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Overexpression of CYP2D6 Attenuates the Toxicity of MPP^+ in Actively Dividing and Differentiated PC12 Cells

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Clonal pheochromocytoma cell lines overexpressing cytochrome P450 2D6 (CYP2D6) were established. CYP2D6 was localized in the endoplasmic reticulum, and its enzymatic activity in the microsomal fraction was confirmed by using high performance liquid chromatography analysis with [*guanidine*-^{14}C]debrisoquine as a substrate. Overexpression of CYP2D6 protected both actively dividing and differentiated cells against the toxic effects of 1-methyl-4-phenylpyridinium ion at the concentration range of 20–40 μM, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The production of reactive oxygen species in the mitochondria was suppressed. The cytotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine was unchanged in both actively dividing and differentiated cells overexpressing CYP2D6 versus mock-transfected controls at concentrations up to 500 μM. These results suggest that the lowered enzyme activity of CYP2D6 in individuals termed "poor metabolizers" may represent a risk factor from exposure to select neurotoxicants.

Debrisoquine hydroxylase MPTP Parkinson's disease Reactive oxygen species

THE etiology of Parkinson's disease (PD) remains an enigma, although a considerable amount of evidence has indicated that environmental or endogenous toxins may be causative agents in genetically susceptible subpopulations (10,21). The environmental etiology of PD was heightened by the discovery that a batch of "street" heroin contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produced a pathological mimic of idiopathic PD in a group of drug abusers in the early 1980s (19). Furthermore, several studies have demonstrated an association between the phenotypic expression of cytochrome P450 2D6 (CYP2D6, debrisoquine hydroxylase) and polymorphism or mutation within the *CYP2D6* gene and PD in Caucasians (1,15,29). It has been reported that organochlorine insecticides are present at a higher concentration in PD tissue, which may explain, in part, the association between PD and rural living, and possibly PD and polymorphism within xenobiotic metabolizing enzymes (8,9).

Variable expression and function of CYP2D6 leads to distinct phenotypes termed ultra rapid, extensive, intermediate, and poor metabolizers (PM) of debrisoquine (12). Whereas the PM phenotype is known to

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be caused by two null alleles leading to an absence of functional CYP2D6 protein, the high degree of variability among individuals with functional alleles remains largely unexplained (13,17).

CYP2D6 substrates are structurally diverse; most are small molecules that interact with the protein via electrostatic forces between a basic nitrogen, which is common to the majority of CYP2D6 substrates, including the neurotoxicant MPTP, and an aspartic acid residue in the active site of the protein (11). Moreover, several studies have identified possible mechanisms that may explain the susceptibility of individuals who are phenotypically PM for developing PD. First, CYP2D6 is localized in the pigmented neurons of the substantia nigra and may be involved in the detoxification of MPTP-like neurotoxicants (11). Second, CYP2D6 metabolizes MPTP to the nontoxic form 4-hydroxy-MPTP in vitro, at biologically relevant concentrations (6). Interestingly, the acute effects of MPTP toxicity in female Dark Agouti (DA) rats, an animal model of the PM phenotype, have been reported versus other strains (16). Additionally, the possible PD-producing agent, tetrahydroisoquinoline, accumulates in the brains of DA rats (22). Thus, the hypothesis has been proposed that PMs are genetically susceptible to PD because of an impaired ability to detoxify exogenous or endogenous neurotoxins that are metabolized by CYP2D6 (1).

The overall goal of the present study was to determine if CYP2D6 overexpression in proliferating and terminally differentiated cells is protective against the neurotoxicants 1-methyl-4-phenylpyridinium ion (MPP^+) or MPTP. Because the brain is comprised of heterogeneous populations of cells, including actively dividing glia and various postmitotic neuronal phenotypes, the pheochromocytoma (PC12) cell line was chosen as a model to allow for the characterization of exposure to the toxicant and the effect of CYP2D6 overexpression in both actively dividing [in the absence of nerve growth factor (NGF)] and differentiated (+ NGF) PC12 cells.

