]-CsA (0.5µCi/ml) at various concentrations of β-LG (0, 0.2, 1, 2 and 20 µM) into the A side of the diffusion cells. Aliquots of 100 µl of the incubation mixture were taken from the B chamber at 0, 30, 60, 90, and 120 min and the radioactivity measured by a liquid scintillation counting (LSC-5200, Aloka, Japan).

The amount of [3H]-CsA (Cr) transported to the receiver side (i.e., the B side) was plotted against time t (min), and the apparent permeability clearance of [3H]-CsA across the cells cultured on a PC filter (PStotal) was calculated according to a previous report [11] as follows:

$$PS_{total}(\mu l/\min) = \left(\frac{dC_r}{dt}\right) \times \frac{1}{C_0} \times V \quad (2)$$

where dCr/dt represents the transport rate of [3H]-CsA, calculated by plotting the amount of [3H]-CsA transported against time and determining the slope of the regression line (µCi/ml/min), V is the volume in the receiver chamber (µl) and C0 represents the initial concentration of [3H]-CsA in the donor chamber (µCi/ml).

In another study, Cr in the absence of the cells (i.e., filter only) at various concentrations of β-LG (0, 0.2, 1, and 20 µM) were also plotted against time t (min), and the permeability clearance of [3H]-CsA across the filter without cells (PSfilter) was calculated by Eq. (2).

The transcellular permeability clearance of [3H]-CsA across the Caco-2 cells (PScell) was calculated as follows[11]:

$$PS_{cell}(\mu l/min) = \frac{1}{\frac{1}{PS_{total}} - \frac{1}{PS_{filter}}} = \frac{PS_{total} \cdot PS_{filter}}{PS_{filter} - PS_{total}} \quad (3)$$

The unbound [3H]-CsA in the donor-side medium (fu) was determined as follows:

$$f_u = \frac{PS_{filter(+\beta-LG)}}{PS_{filter(-\beta-LG)}} \quad (4)$$

where PSfilter(+ -LG) and PSfilter(- -LG) represent the permeability clearances of [3H]-CsA across the filter (without Caco2-cells) in the presence and absence of -LG, respectively.
The intrinsic permeability clearance of unbound [3H]-CsA across the Caco-2 cells (PSu,cell) was calculated by Eq.5 as follows:

$$PS_{u,cell}(\mu l/min) = \frac{PS_{cell}}{f_u} \quad (5)$$

HPLC Analysis

CsA concentrations in whole blood samples were assayed by a high-performance liquid chromatographic (HPLC) system, as previously described[12] with minor modifications. Briefly, aliquots of 100 l of whole blood were dispensed in 10 ml glass tubes. The tube was vortexed and 4 ml of diethyl ether was added into tube for drug extraction. The extracts were centrifuged at 3,000 rpm for 5 min. Supernatants was transferred and evaporated using a gentle stream of air. The extract residues were reconstituted using 1 ml of acetonitrile and 0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v) and washed twice with 3 ml of n-hexane. Sample (70 µl) was injected into the HPLC system composed of a dual pump (DP-8020, Tosoh, Tokyo, Japan), a UV detector (UV-8020, Tosoh), a column oven (CO-8000, Tosoh), a degasser (SD 8022, Tosoh), and a digital recorder (Chromatocorder 21, Tosoh). The reversed-phase column used was a Cadenza CD-C18, 4.6 x 100 mm, 3 µm (Imtakt Co., Kyoto, Japan) equipped with a Nova-Pak® C18 guard column, (Waters Corp., Milford, MA, USA). The mobile phase was a mixture of acetonitrile–0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v) and pumped at a flow rate of 1 ml/min. Detection was set at wavelength of 205 nm. The calibration curves were prepared each time in a concentration range from 0.1–1 µg/ml. The correlation coefficients (linearity) were consistently more than 0.95.

Statistical Analysis

Data were expressed as the mean ± standard deviation (S.D.). Comparisons between two groups and among more than three groups were performed using Student's t-test and one-way ANOVA for multiple comparisons among the groups, followed by Dunnett's post hoc test for the individual differences, respectively. Difference was considered to be statistically significant if the probability value was less than 0.05 ($p<0.05$).

