FUNCTIONAL SIGNIFICANCE OF A NATURAL ALLELIC VARIANT OF HUMAN CARBONYL REDUCTASE 3 (CBR3)

Sukhwinder S. Lakhman, Debashis Ghosh, and Javier G. Blanco

ABSTRACT:

Human carbonyl reductase (CBR) activity accounts for a significant fraction of the metabolism of endogenous and xenobiocarbonyl compounds. It is possible that genetic polymorphisms in CBR1 and CBR3 are key for the wide interindividual variability in the disposition of CBR drug substrates. We pinpointed a single nucleotide polymorphism in CBR3 (CBR3 V244M) that encodes for a V244 to M244 change. Blacks showed a higher frequency of the M244 allele (q = 0.51, n = 49) than did whites (q = 0.31, n = 70; p = 0.003). In addition, DNA variation panels from 10 ethnic groups presented a wide range of CBR3 V244M genotype distributions. Kinetic experiments with the recombinant CBR3 protein variants and menadione revealed that CBR3 M244 has significantly higher $V_{\text{max}}$ than does CBR3 V244 ($V_{\text{max}} \text{CBR3 M244} = 40.6 \pm 1.3 \text{ mol/min} \cdot \text{mg}$ versus $V_{\text{max}} \text{CBR3 V244} = 19.6 \pm 2.0 \text{ mol/min} \cdot \text{mg}$, $p = 0.002$). In contrast, both isoforms presented similar $K_m$ values ($K_m \text{CBR3 M244} = 22.9 \pm 2.9 \text{ \mu}M$ versus $K_m \text{CBR3 V244} = 24.6 \pm 3.2 \text{ \mu}M$, $p = 0.43$). Assays with NADP(H) demonstrated a higher $V_{\text{maxNADP(H)}}$ (1.6-fold) and increased catalytic efficiency ($V_{\text{maxNADP(H)}/K_m \text{NADP(H)}}$) for CBR3 M244 compared with CBR3 V244 ($p = 0.013$). Comparative three-dimensional analyses based on the structure of the homologous porcine carbonyl reductase suggested that the V244M substitution is positioned in a region critical for interactions with the NADP(H) cofactor. These studies demonstrate that the common CBR3 V244M polymorphism encodes for CBR3 isoforms with distinctive enzymatic properties.

Carbonyl reductases (CBRs) are members of the family of short-chain dehydrogenases/reductases. In humans, there are two mono-meric carbonyl reductases, carbonyl reductase 1 (CBR1) and carbonyl reductase 3 (CBR3), which are encoded by different genes located 62 kilobases apart on chromosome 21 (CBR1 and CBR3) (Avramopoulos et al., 1992; Watanabe et al., 1998; Forrest and Gonzalez, 2000). CBR1 and CBR3 have 72% identity and 79% similarity at the amino acid level as indicated by global alignment analysis. CBRs metabolize a broad spectrum of endogenous and xenobiocarbonyl compounds such as prostaglandins, steroids, and various pharmacological agents (Forrest and Gonzalez, 2000). Despite the prominent role of CBR-mediated drug metabolism, there have been no reports on the catalytic properties of CBR3 since the identification of the gene in 1998 (Watanabe et al., 1998). Several studies have described a wide range of interindividual variability in the metabolism of drugs that are CBR substrates (Hermans and Thijsen, 1992; Wong et al., 1992, 1993; Iwata et al., 1993; Ohara et al., 1995; Rady-Pentek et al., 1997; Maser et al., 2000). For example, the anticancer anthracyclines doxorubicin and daunorubicin are extensively reduced by CBRs in normal tissues as well as in tumors into their respective alcohol metabolites doxorubicinol and daunorubicinol (Forrest et al., 1990). Variable CBR activity may contribute to the unpredictable pharmacokinetics and pharmacodynamics of anthracyclines in adult and pediatric cancer patients (Ratain et al., 1991; Krischer et al., 1997; Singal and Iliskovic, 1998; Frost et al., 2002). We hypothesize that genetic polymorphism may play a relevant, although thus far uncharacterized role in modulating CBR activity. Thus, we pinpointed a novel nonsynonymous single nucleotide polymorphism in CBR3 that encodes for a V244 to M244 change in the protein. We determined the frequencies of the variant CBR3 alleles in genomic DNA samples from white and black liver donors, and in DNA variation panels representing different ethnic groups. Furthermore, the recombinant CBR3 V244 and CBR3 M244 isoforms were kinetically characterized with menadione and NADP(H). The distinctive catalytic properties of the CBR3 V244 and CBR3 M244 isoforms support the notion that the common CBR3 V244M genetic polymorphism may impact CBR-mediated biotransformation.

