Dipeptidyl Peptidase-4 Greatly Contributes to the Hydrolysis of Vildagliptin in Human Liver

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ABSTRACT

The major metabolic pathway of vildagliptin in mice, rats, dogs, and humans is hydrolysis at the cyano group to produce a carboxylic acid metabolite M20.7 (LAY151), whereas the major metabolic enzyme of vildagliptin has not been identified. In the present study, we determined the contribution rate of dipeptidyl peptidase-4 (DPP-4) to the hydrolysis of vildagliptin in the liver. We performed hydrolysis assay of the cyano group of vildagliptin using mouse, rat, and human liver samples. Additionally, DPP-4 activity in each liver sample were assessed by DPP-4 activity assay using the synthetic substrate H-glycyl-prolyl-7-amino-4-methylcoumarin (Gly-Pro-AMC). M20.7 formation rates in liver microsomes were higher than those in liver cytosol. M20.7 formation rate was significantly positively correlated with the DPP-4 activity using Gly-Pro-AMC in liver samples (r = 0.917, P < 0.01). The formation of M20.7 in mouse, rat, and human liver S9 fraction was inhibited by sitagliptin, a selective DPP-4 inhibitor. These findings indicate that DPP-4 is greatly involved in vildagliptin hydrolysis in the liver. Additionally, we established stable single expression systems of human DPP-4 and its R623Q mutant, which is the nonsynonymous single-nucleotide polymorphism of human DPP-4, in human embryonic kidney 293 (HEK293) cells to investigate the effect of R623Q mutant on vildagliptin-hydrolyzing activity. M20.7 formation rate in HEK293 cells expressing human DPP-4 was significantly higher than that in control HEK293 cells. Interestingly, R623Q mutation resulted in a decrease of the vildagliptin-hydrolyzing activity. Our findings might be useful for the prediction of interindividual variability in vildagliptin pharmacokinetics.

Introduction

Dipeptidyl peptidase-4 (DPP-4; CD26, EC 3.4.14.5), a serine protease belonging to type II transmembrane glycoproteins, is widely expressed on the surface of epithelial cells of diverse tissues, including liver, kidney, and intestine; on endothelial cells of blood vessels; and on lymphoid cells (Mentlein, 1999; Gorrell et al., 2001). In addition to the integral membrane form, a soluble form of DPP-4 is present in serum (Durinx et al., 2000). By cleaving dipeptides from the N-terminal end of peptides and polypeptides with proline or alanine in the second position, DPP-4 controls the activity of many bioactive molecules, including incretins, cytokines, chemokines, and neuropeptides (Boonacker and Van Noorden, 2003).

Vildagliptin (LAF237) is a potent, orally active inhibitor of DPP-4 for the treatment of type 2 diabetes mellitus (Villhauer et al., 2003). DPP-4 inhibitors, so-called incretin enhancers, are attracting attention among therapeutic agents for type 2 diabetes mellitus, because they improve glucose control with a low risk of hypoglycemia (Scheen, 2010a; Deacon, 2011). Although most DPP-4 inhibitors allow one single oral administration per day for management of type 2 diabetes mellitus, twice-daily administration is recommended for vildagliptin because of its shorter half-life (Deacon, 2011). Vildagliptin is extensively metabolized via at least four pathways before excretion, with the major metabolite M20.7 (LAY151) resulting from cyano group hydrolysis (He et al., 2009a). The parent compound and the major metabolite M20.7, which is pharmacologically inactive, account for the majority of vildagliptin-related materials in human plasma (approximately 25.7 and 55%, respectively) (He et al., 2009a). Minor metabolites, which resulted from amide bond hydrolysis (M15.3), glucuronidation (M20.2), and oxidation on the pyrrolidine moiety of vildagliptin (M20.9 and M21.6), have been identified (He et al., 2009a). Although 85% of an oral dose of vildagliptin is ultimately excreted by the kidney, the liver is the major site of vildagliptin metabolism in humans (He et al., 2007). Although cytochrome P450 (P450), which is a superfamily of microsomal hemoproteins, catalyzes the reduced nicotinamide adenine dinucleotide phosphate-dependent oxidative metabolism of numerous drugs (Meunier et al., 2004), M20.7 was not formed by incubating vildagliptin with recombinant human P450 in the presence of nicotinamide adenine dinucleotide phosphate (He et al., 2009a). These findings suggest that clinically relevant drug-drug interactions with vildagliptin via P450 are very unlikely (He et al., 2009a; Scheen, 2010b; He, 2012). In contrast, non-CYP enzymes, such as uridine diphosphate glucuronosyltransferase and esterase, have attracted recent attention in pharmacokinetics, including metabolism and drug-drug interaction (Nagai, 2010; Akabane et al., 2012; Fukami and Yokoi, 2012). Identification of the major metabolic enzyme responsible for vildagliptin hydrolysis is important in predicting the individual differences in pharmacokinetics and is useful for the prediction of drug-drug interactions with vildagliptin via non-CYP.

