Effect of Genetic Variation on Human Cytochrome P450 Reductase-Mediated Paraquat Cytotoxicity

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Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) is a widely used herbicide and is highly toxic to human and animals. The mechanisms of paraquat toxicity involve the generation of superoxide anion through the process of redox cycling. NADPH-cytochrome P450 oxidoreductase (POR) has been reported to be a major enzyme for one-electron reduction of paraquat that initiates the redox cycling. Recently, a total of six missense variants of human POR have been identified in patients with discorded steroidogenesis. However, the effect of these genetic variations on POR-mediated paraquat toxicity is not known. Using the Flp-In Chinese hamster ovary (CHO) cells stably expressing either mouse or human POR and the cells with POR knockdown by siRNA, we confirmed that POR is responsible for paraquat-induced cytotoxicity. We further used this validated system to compare paraquat-induced toxicity among the cells that stably expressed wild-type human POR and its natural variants. While there was no difference in paraquat-induced toxicity between the cells expressing wild-type human POR and the Cys569Tyr variant, the toxicity in cells expressing all the other variants (Tyr181Asp, Ala287Pro, Arg457His, Val492Glu, and Val608Phe) was significantly decreased. Our results provide further evidence on the important role of POR in paraquat-induced toxicity and suggest that individuals carrying the functional variant POR alleles may have an altered susceptibility to paraquat exposure.

Key Words: NADPH-cytochrome POR; paraquat; genetic variants; Flp-In CHO cells; siRNA knockdown.

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) is a widely used nonselective herbicide for weed and grass control. It is highly toxic and can cause lethal responses in both animals and humans after acute exposure. Paraquat self-poisoning is still a serious problem in many parts of the developing countries, and the major cause of death is respiratory failure (Eddleston, 2000; Onyon and Volans, 1987;

Serra *et al.*, 2003). The most common routes of paraquat exposure in humans are ingestion and skin contact. Inhalation exposure of paraquat during spray in an open field constitutes little risk due to the low vapor pressure of paraquat. However, despite of the routes of exposure, paraquat is rapidly distributed into most tissues and has the highest concentrations in the lung as a result of preferential accumulation. Therefore, the lung in human and animals is the primary target organ of paraquat-induced injury (Suntres, 2002). In addition, the chemical structure of paraquat closely resembles the structure of N-methyl-4-phenyl pyridinium cation, a dopaminergic neurotoxin (Shimizu *et al.*, 2001). Animal studies demonstrate the neurotoxicity of paraquat in nigrostriatal dopaminergic cells (Thiruchelvam *et al.*, 2003), and there is a strong positive correlation between the incidence of Parkinson's disease and the level of paraquat exposure (Andersen, 2003; Liou *et al.*, 1997; Morano *et al.*, 1994). The major mechanism of paraquat toxicity is the production of reactive oxygen species through the process of redox cycling (Bonneh-Barkay *et al.*, 2005).

NADPH-cytochrome POR (EC 1.6.2.4) is a flavoprotein bound to the cytoplasmic surface of the endoplasmic reticulum and the outer membrane of the nuclear envelope in eukaryotic cells. It mediates electron transfer from NADPH to cytochrome P450 enzymes and other non-P450 enzymes that metabolize numerous endogenous and exogenous compounds, including heme oxygenase, fatty acid elongase, cytochrome b_5, and squalene monooxygenase (Enoch and Strittmatter, 1979; Ono and Bloch, 1975; Schacter *et al.*, 1972). In addition to its essential role in supporting these enzymes for their catalytic function, POR is directly involved in the metabolism of certain environmental chemicals including paraquat. POR has been reported to be the major enzyme to catalyze the one-electron reduction of paraquat that initiates the redox cycling, which generates superoxide anion and other reactive oxygen species that are responsible for various cellular damages (Benedetti *et al.*, 1980; Saito *et al.*, 1985).

Recently, a total of six missense variations (Tyr181Asp, Ala287Pro, Arg457His, Val492Glu, Cys569Tyr, and Val608-Phe) in human POR gene have been identified in patients with impaired steroidogenesis (Adachi *et al.*, 2004; Arlt *et al.*, 2004;

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Fluck *et al.*, 2004). However, the effect of these genetic variations on POR-mediated paraquat toxicity is not known. In the present study, we first confirmed the important role of POR in paraquat-induced toxicity in CHO cells with either POR overexpression or knockdown. We then used this validated system to determine if paraquat-induced toxicity was changed in the cells expressing human POR genetic variants. Our results suggest that the susceptibility to paraquat may be altered in individuals who carry the functional POR variant alleles.