Materials and Methods

Samples. DNA samples were procured from 117 liver donors (whites, n = 70, and blacks, n = 47) from the Liver Tissue Resource (Pharmacogenetics of Anticancer Research Group, http://www.pharmacogenetics.org/). Human DNA variation panels were purchased from the Coriell Institute for Medical Research (Camden, NJ). Each panel contained DNA samples from individuals representing the following ethnic groups: Chinese, Indo-Pakistani, Japanese, and African American.

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Mexican, Middle Eastern, South American (Andes region), South East Asian.

Genotyping. We investigated the CBR3 V244M polymorphism with two genotyping techniques. The first approach involved PCR amplification of CBR3 exon 3 followed by SNP detection by direct sequencing analysis (Nickerson et al., 1997). PCR primers were as follows: forward, 5'-TGGAAGGACACAAAAAATT-GAGG-3'; and reverse, 5'-GAGAGAGAGAGAGAGAGAGAGAGAGAG-3'. Each 50-μl PCR contained 10 ng of genomic DNA, nuclease-free water, 5 pmol of each primer, 2.5 units of AmpliTaq Gold DNA polymerase (PerkinElmer Life and Analytical Sciences, Boston, MA), 250 mM each deoxynucleotide-5′-triphosphate (Invitrogen, Carlsbad, CA), and Gene Amp PCR buffer (PerkinElmer Life and Analytical Sciences). Amplification conditions were 92°C for 5 min, 40 cycles of 92°C for 30 s, 56°C for 40 s, and 70°C for 50 s, followed by a final extension at 70°C for 10 min. PCR products (761 base pairs) were incubated with ExoSAP-IT (USB, Cleveland, OH) to remove unincorporated nucleotides and primers. PCR products were sequenced on an ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA) with the forward PCR primer and with the internal sequencing primer 5'-AGCAAGCTCCGAAGCAGA-3'. The resultant trace files were assembled and analyzed using the public National Cancer Institute SNP server (http://lpgwsw.ncl.nih.gov/peror/snp/snp cgi.pl). The second CBR3 V244M genotyping approach utilized the TaqMan technology with specific fluorescent probes labeled with FAM and VIC for discrimination of the G and A alleles, respectively. Each deoxynucleotide-5′-triphosphate (Invitrogen, Carlsbad, CA), and Gene Amp PCR buffer (PerkinElmer Life and Analytical Sciences). Amplification conditions were 92°C for 2 min, 45 cycles of 92°C for 2 min, 54°C for 40 s, and 68°C for 1 min, followed by a final extension at 68°C for 10 min. Purified PCR products were cloned into pET28a expression vectors (Novagen, Madison, WI). The inserts were fully sequenced with vector- and insert-specific primers. The CBR3 V244 and CBR3 M244 constructs were transfected into Escherichia coli BL21 (DE3)-competent cells using the heat shock method. Cells were plated on LB agar supplemented with kanamycin (30 μg/ml) for selection. Five clones were randomly picked and grown overnight in 100 ml of LB broth supplemented with kanamycin (15 μg/ml) to optimize expression. Selected cultures were grown up to 2 liters (A600 nm ~0.2) and grown at 37°C (~A600 nm ~0.6–0.7). Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and the cultures were grown for another 3 to 4 h. Cells were pelleted by centrifugation, washed, and resuspended in lysis buffer (50 mM NaHPO4, 300 mM NaCl, and 10 mM imidazole) supplemented with lysozyme (1 mg/ml), DNase I (5 μg/ml), and ribonuclease (10 μg/ml). Cell lysates were obtained by sonication (three pulses of 30 s at 4°C) and centrifuged for 20 min (24,000g at 4°C). The resulting cell extracts (supernatants) were filtered (0.45-μm membrane) and transferred to a vial with equilibrated nickel-nitrilotriacetic acid resin. Binding occurred in a rotator at 4°C overnight. Proteins were eluted with buffer containing 250 mM imidazole. The proteins were concentrated using Centricron membranes (YM10; 10,000 molecular weight cut-off) and extensively dialyzed for 24 h against phosphate-buffered saline at 4°C with four to five buffer changes (~2.5 liters). After dialysis, protein fractions were desalted by chromatography on a Sephadex G-25 fine column (Amersham Biosciences Inc., Piscataway, NJ). Different fractions (e.g., before and after sonication, wash, and elutions) were collected for analysis by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining and Western blotting with anti-His-tag antibodies. CBR3 M244 and CBR3 V244 were expressed as discrete 39-kDa single bands.