In mice, rats, dogs, and humans, the main metabolite of vildagliptin is M20.7 (He et al., 2009b). In our previous study, we investigated whether human nitritase-like protein (NIT1) and NIT2, which are members of the nitritase superfamily, are involved in the hydrolysis of vildagliptin, because the hydrolysis of the cyano group of vildagliptin is similar to

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ABBREVIATIONS: AMC, 7-Amino-4-methylcoumarin; BNPP, bis(p-nitrophenyl) phosphate; DPP-4, dipeptidyl peptidase-4; Gly-Pro-AMC, H-glycyl-prolyl-7-amino-4-methylcoumarin; hDPP-4, human DPP-4; HEK293, human embryonic kidney 293; NIT, nitritase-like protein; P450, cytochrome P450; PBS, phosphate-buffered saline; SNP, single-nucleotide polymorphism.
kept on ice for the full period before centrifugation. The concentration of M20.7 sample was subjected to liquid chromatography/tandem mass spectrometry assay of protein by centrifugation at 15,000 g. The reaction was terminated by an addition of 200 μL of 10 mM ammonium acetate, pH 8.0 (5/95, v/v), and B was acetonitrile/methanol (10/90, v/v). The flow rate was adjusted to 0.2 mL/min, and an eluent between 0 and 5 minutes was introduced into the mass spectrometer. The ionization conditions were as follows: capillary voltage, 3.6 kV; cone voltage, 32 V; collision energy, 20 V; source temperature, 120°C; desolvation temperature, 400°C; collision gas, argon. The sample was analyzed during the multiple reaction monitoring mode of the mass spectrometer at a dwell time of 0.5 second per channel using m/z 323.1 > 173.3 as the transition.

DPP-4 Activity Assays. DPP-4 activity was determined by cleavage rate of AMC from the synthetic substrate Gly-Pro-AMC (Leiting et al., 2003; Lee et al., 2009). These assays were performed in 96-well flat-bottom plates in a total assay volume of 100 μL. The diluted enzyme sources (0.5–40 μg/mL) were incubated for 15 minutes at room temperature in assay buffer (50 mM glycine, pH 8.7, 1 mM EDTA). After 15-minute incubation, Gly-Pro-AMC was added. The final concentration of Gly-Pro-AMC was 50 μM. The plates were incubated at 25°C for 10 minutes. After incubation, fluorescence was measured using a SpectraMax M5 96-well plate spectrophotometer (excitation, 360 nm; emission, 460 nm; Molecular Devices, Sunnyvale, CA). The standard curve of free AMC was constructed using 0–10 μM solutions of AMC. The DPP-4 activity was expressed as the amount of cleaved AMC per minute per mg protein (nmol/min/mg protein).

Materials and Methods

Chemicals and Reagents. Pooled human liver S9 fraction (50 donors, mixed gender), pooled human liver cytosol (50 donors, mixed gender), pooled rat liver S9 fraction (105 male Fischer 344 rats), pooled rat liver cytosol (105 male Fischer 344 rats) were obtained from XenoTech (Lenexa, KS). Pooled human liver microsomes (105 male Fischer 344 rats) were obtained from BD Gentest (Woburn, MA). 7-Amino-4-methylcoumarin (AMC) was purchased from Sigma-Aldrich (Belmont, MA). Rabbit polyclonal anti-human DPP-4 antibody (AF1180) was purchased from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-human DPP-4 (HEK293) cells expressing human NIT1 or NIT2 suggested that human NIT1 or NIT2 was not involved in the metabolism of vildagliptin. A previous report has shown that, based on findings from the in vivo metabolism data in DPP-4–deficient and DPP-4–normal rats, approximately 20% of the cyano group hydrolysis reaction, which is the formation of M20.7, may be attributable to DPP-4 (He et al., 2009b). Furthermore, M20.7 was formed by incubation of vildagliptin with the recombinant human DPP-4 (He et al., 2009a). Although these data raise the possibility that DPP-4 is partially involved in formation of M20.7 in rats, contribution rate of DPP-4 to the vildagliptin hydrolysis in humans has not been clarified.

In the present study, we determined the contribution rate of DPP-4 to the hydrolysis of vildagliptin in the liver, which is the major site of vildagliptin hydrolysis. First, we performed hydrolysis assays of the cyano group of vildagliptin using mouse, rat, and human liver samples. Additionally, the protein expression levels of DPP-4 in each liver sample were assessed by Western blot analysis. DPP-4 activities in each liver sample were also assessed by DPP-4 activity assay using the synthetic substrate H-glycyl-prolyl-7-amino-4-methylcoumarin (Gly-Pro-AMC).

