Stereoselective Metabolism of Carvedilol by the β-Naphthoflavone-Inducible Enzyme in Human Intestinal Epithelial Caco-2 Cells

Kazuya ISHIDA, Mutsuko HONDA, Takako SHIMIZU, Masato TAGUCHI, and Yukiya HASHIMOTO*

Graduate School of Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.

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Carvedilol is a β-adrenoceptor antagonist, and has been clinically used to treat chronic heart failure as well as hypertension, angina pectoris, and cardiac arrhythmias.1–4) Carvedilol is highly lipophilic and is absorbed rapidly from the gastrointestinal tract after oral administration. Orally administered carvedilol undergoes stereoselective first-pass metabolism, and the blood concentration of R-enantiomer with very low β-blocking activity is approximately 2-fold higher than that of S-enantiomer with high β-blocking activity.5,6) Both enantiomers are eliminated predominantly by hepatic metabolism, with renal excretion accounting for only 0.3% of the administered dose.7) Carvedilol is metabolized extensively via aliphatic side-chain oxidation, aromatic ring oxidation, and conjugation pathways.8) Oldham and Clarke reported that oxidative activity for carvedilol is observed in cytochrome P450 (CYP) 2D6, 2C9, 3A4, and 1A2.9) In addition, Ohno et al. reported that UDP-glucuronosyltransferase (UGT) 2B7, 2B4, and 1A1 are capable of catalyzing the glucuronidation of carvedilol.10) However, it is still unclear which enzyme is responsible for the stereoselective presystemic clearance of carvedilol. Furthermore, although CYP3A4 and several UGTs are expressed in intestinal epithelial cells, it is also unknown whether the intestine as well as the liver is involved in the first-pass metabolism of carvedilol.

The approach for investigating intestinal drug absorption and metabolism is to use human intestinal cell lines, such as the frequently used line Caco-2. This line spontaneously differentiates, after which it shows enterocyte-like morphology and forms polarized monolayers via tight junctions. It also expresses various transporters and efflux pumps such as P-glycoprotein.11,12) Therefore, it has been utilized to examine the mechanism responsible for the intestinal absorption of various compounds.12,13) In addition, several UGTs, such as UGT1A1, 1A6, and 2B7, are expressed in Caco-2 cells, and the expression of some UGTs is induced by aromatic hydrocarbon receptor (AhR) ligands, such as β-naphthoflavone (β-NF).14,15) Therefore, glucuronidation of drugs in the intestine has been studied using Caco-2 cells.16–18) In contrast, the Caco-2 cell line had not been employed to examine the intestinal metabolism of CYP3A4 substrates due to its lack of CYP3A4 expression. Recently, however, it has been reported that CYP3A4 in Caco-2 cells is induced by 1α,25-dihydroxyvitamin D3 (VD3).19,20) The finding suggests that this cell line may also be utilized to investigate intestinal drug metabolism by CYP3A4. In the present study, we investigated whether carvedilol is metabolized stereoselectively in Caco-2 cells, and also evaluated the changes in the metabolic rate of carvedilol in β-NF- and VD3-treated Caco-2 cells.

Key words  Caco-2 cell; carvedilol; UDP-glucuronosyltransferase; β-naphthoflavone; 1α,25-dihydroxyvitamin D3

MATERIALS AND METHODS

Materials  Carvedilol was kindly supplied by Daiichi Pharmaceutical (Tokyo, Japan). β-NF was obtained from Nacalai Tesque (Kyoto, Japan). VD3 and 3′-azido-3′-deoxythymidine (AZT) were purchased from Wako Pure Chemicals (Osaka, Japan). Baicalein, valproic acid, and mfenamic acid were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

Caco-2 Cell Culture  Caco-2 cells at passage 43 were obtained from the Riken Bioresource Center (Tsukuba, Japan). All experiments were carried out with Caco-2 cells between passages 54 to 64. These cells were maintained by serial passage in plastic dishes with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Valley Biochemical Inc., Winchester, VA, U.S.A.) in an atmosphere of 5% CO2–95% air at 37°C. The medium was changed every second or third day, and when the cells reached 80—90% confluence, they were subcultured using a 0.05% trypsin/0.02% EDTA solution.13) Caco-2 cells were seeded at a density 5 × 104 cells/cm2 on a 9.6 cm2 plastic dish using a Falcon® multiwell™ plate (BD Bioscience, Bedford, MA, U.S.A.), and were maintained for 21 d in order to evaluate the metabolism of carvedilol.