Kinetic Analysis. CBR3 V244 and CBR3 M244 enzymatic activities were measured by recording the rate of oxidation of the NADP(H) cofactor at 340 nm (NADPH molar absorption coefficient, 6220 M−1 cm−1). CBR3 concentrations were determined from the absorbance at 280 nm (molar extinction coefficient of 15,220 M−1 cm−1; http://us.expasy.org). Protein concentrations and incubation times were standardized to ensure strict obedience to conditions of initial velocity (V0). Experiments were performed in a Cary-Varian Bio 300 UV-visible spectrophotometer equipped with thermal control and proprietary software for quality control and enzyme kinetics data analysis. Reactions were incubated at 37°C and monitored for 5 min at an acquisition rate of 100s (3000 readings). Assay mixtures (1.0 ml) contained 0.1 mM NADP(H) (Sigma-Aldrich, St. Louis, MO), potassium phosphate buffer (pH 7.4, 0.1 M), and enzyme (CBR3 V244 or CBR3 M244). Menadione (Sigma-Aldrich) concentrations ranged from 3 to 200 μM. For the determination of the kinetic constants of the cofactor, NADP(H) concentrations ranged from 10 to 200 μM.

### TABLE 1

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>n</th>
<th>G/G</th>
<th>G/A</th>
<th>A/A</th>
<th>p</th>
<th>q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africans north of the Sahara</td>
<td>7</td>
<td>1 (14.3)</td>
<td>4 (57.1)</td>
<td>2 (28.6)</td>
<td>0.43</td>
<td>0.57</td>
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<tr>
<td>Africans south of the Sahara</td>
<td>10</td>
<td>5 (50.0)</td>
<td>5 (50.0)</td>
<td>0 (0.0)</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Chinese</td>
<td>10</td>
<td>5 (50.0)</td>
<td>5 (50.0)</td>
<td>0 (0.0)</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Indo-Pakistani</td>
<td>9</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
<td>5 (55.6)</td>
<td>0.87</td>
<td>0.15</td>
</tr>
<tr>
<td>Japanese</td>
<td>10</td>
<td>4 (40.0)</td>
<td>3 (30.0)</td>
<td>0 (0.0)</td>
<td>0.85</td>
<td>0.15</td>
</tr>
<tr>
<td>Mexican</td>
<td>10</td>
<td>7 (70.0)</td>
<td>7 (70.0)</td>
<td>1 (10.0)</td>
<td>0.65</td>
<td>0.45</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>10</td>
<td>2 (20.0)</td>
<td>1 (10.0)</td>
<td>0 (0.0)</td>
<td>0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>Pacific</td>
<td>7</td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
<td>0 (0.0)</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>South American Andes</td>
<td>10</td>
<td>6 (60.0)</td>
<td>3 (30.0)</td>
<td>1 (10.0)</td>
<td>0.70</td>
<td>0.30</td>
</tr>
</tbody>
</table>

n, number of DNA samples from nonrelated individuals.
p, denotes the G allele, and q, denotes the A allele.
with menadione at a fixed concentration of 150 μM. Enzymatic velocities were automatically calculated by linear regression of the ΔA500/A time points. Regression coefficients r ≥ 0.98 were obtained for all substrate concentrations. Kₘ and Vₘₐₓ values were obtained by nonlinear regression analysis with Sigma Plot version 8.0 (SPSS Inc., Chicago, IL). Excellent agreement between the experimental and predicted Kₘ and Vₘₐₓ values was obtained in all cases with regression coefficients r ≥ 0.9 and CV ≤ 10%, respectively.

Results and Discussion

The CBR3 V244M polymorphism was pinpointed from the dbSNP database (rs1056892). First, we sought to confirm the polymorphism in DNA samples from liver donors. Therefore, we analyzed the CBR3 V244M genotype distributions in 70 DNA samples from white donors, and in 47 DNA samples from black donors. In whites, the genotype distribution was as follows: 47.1% were homozygous for the G allele (p = 0.69), 43.3% were heterozygous G/A, and 8.6% were homozygous for the A allele (q = 0.31). In contrast, 17.0% of blacks presented the homozygous G/G genotype (p = 0.49), 69.8% were heterozygous G/A, and 19.1% were homozygous A/A (q = 0.51). Allele distributions were in Hardy-Weinberg equilibrium in both groups (chi square test, p = 0.98 for whites and p = 0.14 for blacks). Comparisons of the allelic frequencies indicated that the CBR3 V244M genotype distributions differed significantly between blacks and whites (chi square = 11.7, p = 0.003) (Fig. 1). We extended our observations by analyzing the CBR3 V244M genotype distributions in 10 human variation panels from the Coriell Institute. CBR3 V244M allele frequencies varied widely among the groups (Table 1). For example, 85.7% of individuals from the Pacific Institute were homozygous for the G allele (V244), whereas 85.7% of the samples from the Indo-Pakistani panel presented the homozygous A/A genotype (M244).