MATERIALS AND METHODS

Chemicals and reagents. Paraquat (98% purity), a rabbit-derived anti-actin polyclonal antibody, horseradish peroxidase conjugated with anti-goat IgG or anti-rabbit IgG, was purchased from Sigma (St. Louis, MO). A goat-derived anti-rat POR polyclonal antibody, which cross-reacts with both mouse and human POR, was purchased from Daiichi Pure Chemicals (Tokyo, Japan). KOD Hot Start DNA Polymerase was obtained from Novagen (Madison, WI). The Cell Titer 96 AQ_{ueous} nonradioactive cell proliferation assay kit was purchased from Promega (Madison, WI). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). Flp-In CHO cells, pcDNA3.1(+), pcDNA5/FRT, pOG44, OPTI-MEMI, Lipofectamine2000, F-12 growth medium, fetal bovine serum, penicillin-streptomycin-glutamine, and hygromycin B were purchased from Invitrogen (Carlsbad, CA). The enhanced chemilunescence (ECL) reagent for immunoblot detection was from Amersham Biosciences Inc. (Piscataway, NJ). All the PCR primers were synthesized by Integrated DNA Technologies (Skokie, IL).

Cloning of mouse POR cDNA. Total RNA was extracted from an A/J mouse liver, and a full-length mouse POR cDNA was obtained by RT-PCR with an iScript cDNA synthesis kit from Bio-Rad (Hercules, CA) and the following specific primers: forward, ATG GGG GAC TCT CAC GAA G; reverse, CTA GCT CCA TAC ATC CAG CGA. The cDNA was then subcloned into a pCR-TOPO2.1 vector (Invitrogen). After excluding any extra mutations (reference sequence NM_007812) by direct sequencing, the cDNA was transferred into a pcDNA5/FRT vector at the *Bam*HI/*Eco*RV restriction sites.

Design and synthesis of mouse POR-specific siRNAs. Two mouse POR-specific siRNA sequences (#1: GGGUCCCGACCUACUGGUUCAUCUU; #2: GGAGCAGAGCCUAUGAGCACUUCAA) were designed using Stealth RNAi Designer (http://www.invitrogen.com), and the duplex siRNA oligoribonucleotides were synthesized by Invitrogen. The oligo nucleotides were dissolved in distilled RNase-free water as the stock solution (100μM).

Construction of human POR variant cDNAs by site-directed mutagenesis. It should be noted that the numbering of the amino acid residues in human POR in this report was based on the National Center of Environmental Health Sciences database for the human POR gene (NC_000007), mRNA (NM_000941), and protein (NP_000932). This numbering is consistent with the terminology used by Fluck *et al.* but different from Arlt *et al.* (2004), who used the GenBank accession number P16435, which did not include the first three N-terminal amino acid residues (Met-Ile-Asn). Therefore, the Ala284Pro, Arg454His, and Cys566Tyr variants reported by Arlt *et al.* were named as Ala287Pro, Arg457His, and Cys569Tyr, respectively, in the report by Fluck *et al.* and in the present study.

A full-length human POR cDNA in pUVI vector was a gift from Dr. Frank J. Gonzalez (National Institutes of Health [NIH], MD). The POR cDNA was excised from the pUVI vector and inserted into a pcDNA3.1(+) vector at the *Eco*RI site. Comparing with the reference GenBank sequence (accession number NM_000941), this human POR cDNA contained two single nucleotide variations (683 T > C and 1508 T > C) that resulted in amino acid substitutions of Pro228Leu and Ala503Val, respectively. Therefore, we first conducted site-directed mutagenesis to correct these two nucleotide variations with the following two pairs of primers: 5′-GAGAGCAGTTCTGGCTGGCCGTGT-GTGAAC-3′ (forward) and 5′-GTTCACACACGGCCAGCCAGAACTGCT-CTC-3′ (reverse) for 683 C>T; 5′-CCAAGGAGCCTGTCGGGGAGAACGG-3′ (forward) and 5′-CCGTTCCCGACAGGCTCCTTGG-3′ (reverse) for 1508 C>T. The primers were designed by Primer X software (Bioinformatics Organization, Inc., MA). We then used the wild-type human POR cDNA as a template to generate the variant POR cDNAs by site-directed mutagenesis as described previously (He *et al.*, 2004). The PCR primer sequences used for construction of the Tyr181Asp, Ala287Pro, Arg457His, and Cys569Tyr variant cDNAs were the same as reported by Arlt *et al.* (2004). For the Val492Glu and Val608Phe variants, the primer sequences were 5′-CATCAACAAGGGCGAG-GCCACCAACTGG-3′ (forward) and 5′-CCAGTTGGTGGCCTCGCCCTTG-TTGATG-3′ (reverse) for Val492Glu and 5′-CCACAAGGTCTACTTCCAGCA-CCTGC-3′ (forward) and 5′-GCAGGTGCTGGAAGTAGACCTTGTGG-3′ (reverse) for Val608Phe. KOD Hot Start DNA Polymerase was used to amplify the PCR products containing the desired mutations with the following conditions: denaturation at 94°C for 2 min, followed by 25 PCR cycles (94°C for 15 s; 60°C for 30 s, 68°C for 7 min 30 s) and extension at 68°C for 10 min. After site-directed mutagenesis, the full-length cDNAs were always completely sequenced to ensure that there were no extra mutations.

