ISOFORM SPECIFICITY OF N-DEACETYL KETOCONAZOLE BY HUMAN AND RABBIT FLAVIN-CONTAINING MONOOXYGENASES

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ABSTRACT:

N-Deacetyl ketoconazole (DAK) is the major metabolite of orally administered ketoconazole. This major metabolite has been demonstrated to be further metabolized predominately by the flavin-containing monooxygenases (FMOs) to the secondary hydroxylamine, N-deacetyl-N-hydroxyketoconazole (N-hydroxy-DAK) by adult and postnatal rat hepatic microsomes. Our current investigation evaluated the FMO isoform specificity of DAK in a pyrophosphate buffer (pH 8.8) containing the glucose 6-phosphate NADPH-generating system. cDNA-expressed human FMOs (FMO1, FMO3, and FMO5) and cDNA-expressed rabbit FMOs (FMO1, FMO2, FMO3, and FMO5) were used to assess the metabolism of DAK to its subsequent FMO-mediated metabolites by HPLC analysis. Human and rabbit cDNA-expressed FMO3 resulted in extensive metabolism of DAK in 1 h (71.2 and 64.5%, respectively) to N-hydroxy-DAK (48.2 and 47.7%, respectively) and two other metabolites, metabolite 1 (11.7 and 7.8%, respectively) and metabolite 3 (10.5 and 10.0%, respectively). Previous studies suggest that metabolite 1 is the nitrene formed after successive FMO-mediated metabolism of N-hydroxy-DAK. Moreover, these studies display similar metabolic profiles seen with adult and postnatal rat hepatic microsomes. The human and rabbit FMO1 metabolized DAK predominately to the N-hydroxy-DAK in 1 h (36.2 and 25.3%, respectively) with minimal metabolism to the other metabolites (<5%). Rabbit FMO2 metabolized DAK to N-hydroxy-DAK (15.9%) and metabolite 1 (6.6%). Last, DAK did not appear to be a substrate for human or rabbit FMO5. Heat inactivation of cDNA-expressed FMOs abolished DAK metabolite formation. These results suggest that DAK is a substrate for human and rabbit FMO1 and FMO3, rabbit FMO2, but not human or rabbit FMO5.

Ketoconazole (KT) is a prominent broad-spectrum oral antifungal agent used in the treatment of systemic mycoses (Ringel, 1990). KT exerts its antifungal activity by inhibiting lanosterol 14α-demethylase (Van Den Bossche et al., 1980). Also, the inhibitory effect of KT on the synthesis of testosterone in both testicular and adrenal cells has made KT a suitable candidate for treatment of androgen-dependent diseases such as advanced prostate cancer (Pont et al., 1984; Jubelirer and Hogan, 1989; Vogelzang and Kennealey, 1992). Due to the extensive therapeutic usage of KT, there have been numerous documented cases of KT-induced hepatotoxicity (Bercoff et al., 1985; Benson et al., 1988; Brusko and Marten, 1991). Biochemical features related to KT hepatotoxicity tend to be hepatocellular injury in 57% of the patients and cholestatic injury in 43% of the patients (Stricker et al., 1986) and that the type of hepatic injury is zone 3 necrosis (Stricker et al., 1986; Benson et al., 1988). The hepatotoxicity was usually reversible when the drug was discontinued, with recovery occurring within 3 to 6 months (Janssen and Symoens, 1983; Benson et al., 1988). Apoptosis through the p53-dependent pathway (Ho et al., 1998) and metabolic bioactivation to reactive metabolites (Rodriguez and Acosta, 1997a; Rodriguez et al., 1999) have been suggested for the toxicity. KT appears to be extensively metabolized by hepatic microsomal enzymes that are primarily responsible for the biotransformation reactions (Gascoigne et al., 1981; Daneshmend and Warnock, 1988). The metabolic pathways suggested include cytochrome P450 (CYP)-mediated oxidation, cleavage, degradation, and scission of the imidazole and piperazine rings; oxidative O-dealkylation; and aromatic hydroxylation (Heel et al., 1982; Daneshmend and Warnock, 1988). N-Deacetyl ketoconazole (DAK), Fig. 1, has been reported to be the major metabolite in mice that accumulates to significant levels in hepatic tissues (Whitehouse et al., 1994a,b). Also, two flavin-containing monoxygenase (FMO)-mediated N-oxide metabolites of KT have been isolated from mouse liver (Whitehouse et al., 1994a). Moreover, a recent study demonstrated that DAK was further metabolized by FMO to produce a secondary hydroxylamine, N-deacetyl-N-hydroxyketoconazole (N-hydroxy-DAK, Fig. 1) from rat hepatic microsomes (Rodriguez et al., 1999). The formation of the aforementioned metabolites appears to be CYP- and/or FMO-mediated.