The treatment of Caco-2 cells with VD3 and β-NF was performed as described by Schmedlin-Ren et al. and Paine et al., respectively.19,20) That is, Caco-2 cells were maintained with the culture medium supplemented with 50 μM β-NF for

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the last 3 d of the 21-d culture period. β-NF was dissolved in dimethylsulfoxide (DMSO), and final concentration of DMSO in the culture medium of β-NF-treated and non-treated Caco-2 cells was 0.2%. On the other hand, VD3 was added at a concentration of 250 nm to the culture medium supplemented with 45 nm (−)-α-tocoferol, 0.1 µM sodium selenite, 3 µM zinc sulfate, and 5 µM ferrous sulfate. VD3 was dissolved in ethanol, and final concentration of ethanol in the culture medium of VD3-treated and non-treated Caco-2 cells was 1%. The cells were maintained with the VD3-containing culture medium for the last 14 d of the 21-d culture period.

Metabolism of Carvedilol Caco-2 cells grown on a 9.6 cm² plastic dish were washed with phosphate buffer, and 1 ml of the culture medium supplemented with 1 µM racemic carvedilol and 4% bovine serum albumin was added to the cells. Carvedilol was dissolved in a mixture of dimethylformamide and acetonitrile, and final concentration of dimethylformamide and acetonitrile in the culture medium was 0.05% and 0.02%, respectively. To evaluate the metabolism of carvedilol, the cells were incubated for 24 h in an atmosphere of 5% CO₂–95% air at 37 °C. We also evaluated the effect of mefenamic acid, valproic acid, AZT, and baicalein on the metabolism of carvedilol in β-NF-treated Caco-2 cells. The cells and culture medium were collected at 24 h after the addition of carvedilol, and stored at −30 °C for the assay of carvedilol. The metabolized amount of R- and S-carvedilol was calculated by subtracting the amount remaining in the sample (cells and medium) from the amount applied.

Assay of Carvedilol The amount of carvedilol in the sample was measured using chiral high performance liquid chromatography (HPLC) as described previously. Briefly, the samples (0.2 ml) were mixed with 0.3 ml distilled water. After alkalization with 3 ml of 0.1 M Britton–Robinson buffer (pH 8.5), the samples were extracted with 5 ml of diethyl ether and back-extracted from the organic phase with 0.3 ml of 50 mM H₂SO₄. The organic layer was discarded, and the remaining aqueous phase was alkalized with 3 ml of 0.1 M Britton–Robinson buffer (pH 8.5). This mixture was then reextracted with 5 ml of diethyl ether. The organic phase was evaporated dry in a water bath at 45 °C. The residue was dissolved in 500 µl of mobile phase, and 70 µl was injected into the HPLC column. The HPLC system was a Shimadzu LC-10AS (Kyoto, Japan). Separation was achieved with a chiral stationary phase column (CHIRALPACK AD-H: 5 µm particle size, 2 mm i.d. ×25 cm; Daicel Chemical Industries, Tokyo, Japan). The temperature of the column oven was set at 40 °C. The mobile phase consisted of 73% hexane, 27% iso-propanol, and 0.1% (v/v) diethylamine, and the flow rate was 0.3 ml/min. The peaks were monitored at an excitation wavelength of 284 nm and an emission wavelength of 343 nm (Shimadzu RF-10A). The detection limit for each enantiomer was 0.5 nm for the concentration in the samples. The coefficient of intra-day variation for the assay of R- and S-carvedilol was 6.4% and 7.5%, respectively, at the concentration of 500 nm.