Second, to clarify the contribution rate of DPP-4 to the metabolism of vildagliptin, we assessed inhibitory effect of sitagliptin, a selective DPP-4 inhibitor, on vildagliptin-hydrolyzing activity of mouse, rat, and human liver S9 fractions. Finally, we established stable single expression systems of human DPP-4 and its mutants (R623Q and S630A) in HEK293 cells to assess the vildagliptin-hydrolyzing activity of human DPP-4 and to investigate the effect of the mutations on vildagliptin-hydrolyzing activity.
construct the expression vector of full-length human DPP-4, we subcloned the 2.95-kb StuI fragment of DPP-4 vector 2 into the 5.56-kb StuI fragment of DPP-4 vector 1. Additionally, we constructed the two expression vectors of human DPP-4 mutant. The expression vector of DPP-4 vector 2 was used as the template for constructing mutants. A KOD-plus-Neo DNA polymerase (Toyobo, Osaka, Japan) was used to create two single mutants of human DPP-4 (R623Q or S630A) in this study. The forward and reverse primers used for the mutagenesis were shown as follows: 5′-CAA ACA AAT TGC AAT TTG GGG CTG GTC-3′ and 5′-CCC AAA TTG CAA TTT GTT TGT TGT CCACAA ATC CC-3′ for the R623Q mutant, and 5′-GCA ATT TGG GCC TGG GCA TAT GGA GGG TAC GTA ACC-3′ and 5′-GGT TAC GTA CCC TCC ATG ATG CCA GCC CCA AAT TGC GTC-3′ for the S630A mutant. To construct the expression vector of full-length R623Q or S630A, we subcloned the 2.95-kb StuI fragment of DPP-4 vector 2 mutated R623Q or S630A into the 5.56-kb StuI fragment of DPP-4 vector 1, respectively. To confirm the desired mutation and verify the absence of unintended mutations, the constructs were sequenced.

HEK293 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cells were grown in Dulbecco’s modified Eagle’s medium containing 100 μM penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum with 5% CO2 at 37°C. The wild-type human DPP-4, R623Q, or S630A-expressing HEK293 cells and control cells (Mock) were constructed by transfecting the expression vector and control pTARGET vector, respectively, into cells using a Lipofectamine LTX & Plus Reagent (Invitrogen, Carlsbad, CA). Stable transfectants were selected in medium containing 600 μg/mL G418, and several clones were isolated. Preparation of Cell S9 Fraction. HEK293 cells expressing single wild-type human DPP-4, R623Q, or S630A were suspended in cold PBS and homogenized with a teflon-glass homogenizer for 40 strokes. The total cell homogenate was centrifuged at 9000 g for 10 minutes at 4°C. The supernatant, which contained nuclear fraction, was discarded. The pellet, which contained mostly cell protein, was collected as the cell homogenate. Additionally, the cell homogenate was centrifuged at 600 g for 10 minutes at 4°C to produce the cell homogenate. The cell homogenate was centrifuged at 600 g for 10 minutes at 4°C to produce the supernatant, which contained cytosol. The supernatant, which contained microsomes, was collected as the cytosol. Additionally, the cell homogenate was centrifuged at 9000 g for 20 minutes at 4°C. The supernatant was used as the S9 fraction.

Kinetic Analysis of DPP-4 Activity and Vildagliptin-Hydrolyzing Activity. The kinetic analysis of DPP-4 activity using the synthetic substrate Gly-Pro-AMC was performed using human liver samples, human DPP-4-expressing HEK293 cells, and DPP-4 mutant-expressing HEK293 cells with substrate concentrations from 1 to 200 μM. The kinetic analysis of vildagliptin-hydrolyzing activity was performed using human liver samples with substrate concentrations from 1 to 1000 μM and using HEK293 cells expressing human DPP-4 and its mutants with substrate concentrations 1–500 μM, respectively. Kinetic parameters were estimated from the fitted curves using a Kalesid撰写方格程序 (Synergy Software, Reading, PA) designed for nonlinear regression analysis. The following equations were applied for Michaelis-Menten kinetics (eq. 1) or biphasic kinetics (eq. 2):

$$V = \frac{V_{\text{max}} \times S}{K_m + S}$$

(1)

$$V = \frac{V_{\text{max}1} \times S}{K_m1 + S} + \frac{V_{\text{max}2} \times S}{K_m2 + S}$$

(2)

where $V$ is the initial velocity of the reaction, $V_{\text{max}}$ is the maximum velocity, $S$ is the substrate concentration, and $K_m$ is the Michaelis constant, which is defined as the substrate concentration at one-half the maximum velocity. $K_{m1}$ and $K_{m2}$ are affinity constants for high- and low-affinity sites, respectively, and $V_{\text{max}1}$ and $V_{\text{max}2}$ are maximum velocities for high- and low-affinity sites, respectively. Kinetic data were also analyzed using Eadie-Hofstee plots.

Statistical Analysis. Data were presented as means ± S.D. and were assessed for statistical significance using the unpaired t test. The correlation analysis was performed using the Spearman rank method. A value of $P < 0.05$ was considered statistically significant.