Results and Discussion

β-LG is a lipocalin protein family which has a β-barrel of eight anti-parallel β-strands with a hydrophobic interior. β-LG exhibits the ability to bind a variety of compounds including retinol, phospholipids, phenolic compounds, fatty acids, and other small hydrophobic molecules via tight binding inside its hydrophobic calyx. [1]

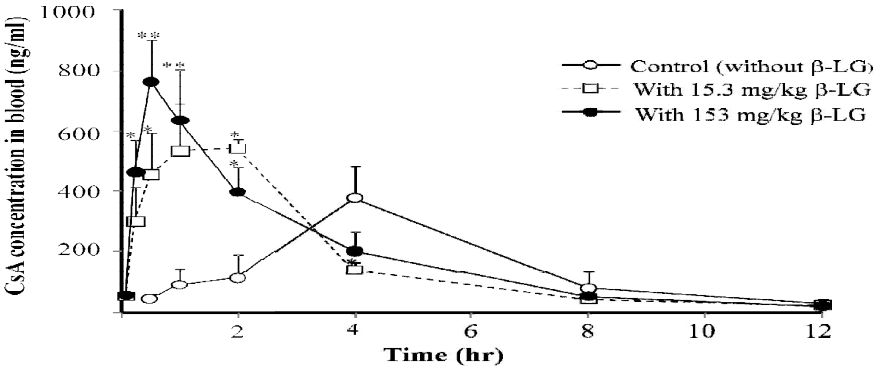

Figure. 1 The blood concentration-time profiles of CsA in rats after intraduodenal administration of CsA with or without β-LG (15 and 150 mg/kg) Each value is expressed as the mean ± S.D. (n=6).
*, ** represent $p<0.05$ and $p<0.01$, respectively as compared with the control group (without β-LG)

Certain compositions of milk such as milk fat globule membranes (MFGM) have been reported to enhance the intestinal absorption of lipophilic drugs including CsA after administered concomitantly. [13] In accordance, our *in vivo* data show that β-LG increased blood concentration of CsA in concentration dependent manner (Fig. 1). Co-administration of 150 mg/kg β-LG significantly increased AUC and C_{max} of CsA by about 30% and 100%, respectively, as compared with control (p<0.05) (Table 1). T_{max} after co-administration of CsA with 15 and 150 mg/kg β-LG was significantly lower than that of control (p<0.05). Moreover, we calculated the intestinal absorption rate by using a deconvolution method using the profiles of the CsA concentrations in blood after intravenous and intraduodenal administrations. As a result, the average rates of CsA absorption were determined to be 9.3 % dose/hr with 15 mg/kg β-LG, 24 % dose/hr with 150 mg/kg β-LG, and 1.9 % dose/hr with the control. Therefore, the rates of CsA absorption in the presence of 15 and 150 mg/kg β-LG were greater than that of control by about 4 and 12 times, respectively. In contrast, $T_{1/2}$ was not significantly different among the β-LG and control groups, indicating that the hepatic clearance of CsA did not change. These results clearly indicate that β-LG enhances the rate and extent of the gastrointestinal CsA absorption.

Table 1 : Pharmacokinetic parameters of CsA after intraduodenal administration in rats

Parameters (unit)	Control (5mg/kg CsA)	Co-administration with 15 mg/kg β-LG	Co-administration with 150 mg/kg β-LG
$T_{1/2}$ (hr)	3.20±1.20	3.20± 0.49	2.55 ± 0.25
C_{max} (ng/ml)	379 ± 208	604 ± 79	763 ± 140*
T_{max} (hr)	4.0 ± 0.0	1.50± 0.58*	0.50 ± 0.0*
AUC(ng/ml·hr)	1753 ± 214	2097 ± 310	2297± 247*
CL_{tot}/F (L/hr/kg)	3.56 ± 1.94	2.43 ± 0.37	2.25 ± 0.49*
V_d/F (L/kg)	19.1 ± 9.60	8.97 ± 1.47	7.39 ± 1.0*
MRT (hr)	5.86 ± 0.38	3.71± 0.44*	3.35 ± 0.50*

Each value is expressed as the mean ± S.D. (n=6). * p<0.05 as compared with the control group (without β-LG).