Heterologous expression of mouse and human POR in Flp-In CHO cells. The human POR cDNA in the pcDNA3.1(+) vector was transferred into an Flp-In expression vector pcDNA5/FRT using *Hind*III and *Not*I restriction sites. Transfection of the Flp-In CHO cells with the POR cDNAs and selection of the cells stably expressing mouse P450 oxidoreductase (mPOR-CHO) or human P450 oxidoreductase (hPOR-CHO) were conducted by our established protocols (He *et al.*, 2001; Wang *et al.*, 2001).

Transfection of siRNAs into mPOR-CHO cells. The mPOR-CHO cells were added into a 24-well plate (1×10^5 cells per well) and were grown with F12 growth medium 24 h before transfection. A mixture of #1 and #2 siRNA (25 pmol of each) that are specific for mouse POR was combined with OPTI-MEMI (62.5 μl) and Lipofectamine2000 (1.25 μl) for the formation of liposomes. After a 20-min incubation at room temperature, the siRNA/Lipofectamine mixture was added into OPTI-MEMI (125 μl). The cells were then incubated with the siRNA solution for 12 h before the replacement of fresh growth medium. A nonspecific scrambled siRNA (Dharmacon, Chicago, IL) was used as the negative control.

Immunoblot analysis. Expression of mouse or human POR protein in the stable transfectant cells was determined by immunoblotting. The immunoblot assay used a polyclonal antibody against rat POR as the primary antibody (1:5000 dilution) and an anti-goat IgG conjugated with horseradish peroxidase as the secondary antibody (1:4000 dilution). The immunoblot was visualized by ECL detection according to the manufacturer's protocol (Amersham Biosciences Inc.).

POR activity assay. POR activity was assayed spectrophotometrically as the NADPH-dependent reduction of cytochrome *c* according to a modified protocol (Patterson *et al.*, 1997). The assay system consisted of cytochrome *c* (100 μl, final concentration 80μM), reductase assay buffer (840 μl, 50mM potassium phosphate, 0.1mM tetrasodium dihydrate, 0.3M potassium chloride, pH 7.4), and cell lysate (10 μl). The reaction was initiated by the addition of 4mM NADPH (50 μl, final concentration 200μM). The initial rate of cytochrome *c* reduction was monitored at λ_{550nm} for 3 min against a blank with the assay buffer only. The activity was expressed as unit per milligram protein. One unit of reductase activity is defined as 1 μmol cytochrome *c* reduced per minute.

Reverse transcription–polymerase chain reaction. Total RNA was extracted from CHO cells by chloroform and precipitated with 70% final ethanol. The cDNAs were obtained by reverse transcription using an iScript cDNA synthesis kit from Bio-Rad according to the manufacturer's protocol. A pair of human POR-specific primers (forward, TTG GGA ACA AGA CCG ACG AGC ACT TCA AT; reverse, GCA GGT GCT GGA AGT AGA CCT TGT

GG) were used for PCR amplification. A pair of β-actin–specific primes (forward, CGT ACC ACT GGC ATC GTG AT; reverse, GTG TTG GCG TAC AGG TCT TTG) (Rodriguez-Antona *et al.*, 2001) were also used as the internal control for RT-PCR analysis.