MATERIALS AND METHODS

Preparation of Clonal Cell Lines Overexpressing CYP2D6

Human CYP2D6 cDNA was made from human liver mRNA by reverse transcription-polymerase chain reaction with CYP2D6-specific oligomers encompassing the coding region of CYP2D6. The sequence of the forward primer was ATGGGGCTAGAAGCA CTGGTG, corresponding to the sequence from 89 to 109 of CYP2D6 cDNA (Accession No. NM000106, GenBank, National Center for Biotechnology Information, Bethesda, MD, USA). The reverse primer was GGGCACAGCACAAAGCTCATA and complementary to the sequence from 1576 to 1556. The resultant 1.5-kb fragment was inserted into a pcDNA3.1 (–) vector (Invitrogen Corporation, Carlsbad, CA, USA) containing the G418 (geneticin) antibiotic resistance marker. This CYP2D6 expression construct was transfected into PC12 cells, which were subsequently grown in medium containing 1 mg/ml Geneticin® (Gibco, Rockville, MD, USA, a subsidiary of Invitrogen Corporation) for selection. After a 20-day incubation with Geneticin®, the resistant colonies were examined by immunoblotting with mouse monoclonal antibody against human CYP2D6, MAB-2D6 (BD Gentest™, Discovery Labware, Inc., Woburn, MA, USA, a subsidiary of Becton, Dickinson and Company), for selection as CYP2D6-expressing clones. Enhanced chemiluminescence system, ECL™ (Amersham Biosciences Corp., Piscataway, NJ, USA), was used for detection with horseradish peroxidase-coupled rabbit polyclonal anti-mouse IgG antibody (Amersham Biosciences Corp.). The selected clones were double stained with ER-Tracker™ Blue-White DPX (Molecular Probes, Inc., Eugene, OR, USA) and MAB-2D6 antibody, followed by detection with fluorescein iso-

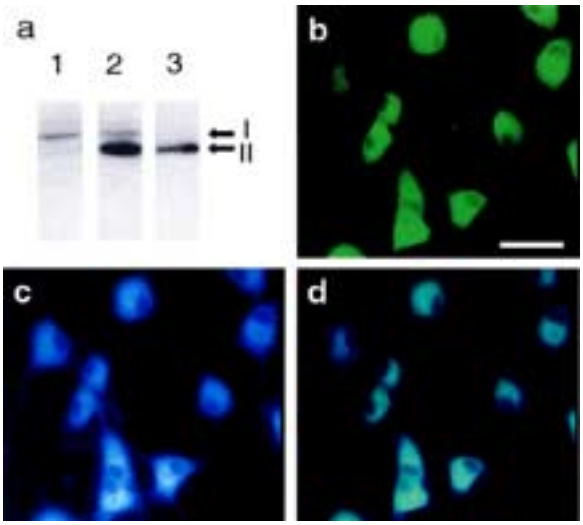

Figure 1. Expression of CYP2D6 in clonal PC12 cells. (a) Immunoblot analysis of CYP2D6 expression was performed with anti-CYP2D6 antibody. The results of representative cell lines of mock (lane 1) and CYP2D6 (lane 2), and also that of COS7 cells (monkey; kidney, epithelial) transiently transfected with CYP2D6 cDNA (lane 3) are shown. The bands for endogenous or transfected CYP2D6 were detected at 65 kDa (arrow I) and 62 kDa (arrow II), respectively. (b–d) Clonal cells of CYP2D6 were immunostained with anti-CYP2D6 antibody. CYP2D6 (b) and ER-Tracker™ Blue-White DPX (c) are colocalized intracellularly (d). Scale bar: 50 μM.