Results

Vildagliptin-Hydrolyzing Activity of Mouse, Rat, and Human Liver Samples. M20.7 formation rates in mouse, rat, and human liver samples were measured. Time and protein linearity studies were performed using human liver S9 fraction. It was demonstrated that formation of M20.7 in human liver S9 fraction increased linearly for at least 2 hours and 2 mg/mL, respectively (data not shown). M20.7 formation rate in mouse liver S9 fraction, cytosol, and microsomes was 0.21 ± 0.03, 0.13 ± 0.01, and 0.64 ± 0.05 pmol/h/mg protein, respectively, at a substrate concentration of 10 μM (Fig. 1A). M20.7 formation rate in human liver microsomes was statistically higher than that in the liver cytosol ($P < 0.01$). M20.7 formation rate in mouse liver samples was statistically lower than that in the liver cytosol and microsomes ($P < 0.01$). M20.7 formation rate in human liver S9 fraction, cytosol, and microsomes was 0.21 ± 0.03, 0.13 ± 0.01, and 0.64 ± 0.05 pmol/h/mg protein, respectively, at a substrate concentration of 10 μM (Fig. 1A). M20.7 formation rate in human liver microsomes was statistically higher than that in the liver cytosol ($P < 0.01$). M20.7 formation rate in mouse liver samples was lower than that in the liver cytosol and microsomes ($P < 0.01$).

TABLE 1

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Affinity Site</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/h/mg protein)</th>
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<tbody>
<tr>
<td>Human liver S9 fraction</td>
<td>Low</td>
<td>1.95 ± 0.57</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Human liver S9 fraction</td>
<td>Low</td>
<td>335 ± 5.9</td>
<td>4.97 ± 0.10</td>
</tr>
<tr>
<td>Human liver cytosol</td>
<td>Low</td>
<td>2.34 ± 0.96</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>Low</td>
<td>189 ± 5.0</td>
<td>3.18 ± 0.07</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>High</td>
<td>0.75 ± 0.42</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>High</td>
<td>448 ± 104</td>
<td>10.8 ± 2.82</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>High</td>
<td>6.02 ± 0.10</td>
<td>11.4 ± 0.79</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>Low</td>
<td>151 ± 71.2</td>
<td>11.5 ± 1.55</td>
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*Calculated from Eadie-Hofstee plots.

*Calculated using biphasic equation.
liver S9 fraction, cytosol, and microsomes was 0.41 ± 0.04, 0.36 ± 0.01, and 0.51 ± 0.02 pmol/h/mg protein, respectively. M20.7 formation rate in rat liver S9 fraction, cytosol, and microsomes was 0.50 ± 0.01, 0.32 ± 0.02, and 0.95 ± 0.03 pmol/h/mg protein, respectively. M20.7 formation rate in mouse and rat liver microsomes was also statistically higher than that in the liver cytosol, respectively (P < 0.01). These results indicate that the cyano group hydrolysis reaction of vildagliptin can be catalyzed by enzymes present in the liver, particularly in liver microsomes. A kinetic analysis of vildagliptin-hydrolyzing activity was performed using human liver samples (Fig. 1B). The Eadie-Hofstee plots of vildagliptin-hydrolyzing activities in human liver S9 fraction, cytosol, and microsomes were not linear at the low substrate concentration, indicating that multiple enzymes might be responsible for the hydrolysis of vildagliptin (Supplemental Fig. 2A). Because the kinetic parameters of human liver samples could not be calculated using the KaleidaGraph computer program, these parameters were calculated from the Eadie-Hofstee plots (Supplemental Fig. 2A). The \( K_m \) value for high-affinity sites of the vildagliptin-hydrolyzing activity in human liver S9 fraction, cytosol, and microsomes was 1.95 ± 0.6, 2.34 ± 1.0, and 0.75 ± 0.4 \( \mu \)M, respectively (Table 1). The \( K_m \) value for low-affinity sites of the vildagliptin-hydrolyzing activity in human liver S9 fraction, cytosol, and microsomes was 335 ± 10, 189 ± 5.0, and 448 ± 104 \( \mu \)M, respectively (Table 1).

**DPP-4 Activities and Protein Expression Levels of DPP-4 in Mouse, Rat, and Human Liver Samples.** We performed DPP-4 activity assay using Gly-Pro-AMC, which is a synthetic DPP-4-specific substrate (Leiting et al., 2003; Lee et al., 2009), and Western blot analysis using mouse, rat, and human liver samples. The DPP-4 activity in human liver S9 fraction, cytosol, and microsomes was 1.9 ± 0.2, 1.4 ± 0.2, and 3.3 ± 0.1 nmol/min/mg protein, respectively, at a substrate concentration of 50 \( \mu \)M (Fig. 2A). The DPP-4 activity in human liver microsomes was statistically higher than that in the liver cytosol (P < 0.01). Additionally, DPP-4 activity in mouse and rat liver microsomes was also statistically higher than that in the liver cytosol, respectively. These results suggest that DPP-4 is abundantly present in liver microsomes. Indeed, the protein of DPP-4 was highly detected in liver microsomes (Fig. 2B). The protein expression level of DPP-4 in liver samples correlated well with the DPP-4 activity in each liver sample (Fig. 2, A and B). A kinetic analysis of the DPP-4 activity was performed using human liver samples. The DPP-4 activity by human liver S9 fraction, cytosol, and microsomes followed the Michaelis-Menten equation (Fig. 2C), as the Eadie-Hofstee plots were almost linear, respectively (Supplemental Fig. 3A). The \( K_m \) value of the DPP-4 activity in human liver S9 fraction, cytosol, and microsomes was 26.2 ± 2.5, 21.3 ± 1.5, and 24.6 ± 1.1 \( \mu \)M, respectively (Table 2).