Paraquat treatment and cytotoxicity determination. Cells were treated with different concentrations of paraquat for 24 h. For the cells transfected with siRNA, the paraquat treatment was started 48 h after the siRNA transfection. A modified MTS assay was used to determine the cytotoxicity of paraquat as described previously (Wang *et al.*, 2005). LD_{50} values were estimated from the linear scale model based on the paraquat concentrations from 1 to 500μM.

RESULTS

Expression of Mouse POR in CHO Cells and Its Knockdown by siRNA

The Flp-In CHO cells contain an Flp recombination target (FRT) site in their genome and allow for the integration of the pcDNA5/FRT expression vector at the FRT site via Flp recombinase-mediated DNA recombination (O'Gorman *et al.*, 1991). This process results in the generation of isogenic stable cell lines and ensures that different colonies obtained from transfection will express the same level of the transgene. This expression system has been validated by our previous studies (He *et al.*, 2001; Wang *et al.*, 2005). In the present study, we first generated the stable transfectant cells that expressed mouse POR. As shown in Figure 1, successful expression of mouse POR in the Flp-In CHO cells was confirmed by immunoblotting. There was no detectable POR protein in the cells transfected with the vector alone (data not shown). Treatment of the mPOR-CHO cells with a mixture of two mouse POR-specific siRNAs significantly reduced the expression of POR protein in a time-dependent pattern (Fig. 1). In contrast, treatment with a nonspecific scrambled siRNA caused no changes in POR protein expression in the mPOR-CHO cells (Fig. 1). In the mPOR-CHO cells pretreated with mouse POR-specific siRNAs for 24, 48, and 72 h, the POR protein level was reduced to 71, 37, and 28%, respectively, of the corresponding controls. There was no change in the protein level of β-actin by the treatment with mPOR-specific siRNAs (Fig. 1). Consistent with the POR protein expression data, the POR activity (assayed as cytochrome *c* reduction) was also significantly decreased by the siRNA treatment. After 48 or 72 h of mPOR-specific siRNA treatment, approximately 80–85% POR activity was lost in the mPOR-CHO cells (Fig. 2). These results demonstrated that the siRNAs we used can specifically and effectively inhibit expression of mouse POR.

Paraquat Toxicity in mPOR-CHO Cells

The viability of the CHO cells transfected with the pcDNA5/FRT vector alone (without POR cDNA) was not significantly changed by a 24-h treatment with paraquat (Fig. 3). At the highest concentration (200μM), survival of the treated cells was approximately 85%. However, paraquat treatment, starting
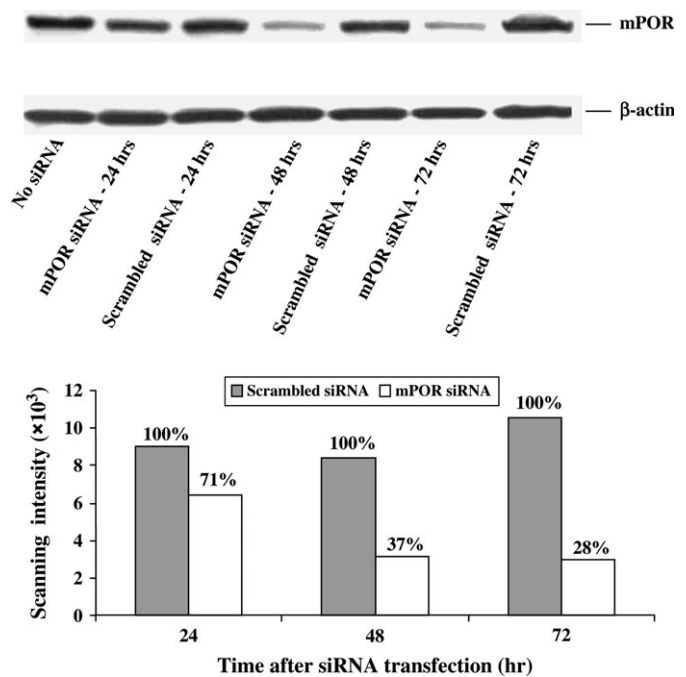

FIG. 1. Expression of mPOR proteins and its knockdown by mPOR-specific siRNAs. Flp-In CHO cells were transfected with wild-type mouse POR cDNA. The stable transfectant cells (mPOR-CHO) were then treated with mouse POR-specific siRNA. The mPOR-CHO cells treated with nonspecific scrambled siRNA were used as a negative control. Cell lysate proteins were prepared for immunoblot analysis (30 μg per lane). The mouse POR protein was detected using an anti-rat POR polyclonal antibody, which cross-reacts with mouse and human POR, as the primary antibody (1:5000 dilution) and an anti-goat IgG conjugated with horseradish peroxidase as the secondary antibody (1:4000 dilution). The β-actin protein was detected using anti-actin rabbit antibodies as the primary antibody (1:2000 dilution) and an anti-rabbit IgG conjugated with horseradish peroxidase as the secondary antibody (1:5000 dilution).