To evaluate the functional impact of the CBR3 V244M polymorphism, we first characterized the catalytic properties of the recombinant CBR3 V244 and CBR3 M244 isoforms with the prototypical quinone substrate menadione. Menadione or vitamin K₃ is considered a faster association-dissociation rate for NADP(H)/NADP per catalytic cycle. To explore whether the three-dimensional structure of the enzyme can provide any clues to this possibility, we constructed a model of human CBR3 and examined the effect of the V244M polymorphism. The human CBR3 structure was derived from the crystal structure of porcine testicular carbonyl reductase (PTCR) (Ghosh et al., 2001), with which it shares about 70% sequence identity (global alignment analysis; GCG-SeqWeb version 2.0, Accelrys, San Diego, CA), by substituting amino acids in the regions surrounding the coenzyme and the substrate binding sites, and then optimizing conformations of the backbone and side chains in those regions.

The molecular modeling software CHAIN (Sack, 1988) was used for this purpose. Figure 2 shows a modeling of V244 and M244 side chains on the three-dimensional structure of the NADP(H) binding region of human CBR3. The residue at position 244 is a proline in PTCR (P243), but a valine in CBR3 (V244). This structural variation may influence the docking and/or direct interactions between the NADP(H) cofactor and the neighboring amino acids on the CBR3 peptide backbone. Instead, the higher Vₘₐₓ values were obtained by nonlinear regression analysis with Sigma Plot version 8.0 (SPSS Inc., Chicago, IL). Excellent agreement between the experimental and predicted Kₘ and Vₘₐₓ values was obtained in all cases with regression coefficients r ≥ 0.9 and CV ≤ 10%, respectively.

To further characterize the impact of the V244M substitution, we performed kinetic studies with varying concentrations of NADP(H) (Sciotti and Wermuth, 2001; Sugiyama et al., 2004). Interestingly, Kₘ values were similar for both CBR3 isoforms, but the Vₘₐₓ values of CBR3 M244 was 1.6-fold higher than the Vₘₐₓ of CBR3 V244 (Student’s t test, p = 0.013; Table 2). Thus, the kinetic data suggest that the V244M substitution may not affect direct interactions between the NADP(H) cofactor and the neighboring amino acids on the CBR3 peptide backbone. Instead, the higher Vₘₐₓ values were obtained by nonlinear regression analysis with Sigma Plot version 8.0 (SPSS Inc., Chicago, IL). Excellent agreement between the experimental and predicted Kₘ and Vₘₐₓ values was obtained in all cases with regression coefficients r ≥ 0.9 and CV ≤ 10%, respectively.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>CBR3 V244</th>
<th>CBR3 M244</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ (μM)</td>
<td>Vₘₐₓ (μmol/min · mg)</td>
</tr>
<tr>
<td>Menadione</td>
<td>24.6 ± 3.2</td>
<td>19.6 ± 2.0</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>90.5 ± 2.5</td>
<td>32.4 ± 1.0</td>
</tr>
<tr>
<td>Vₘₐₓ/Kₘ</td>
<td>22.9 ± 2.9</td>
<td>40.6 ± 1.3</td>
</tr>
<tr>
<td>kₐcat</td>
<td>85.0 ± 2.0</td>
<td>53.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Each value corresponds to the mean ± S.D. obtained from eight experiments performed with two independent protein preparations for each isoform.

* Menadione was held constant at 150 μM. Each value represents the mean ± S.D. obtained from four experiments with two independent protein preparations.
CBR3 protein isoforms with distinctive catalytic properties. Our observations warrant further research to elucidate the role of polymorphic CBR3 in the variable metabolism of CBR drug substrates.

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References


Address correspondence to: Dr. Javier G. Blanco, Department of Pharmaceutical Sciences, The State University of New York at Buffalo, 545 Cooke Hall, Buffalo, NY 14260-1200. E-mail: jgblanco@buffalo.edu