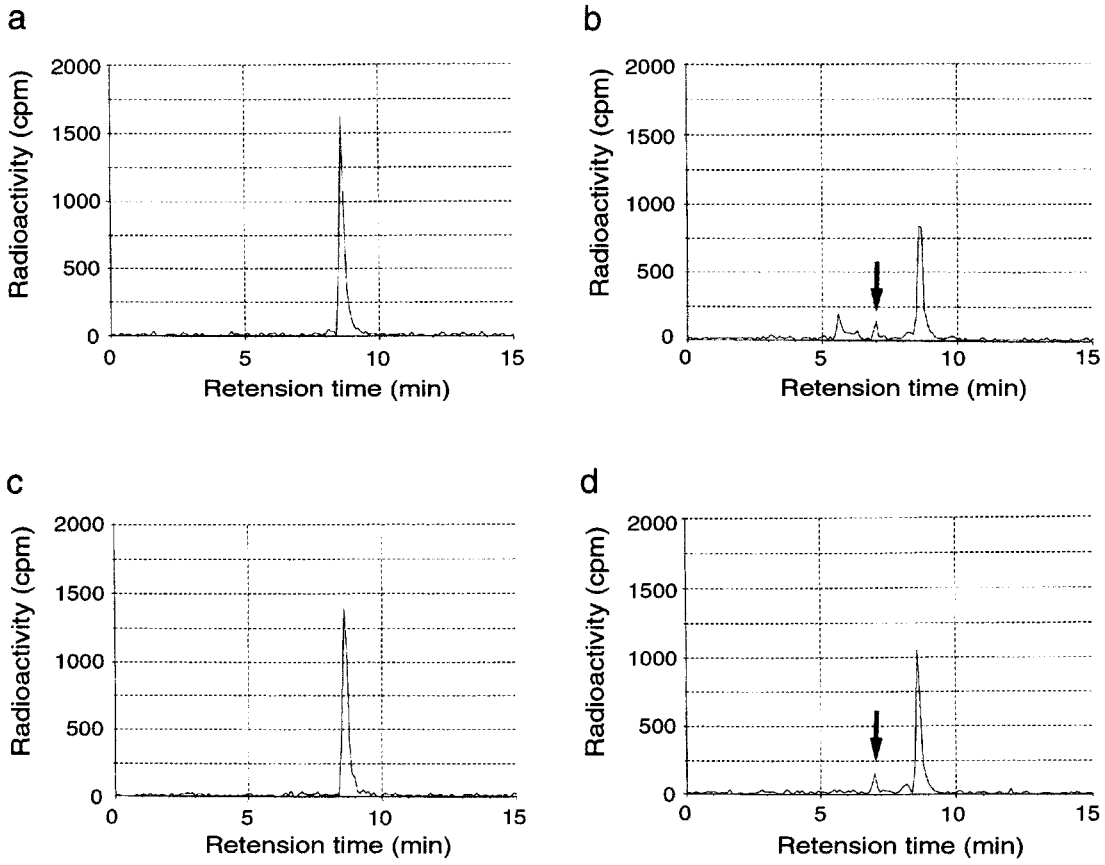

Figure 2. Enzyme activity of CYP2D6 in clonal PC12 cells. Enzyme activity of CYP2D6 was assayed by HPLC with [*guanidine*-^{14}C]debrisoquine as substrate. Profiles of negative control (before incubation) (a), positive control (after a 3-h incubation with recombinant CYP2D6) (b), and those after a 3-h incubation with microsomal fraction of clonal cells of mock (c) and CYP2D6 (d) are shown.

thiocyanate-labeled rabbit polyclonal anti-mouse IgG antibody (DakoCytomation Denmark A/S, Glostrup, Denmark). Intracellular localization was examined by a fluorescent microscope, Axioskop (Carl Zeiss, Oberkochen, Germany), and overlay views were made using Adobe Photoshop® 5.0 software (Adobe Systems Incorporated, San Jose, CA, USA). PC12 cells were differentiated by addition of mouse NGF 7S subunit (Gibco) at a concentration of 50 ng/ml.

