Regular Article

In Vitro Enhancement of Carvedilol Glucuronidation by Amiodarone-Mediated Altered Protein Binding in Incubation Mixture of Human Liver Microsomes with Bovine Serum Albumin

Makoto Sekimoto, Toru Takamori, Saki Nakamura, and Masato Taguchi*

Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.
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Carvedilol is mainly metabolized in the liver to O-glucuronide (O-Glu). We previously found that the glucuronidation activity of racemic carvedilol in pooled human liver microsomes (HLM) was increased, R-selectively, in the presence of amiodarone. The aim of this study was to clarify the mechanisms for the enhancing effect of amiodarone on R- and S-carvedilol glucuronidation. We evaluated O-Glu formation of R- and S-carvedilol enantiomers in a reaction mixture of HLM including 0.2% bovine serum albumin (BSA). In the absence of amiodarone, glucuronidation activity of R- and S-carvedilol for 25 min was 0.026, and 0.51 pmol/min/mg protein, and that was increased by 6.15 and 1.60-fold in the presence of 50 µM amiodarone, respectively. On the other hand, in the absence of BSA, or when BSA was replaced with human serum albumin, no enhancing effect of amiodarone on glucuronidation activity was observed, suggesting that BSA played a role in the mechanisms for the enhancement of glucuronidation activity. Unbound fraction of S-carvedilol in the reaction mixture was greater than that of R-carvedilol in the absence of amiodarone. Also, the addition of amiodarone caused a greater increase of unbound fraction of R-carvedilol than that of S-carvedilol. These results suggest that the altered protein binding by amiodarone is a key mechanism for R-selective stimulation of carvedilol glucuronidation.

Key words drug interaction; protein binding; human liver microsome; glucuronidation; carvedilol; amiodarone

The nonselective β- and α1-adrenoceptor antagonist carvedilol has been clinically used to treat chronic heart failure, as well as hypertension, angina pectoris, and cardiac arrhythmia.1) Carvedilol is administered orally as a racemate mixture, but undergoes enantioselective first-pass metabolism. The blood concentration of the S-enantiomer, which has high β-blocking activity, is approximately one-half of that of the R-enantiomer, which has low β-blocking activity.2,3) Both enantiomers are mostly eliminated by hepatic metabolism, with renal excretion accounting for only 0.3% of the administered dose.3) Carvedilol is metabolized extensively via aliphatic side-chain oxidation, aromatic ring oxidation, and conjugation pathways.4) We previously demonstrated that R-carvedilol is metabolized mainly by CYP 2D6 and partly by CYP1A2, 2C9, and 3A4, and that S-carvedilol is metabolized mainly by CYP1A2 and partly by CYP2C9, 2D6, and 3A4.5–9) On the other hand, Ohno et al. found that uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) 2B7, 2B4, and 1A1 are capable of catalyzing the glucuronidation of carvedilol using microsomes from insect cells expressing human UGT.10) They also reported that glucuronidation of R-carvedilol is mediated by UGT1A1 and 2B4, and glucuronidation of S-carvedilol is mediated by UGT2B7 and 2B4.10)

In 2005, Fukumoto et al. reported that coadministration of amiodarone affects the enantioselective pharmacokinetics of carvedilol in patients with heart failure.11) That is, the mean serum concentration to dose (C/D) ratio of S-carvedilol in 54 patients received amiodarone concomitantly with carvedilol was 2-fold higher than that in 52 patients received carvedilol alone. However, there was no significant difference in the mean C/D values of R-carvedilol between the two groups.11) We have previously evaluated the effect of amiodarone on the metabolism of racemic carvedilol (1 μM) in pooled human liver microsomes (HLM).12) The oxidation activity for both R- and S-carvedilol decreased by amiodarone (50 µM) and/or desethyl-amiodarone (25 µM) significantly,12) because amiodarone and/or desethylamiodarone are potent inhibitors of CYP1A2, 2C9, 2D6, and 3A4.13,14) In contrast, the glucuronidation activity for R-carvedilol was increased 1.6- and 1.4-fold by amiodarone and desethylamiodarone, respectively, whereas that for S-carvedilol was only slightly increased by amiodarone and desethylamiodarone.12) Based on these results, we speculate that the stimulative effects of amiodarone and/or desethyl-amiodarone on the glucuronidation of R-carvedilol may compensate for the inhibitory effects they have on the oxidation of R-carvedilol.12) In our previous study, however, we could not determine the metabolite formation in the incubation mixture. That is, the metabolized amount of R- and S-carvedilol was calculated by subtracting the amount remaining in the sample from the amount applied. In addition, there is less evidence supporting such a mechanism responsible for the increased C/D ratio of S-carvedilol associated with coadministration of amiodarone in patients.