Real-Time PCR Assay of UGTs mRNA Total RNA was isolated from Caco-2 cells treated with or without β-NF using an RNeasy® Mini Kit and RNase-Free DNase Set (QIA-GEN, Valencia, CA, U.S.A.) according to the manufacturer’s instructions. Reverse transcription of extracted total RNA was performed using an Omniscript® RT Kit (QIAGEN) and random hexamer (QIAGEN) according to the manufacturer’s instructions. PCR was carried out on the MX3000P® QPCR System (Stratagene, La Jolla, CA, U.S.A.) using SYBR® Premix Ex Taq™ (TaKaRa, Shiga, Japan) according to the manufacturer’s instructions. Primer sequences for UGT1A1, 1A6, 1A9, 2B4, 2B7, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been already reported elsewhere. Cycling conditions were 1 cycle for 30 s at 95 °C, followed by 45 cycles of 5-s denaturation at 95 °C, 20-s annealing at 60 °C, and 15-s extension at 60 °C. The mRNA level of UGTs was normalized according to GAPDH mRNA levels, and the ratio was presented using a common logarithm.

Data Analysis Values are expressed as the mean±S.E. Multiple comparisons were performed using Scheffé’s test following one-way ANOVA provided that the variances of groups were similar. If this was not the case, a Scheffé-type test was applied following Kruskal–Wallis analysis. p<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Several UGTs and CYPs are capable of metabolizing carvedilol; however, it is unclear which enzyme is mainly responsible for the stereoselective first-pass metabolism of carvedilol. Moreover, in spite of the expression of UGTs and CYP3A4 in the intestine, it is also unclear whether the intestine is involved in the presystemic metabolism of carvedilol. In the present study, we investigated the metabolism of R- and S-carvedilol in human intestinal epithelial Caco-2 cells. Treatment of Caco-2 cells with β-NF and VD3 induces UGTs and CYP3A4, respectively. We, therefore, also evaluated changes in the metabolic rate of R- and S-carvedilol in β-NF- and VD3-treated Caco-2 cells.

We first examined the metabolism of carvedilol in non-treated Caco-2 cells. In the present study, Caco-2 cells grown on a 9.6 cm² plastic dish were incubated with 1 ml of culture medium containing 1 µM racemic carvedilol. Figure 1A shows that carvedilol was metabolized stereoselectively in non-treated Caco-2 cells. That is, although the metabolism of R-carvedilol was not significant, S-enantiomer was metabolized significantly (Fig. 1A). The next examined the metabolism of R- and S-carvedilol in Caco-2 cells treated with 50 µM β-NF for 3 d. The metabolism of R- and S-carvedilol was significantly increased in β-NF-treated Caco-2 cells, as compared with non-treated cells (Fig. 1A). We further examined the metabolism of carvedilol in Caco-2 cells treated with 250 nm VD3 for 2 weeks. Figure 1B shows that the percentage of metabolized R- and S-carvedilol following 24-h incubation in non-treated and VD3-treated Caco-2 cells. The treatment of Caco-2 cells with VD3 did not induce a significant change in the metabolism of R- and S-carvedilol (Fig. 1B). These results indicated that carvedilol was metabolized stereoselectively by β-NF-inducible enzymes, but not significantly by the VD3-inducible enzyme (CYP3A4) in Caco-2 cells.

CYP3A4 is expressed in the intestine as well as the liver, and is responsible for the first-pass metabolism of several drugs. Wu et al. reported that the extraction ratio for orally administered cyclosporine in the gut is approximately
2-fold higher than that in the liver in kidney transplant patients. Hebert reported that tacrolimus is metabolized primarily by CYP3A4 in the liver and small intestine. In addition, Paine et al. reported that intestinal CYP3A4 contributes to the first-pass metabolism of midazolam in liver transplant patients. Recently, Caco-2 cells treated with VD₃ have been utilized to investigate intestinal drug metabolism by CYP3A4. That is, we previously reported that the expression level of CYP3A4 mRNA in Caco-2 cells treated with 250 nM VD₃ was more than 300-fold higher than that in non-treated cells. In fact, Schmiedlin-Ren et al. reported that midazolam was metabolized significantly in Caco-2 cells treated with VD₃. The present finding that the metabolism of S-enantiomer was not changed in Caco-2 cells treated with VD₃ (Fig. 1B), suggested that CYP3A4 is not significantly responsible for the metabolism of carvedilol in the cells.