TABLE 1
CYTOTOXICITY OF MPP+ AND MPTP ON PC12 CELLS

	Differentiated (IC_{50}, μM)*		Undifferentiated (IC_{50}, μM)*	
PC12 Cells	MPP^+	MPTP	MPP^+	MPTP
Mock transfected	15	680	24	830
Overexpressing human CYP2D6	38	450	42	500

*Assessed by MTT assay.

CYP2D6 Activity Assay

Microsomal fractions from PC12 cells were isolated using previously published methods, with several modifications (26). Briefly, cells (5×10^6) were washed twice with phosphate-buffered saline (PBS) and collected by scraping in sonication buffer [10 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, and 1 μg/ml pepstatin]. The cell suspension was sonicated on ice 5 times for 5 s each with 30-s intervals by an ultrasonic homogenizer, Astrason™ W-385 (Misonix, Farmingdale, NY, USA; formerly Heat Systems Ultrasonics Inc.). The cell lysate was centrifuged at $8000 \times g$ for 10 min at 4°C. The supernatant was saved and centrifuged at $100{,}000 \times g$ for 1 h at 4°C. The pellet (microsomal fraction) was resuspended in 200 μl of 200 mM potassium phosphate (pH 7.4). A microsomal fraction prepared from insect cells that were infected with a baculovirus containing human CYP2D6 cDNA, CYP2D6 Baculosomes™ (PanVera Corporation, Madison, WI, USA), was used as a positive control.