We further performed correlation analyses between M20.7 formation rates and DPP-4 activities in the liver samples (Fig. 3). M20.7 formation rate was significantly positively correlated with the DPP-4 activity using Gly-Pro-AMC in liver samples (\( r = 0.917, P < 0.01 \)). This finding suggests that DPP-4 is primarily responsible for vildagliptin hydrolysis in the liver.

**Inhibition Study of Vildagliptin-Hydrolyzing Activity.** The inhibitory effect of sitagliptin, a selective DPP-4 inhibitor, on vildagliptin-hydrolyzing activity of mouse, rat, and human liver S9 fractions was assessed. The formation of M20.7 was inhibited by sitagliptin in a concentration-dependent manner (Fig. 4A). The formation of M20.7 in human liver S9 fraction was approximately 60% inhibited by 1000 \( \mu \)M sitagliptin. The formation of M20.7 in mouse and rat liver S9 fraction was approximately 50 and 90% inhibited by 1000 \( \mu \)M sitagliptin, respectively.

We also assessed inhibitory effect of BNPP (a carboxylesterase inhibitor) and ethopropazine (a butyrylcholinesterase inhibitor) on vildagliptin-hydrolyzing activity of human liver S9 fractions, because it has been reported that anaglinptin, which has the same cyanopyridoline

<table>
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<tr>
<th>Enzyme Source</th>
<th>( K_m ) ( \mu )M</th>
<th>( V_{max} ) nmol/min/mg protein</th>
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<tbody>
<tr>
<td>Human liver S9 fraction</td>
<td>26.2 ± 2.48</td>
<td>2.70 ± 0.16</td>
</tr>
<tr>
<td>Human liver cytosol</td>
<td>21.3 ± 1.53</td>
<td>1.71 ± 0.06</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>24.6 ± 1.11</td>
<td>5.06 ± 0.18</td>
</tr>
<tr>
<td>Wild-type hDPP-4</td>
<td>54.0 ± 2.59</td>
<td>281 ± 11.0</td>
</tr>
<tr>
<td>hR623Q</td>
<td>45.6 ± 8.03</td>
<td>251 ± 12.6</td>
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is an active site mutant that lacked the catalytic serine 630 residue required for DPP-4 activity (Tanaka et al., 1993; Metzler et al., 2008; Greene et al., 2011). The protein expression levels of DPP-4 were assessed by Western blot analysis. We selected the clones with the highest DPP-4 protein levels for the subsequent analyses. Significant DPP-4 protein expression was observed in the cell S9 fraction of the wild-type human DPP-4 transfectant compared with that of Mock cells (Fig. 5A). The R623Q or S630A transfectant also showed significant DPP-4 protein expression compared with Mock, respectively. In addition, the protein expression levels of DPP-4 in HEK/R623Q and HEK/S630A were similar and higher than that in HEK/DPP-4, respectively. The two bands reacting with the anti-human DPP-4 antibody were observed in HEK/DPP-4 and HEK/R623Q. To elucidate whether the two bands were different glycosylation forms of DPP-4, the S9 fraction of HEK/DPP-4 was deglycosylated under denaturing conditions and analyzed by Western blotting with the anti-human DPP-4 antibody. Glycosidase treatment reduced the size of two bands to a single 110-kDa band in HEK/DPP-4 (Supplemental Fig. 4). This result suggested that the two bands reacting with the anti-human DPP-4 antibody in HEK/DPP-4 and HEK/R623Q were different glycosylation forms of DPP-4.