Very low solubility of CsA in aqueous solution is considered to limit the oral bioavailability of CsA. β-LG has been reported to bind and solubilize several compounds, such as resveratrol, theophylline and sulfamethoxazole. [14,15] We preliminarily expected that β-LG binds CsA and thus may increase the drug solubility. However, we found that β-LG did not actually affect the solubility of CsA, as compared with control (63.2 ± 0.31 *vs* 67.7 ± 11.2 μg/ml in the absence and presence of β-LG, respectively).

Table 2 : The permeability clearances of [^{3}H]-CsA without and with β-LG (0.2, 1 and 20 μM) in Caco-2 cell monolayer model

Group	PS_{filter} (μl/min)	PS_{cell} (μl/min)	f_u	$PS_{u,cell}$ (μl/min) (%of control)
Without β-LG	156.3±19.2	3.80± 1.12	1	3.8 (100%)
0.2μM β-LG	108.2± 10.3	3.45± 0.68	0.69	5.0 (131%)
1 μM β -LG	59.2± 6.4	4.25± 1.02	0.55	7.8 (204%)
20 μM β-LG	15.9± 2.1	7.15± 0.67	0.38	18.5 (486%)

Each value of PS_{filter} and PS_{cell} is expressed as the mean ± S.D. of 3-4 experiments.

f_u and $PS_{u,cell}$ represent the fraction unbound and the intrinsic permeability clearance of unbound [^{3}H]-CsA, respectively, calculated as the mean values by Eq. 4 and 5.

As shown in Table 2, PS_{filter} and PS_{cell} of [^{3}H]-CsA were calculated to be 156.3 ±19.2 and 3.80 ± 1.12 μl/min in the absence of β-LG, respectively, indicating that the cell permeability is the rate-limiting barrier of overall [^{3}H]-CsA transport across the Caco-2 monolayer cultured on a polycarbonate filter. PS_{filter} of [^{3}H]-CsA was extensively decreased in the presence of β-LG in a concentration-dependent manner, indicating that the binding of [^{3}H]-CsA with β-LG resulted in the reduction of unbound fraction of [^{3}H]-CsA in the donor chamber. In contrast, PS_{cell} of [^{3}H]-CsA was significantly

increased in the presence of β-LG in a concentration-dependent manner, indicating that the binding of [^{3}H]-CsA with β-LG enhanced the transcellular transport of [^{3}H]-CsA across the Caco-2 cells. It was also shown that β-LG increased the apical-to-basolateral intrinsic permeability clearance of unbound CsA ($PS_{u,cell}$). Therefore, it was suggested that β-LG binding with CsA may contribute to the accelerated transport of the drug across the Caco-2 cells, probably by efficiently carrying lipophilic CsA molecules across the unstirred water-layer adjacent to the apical side of Caco-2 cells and by rapidly dissociating CsA molecules at the proximity of Caco-2 cell membranes (generally referred to as protein-mediated transport). Thus, β-LG may act as a carrier-protein of CsA, in consistent with a previous report that β-LG enhanced the intestinal absorption of palmitate[16], retinol [17], and fatty acid [17]. Another example of protein-mediated transport can be seen with fatty acids, as Burczynski *et al.*[18] reported that the uptake of [^{3}H]-palmitate by hepatocytes was statistically greater in the presence of chemically modified albumin which has higher pI values, and therefore concluded that the protein-mediated complex does interact with hepatocyte membrane. Moreover, Yi *et al.*[19]recently demonstrated that -carotene-encapsulated β-lactoglobulin–dextran-conjugated nanoparticles are stable under gastric pH conditions and can release -carotene with high permeability to Caco-2 cells; thus, β-LG was suggested to be a good carrier for oral delivery of the very lipophilic vitamin A precursor.

Taken altogether, the present study suggested that β-LG-mediated transport of CsA is a possible mechanism to explain the enhancing effect of β-LG on the intestinal absorption of CsA *in vivo*.

Conclusion

In summary, we provided in vivo and in vitro evidence to show that -LG increases the rate and extent of gastrointestinal absorption of CsA, suggesting that -LG could be used as an oral absorption enhancer for lipophilic drugs.

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