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Kinetic mechanism of time-dependent inhibition of CYP2D6 by 3,4-methylenedioxymethamphetamine (MDMA): Functional heterogeneity of the enzyme and the reversibility of its inactivation

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ABSTRACT

We investigate the mechanism of time-dependent inhibition (TDI) of human cytochrome P450 2D6 (CYP2D6) by 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), one of the most widespread recreational drugs of abuse. In an effort to unravel the kinetic mechanism of the formation of metabolic inhibitory complex (MIC) of CYP2D6 with MDMA-derived carbene we carried out a series of spectrophotometric studies paralleled with registration of the kinetics of time-dependent inhibition (TDI) in CYP2D6-incorporated proteoliposomes. The high amplitude of spectral signal in this system allowed us to characterize the spectral properties of the formed MIC in details and obtain an accurate spectral signature of MIC formation. This information was then used in the studies with CYP2D6-containing microsomes of insect cells (CYP2D6 Supersomes™). Our results demonstrate that in both systems the formation of the ferrous carbene-derived MIC is relatively slow, reversible and is not associated with the accumulation of the ferric carbene intermediate, as takes place in the case of CYP3A4 and podophylotoxin. Furthermore, the limited amplitude of MIC formation suggests that only a fraction (~50%) of spectrally detectable CYP2D6 in both proteoliposomes and Supersomes participates in the formation of MIC and is therefore involved in the MDMA metabolism. This observation reveals yet another example of a cytochrome P450 that exhibits persistent functional heterogeneity of its population in microsomal membranes. Our study provides a solid methodological background for further mechanistic studies of MIC formation in human liver microsomes and demonstrates that the potency and physiological relevance of MDMA-dependent TDI of CYP2D6 may be overestimated.

1. Introduction

The central role the cytochromes P450 (P450) play in human drug metabolism makes these enzymes a major subject for studies of drug disposition, adverse drug effects and drug-drug interactions (DDIs). One of important P450-related problems of high pharmacological importance is the problem of prediction and analysis of DDIs with drugs capable of metabolism-based inhibition (MBI) of P450s. Metabolism of these compounds results in a loss of enzyme activity over time and therefore is associated with time-dependent inhibition (TDI) of the metabolizing P450 enzymes [1–3]. Compounds that show TDI are usually avoided in selection of drug candidates due to complexity and uncertainty associated with DDI prediction. Despite of these reservations, there are quite a few drugs on the market that are known to cause TDI of the P450 enzymes that metabolize them [4,5].

The most complex type of TDI is the one associated with quasi-reversible formation of metabolic intermediate complexes (MIC) [6,7]. One of the most representative groups of P450 inhibitors of this type includes methylenedioxyphenyl (MDP) compounds, such as the insecticide synergist piperonyl butoxide and the drugs tadalafil, paroxetine, and podophyllotoxin. This group also includes 3,4-methylenedioxyamphetamine (MDMA) also known as “ecstasy”. This widespread drug of abuse is metabolized by several cytochrome P450 species, of whose CYP2D6 plays the key role [8]. The structure of MDMA and the main routes of its transformation by CYP2D6 are shown in Fig. 1.

The mechanism of formation of inhibitory MIC with MDP compounds involves an oxidative conversion of their methylenedioxy group to a carbene. The formed carbene intermediate then binds to the heme

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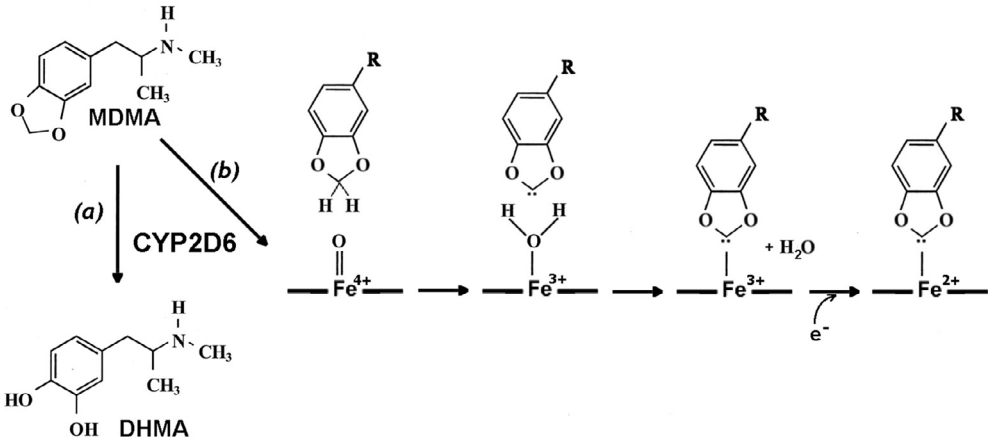

Fig. 1. The structure of MDMA and the main routes of its transformation by CYP2D6: the demethylenation path ***(a)*** resulting in the formation of 3,4-dihydroxymethamphetamine (DHMA) and the path associated with the in mechanism-based inhibition ***(b)*** that involves the formation of MDMI-derived carbene, its binding to the CYP2D6 heme iron(3^+) as an axial ligand and the subsequent reduction of the formed MIC.

iron of ferric cytochrome P450 as an axial ligand. Subsequent reduction stabilizes the carbene-bound porphyrin in ferrous (Fe^{2+}) state [9]. The implied mechanism of this process for the case of MDMA-dependent inactivation of CYP2D6 is shown as path (b) in Fig. 1.