at 5μM, caused a remarkable reduction in viability in the CHO cells expressing mouse POR. While paraquat-induced cytotoxicity was not altered in mPOR-CHO cells by the pretreatment with a nonspecific scrambled siRNA, pretreatment with mPOR-specific siRNAs for 48 h remarkably increased the cell viability (Fig. 3). The LC_{50} of paraquat in the POR knockdown cells was 5.5-fold higher than that in the control mPOR-CHO cells that received nonspecific siRNA (105μM vs. 19μM). All these results clearly demonstrate that POR plays a critical role in paraquat-induced cytotoxicity. As further support to this conclusion, we also observed that the paraquat-induced toxicity was decreased in BEAS-2B human bronchial epithelial cells after their endogenous POR activity was knocked down by siRNA approach (data not shown).

Expression of Human POR and Its Variants in CHO Cells

The cDNAs of wild-type human POR and the six reported variants were used to transfect Flp-In CHO cells for stable expression. Immunoblot analysis detected a single protein band
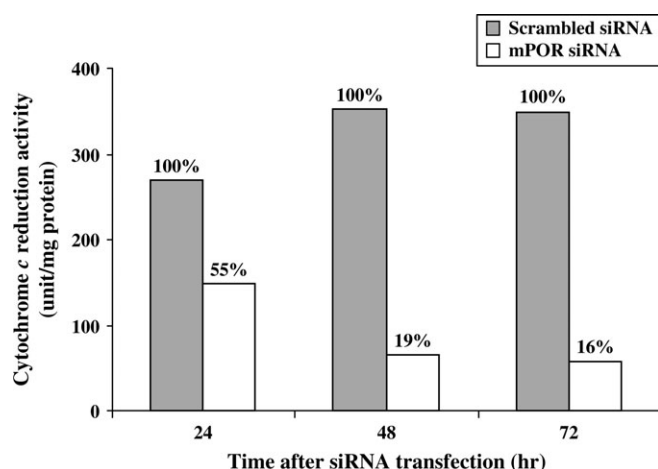
FIG. 2. POR activity in mPOR-CHO cells. The activity was determined as cytochrome *c* reduction, which is a routine assay in our laboratory with little interassay variations. The mPOR-CHO cells were treated with either mouse POR-specific siRNA or scrambled siRNA for different times.

FIG. 4. Expression of human POR proteins in Flp-In CHO cells. The expression level of human POR proteins was determined by immunoblot analysis with the same anti-rat POR polyclonal antibody as described in Figure 1. Cell lysate proteins (50 μg per lane) were prepared from the CHO cells expressing wild-type or variant human POR. Cells transfected with expression vector alone (without human POR cDNA) were used as a negative control.