The aim of the present study was to clarify the relevance of the stimulative effect of amiodarone on glucuronidation of carvedilol in HLM. Therefore, in the present study, we developed approaches for analyzing the stereoselective effect of amiodarone on R- and S-carvedilol glucuronidation. That is, we first evaluated the effect of amiodarone in several substrate concentrations of both its racemic and enantiomeric form. Second, we also evaluated whether amiodarone was capable of stimulating an in vitro glucuronidation reaction, based on the
determination of carvedilol O-glucuronide (O-Glu) formation in the incubation mixture. Third, to understand simply why amiodarone stimulates R-carvedilol rather than S-carvedilol, we evaluated the effect of amiodarone on glucuronidation of each enantiomer separately. Finally, we demonstrated that amiodarone increases the generation rate of carvedilol glucuronide as a consequence of altered protein binding in an incubation mixture of human liver microsomes, and that bovine serum albumin (BSA) has idiosyncratic contribution to the mechanism of the effect of amiodarone.

MATERIALS AND METHODS

Materials  R- and S-carvedilol enantiomers and carvedilol β-0-glucuronide (O-Glu) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). UDP-glucuronic acid (UDPGA) trisodium salt and amiodarone hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

Human Liver Microsomes  HLM (lot as #88114) was purchased from BD Biosciences (Woburn, MA, U.S.A.). Microsomes were stored at –85°C until use. HLM were pooled from 50 donors; 86% Caucasian, 46% female, and a mean age of 51 years (range, 26–66 years). Activity for HLM was reported as 1300, 730, 10000, 3300, and 580 pmol min⁻¹mg protein⁻¹ for UGT1A1-, UGT1A4-, UGT1A6-, UGT1A9-, and UGT2B7-specific substrates, respectively.

Glucuronidation of Racemic Carvedilol in HLM  Glucuronidation of racemic carvedilol in HLM was evaluated in the presence of UDPGA, as described previously, with minor modification.⁸,¹² That is, the mixture consisting of racemic carvedilol, 50 µM amiodarone, 1.0 mg/mL microsomal protein, 0.2% BSA, 10 mM MgCl₂, and 25 µg/mL alamethicin in 50 mM Tris–HCl buffer (pH 7.4) was preincubated for 5 min at 37°C. The reaction was initiated by the addition of UDPGA, and the reaction mixture was incubated for 25 min at 37°C. The total volume of the incubation mixture was 150 µL, and the final concentration of racemic carvedilol was 0.003–3.0 µM. The reaction was terminated by the addition of ice-cold 0.1 M Britton–Robinson buffer (pH 8.5). The amount of carvedilol in the samples was measured by HPLC with fluorescence detection, as described previously.⁸,¹² In brief, carvedilol was extracted from samples with 5 mL diethylether after alkalization in 3 mL 0.05 M H₂SO₄ and 150 µL water were added to 250 µL of the supernatant to remove unreacted carvedilol. The mixture was stirred, and then centrifuged at 3000×g for 5 min at 4°C. Fifty microliters of the supernatant was injected into an HPLC system. The HPLC system consisted of an LC-10ATvp Liquid Chromatograph Series (Shimadzu, Kyoto, Japan) with a model RF-20 A fluorescence detector (Shimadzu) and L-column 2 ODS (Chemical Evaluation and Research Institution, Saitama, Japan). The mobile phase consisted of 25% acetonitrile, 75% 10 mM KH₂PO₄, and 0.59% (w/v) triethylamine.¹³ Flow rate was 0.7 mL/min and column temperature was 40°C. The peaks were monitored at an excitation wavelength of 240 nm and an emission wavelength of 340 nm, and the retention times were approximately 18 and 20 min for R- and S-carvedilol glucuronide, respectively.

Unbound Fraction of R- and S-Carvedilol in Incubation Medium  The unbound fraction of R- and S-carvedilol in the incubation medium was determined by ultrafiltration using Centrifree™ Ultrafiltration Devices (Merck Millipore, Carrigtwohill, Ireland). The incubation mixture (final volume 1000 µL) consisted of 30 mM R- or S-carvedilol, 50 µM amiodarone, 0.05 mg protein/mL microsomal protein, 0.2% BSA, 10 mM MgCl₂, and 12.5 µg/mL alamethicin in 50 mM Tris–HCl buffer (pH 7.4). The sample was ultrafiltrated at 1000×g, 37°C until 250 µL of the filtrate was collected. Concentration of R- and S-carvedilol in the filtrate was measured by HPLC, as described above.⁸,¹²

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

RESULTS AND DISCUSSION

We have previously found a stimulative effect of amiodarone (50 µM) on the metabolism of racemic carvedilol (1 µM) in HLM.¹⁵ In the present study, a concentration-dependent manner of substrate in the amiodarone effect was further evaluated at the racemic concentration range of 0.03–3.0 µM (Fig. 1). The glucuronidation of racemic carvedilol in HLM was stimulated greater for the R-enantiomer than the S-enantiomer by the presence of 50 µM amiodarone. That is, the glucuronidation activity for R- and S-carvedilol in HLM increased up to 3.17- and 1.65-fold, respectively. The stimulative effect of amiodarone in HLM was significant at lower substrate concentrations, whereas no stimulative effect was observed at the racemic carvedilol concentration of 3.0µM (Fig. 1).