Effects of DPP-4 Mutants on DPP-4 Activity. To assess the DPP-4 activity of wild-type human DPP-4, we performed DPP-4 activity assays using the synthetic substrate Gly-Pro-AMC. To assess the effects of R623Q and S630A on DPP-4 activity, we also performed DPP-4 activity assays using HEK/R623Q and HEK/S630A. The DPP-4 activity in Mock, HEK/DPP-4, HEK/R623Q, and HEK/S630A was 1.2 ± 0.1, 134 ± 5.3, 132 ± 6.2, and 1.0 ± 0.1 nmol/min/mg protein, respectively, at a substrate concentration of 50 μM (Fig. 5B). The DPP-4 activity in HEK/DPP-4 was approximately 100-fold higher than that in Mock. The DPP-4 activity in HEK/R623Q was comparable with that in HEK/DPP-4. Although S630A transfectant showed significant DPP-4 protein expression compared with Mock, the DPP-4 activity in HEK/S630A was comparable with that in Mock. A kinetic analysis of DPP-4 activity was performed using Mock, HEK/DPP-4, and HEK/R623Q. The DPP-4 activity in recombinant human DPP-4 (hDPP-4) and R623Q (hR623Q) was expressed as the DPP-4 activity (nmol/min/mg protein) in which the DPP-4 activity obtained with Mock was subtracted from that obtained with HEK/DPP-4 and HEK/R623Q, respectively. The DPP-4 activity by wild-type hDPP-4 and hR623Q followed the Michaelis-Menten equation (Fig. 5C), as the Eadie-Hofstee plots were almost linear, respectively (Supplemental Fig. 3B). The $K_m$ value of the DPP-4 activity in wild-type hDPP-4 and hR623Q followed the Michaelis-Menten equation (Fig. 5C), as the Eadie-Hofstee plots were almost linear, respectively (Supplemental Fig. 3B). The $K_m$ value of the DPP-4 activity in hDPP-4 was 34.0 ± 2.6 and 45.6 ± 8.0 μM, respectively (Table 2). The $K_m$ value of the DPP-4 activity in hR623Q was close to that in wild-type hDPP-4.

Establishment of Single Expression Systems of Wild-Type Human DPP-4, R623Q, and S630A in HEK293 Cells. To assess the vildagliptin-hydrolyzing activity of human DPP-4, we constructed the stable single expression systems of wild-type human DPP-4 in HEK293 cells (HEK/DPP-4). To assess the effects of two DPP-4 mutants on DPP-4 activity and vildagliptin-hydrolyzing activity, stable single expression systems of R623Q and S630A in HEK293 cells (HEK/R623Q and HEK/S630A) were also constructed. R623Q (rs147614497) is the nonsynonymous human DPP-4 single-nucleotide polymorphism (SNP) with the highest minor allele frequency (=0.0248) in December 2013, which has been reported in the National Center for Biotechnology Information SNP database (build 141; http://www.ncbi.nlm.nih.gov/projects/SNP/). S630A is an active site mutant that lacked the catalytic serine 630 residue required for DPP-4 activity. The protein expression levels of DPP-4 were assessed by Western blot analysis. We selected the clones with the highest DPP-4 protein levels for the subsequent analyses. Significant DPP-4 protein expression was observed in the cell S9 fraction of the wild-type human DPP-4 transfectant compared with that of Mock cells (Fig. 5A). The R623Q or S630A transfectant also showed significant DPP-4 protein expression compared with Mock, respectively. In addition, the protein expression levels of DPP-4 in HEK/R623Q and HEK/S630A were similar and higher than that in HEK/DPP-4, respectively. The two bands reacting with the anti-human DPP-4 antibody were observed in HEK/DPP-4 and HEK/R623Q. To elucidate whether the two bands were different glycosylation forms of DPP-4, the S9 fraction of HEK/DPP-4 was deglycosylated under denaturing conditions and analyzed by Western blotting with the anti-human DPP-4 antibody. Glycosidase treatment reduced the size of two bands to a single 110-kDa band in HEK/DPP-4 (Supplemental Fig. 4). This result suggested that the two bands reacting with the anti-human DPP-4 antibody in HEK/DPP-4 and HEK/R623Q were different glycosylation forms of DPP-4.

Correlation between M20.7 formation rates and DPP-4 activities in the liver samples. Nine data points for each liver fraction (S9 fraction, cytosol, and microsomes) of three species (mouse, rat, and human) were employed to show the correlation between M20.7 formation rates and the DPP-4 activities. The correlation analysis was performed using the Spearman rank method. Data represent the means of triplicate determinations.

**Fig. 3.** Establishment of Single Expression Systems of Wild-Type Human DPP-4, R623Q, and S630A in HEK293 Cells. To assess the vildagliptin-hydrolyzing activity of human DPP-4, we constructed the stable single expression systems of wild-type human DPP-4 in HEK293 cells (HEK/DPP-4). To assess the effects of two DPP-4 mutants on DPP-4 activity and vildagliptin-hydrolyzing activity, stable single expression systems of R623Q and S630A in HEK293 cells (HEK/R623Q and HEK/S630A) were also constructed. R623Q (rs147614497) is the nonsynonymous human DPP-4 single-nucleotide polymorphism (SNP) with the highest minor allele frequency (=0.0248) in December 2013, which has been reported in the National Center for Biotechnology Information SNP database (build 141; http://www.ncbi.nlm.nih.gov/projects/SNP/). S630A is an active site mutant that lacked the catalytic serine 630 residue required for DPP-4 activity. The protein expression levels of DPP-4 were assessed by Western blot analysis. We selected the clones with the highest DPP-4 protein levels for the subsequent analyses. Significant DPP-4 protein expression was observed in the cell S9 fraction of the wild-type human DPP-4 transfectant compared with that of Mock cells (Fig. 5A). The R623Q or S630A transfectant also showed significant DPP-4 protein expression compared with Mock, respectively. In addition, the protein expression levels of DPP-4 in HEK/R623Q and HEK/S630A were similar and higher than that in HEK/DPP-4, respectively. The two bands reacting with the anti-human DPP-4 antibody were observed in HEK/DPP-4 and HEK/R623Q. To elucidate whether the two bands were different glycosylation forms of DPP-4, the S9 fraction of HEK/DPP-4 was deglycosylated under denaturing conditions and analyzed by Western blotting with the anti-human DPP-4 antibody. Glycosidase treatment reduced the size of two bands to a single 110-kDa band in HEK/DPP-4 (Supplemental Fig. 4). This result suggested that the two bands reacting with the anti-human DPP-4 antibody in HEK/DPP-4 and HEK/R623Q were different glycosylation forms of DPP-4.