with the size of 78 kD in the lysate of the CHO cells transfected with the expression vectors containing wild-type and most variant cDNAs of POR. This band was not shown in the negative control cells transfected with the vector alone (containing no POR cDNA; Fig. 4). There was no significant difference in the POR protein expression level among the stable transfectants expressing wild-type human POR and four variants: Arg457His, Val492Glu, Cys569Tyr, and Val608Phe (Fig. 4). However, the level of the Ala287Pro variant protein was always significantly lower, and there was always little
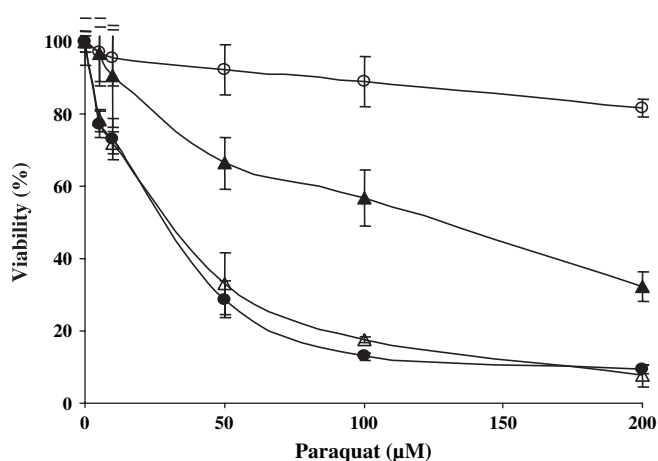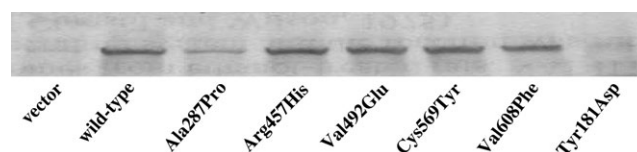
expression of the Tyr181Asp variant protein, as demonstrated by three independent transfection experiments (Fig. 4 and data not shown). To understand the mechanisms involved in the low expression of these two variants, we conducted RT-PCR analysis to determine the mRNA expression of POR in the corresponding transfectants. While the mRNA level of the Tyr181Asp variant in the transfected cells was very low, the Ala287Pro variant mRNA appeared to be expressed at the level similar to that in the cells transfected with the wild-type and the Val608Phe variant POR cDNAs (Fig. 5). There was no detectable POR mRNA in the cells transfected with the expression vector containing no POR cDNA. Consistent with the protein expression data, corresponding changes in POR activity (assayed by cytochrome *c* reduction) were observed. There was little endogenous POR activity in the control cells transfected with vector alone, and the activity was increased 30-fold in the cells expressing wild-type human POR (Fig. 6). For the Cys569Tyr variant, POR activity was slightly higher (25% increase) than the wild-type POR, but the difference was not statistically significant. In contrast, POR activities in the cells expressing the Ala287Pro, Arg457His, Val492Glu, and Val608Phe variants were only 24, 44, 13, and 9%, respectively, of that in the cells expressing the wild-type POR. POR activity in the Tyr181Asp variant cells was similar to the background
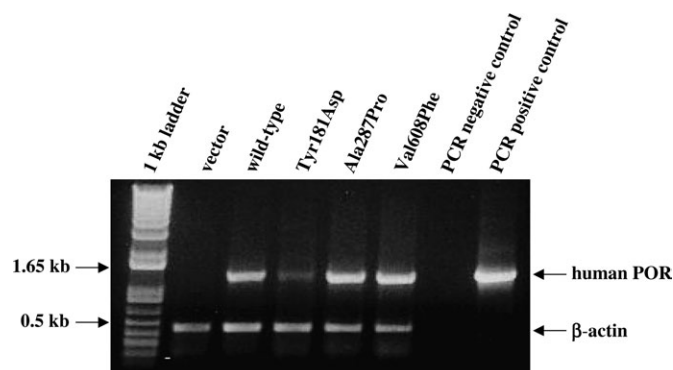

FIG. 3. Paraquat-induced toxicity in mPOR-CHO cells. The cells were treated with different concentrations of paraquat and cultured at 95% humidity and 5% CO_2 at 37°C for 24 h. Cell variability was determined by an MTS assay. For the siRNA experiment, cells were treated with paraquat at 48 h after siRNA transfection. Cells without siRNA treatment or treated with scrambled siRNA were used as negative controls. The values are mean ± SD of triplicate samples (●: mPOR-CHO cells; ○: control CHO cells transfected with vector alone (no POR); △: mPOR-CHO cells pretreated with scrambled siRNA as a negative control; and ▲: mPOR-CHO cells pretreated with mPOR-specific siRNA).

FIG. 5. RT-PCR analysis of human POR mRNA expression in hPOR-CHO cells. Total RNA was prepared from the cells transfected with different human POR cDNAs. Cells transfected with vector alone (no POR) were used as a negative control. Additional controls include β-actin mRNA expression in the same cells and the controls for PCR reaction.
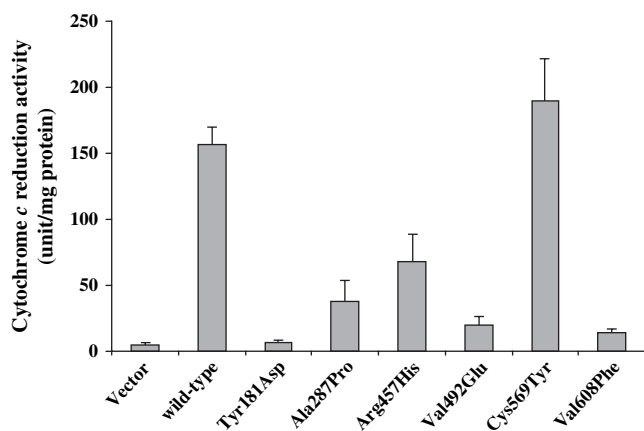
FIG. 6. POR activity in hPOR-CHO cells expressing wild-type and variant human POR. The activity was determined by cytochrome c reduction assay. The values are mean ± SD of three separate experiments.