An important advantage of studying formation of MICs with MDP compounds is the pronounced changes in the absorbance spectra of P450 caused by the formation of the ferrous carbene-bound intermediate. It is well established that this intermediate has the main Soret band positioned at 455 nm with an additional band at 425–427 nm ("type III" spectrum) [9–13]. This is in contrast to the parent low- and high-spin states of ferric P450, where the Soret band is centered at 416–418 and 396–398 nm respectively. The 455 nm absorbance is considered the defining characteristic of MIC formation [9,14]. A pronounced pH dependence of the 425 nm band prompted a hypothesis that this band is due to partial conversion of the 5th axial thiolate ligand of the heme iron to unionized thiol state [9], similar to what is observed in inactivated "cytochrome P420" state of P450 heme proteins [15–17]. Moreover, an additional transient peak at 438 nm was attributed to absorbance of a supposed transitory complex of carbene with ferric cytochrome P450 (Fe^{3+}:carbene metabolite) [18].

In our recent study [19] we used these unique spectral properties of carbene-derived MICs to explore the mechanism of TDI of CYP3A4 by podophylotoxin with UV/VIS absorbance spectroscopy. These studies illustrate the potential of singular value decomposition and/or principal component analysis (PCA) for the studies of the mechanism of MIC formation. Application of these advanced methods of spectral analysis allowed us to demonstrate that the formation of MIC of CYP3A4 with podophylotoxin goes through a reversible formation of Fe^{3+}:carbene intermediate that undergoes a slow conversion to the Fe^{2+}:carbene complex, which is virtually irreversible.

In the present study, we extend the application of this strategy to CYP2D6 and its interactions with MDMA, which recreational use is known to have potential of causing acute liver failure. MDMA was reported among the most common causes of liver injury in young people [20–22]. Hepatotoxicity of MDMA is mediated by its metabolism on cytochrome P450 2D6 (CYP2D6) [8,23]. Furthermore, CYP2D6-dependent metabolism of MDMA results in a physiologically significant MBI [24–27]. However, the *in vitro* estimates of the importance of MDMA-dependent TDI were made based on the studies with the classical replot method [24,25]. In previous work, we have shown that the use of this simplistic approach can lead to an overestimation of the potency of TDI and its physiological relevance in the instances of quasi-reversible formation of MIC [6,7]. Therefore, a detailed study of the mechanisms and kinetics of MDMA-dependent MIC formation and the associated TDI of CYP2D6 is warranted.

In an effort to unravel the kinetic mechanism of MIC formation and gain a quantitative assessment of the maximal depth of enzyme conversion to the carbene-derived MIC, we carried out most of our studies in CYP2D6-incorporated proteoliposomes (PLS) containing NADPH-cytochrome P450 reductase (CPR). High amplitude of the spectral signal in this model system allowed us to characterize the spectral properties of the formed MIC in details and obtain an accurate spectral signature of MIC formation. This information was then successfully used in the studies with CYP2D6-containing microsomes of insect cells (CYP2D6 Supersomes™). Besides providing a solid methodological background for further mechanistic studies of MIC formation in human liver microsomes, our results demonstrate that the potency and physiological relevance of MDMA-dependent TDI of CYP2D6 may be overestimated. Furthermore, our study demonstrates that MDMA-dependent MBI affects only a limited fraction (~50%) of spectrally-detectable CYP2D6 in both PLS and Supersomes. The remaining portion of the enzyme is inactive toward MDMA metabolism and remains unaffected by the associated MIC formation. This observation divulges yet another example of cytochrome P450 that exhibits persistent functional heterogeneity of its population in the microsomal membranes [28,29].

2. Methods and materials

2.1. Materials

Glucose-6-phosphate, protocatechuate 3,4-dioxygenase from *Pseudomonas* sp., protocatechuic acid, DL-dithiothreitol (DTT) and L-α-phosphatidylcholine (PC) from egg yolk were the products of Sigma-Aldrich (St. Louis, MO). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and NADPH tetrasodium salt were from EMD Millipore (Billerica, MA). L-α-phosphatidylethanolamine (PE) from bovine liver and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (phosphatidic acid, PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). CYP2D6-containing Supersomes™ and 7-hydroxy-4-(aminomethyl)-coumarin (HAMC) were the products of Corning Life Sciences (Corning, NY). Octyl-β-D-glucopyranoside (octylglucoside) was the product of Fluka Honeywell Specialty Chemicals (Seelze, Germany). 4-(2- Hydroxyethyl)-1- piperazine ethanesulfonic acid (HEPES) was obtained from Indofine Chemical Company (Hillsborough, NJ). Dextromethorphan (DXM) hydrobromide hydrate, dextrorphan (DXP) tartrate, 3,4-methylenedioxymethylamphetamine (MDMA) hydrochloride and 3,4-dihydroxymethamphetamine (DHMA) were the products of Cayman Chemical (Ann Arbor, MI). MDMA was obtained as a certified reference material (CRM) solution in methanol (1 mg/ml), which was evaporated and the chemical was re-dissolved in acetone to the concentration of 25 mM. Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Gold Biotechnology (St. Louis, MO). *N*-butyl 7-methoxy-4-(aminomethyl)-coumarin (BMAMC) was synthesized from 7-methoxy-4-(bromomethyl)-coumarin (Sigma-Aldrich, St. Louis, MO) and purified as previously described [30]. All other chemicals (KCl,

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