**Fig. 4.** Inhibition study of vildagliptin-hydrolyzing activity. (A) The inhibitory effect of sitagliptin, a selective DPP-4 inhibitor, on vildagliptin-hydrolyzing activity of mouse, rat, and human liver S9 fractions was assessed. (B) The inhibitory effect of BNPP and ethopropazine, a carboxylesterase and butyrylcholinesterase inhibitor, respectively, on vildagliptin-hydrolyzing activity of human liver S9 fractions was also assessed. The substrate concentration was 10 μM. Data represent the means of triplicate determinations.
Effects of DPP-4 Mutants on Vildagliptin-Hydrolyzing Activity.

To assess the vildagliptin-hydrolyzing activity of wild-type human DPP-4, we performed hydrolysis assays of the cyano group of vildagliptin using HEK/DPP-4. To assess the effects of R623Q and S630A on vildagliptin-hydrolyzing activity, we also performed hydrolysis assays of the cyano group of vildagliptin using HEK/R623Q and HEK/S630A. The M20.7 formation rate in HEK/R623Q and HEK/S630A was 0.18 ± 0.01, 7.4 ± 0.2, 5.2 ± 0.1, and 0.13 ± 0.01 pmol/h/mg protein, respectively, at a substrate concentration of 1 μM (Fig. 5D). The M20.7 formation rate in HEK/DPP-4 was statistically higher than that in Mock (P < 0.01). Interestingly, although DPP-4 activity in HEK/R623Q was comparable with that in HEK/DPP-4, M20.7 formation rate in HEK/R623Q was statistically lower than that in HEK/DPP-4 (P < 0.01). Furthermore, M20.7 formation rate in HEK/S630A was comparable with that in Mock. These results suggest that the catalytic serine 630 residue is directly involved in the vildagliptin hydrolysis in human DPP-4 and that R623Q mutation results in a decrease of the vildagliptin-hydrolyzing activity. A kinetic analysis of vildagliptin-hydrolyzing activity was performed using Mock and HEK/DPP-4. The vildagliptin-hydrolyzing activity in hDPP-4 was expressed as a M20.7 formation rate (pmol/h/mg protein) in which the vildagliptin-hydrolyzing activity obtained with Mock was subtracted from that obtained with HEK/DPP-4. Data represent the means ± S.D. of triplicate determinations.

Effects of DPP-4 Mutants on Vildagliptin-Hydrolyzing Activity.

Stable single expression systems of wild-type human DPP-4, R623Q, and S630A in HEK293 cells (HEK/DPP-4, HEK/R623Q, and HEK/S630A) were constructed. (A) The S9 fraction of Mock, HEK/DPP-4, HEK/R623Q, and HEK/S630A (3 μg protein) were subjected to NuPAGE 4–12% Bis-Tris Gel and probed with the anti-DPP-4 antibody. Actin was used as the protein-loading control. (B) DPP-4 activity of HEK/DPP-4, HEK/R623Q, and HEK/S630A was measured using Gly-Pro-AMC as a substrate. Data represent the means ± S.D. of triplicate determinations. (C) The substrate concentration-velocity curves of DPP-4 activities are shown. The DPP-4 activity in wild-type hDPP-4 and hR623Q was expressed as the DPP-4 activity (nmol/min/mg protein) in which the DPP-4 activity obtained with Mock was subtracted from that obtained with HEK/DPP-4 and HEK/R623Q, respectively. Data represent the means ± S.D. of triplicate determinations. (D) The vildagliptin-hydrolyzing activity of HEK/DPP-4, HEK/R623Q, and HEK/S630A was measured. The substrate concentration was 1 μM. Data represent the means ± S.D. of triplicate determinations. **P < 0.01. (E) The substrate concentration-velocity curves of vildagliptin-hydrolyzing activities in hDPP-4 are shown. The vildagliptin-hydrolyzing activity in hDPP-4 was expressed as a M20.7 formation rate (pmol/h/mg protein) in which the vildagliptin-hydrolyzing activity obtained with Mock was subtracted from that obtained with HEK/DPP-4. Data represent the means ± S.D. of triplicate determinations.
DPP-4 was approximately 50% consistent with previous reports (Leiting et al., 2003; Aertgeerts et al., 2007). These findings indicate that the hydrolysis of vildagliptin in human liver was not only DPP-4 but also in other tissues (Mentlein, 1999; Gorrell et al., 2001). It has been suggested that the amino acid residue serine 630 of DPP-4 is involved in formation of M20.7 (He et al., 2009a; Ahrén et al., 2011). The contribution rate of DPP-4 to vildagliptin hydrolysis at 50 mg twice daily is approximately 1% (He, 2012). Human DPP-4 was greatly involved in vildagliptin hydrolysis at 1 μM vildagliptin, whereas the enzymes, which have expressed in Mock cells, were involved in vildagliptin hydrolysis at 50–500 μM vildagliptin (Fig. 5D; Supplemental Fig. 7). These findings also indicate that human DPP-4 could be greatly involved in vildagliptin hydrolysis in vivo.