In the case of racemic carvedilol, the glucuronidation activ-
ity of S-carvedilol in HLM without amiodarone was 3.6-fold higher than that of R-carvedilol (Fig. 2A). Takekuma et al.\textsuperscript{16} reported that the stereoselectivity for R- and S-carvedilol glucuronidation estimated in HLM differed greatly depending on the substrate form, namely racemic carvedilol and each enantiomer. This phenomenon is thought to be caused by mutual inhibition between carvedilol enantiomers during racemate glucuronidation.\textsuperscript{16} Therefore, to understand simply why amiodarone stimulates glucuronidation for R-carvedilol, rather than S-carvedilol, we compared the effects of amiodarone on the glucuronidation of each enantiomer separately (Fig. 2B). Because the glucuronide formation was supposed to be increased without the mutual inhibition between carvedilol enantiomers, a lesser concentration of microsomal protein (0.5 mg/mL) was applied for the enantiomer glucuronidation. In the case of enantiomer separations, the glucuronide formations from R- and S-enantiomers were slightly higher than those of racemic carvedilol. However, the stereoselectivity of each enantiomer was comparable to that of racemate (Fig. 2). In addition, glucuronide formation increased linearly suggesting that the microsomal activity was more than enough to evaluate the mechanism of this effect (Fig. 2). Therefore, in the subsequent experiments, we determined the glucuronidation activity with 0.05 mg/mL of microsomal protein based on the formation of metabolites derived from each enantiomer.

To confirm that the stimulative effect of amiodarone on the carvedilol glucuronidation can be observed in the case of enantiomer separations, we evaluated the effect of amiodarone (50 µM) at several substrate concentrations (Fig. 3). The glucuronide formation for R- and S-carvedilol in HLM increased up to 5.26- and 2.13-fold, respectively, in the presence of 50 µM amiodarone. The stimulative effects observed were

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**Fig. 1. Effect of 50 µM Amiodarone on Racemic Carvedilol Glucuronidation Activity in HLM**

HLM was incubated with substrate for 25 min in the presence of 2 mM UDPGA. Final concentration of microsomal protein was 1.0 mg/mL. Open and closed columns represent R- and S-carvedilol, respectively. Each column represents the mean±S.E. for 5–7 experiments. *p<0.05 compared with controls.

**Fig. 2. Comparison of Glucuronidation Activity between Racemic and Enantiomeric Carvedilol in HLM**

HLM was incubated with racemic (A) and enantiomeric (B) carvedilol for 25 min in the presence of 2 mM UDPGA. Final concentrations of microsomal protein were 1.0 and 0.5 mg/mL for racemic and enantiomeric carvedilol, respectively. The metabolized amount of racemic carvedilol was calculated by subtracting the amount remaining in the sample from the amount applied. The metabolized amount of enantiomer carvedilol was calculated by evaluating the amount glucuronide formation in the incubation mixture. Open and closed circles represent R- and S-carvedilol, respectively. Each point represents the mean±S.E. for 3–7 experiments.

**Fig. 3. Effect of 50 µM Amiodarone on Glucuronide Formation Derived from Each Carvedilol Enantiomer in HLM**

HLM was incubated with R-carvedilol (A) and S-carvedilol (B) for 25 min in the presence of 2 mM UDPGA. Final concentration of microsomal protein was 0.5 mg/mL. Each column represents the mean±S.E. for 3 experiments. *p<0.05 compared with controls.
more significant in R-carvedilol (Fig. 3). The effect of amiodarone on glucuronide formation derived from each enantiomer was marked at lower substrate concentrations, and no stimulative effect was observed at substrate concentrations of 1000 or 3000 nM (Fig. 3). These results corresponded to those of the racemate (Fig. I), suggesting that the mutual effect between the two carvedilol enantiomers in the glucuronidation reaction is less involved in the key mechanisms of the amiodarone effect.

