Involvement of the CYP1A Subfamily in Stereoselective Metabolism of Carvedilol in β-Naphthoflavone-Treated Caco-2 Cells

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We have previously reported that the metabolism of S-carvedilol in β-naphthoflavone (β-NF)-treated Caco-2 cells is faster than that of R-carvedilol. The aim of the present study was to identify the enzyme responsible for the stereoselective metabolism of carvedilol in the cells. The expression of cytochrome P450 (CYP) 1A1 and CYP1A2 mRNA, but not CYP2D6, CYP3A4, and CYP2C9 mRNA, was increased in β-NF-treated Caco-2 cells, as compared with non-treated cells. Furafylline, an inhibitor of the CYP1A subfamily, decreased the metabolism of S-carvedilol in Caco-2 cells cultured on plastic dishes. In addition, the glucuronidation of carvedilol was not significant in microsomes of β-NF-treated Caco-2 cells. On the other hand, the oxidation of S-carvedilol in microsomes of β-NF-treated Caco-2 cells was faster than that of R-carvedilol, and furafylline decreased the oxidative activity of S-carvedilol. These findings suggested that the CYP1A subfamily was responsible for the stereoselective metabolism of carvedilol in β-NF-treated Caco-2 cells.

Key words carvedilol; Caco-2 cell; stereoselective metabolism; cytochrome P450

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.1,2) Orally administered carvedilol undergoes stereoselective first-pass metabolism, and the blood concentration of R-enantiomer with low β-blocking activity is approximately 2-fold higher than that of S-enantiomer with high β-blocking activity.3–5) Carvedilol is metabolized extensively via aliphatic side-chain oxidation, aromatic ring oxidation, and conjugation pathways.6) Oldham and Clarke reported that oxidative activity of carvedilol is observed in cytochrome P450 (CYP) 2D6, 1A2, 3A4, and 2C9.7) In addition, Ohno et al. reported that UDP-glucuronosyltransferase (UGT) 2B7, 1A1, and 2B4 are capable of catalyzing the glucuronidation of carvedilol.8) However, it has been unknown whether the intestine also plays a role in the first-pass presystemic metabolism of carvedilol, although several CYP and UGT isoforms are expressed in human intestinal epithelial cells.9,10)

We previously investigated the metabolism of carvedilol in human intestinal epithelial Caco-2 cells.9) The metabolism of R-carvedilol was not significant in Caco-2 cells cultured on plastic dishes, whereas S-carvedilol was significantly metabolized in the cells. The metabolism of S-carvedilol was further increased by the treatment of Caco-2 cells with 50 μM β-naphthoflavone (β-NF) for 3 d. In addition, the mRNA expression level of the UGT1A subfamily in β-NF-treated Caco-2 cells was higher than that in non-treated cells, whereas the expression level of UGT2B4 and UGT2B7 mRNA in β-NF-treated Caco-2 cells were lower than that in non-treated cells.9) On the other hand, Takekuma et al. have recently evaluated the glucuronidation of racemic carvedilol by recombinant UGT1A1 and UGT2B7, and reported that UGT1A1 and UGT2B7 was mainly involved in the glucuronidation of R- and S-carvedilol, respectively.11) These findings suggested that UGT was not involved in the stereoselective metabolism of carvedilol in β-NF-treated Caco-2 cells.

The aim of the present study was to identify the CYP enzyme responsible for the stereoselective metabolism of carvedilol in β-NF-treated Caco-2 cells. We evaluated the effect of β-NF-treatment on the expression of CYP mRNA in Caco-2 cells, and then investigated the effect of CYP-specific inhibitors on the metabolism of carvedilol in β-NF-treated Caco-2 cells cultured on plastic dishes. Moreover, we examined whether the oxidation of carvedilol in microsomes of β-NF-treated Caco-2 cells was faster than glucuronidation of the drug. That is, the oxidation of carvedilol in microsomes was evaluated in the presence of NADPH, whereas glucuronidation was evaluated in the presence of UDP-glucuronic acid (UDPGA).10)