FIG. 7. Paraquat-induced toxicity in Flp-In CHO cells expressing human POR. The cells were treated with different concentrations of paraquat and cultured at 95% humidity and 5% CO_2 at 37°C for 24 h. Flp-In CHO cells transfected with vector alone (no POR) were used as a negative control. Cell variability was determined by an MTS assay. The values are mean ± SD of triplicate samples (◇: vector; ◆: WT; △: Tyr181Asp; ▲: Ala287Pro; ○: Arg457His; ●: Val492Glu; □: Cys569Tyr; and ■: Val608Phe).

The table in Fig. 7:

	LC$_{50}$ (μM)
Vector	>500
WT	26
Tyr181Asp	>500
Ala287Pro	177
Arg457His	105
Val492Glu	445
Cys569Tyr	25
Val608Phe	>500

level of the cells transfected with vector alone, which is consistent with the observation that there was little POR protein expression in these cells (Fig. 4).

Paraquat-Induced Toxicity in CHO Cells Expressing Human POR

To examine the effect of genetic variation of human POR on the metabolic activation of paraquat, the Flp-In CHO cells stably expressing wild-type or variant human POR were treated with different concentrations of paraquat for 24 h. Cells transfected with vector alone (containing no POR cDNA) were used as a negative control. The MTS assay demonstrated that for the negative control cells, there was no significant cell death induced by 24 h paraquat treatment even at the highest concentration (100μM; Fig. 7). The cell viability, however, was significantly decreased in the cells expressing wild-type human POR. At 50μM of paraquat, the viability was only approximately 30% of the negative control (Fig. 7). The paraquat-induced cell death in the Cys569Tyr cells was not different from the wild-type POR cells. However, the viability was significantly increased in the cells expressing the other POR variants. For the Val608Phe and Tyr181Asp variant cells, the cell viability was the same as that of the negative control cells (Fig. 7).

DISCUSSION

The present study clearly demonstrates that the expression level of POR in the cells is well correlated with paraquat-induced cytotoxicity, thus confirming the direct role of POR in paraquat bioactivation and toxicity. Our study also demonstrates that except for the Cys569Tyr variant, stable transfectant cells expressing all the other human POR variants have a significant increase in their resistance to paraquat toxicity,
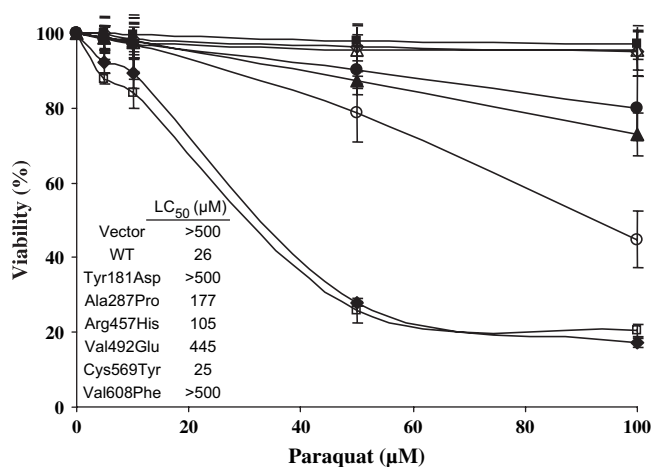
suggesting that these missense variations affect the catalytic function and/or expression of human POR. This suggestion is strongly supported by the corresponding POR activity changes in these cells.

Although the CHO cells expressing the human POR natural variants (Tyr181Asp, Ala287Pro, Arg457His, Val492Glu, and Val608Phe) all showed a significant activity decrease in paraquat bioactivation and cytochrome c reduction, it appears that different molecular mechanisms are involved. For the Arg457His, Val492Glu, and Val608Phe variants, their protein expression levels were the same as the wild-type POR in the stable transfectant cells, suggesting that the activity decrease was caused by the alteration in protein structure. However, repeated transfection experiments consistently showed that the protein expression level in the cells was lower for the Ala287Pro variant, while there was no detectable expression of the Tyr181Asp variant protein. Since the cellular mRNA level of Ala287Pro variant appears to be not significantly different from that of wild-type POR or the Val608Phe variant, it is possible that the amino acid substitution may affect the translation efficiency and/or protein stability. On the other hand, the cellular mRNA level of the Tyr181Asp variant was much lower in the stable transfectant cells, suggesting that the mRNA of this variant might be less stable. The lack of mRNA and protein expression of the Tyr181Asp variant in our CHO cell system is consistent with the observation by Arlt et al. (2004) that there was no detectable cytochrome c reductase activity in Escherichia coli transformed by a bacterial expression vector containing this variant cDNA.