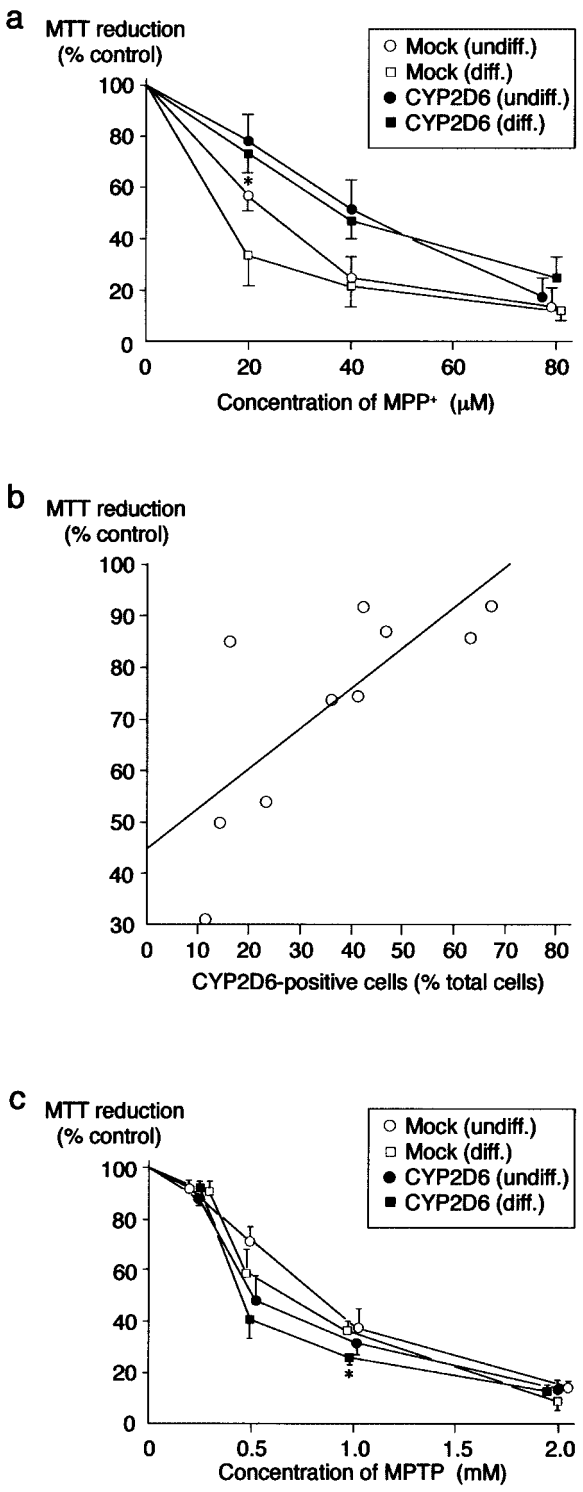

Figure 3. Mitochondrial activity to reduce MTT after exposure to MPP^+ or MPTP. Clonal cells of CYP2D6 and mock, undifferentiated or differentiated by NGF, were incubated with MPP^+ (a) and MPTP (c) for 24 h. The activity is expressed as percentage of the mean value of control (vehicle alone). Each point represents the mean value ± SEM ($n = 4$) for mock and 10 for CYP2D6. $*p <$ 0.05 by unpaired Student's t-test for mock versus CYP2D6. (b) MTT-reducing activity after exposure to MPP^+ and percentage of CYP2D6-positive cells are positively correlated. The regression line is $y = 0.78x + 44.51$ ($r = 0.736$).

CYP2D6 activity was assayed by using [*guanidine*-^{14}C]debrisoquine (Amersham Biosciences Corp.) as substrate. To a 50-μl aliquot of the microsomal fraction, 20 μl of 10 mM NADPH, 20 μl of [*guanidine*-^{14}C]debrisoquine (0.5 μCi), and 10 μl H_2O were added. The reaction mixture was incubated for 3 h at 37°C and was stopped by the addition of 15 μl of 70% perchloric acid. After centrifugation at $10{,}000 \times g$ for 15 min, the supernatant was saved and subjected to an LC-10A high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with Ultrasphere™ ODS (C-18, 4.6×250 mm) high performance column (Beckman Coulter Inc., Fullerton, CA, USA). Elusion was performed at a flow rate of 1.0 ml/min at room temperature using 10 mM trifluoroacetic acid as solvent A and a mixture of 10 mM trifluoroacetic acid and acetonitrile (1:3) as solvent B. The metabolites of [*guanidine*-^{14}C]debrisoquine were separated by a linear gradient of 20–100% solvent B for 10 min. The eluted ^{14}C-labeled debrisoquine metabolites were detected by a flow scintillation analyzer Radiomatic™ Model C515TR with FLO-ONE® for Windows (PerkinElmer, Inc., Wellesley, MA, USA).

Quantification of Cell Viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich Co., St. Louis, MO, USA) assay (20). In viable cells, the mitochondrial complex II enzyme, succinate dehydrogenase, metabolizes the substrate MTT, a tetrazolium salt, to a blue-colored water-insoluble formazan. After incubation of undifferentiated or differentiated PC12 cells with various concentrations of MPP^+ iodide or MPTP (both from Sigma-Aldrich Co., St. Louis, MO, USA) for 24 h, the culture medium was changed to fresh medium without the respective toxicant and 0.1 vol of 5 mg/ml MTT solution was added. After 4 h of incubation at 37°C, an equal volume of isopropanol containing 40 mM HCl was added to dissolve the formazan. Optical density was measured by a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) at 570 nm.