It is possible that the hepatotoxicity associated with KT may be due to the bioactivation of DAK by successive oxidative attacks by FMO on the piperazine ring to generate a secondary hydroxylamine, N-hydroxy-DAK, which may be further metabolized by FMO to generate a nitrene, an aldehyde, oxime, and a ring-opened dialdehyde that...
could eventually result in toxic consequences producing hepatic injury. To date, our studies have been the first to demonstrate that DAK undergoes extensive metabolism to several FMO-mediated metabolites by rat hepatic microsomes; in particular, N-hydroxy-DAK (Rodriguez and Acosta, 1997a; Rodriguez et al., 1999). Also, our previous study in adult rats suggested that gender differences may have produced different metabolic profiles (Rodriguez et al., 1999). N-Hydroxy-DAK was the predominant metabolite of DAK formed from the male and female hepatic microsomes; however, the formation of the two metabolites, metabolite 1 and metabolite 3, was significantly less in the female rats than in the male rats (Rodriguez et al., 1999). It has been reported that FMO3 and FMO5 are gender-independent in rat, whereas FMO1 appears to be selective to the male rat (Cherrington et al., 1998). Thus, our current objective was to evaluate the FMO isoform specificity and species differences of DAK from cDNA-expressed human and rabbit FMOs.

Materials and Methods

Chemicals. All chemicals were from Sigma Co. (St. Louis, MO) unless otherwise stated. cDNA-expressed human FMOs, Supersomes, were purchased from Gentest Corp. (Woburn, MA). DAK was a generous gift from Janssen Pharmaceutica (Beerse, Belgium). cDNA-expressed rabbit FMOs were a generous gift from Dr. Richard M. Philpot (National Institute of Environmental Health Sciences, Laboratory of Signal Transduction, Research Triangle Park, NC). Solvents used for HPLC analysis were HPLC grade.

Metabolic Assays. In vitro metabolism of DAK by cDNA-expressed FMOs was a modified procedure (Miranda et al., 1991). The incubation mixture consisted of 0.1 mg of cDNA-expressed FMO protein, 100 mM glycine-25 mM pyrophosphate buffer, pH 8.8, 50 and 100 μM DAK, and the NADPH-generating system (10 mM glucose 6-phosphate, 1.0 U/ml of glucose-6-phosphate dehydrogenase, and 1 mM NADP+) in a total volume of 0.5 ml. After 0.25, 0.5, and 1 h of incubation at 37°C with shaking, the reaction was terminated with 0.5 ml of ice-cold methanol with 0.5% v/v formic acid and cooled on ice. Proteins were precipitated by centrifugation at 14,000g for 15 min at 4°C, and aliquots of the supernatant were analyzed by HPLC as described below. cDNA-expressed FMO activity was inhibited by heat inactivation to determine the effect of heat treatment on FMO activity (Ziegler, 1980). The cDNA-expressed FMOs were heated at 50°C for 90 s before adding them to the incubation mixture. Negative controls without the NADPH-generating system and the control insect cell line microsomes (nonexpressed FMO) with the NADPH-generating system were performed. In addition, positive controls using 1 mM thiobenzamide with and without the NADPH-generating system were performed using the method of Cashman and Hanzlik (1981).