Using truncated human POR cDNAs and the E. coli expression system, both Fluck et al. and Arlt et al. reported

that the catalytic efficiency (V_{max}/K_m) for cytochrome c reduction of the human POR variants Ala287Pro and Arg457His (named as Ala284Pro and Arg454His by Artl *et al.*) was decreased compared to the wild-type POR, although there was a substantial difference between these two reports on the V_{max} value of the wild-type human POR and the catalytic efficiency of the variants. In addition, Fluck *et al.* reported that the Val492Glu and Val608Phe variants also had a decreased catalytic efficiency for cytochrome c reduction (Fluck *et al.*, 2004). Our results on the bioactivation of paraquat, a substrate of POR, provide further evidence that these human genetic variants of POR are of functional significance.

In the reports by Fluck *et al.* and Artl *et al.* the Cys569Tyr variant was found to display a significant decrease in the cytochrome c reductase activity (Arlt *et al.*, 2004; Fluck *et al.*, 2004). However, in the present study, the Cys569Tyr variant did not show activity change in both paraquat bioactivation and cytochrome c reduction activity. It should be noted that there is a major difference in the functional characterization of human POR variants between the present study and the ones by Fluck *et al.* and Artl *et al.* For the cytochrome c reduction activity, both the reported studies used truncated wild-type or variant human POR cDNAs for bacterial expression, and the expressed POR proteins lacked either 27 or 46 N-terminal amino acid residues (Arlt *et al.*, 2004; Fluck *et al.*, 2004). In contrast, we used the full-length human POR cDNAs for the expression in mammalian cells, which should be more closely relevant to the physiological situation. In addition, we did not only use the reduction of cytochrome c, which is not a substrate of POR *in vivo*, for POR activity but also used the bioactivation of paraquat, a direct substrate of POR, for the functional characterization of the POR variants. These may explain why our results on the Cys569Tyr variant activity are different from the reported data (Arlt *et al.*, 2004; Fluck *et al.*, 2004). A recent study demonstrated that the N-terminal hydrophobic sequence (residues 1–56) is critical for POR to transfer electrons from NADPH to cytochrome P450 (Bonina *et al.*, 2005). Indeed, the possible effect of protein truncation on POR activity appears to be also supported by the observation of Fluck *et al.* (2004). Although the bacteria-expressed Cys569Tyr variant protein (lacking 24 N-terminal amino acid residues) showed a significant decrease in catalytic efficiency (V_{max}/K_m) for cytochrome c reduction (2.5 compared to 80 for wild-type POR), there was no significant difference between the wild-type POR and the Cys569Tyr variant in supporting the catalytic function of human P450c17 when the full-length POR cDNAs were expressed in a yeast system (Fluck *et al.*, 2004).

In recent years, the important role of gene-environment interaction in etiology and pathogenesis of human diseases has received a great attention. Functional characterization of genetic variations is critical for the identification of susceptibility biomarkers that could be used in molecular epidemiological studies of environmental diseases (Hong and Yang, 1997). The present study demonstrates that the activity of several human POR genetic variants in paraquat bioactivation is significantly decreased and suggests that individuals carrying these variant POR alleles may have an altered susceptibility to paraquat toxicity. Although there were case reports indicating a difference in patient survival after acute paraquat poisoning (Philippe *et al.*, 1995; National Poisons Information Service 86/06961, http://www.inchem.org/documents/pims/chemical/pim399.htm), substantial interindividual differences to paraquat toxicity in general populations have not been reported. However, such differences are anticipated to exist based on our general knowledge of human responses to most, if not all, environmental toxicants. Further studies are needed to determine the distribution frequency of these POR variants in general populations and its relationship with paraquat-induced toxic responses in humans. It would also be of interest to determine the effects of these genetic variations on the metabolism of other environmental toxins that are either direct substrates of POR or the substrates of cytochrome P450 enzymes that require POR-mediated electron transfer for their catalytic function.

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