MATERIALS AND METHODS

Materials Carvedilol was kindly supplied by Daiichi Pharmaceutical (Tokyo, Japan). 1α,25-Dihydroxyvitamin D3 (VD3), β-NF, and ketoconazole were purchased from Wako Pure Chemicals (Osaka, Japan). Quinidine hydrochloride, furafylline, sulfaphenazole, and baicalein were purchased from Sigma-Aldrich (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). The cells were seeded at a density 5×10^5 cells/cm^2 on a 100 mm plastic dish (BD Bioscience, Bedford, MA, U.S.A.) or 9.6 cm^2 plastic dish using a Falcon™ multwell™ plate (BD Bioscience). They were maintained with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Biowest, Nuallé, France) in an atmosphere of 5% CO2-95% air at 37°C for 21 d.9) All experiments were carried out between passages 55—74.

The treatment of Caco-2 cells with β-NF or VD₃ (an inducer of CYP3A4) was performed as described previously.9) Briefly, Caco-2 cells were maintained with culture medium supplemented with 50 μM β-NF for the last 3 d of the 21-d culture period. β-NF was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the culture medium of β-NF-treated and non-treated Caco-2 cells was 0.2% (v/v). On the other hand, VD₃ was added at a concen-
tration of 250 nm to culture medium supplemented with 45 nm (±)-α-tocopherol, 0.1 μM sodium selenite, 3 μM zinc sulfate, and 5 μM ferrous sulfate. VD₃ was dissolved in ethanol, and the final concentration of ethanol in the culture medium of VD₃-treated and non-treated Caco-2 cells was 1% (v/v). The cells were maintained with VD₃-containing culture medium for the last 14 d of the 21-d culture period. The cells, which were cultured in the medium containing 0.2% DMSO or 1% ethanol, served as the non-treated control.

**Real-Time PCR Assay of mRNA of CYPs in Caco-2 Cells** Total RNA was isolated from non-treated, VD₃-treated, and β-NF-treated Caco-2 cells using an RNeasy® Mini Kit and RNase-Free DNase Set (QIAGEN, 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 CYP2D6, CYP3A4, CYP2C9, CYP1A1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been already reported elsewhere. In brief, the metabolites 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 carvedilol, the cells were incubated for 24 h in an atmosphere of 5% CO₂-95% air at 37 °C. The metabolized amount of carvedilol was dissolved in a mixture of dimethylformamide and acetone, and the final concentration of carvedilol in the microsomes. Furafylline and baicalein were dissolved in methanol, and the final concentration of ethanol in the culture medium supplemented with 1 μM racemic carvedilol, 0.3 mg/ml microsomal proteins, 10 mM MgCl₂, 2 mM UDPGA, 7.5 μg/ml alamethicin, and 50 mM Tris–HCl buffer (pH 7.4). The reaction was allowed to run for 40 min at 37 °C. The metabolized amount of R- and S-carvedilol was calculated by subtracting the amount remaining in the sample from the amount applied.

**Assay of Carvedilol** The amount of carvedilol in the samples was measured using chiral high performance liquid chromatography (HPLC) as described previously. Briefly, after alkalization in 3 ml of 0.1 M Britton–Robinson buffer (pH 8.5), samples (0.1 ml) were extracted with 5 ml of diethyl ether. The organic phase was transferred and 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 (CHIRALPAK 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% isopropanol, 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 of each enantiomer was 0.5 nm for the concentration in the samples. The coefficient of inter-day variation for the assay of R- and S-carvedilol was 7.5% and 7.7%, respectively, at a concentration of 500 nm.

**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**

**Expression of mRNA of CYPs in VD₃ or β-NF-Treated Caco-2 Cells** We first evaluated the effect of β-NF and also VD₃ (a well-known inducer of CYP3A4) on the expression of mRNA of CYP2D6, CYP1A2, CYP3A4, and CYP2C9 in Caco-2 cells. In addition, we evaluated the expression of