HPLC. HPLC analysis for DAK and its metabolites was performed with a Waters system (Milford, MA) that consisted of a Waters C18 symmetry column connected to a Waters Alliance 2690 solvent delivery system, Waters 996 photodiode array detector, Waters 2690 autosampler, and Millennium Chromatography software version 3.0. DAK and its metabolites were eluted with 0.005 M ammonium formate (pH 4.5) and 20 to 60% acetonitrile at a flow rate of 1.0 ml/min, with UV detection at 220 nm (Rodriguez et al., 1999).

Results

Figure 2 shows a representative HPLC chromatogram of the metabolism of DAK after a 1-h incubation period of DAK and the cDNA-expressed human FMO3 in the NADPH-generating system, pH 8.8. The retention times for DAK and its metabolites were: DAK, 7.5 min; metabolite 1 (M1), 8.0 min; N-hydroxy-DAK, 8.9 min; and metabolite 3 (M3), 9.5 min. N-Hydroxy-DAK was the predominant metabolite peak formed at all time points evaluated from both the cDNA-expressed human and rabbit FMO1 and FMO3 incubations. Minimal formation of M1 and M3 was seen with the cDNA-expressed human and rabbit FMO1.

Figure 3 demonstrates the disappearance of DAK and the formation of its metabolites from the cDNA-expressed human FMO1 over the 1-h incubation period. DAK was metabolized by 43.7 ± 2.0% primarily to N-hydroxy-DAK (36.2 ± 0.8%). Minimal metabolism to M1 and M3 occurred with FMO1 (<4%). In Fig. 4, extensive metabolism of DAK (71.2 ± 7.7%) to N-hydroxy-DAK (48.2 ± 4.4%) and the two other metabolites, M1 (11.7 ± 1.2%) and M3 (10.5 ± 1.2%), occurred with cDNA-expressed human FMO3. There was no metabolism of DAK with the cDNA-expressed human FMO5. In addition, incubations with 50 μM DAK produced an identical metabolic profile as the 100 μM DAK incubation (data not shown).

In the cDNA-expressed rabbit FMO1 1-h incubation period, DAK was metabolized primarily by 30.5 ± 6.2% to N-hydroxy-DAK (25.3 ± 1.6%), although there was no M3 formation. In Fig. 5, rabbit FMO2 metabolized DAK to N-hydroxy-DAK (15.9 ± 1.6%), M1 (6.6 ± 0.6%), and M3 (1.3 ± 0.2%). Extensive metabolism of DAK to N-hydroxy-DAK (47.7 ± 6.6%), M1 (7.8 ± 0.3%), and M3 (10.0 ± 7.0%) occurred with cDNA-expressed rabbit FMO3. Like the cDNA-expressed human FMO5, there was no metabolism of DAK with the cDNA-expressed rabbit FMO5 (data not shown).

Discussion

KT appears to be extensively metabolized by hepatic microsomal enzymes that are primarily responsible for the biotransformation reactions (Gascoigne et al., 1981; Daneshmend and Warnock, 1988). DAK has been reported to be the major metabolite of KT in mice that accumulates to significant levels in hepatic tissues (Whitehouse et al., 1994a,b). Previous studies suggest that DAK may be responsible, in part, for the hepatotoxicity associated with KT (Rodriguez and Acosta, 1997b). DAK was more cytotoxic than KT in a time- and dose-response relationship using postnatal (8–10-day-old) rat hepatocytes (Rodriguez and Acosta, 1997b). Furthermore, the toxicity of DAK was enhanced with octylamine, a known positive effector for FMO as well as a CYP-inhibitor (Cashman and Ziegler, 1986), and suppressed with methimazole, a competitive substrate for FMO (Rodriguez and Acosta, 1997b). It is possible that the hepatotoxicity associated with KT may be due to the bioactivation of DAK by successive oxidative attacks by FMO on the piperazine ring to gen-
erate a secondary hydroxylamine (N-hydroxy-DAK) (Rodriguez et al., 1999) that may be further metabolized by FMO to generate a nitrone, an aldehyde, oxime, and a ring-opened dialdehyde that could eventually result in toxic consequences producing hepatic injury. Our earlier studies demonstrated that adult (male and female) and postnatal rat hepatic microsomes metabolize DAK primarily to the N-hydroxy-DAK (Rodriguez and Acosta, 1997a; Rodriguez et al., 1999). Also, our previous studies suggest that M1 had a molecular weight of 503, which is consistent with the formation of a nitrone from the FMO-mediated metabolism of N-hydroxy-DAK (Rodriguez et al., 1999). Formation of M1 and M3 increased in a time-dependent manner after formation of N-hydroxy-DAK (Figs. 3–5). Last, the metabolic profile for the 50 μM DAK incubation was identical with the 100 μM DAK incubation (data not shown). This study supports further FMO-mediated metabolism of N-hydroxy-DAK from human and rabbit FMOs; however, it is possible that the unidentified M3 may be a decomposition product. Continued efforts are being made to identify M3.