The result of inhibition study using rat liver S9 fraction (Fig. 4A), which suggested that DPP-4 mainly contributes to the hydrolysis of vildagliptin in rats, was not consistent with the previous report, which suggested that approximately 20% of the formation of M20.7 in rats is most likely attributable to DPP-4 (He et al., 2009b). This inconsistency might be due to the fact that the two sets of studies are very different experimental designs. To understand the pharmacokinetics of vildagliptin in humans and other animals, the exploration of multiple enzymes responsible for hydrolysis of vildagliptin needs to be conducted in the future.

We examined the effects of two DPP-4 mutants, R623Q and S630A, on DPP-4 activity and vildagliptin-hydrolyzing activity. It has been reported that vildagliptin forms reversible covalent bounds with DPP-4 to inhibit DPP-4 enzymatic activity (Ahrén et al., 2011). The covalent binding between vildagliptin and DPP-4 is a consequence of the formation of an unstable complex by reaction of the cyano group hydrolysis reaction of vildagliptin was first demonstrated in the present study. The Cmax of vildagliptin after the therapeutic dose of 50 mg twice daily is approximately 1 μM vildagliptin, whereas the enzymes, which have expressed in Mock cells, were involved in vildagliptin hydrolysis at 50–500 μM vildagliptin (Fig. 5D; Supplemental Fig. 7). These findings also indicate that human DPP-4 could be greatly involved in vildagliptin hydrolysis in vivo.

In conclusion, DPP-4 was greatly involved in vildagliptin hydrolysis at 50 mg twice daily is approximately 1% (He, 2012). Human DPP-4 was greatly involved in vildagliptin hydrolysis at 1 μM vildagliptin, whereas the enzymes, which have expressed in Mock cells, were involved in vildagliptin hydrolysis at 50–500 μM vildagliptin (Fig. 5D; Supplemental Fig. 7). These findings also indicate that human DPP-4 could be greatly involved in vildagliptin hydrolysis in vivo.

We examined the effects of two DPP-4 mutants, R623Q and S630A, on DPP-4 activity and vildagliptin-hydrolyzing activity. It has been reported that vildagliptin forms reversible covalent bounds with DPP-4 to inhibit DPP-4 enzymatic activity (Ahrén et al., 2011). The covalent binding between vildagliptin and DPP-4 is a consequence of the formation of an unstable complex by reaction of the cyano group hydrolysis reaction of vildagliptin was first demonstrated in the present study. The Cmax of vildagliptin after the therapeutic dose of 50 mg twice daily is approximately 1 μM vildagliptin, whereas the enzymes, which have expressed in Mock cells, were involved in vildagliptin hydrolysis at 50–500 μM vildagliptin (Fig. 5D; Supplemental Fig. 7). These findings also indicate that human DPP-4 could be greatly involved in vildagliptin hydrolysis in vivo.

In conclusion, DPP-4 was greatly involved in vildagliptin hydrolysis in mouse, rat, and human liver. The contribution rate of DPP-4 to vildagliptin hydrolysis in human liver was approximately 60%. Additionally, R623Q mutant, which is the nonsynonymous SNP of human DPP-4, led to a decrease of the vildagliptin-hydrolyzing activity. There

**Fig. 6.** Proposed metabolic pathways of vildagliptin in humans. The major metabolic pathway of vildagliptin in humans is hydrolysis at the cyano group to produce a carboxylic acid metabolite M20.7. Vildagliptin is also metabolized to form amide bond hydrolysis (M15.3) and glucuronidation (M20.2). Reported amount of vildagliptin and metabolites in urine and feces is indicated as percentage of dose (He et al., 2009a).
have been rare cases of hepatic dysfunction in patients treated with vildagliptin (Deacon, 2011; Karagiannis et al., 2014; Kurita et al., 2014). Our findings might be useful for the prediction of liver injury induced by vildagliptin.

**Authorship Contributions**

*Participated in research design:* Asakura, Fuji, Atsuda, Itoh, Fujiwara.

*Conducted experiments:* Asakura, Fujiwara.

*Performed data analysis:* Asakura, Fujiwara.

*Wrote or contributed to the writing of the manuscript:* Asakura, Fuji, Atsuda, Itoh, Fujiwara.

**References**


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