Fujimaki et al.\textsuperscript{4)} reported that the unbound fraction of S-carvedilol in human plasma was 1.4-fold higher than that of R-carvedilol. That is, the fractions of the drug present in the free form in plasma for R- and S-enantiomer were 0.0045 and 0.0063, respectively.\textsuperscript{4)} In the present study, BSA was included at 0.2\% in the reaction mixture of HLM to prevent adsorption and/or as a solubilizing agent. On the other hand, it was reported that the plasma protein binding of amiodarone was marked at 99.977\%.\textsuperscript{17)} Thus, to clarify the possible effect of amiodarone on the protein binding of carvedilol in the reaction mixture, we conducted the same experiments in the absence of BSA (Fig. 4). As a result, the effect of amiodarone on the glucuronide formation disappeared in the absence of BSA, suggesting that the presence of BSA was essential for the effect of amiodarone (Fig. 4). In addition, to evaluate whether the stimulative effect of amiodarone on glucuronide formation is specific to BSA, we conducted the same experiments, replacing BSA with HSA (Fig. 5). In the presence of HSA, the glucuronide formation from each enantiomer decreased more than those in the absence of HSA. These results suggest that BSA, not HSA, mediated the effect of amiodarone on glucuronide formation of carvedilol in HLM (see closed column in Figs. 4, 5).

To clarify whether there is a difference in protein-binding characteristics between the two enantiomers to BSA, and their

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Effect of BSA on the Amiodarone-Related Change in the R- and S-Carvedilol Glucuronidation in HLM}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Effect of HSA on the Amiodarone-Related Change in the R- and S-Carvedilol Glucuronidation in HLM}
\end{figure}

| Table 1. Effect of 50 \( \mu \)M Amiodarone on Unbound Fraction (\( f_u \)) and Velocity of Glucuronide Formation (\( v \)) of R- or S-Carvedilol in Reaction Mixture |
|-----------------|-----------------|
|                 | R-Carvedilol    | S-Carvedilol    |
| Control         | +Amiodarone     | Control         | +Amiodarone     |
| \( f_u \) (%)   | 0.31±0.02       | 7.79±0.72\*     | 1.24±0.23       | 9.26±0.50\*     |
| \( v \) (pmol/min/mg protein) | 0.026±0.001 | 0.16±0.004\*   | 0.51±0.10       | 0.82±0.11       |

The concentrations of R- and S-carvedilol were 30 nM, respectively. Final concentration of microsomal protein was 0.05 mg/mL. The velocity of glucuronide formation was determined based on the amount of glucuronide generated at 25 min of incubation. Each value represents the mean±S.E. for 3–4 experiments. \(* p<0.05\) compared with controls.
interaction with amiodarone, the unbound fraction of \( R \)- and \( S \)-carvedilol in the reaction mixture was determined (Table 1). Unbound fraction of \( R \)-carvedilol was 0.31 and 7.79% in the control and in the presence of amiodarone, respectively. Unbound fraction of \( S \)-carvedilol was 1.24 and 9.26% in the control and in the presence of amiodarone, respectively. That is, amiodarone increased the unbound fraction of \( R \)-carvedilol (25-fold) much greater than that of \( S \)-carvedilol (7.5-fold). In addition, the increase in the glucuronidation rate by amiodarone for \( R \)- and \( S \)-carvedilol was 6.15- and 1.60-fold, respectively (Table 1). In conjunction with the inhibitory effect of amiodarone on the glucuronidation activity in HLM, the stimulative effect of amiodarone on the carboxidil glucuronidation may be mainly explained by the increased unbound fraction of substrates.

In our previous study, 0.2% BSA was used to prevent adsorption of drugs to glass-ware because lower concentrations of substrate may produce results confounded by non-specific binding.\(^{12,13}\) On the other hand, Rowland et al.\(^{19}\) proposed that the addition of albumin (at concentrations of 0.05–4%) is useful to evaluate glucuronidation clearance in HLM incubations. They found markedly improved predictivity of \textit{in vitro}–\textit{in vivo} clearance extrapolation for microsomal incubations conducted in the presence of BSA, and demonstrated that BSA increased the rate of glucuronidation by HLM due to a decrease in \( K_{m} \), without a significant effect on \( V_{\text{max}} \). Moreover, the authors suggested that the effect of BSA was not always consistent with that of HSA. That is, long-chain fatty acids released from the microsomal membrane competitively inhibit the UGTs, and that BSA has the capacity to sequester inhibitory fatty acids, whereas fatty acid binding sites are presumably saturated in HSA.\(^{18}\)

In conclusion, higher protein binding of \( R \)-carvedilol compared to \( S \)-carvedilol, and the addition of amiodarone, which highly binds to BSA, lead to an increase in the unbound fraction of substrate in the reaction mixture. These results may explain the mechanism responsible for the amiodarone-mediated \( R \)-selective enhancement of the glucuronide formation in HLM. Although the \textit{in vitro} data appear not to support our previous proposal\(^{11,12}\) for the mechanisms involved in the clinical interaction between carvedilol and amiodarone in humans, our observations described here may provide new insight into the idiosyncratic effect of BSA on drug–drug interactions in HLM.

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Conflict of Interest The authors declare no conflict of interest.

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