To evaluate the involvement of UGTs in the metabolism of carvedilol in Caco-2 cells, we examined the inhibitory effect of several substrates of UGTs on the metabolism of carvedilol in β-NF-treated Caco-2 cells. In the present study, we used mefenamic acid (a substrate of UGT1A9 and an inhibitor of UGT2B7), valproic acid (a substrate of UGT1A6, 1A9, and 2B7), AZT (a substrate of UGT2B7), and baicalein (a substrate of the UGT1A subfamily). Figure 2 shows the percentage of metabolized R- and S-carvedilol following 24-h incubation with β-NF-treated Caco-2 cells in the absence and presence of 500 μM substrates of UGTs. Mefenamic acid and valproic acid had no effect on the metabolism of R- and S-carvedilol. In addition, AZT slightly decreased the metabolism of carvedilol, but the difference was not statistically significant (Fig. 2). On the other hand, baicalein significantly decreased the metabolism of R- and S-carvedilol in β-NF-treated Caco-2 cells (Fig. 2). Zhang et al. reported that baicalein 7-O-glucuronide, the predominant metabolite of baicalein, was extensively generated in human liver and jejunum microsomes, and that its formation was mainly catalyzed by the UGT1A1 subfamily. The present findings suggested that glucuronidation by UGTs is a major metabolic pathway of R- and S-carvedilol in β-NF-treated Caco-2 cells.

Ohno et al. reported that UGT2B7, 2B4, and 1A1 are capable of catalyzing the glucuronidation of carvedilol. Therefore, we further evaluated the expression level of UGT2B7, 2B4, and 1A1 mRNA in non-treated and β-NF-treated Caco-2 cells. In addition, Münzel et al. reported that UGT1A6 and 1A9 are expressed in Caco-2 cells. We, therefore, also evaluated the expression level of UGT1A6 and 1A9 mRNA in Caco-2 cells treated with 50 μM β-NF for 3 d. Table 1 shows the UGT/GAPDH ratio of mRNA in non-treated and β-NF-treated Caco-2 cells. The expression of UGT1A1, 1A6, and 1A9 mRNA in β-NF-treated Caco-2 cells was higher than that in non-treated cells (Table 1). On the other hand, the expression of UGT2B4 and 2B7 mRNA in β-NF-treated cells was lower than that in non-treated cells (Table 1). The finding also suggested that the UGT1A subfamily is responsible for the metabolism of carvedilol in Caco-2 cells.

In contrast to intestinal Caco-2 cells, UGTs other than the UGT1A subfamily may be involved in the glucuronidation of carvedilol in the human liver. Ohno et al. reported that carvedilol was metabolized to two forms of its glucuronides, G1 and G2, in pooled human liver microsomes (HLM), and that the formation rate of G1 by pooled HLM at 100 μM carvedilol was approximately 2.5-fold higher than that of G2. Microsomes from insect cells expressing human UGT2B4 formed both G1 and G2, with the G1/G2 ratio and Kᵣ value comparable to those of HLM. On the other hand, recombinant UGT2B7 catalyzed the formation of G1 (a major metabolite) only, whereas recombinant UGT1A1 catalyzed the formation G2 (a minor metabolite) only. The findings suggested that UGT2B4 and/or UGT2B7 is mainly responsible for the glucuronidation of carvedilol in the human liver. However, it is still unclear whether the major
glucuronide (G1) of HLM is derived from S-carvedilol, which undergoes more extensive first-pass effect than R-carvedilol. We are now planning to investigate which UGT isoform is responsible for the stereoselective metabolism of carvedilol.

In conclusion, the present study indicated that carvedilol was metabolized stereoselectively by β-NF-inducible enzymes in Caco-2 cells. The UGT1A subfamily in intestinal epithelial cells may be partly responsible for first-pass metabolism of the drug. Further investigations are needed to clarify the UGT isoform responsible for the stereoselective metabolism of carvedilol.

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REFERENCES