Detection of ROS in Mitochondria

Mitochondrial ROS generation was determined using reduced chloromethyltetramethylrosamine, MitoTracker® Red CM-H_2XRos (Molecular Probes, Inc.). This selectively accumulates in the mitochondria of viable cells and is oxidized by hydrogen peroxide to its fluorescent form (32). Following a 24-h exposure to MPP^+ or MPTP, cells were incubated with 500 nM of reduced MitoTracker® Red in phenol red-free medium for 45 min at 37°C, washed twice with PBS,

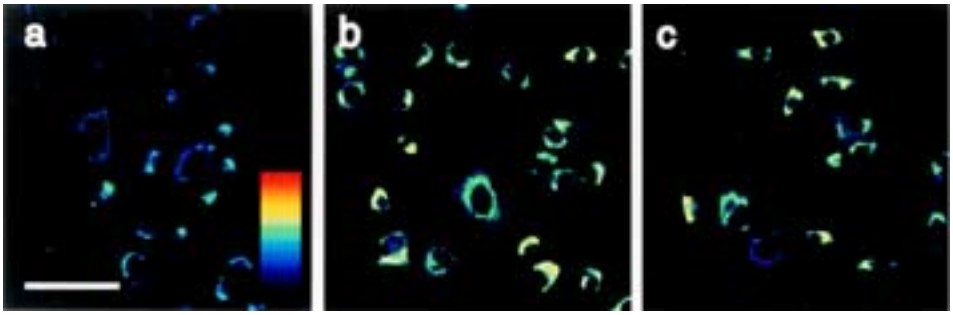

Figure 4. Mitochondrial generation of ROS after exposure to MPP^+ or MPTP. Clonal cells were treated with a reduced form of chloromethyltetramethylrosamine before (a) after a 24-h incubation with 20 μM MPP^+ (b) or 500 μM MPTP (c). Mitochondrial ROS were visualized by oxidized chloromethyltetramethylrosamine. The fluorescence intensity was semiquantified by linear color scale. Relative levels of the fluorescence are indicated by the color scale. Scale bar: 40 μm.

and fixed with acetone/methanol (1:1). Cellular fluorescence was quantified using a confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany) with a krypton/argon laser and a 590-nm bandpass filter. The intensity of the laser beam and the sensitivity of the photodetector were held constant to allow quantitative comparisons of relative fluorescence intensity of oxidized MitoTracker® Red.

Data Analysis and Statistics

Unpaired Student's *t*-test was used to compare MTT reduction between the two groups using StatView 4.0 software (SAS Institute Inc., Cary, NC, USA). A probability of less than 5% ($p < 0.05$) was considered as statistically significant.

RESULTS

Expression of CYP2D6 in Clonal PC12 Cells Lines

Fourteen PC12 clones overexpressing CYP2D6 were obtained. Overexpressed human CYP2D6 and endogenous rat CYP2D5 (debrisoquine-specific homologue to human CYP2D6) were detectable at the size of 62 and 65 kDa, respectively, by immunoblotting with anti-CYP2D6 antibody (MAB-2D6) (Fig. 1a). Expression of transfected CYP2D6 in these clones was localized to endoplasmic reticulum (ER) by overlay view with ER-Tracker™ (Fig. 1b–d). The expression of transfected *CYP2D6* was 10-fold higher than endogenous levels of CYP2D5.

Enzyme Activity of Clonal PC12 Cell Lines Overexpressing CYP2D6

The enzyme activity of CYP2D6 was measured by detection of the metabolites of [*guanidine*-^{14}C]debrisoquine by HPLC. Control microsomes (from insect cells containing human CYP2D6) were incubated with [*guanidine*-^{14}C]debrisoquine for 3 h. Control versus microsomes incubated with debrisoquine revealed that the [*guanidine*-^{14}C]debrisoquine peak (retention time 8.65 min) was decreased in the microsomal preparation with the appearance of several metabolites (Fig. 2a, b). After incubation with microsomal fraction from clonal cell lines of CYP2D6, but not mock, the minor peak (retention time 6.95 min) increased with a decrease of the major peak (Fig. 2c, d).