The metabolism of DAK appears to be nonlinear after 0.25 h (Fig. 4). It is possible that the end-product, N-hydroxy-DAK, may have inhibited the human and rabbit FMO3 reaction of DAK to N-hydroxy-DAK. N-Hydroxy-DAK does not appear to have an inhibitory effect on human and rabbit FMO1. DAK was a substrate for rabbit FMO2 that formed N-hydroxy-DAK, M1, and M3 (Fig. 5). Last, no metabolism of DAK was seen with either the human or rabbit FMO5. Because minimal M1 and M3 metabolites were formed with the human and rabbit FMO1, N-hydroxy-DAK did not appear to be a substrate for FMO1. Although there was an increase in N-hydroxy-DAK formation from DAK, it is possible that N-hydroxy-DAK may
have inhibited the reaction to prevent further metabolism to M1 and M3. On the other hand, human and rabbit FMO3 and rabbit FMO2 appeared to further metabolize N-hydroxy-DAK to M1 and M3. This substrate specificity of DAK and N-hydroxy-DAK may explain the gender differences seen in the metabolic profile of adult rats (Rodriguez et al., 1999). In the Rodriguez et al. (1999) study, the metabolite formation of M1 and M3 from N-hydroxy-DAK from female hepatic microsomes was less than the male. Previous studies have shown that male rats have higher FMO activity than female (Skett et al., 1980; Dannan et al., 1986; Lemoine et al., 1991). The higher activity may be due to the fact that FMO3 and FMO5 are gender-independent in rat, whereas FMO1 appears to be selective to the male rats (Cherrington et al., 1998). DAK appears to be a substrate for FMO1, FMO2, and FMO3, whereas N-hydroxy-DAK appears to be a substrate for FMO2 and FMO3. It still needs to be determined whether N-hydroxy-DAK is a substrate for FMO5. These studies support the theory that DAK would still be metabolized to N-hydroxy-DAK in female rats but not to the same extent as seen in the male rats due to the isoform specificity.

In conclusion, this study supports our original proposal that bioactivation of DAK through FMO-mediated oxidation on the piperazine ring generates a secondary hydroxyamine, N-hydroxy-DAK, that is susceptible to further FMO-mediated oxidative attacks. Moreover, FMO isoform specificity of the substrate appears to play a role in the metabolism of DAK and N-hydroxy-DAK. If both oxidation and reduction reactions of DAK and N-hydroxy-DAK occur rapidly, the tissue NADPH concentrations could be depleted (Ziegler, 1988), thereby resulting in a loss of cellular NADPH that would affect a number of cellular processes resulting in toxic consequences. Last, this study also supports that the FMO-mediated metabolism of DAK to N-hydroxy-DAK seen in adult and postnatal rat hepatic microsomes also occurs with human and rabbit FMOs. In summary, FMO metabolism of DAK and N-hydroxy-DAK appears to be isoform-specific in both the cDNA-expressed human and rabbit FMOs.

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References