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mRNA of CYP1A1 in Caco-2 cells, since CYP1A1 is expressed in the cells. Figure 1 shows the CYP/GAPDH ratio of mRNA in non-treated, VD$_3$-treated, and β-NF-treated Caco-2 cells. In non-treated Caco-2 cells, the expression of CYP1A1 mRNA was greater than that of any other CYP mRNA. The expression level of CYP3A4 mRNA in VD$_3$-treated Caco-2 cells was much higher than that in non-treated cells, whereas the expression of CYP2D6, CYP1A2, CYP2C9, and CYP1A1 mRNA was not altered by VD$_3$ treatment. On the other hand, the expression of CYP1A1 mRNA, but not CYP2D6, CYP3A4, and CYP2C9 mRNA, was markedly increased in β-NF-treated Caco-2 cells, as compared with non-treated cells. In addition, the expression of CYP1A2 mRNA was slightly increased in β-NF-treated Caco-2 cells (Fig. 1). These results indicated that the CYP1A subfamily was one of the main enzymes induced by β-NF treatment of Caco-2 cells.

**Effect of CYP-Specific Inhibitors on the Stereoselective Metabolism of Carvedilol in β-NF-Treated Caco-2 Cells**

Caco-2 cells were incubated with 1 μM racemic carvedilol for 24 h. Open and hatched columns indicate R-carvedilol and S-carvedilol, respectively. Each column expresses the mean±S.E. for 4—11 experiments. *p<0.05, significantly different from the control.

Fig. 2. Effect of CYP-Specific Inhibitors on the Stereoselective Metabolism of Carvedilol in β-NF-Treated Caco-2 Cells

Microsomes were incubated with 1 μM racemic carvedilol for 40 min. Open and hatched columns indicate R-carvedilol and S-carvedilol, respectively. Each column expresses the mean±S.E. for 5—10 experiments. *p<0.05, significantly different from the control with NADPH (+) and inhibitor (−).

DISCUSSION

In our previous report, we demonstrated that the metabolism of carvedilol in β-NF-treated Caco-2 cells was stereoselective for S-enantiomer, and that baicalein significantly inhibited the metabolism of S-carvedilol. Baicalein is not only a substrate of the UGT1A subfamily, but also an inhibitor of the CYP1A subfamily. Therefore, it was unknown which enzyme is responsible for the stereoselective metabo-
lism of carvedilol in Caco-2 cells. The findings in the present study indicated that the CYP1A subfamily was involved in the metabolism of carvedilol in Caco-2 cells, and that baicalein inhibited the CYP1A subfamily very strongly (Figs. 1, 2, 3).

Several reports have shown that baicalein is not only a notable substrate of the UGT1A subfamily, but also an potent inhibitor of the CYP1A subfamily. Zhang et al. investigated the glucuronidation of baicalein in human liver microsomes (HLM) and human intestinal microsomes (HIM), and reported that baicalein was glucuronidated in HLM and HIM with a $K_m$ value of 23 $\mu M$ and 94 $\mu M$, respectively. In addition, they also reported that the UGT1A subfamily, including UGT1A1, 1A3, 1A7, 1A8, and 1A9, catalyzed the glucuronidation of baicalein. On the other hand, Chan et al. investigated the effect of baicalein on ethoxyresorufin O-deethylase (EROD) activities using MCF-7 cells, and reported that EROD activities were reduced by baicalein with an IC$_{50}$ of around 1 $\mu M$. They also conducted subsequent kinetic assays, and reported that baicalein was a competitive inhibitor of EROD.

We previously investigated the stereoselective metabolism of carvedilol in HIM. The oxidation of S-carvedilol in HIM was faster than that of R-carvedilol. However, the oxidation of S and R-carvedilol in HIM was inhibited by ketoconazole, but not quinidine, furafylline, and sulfaphenazole, indicating that CYP3A4 was involved in the oxidation of carvedilol in HIM. Furthermore, the glucuronidation of S-carvedilol in HIM was faster than that of R-carvedilol. These findings suggested that the enzymes responsible for the stereoselective metabolism of carvedilol in the human intestine are different from those in β-NF-treated Caco-2 cells.

In conclusion, the present findings indicated that the CYP1A subfamily is involved in the stereoselective metabolism of carvedilol in Caco-2 cells. The present findings will provide an insight into the usefulness (uselessness) of Caco-2 cells to study the first-pass metabolism of drugs in the intestine.

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