Effects of CYP2D6 Overexpression on Cytotoxicity of MPP^+ or MPTP

Cell viability of PC12 cells, as assessed by the MTT assay, decreased in a dose-dependent manner following exposure to MPP^+ and MPTP. Differentiated PC12 cells were more susceptible to the cytotoxic effects of MPP^+ than undifferentiated PC12 cells and mock-transfected cells (Table 1, Fig. 3a). The cytotoxicity of MPP^+ was decreased in differentiated and undifferentiated PC12 cells overexpressing CYP2D6 (Table 1). The viability of clonal cell lines of CYP2D6 after exposure to MPP^+ positively correlated with the percentage of CYP2D6-positive cells (Fig. 3b), supporting the protective role of CYP2D6 overexpression against the cytotoxicity of MPP^+. Although the cytotoxic effects of MPTP were much lower than that of MPP^+, a marked increase in MPTP toxicity was observed at higher doses in differentiated and undiferented PC12 cells overexpressing CYP2D6 versus mock-transfected controls (Table 1, Fig. 3c).

ROS Generation by MPP^+ and MPTP in Mitochondria

The results of the MTT assays indicated an impairment of the energy charge or redox state in mitochondria in the early phase of the cytotoxic action of MPP^+ and MPTP. To further specify the subcellular localization of ROS generation, cells were stained

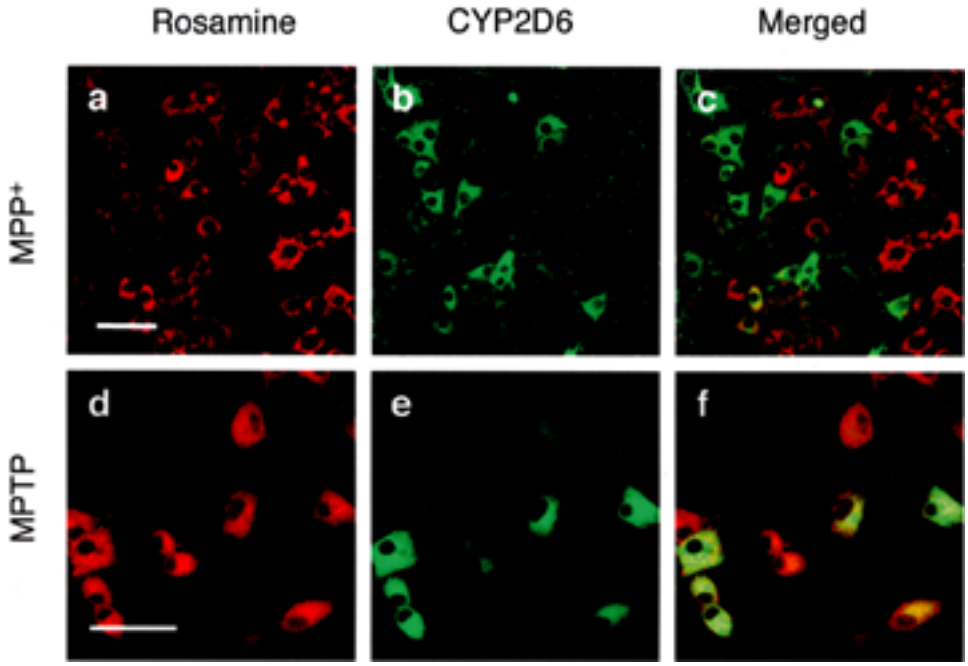

Figure 5. Mitochondrial ROS generation and CYP2D6 expression. Mitochondrial ROS generation in CYP2D6-transfected PC12 cells was visualized by oxidized chloromethyltetramethylrosamine after a 24-h exposure to 20 μM MPP^+ (a) or 500 μM MPTP (d). Overexpression of CYP2D6 was detected with anti-CYP2D6 antibody (b, e). The merged images of mitochondrial ROS generation and CYP2D6 expression are shown (c, f). Scale bar: 40 μm.

with a mitochondria-specific agent (i.e., reduced MitoTracker® Red). The exposure to 20 μM MPP^+ and 500 μM MPTP enhanced the generation of ROS in mitochondria of mock-transfected differentiated PC12 cells (Fig. 4). But the fluorescent intensity of oxidized MitoTracker® Red after exposure to MPP^+ was suppressed in differentiated CYP2D6-positive cells (Fig. 5a–c). By contrast, no significant change in mitochondrial ROS generation was observed in cells overexpressing CYP2D6 following exposure to MPTP (Fig. 5d–f).

DISCUSSION

The results of the present study show that both actively dividing and differentiated PC12 cells overexpressing CYP2D6 are less susceptible to MPP^+ toxicity versus mock-transfected controls. Inside the cell, MPP^+ accumulates in the mitochondria and disrupts mitochondrial respiration by inhibiting complex I in the electron transport chain, leading to impaired ATP production, loss of mitochondrial membrane potential, and formation of ROS (3,28). A secondary source of ROS results in the cytoplasm from the metabolism and redox cycling of MPP^+ by xanthine oxidase (18). Our results show that CYP2D6 overexpression reduces the fluorescent intensity of oxidized chloromethyltetramethylrosamine following MPP^+ exposure, indicating a decrease in the generation of ROS in mitochondria. Further, the suppression of ROS in the mitochondria by CYP2D6 overexpression is consistent with the results of the MTT assay, or its protective role against the cytotoxic effects of MPP^+.

In the brain, MPTP is metabolized to MPP^+ by monoamine oxidase B (MAO-B), mainly in astrocytes versus neurons. Dopamine transporters selectively pass MPTP and its metabolite MPP^+ across the plasma membrane of dopaminergic neurons and differentiated (NGF-treated) PC12 cells; however, the low activity of MAO-B in PC12 cells limits the bioactivation of MPTP to MPP^+ by this pathway (30). In the present study, cell viability, as assessed by MTT assay, after exposure to MPTP was higher than that after MPP^+ exposure in mock-transfected controls by 34.6-fold for undifferentiated and 45.3-fold for differentiated PC12 cells. This decreased toxicity is notably due to the low expression levels of MAO-B. However, a cytotoxic effect became evident at concentrations above 500 μM in cells overexpressing CYP2D6. This increase in toxicity may be explained by the enzyme systems, other than MAO-B, that metabolize MPTP.

The microsomal flavin-containing monoxygenase system (FMO) oxidizes MPTP to MPTP N-oxide.

This pathway is the major detoxification pathway in cells, with a metabolic capacity 25 times that of CYP2D6 (2,5,31). CYP2D6 can also metabolize MPTP to several metabolites including: 4-phenyl-1,2,3,6-tetrahydropyridine, a nontoxic metabolite, and 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and MPP^+, depending on the concentration of MPTP utilized (5,23,27,31). Previous studies have demonstrated that FMO inhibitors enhance MPTP neurotoxicity by increasing the concentration of MPP^+ in the brain (4,5). Therefore, our results, indicating a higher degree of toxicity following exposure to concentrations of MPTP exceeding 500 μM in cells overexpressing CYP2D6, suggest that by saturating the FMO system, MPTP is shunted to the CYP2D6 pathway, resulting in an increase in the ratio of toxic metabolites, namely $MPDP^+$ and MPP^+. The decreased generation of ROS in the mitochondria of transfected cells treated with MPTP would suggest that this increased level of cytotoxicity may result from redox cycling of MPP^+/MPP by xanthine oxidase and the inherent production of ROS that accompanies enzymatic activity of the microsomal P450 system (25,33). It is worth mentioning that the relevance of this concentration in relation to the expected levels of MPTP utilized in in vivo studies is excessive considering most whole-animal studies rarely use a dose of MPTP higher than 40 mg/kg, which would be equivalent to a systemic concentration of 190 μM (7,14,24).

In summary, our results provide a possible rationale for the association of defective drug metabolism with increased susceptibility of postmitotic cells to neurotoxicants, such as dopaminergic neurons and MPP^+ toxicity.

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