Inhibition of Mitochondrial Function in Isolated Rat Liver Mitochondria by Azole Antifungals

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ABSTRACT: Ketoconazole is an imidazole oral antifungal agent with a broad spectrum of activity. Ketoconazole has been reported to cause liver damage, but the mechanism is unknown. However, ketoconazole and a related drug, miconazole, have been shown to have inhibitory effects on oxidative phosphorylation in fungi. Fluconazole, another orally administered antifungal azole, has also been reported to cause liver damage despite its supposedly low toxicity profile. The primary objective of this study was to evaluate the metabolic integrity of adult rat liver mitochondria after exposure to ketoconazole, miconazole, fluconazole, and the deacetylated metabolite of ketoconazole by measuring ADP-dependent oxygen uptake polarographically and succinate dehydrogenase activity spectrophotometrically. Ketoconazole, N-deacetyl ketoconazole, and miconazole inhibited glutamate-malate oxidation in a dose-dependent manner such that the 50% inhibitory concentration ($I_{50}$) was 32, 300, and 110 µM, respectively. In addition, the effect of ketoconazole, miconazole, and fluconazole on phosphorylation coupled to the oxidation of pyruvate/malate, ornithine/malate, and succinate was evaluated. The results demonstrated that ketoconazole and miconazole produced a dose-dependent inhibition of NADH oxidase in which ketoconazole was the most potent inhibitor. Fluconazole had minimal inhibitory effects on NADH oxidase and succinate dehydrogenase, whereas higher concentrations of ketoconazole were required to inhibit the activity of succinate dehydrogenase. N-deacetylated ketoconazole inhibited succinate dehydrogenase with an $I_{50}$ of 350 µM. In addition, the reduction of ferricyanide by succinate catalyzed by succinate dehydrogenase demonstrated that ketoconazole caused a dose-dependent inhibition of succinate activity ($I_{50}$ of 74 µM). In summary, ketoconazole appears to be the more potent mitochondrial inhibitor of the azoles studied; complex I of the respiratory chain is the apparent target of the drug’s action.

KEYWORDS: Azole Antifungals, Mitochondria, NADH Oxidase, Oxidative Phosphorylation, Rat Liver, Succinate Dehydrogenase.

INTRODUCTION

Ketoconazole (KT), miconazole (MC), and fluconazole (FL), shown in Figure 1, are antifungal agents in a series of azole-derivatives with a broad spectrum of activity against superficial and systemic mycoses. The antifungal actions of these drugs are related to their inhibition of cytochrome P-450-dependent 14α-demethylation of lanosterol or 24-methylatedihydrolanosterol in the biosynthetic pathway of ergosterol in fungi (1). However, KT’s action is not limited to the cytochromes of fungi because it also inhibits a number of mammalian cytochromes; in contrast, FL is specific for 14α-demethylase. Nonetheless, the literature clearly indicates that KT and FL are toxic to the liver (2–6). In addition, in vitro studies demonstrated that KT was toxic to liver cells (7,8). It has also been reported that KT inhibited mitochondrial oxidative phosphorylation in Candida albicans (9,10) and in rat liver mitochondria (11). Similar effects were seen with MC on isolated rat liver mitochondria (11). Presently, the mechanism of azole-induced hepatic necrosis is unknown (12).

It has been reported that inhibition of mitochondrial NADH (nicotinamide adenine dinucleotide) oxidase was KT’s primary mechanism of toxicity in C. albicans (10), but higher concentrations of KT were required to inhibit mitochondrial activity of succinate oxidase (SDH) in rat liver. MC and clotrimazole directly damaged membranes of C. albicans cells at lower concentrations than KT; however, KT’s inhibition of respiratory function occurred at lower concentrations than the other azole compounds studied (10). MC was shown to exhibit uncoupling/inhibition patterns for both glutamate/malate and succinate (11). Shigematsu et al. (9) reported that KT blocked the transport of electrons in the respiratory chain of C. albicans under aerobic conditions with such substrates as NADH and
The addition of KT to C. albicans mitochondria without substrates resulted in strong reduction of cytochrome $a_3$, indicating that there was a specific interaction between KT and cytochrome $c$ oxidase (9). These results suggest that the primary site of KT inhibition on mitochondria isolated from C. albicans is at the most distal portion of the respiratory chain. Because the aforementioned studies indicate that antifungals may inhibit mitochondrial function in fungi (9–11), the objective of this investigation is to assess mitochondrial dysfunction in mammalian mitochondrial preparations, rat liver, after exposure to KT, MC, FL, and KT’s deacetylated metabolite by measuring oxidative phosphorylation polarographically and by monitoring SDH activity spectrophotometrically.

**MATERIALS AND METHODS**

**Materials**

KT and deacetylated KT (DAK) were generous gifts from Janssen Pharmaceutica (Beerse, Belgium). FL was a gift from Pfizer Pharmaceuticals (Groton, CT). Albumin fraction V, adenosine 5’-diphosphate (ADP), carbonyl cyanide $m$-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), miconazole, and rotenone were obtained from Sigma Chemical Co. (St. Louis, MO). Thenoyltrifluoroacetone (TTFA) was obtained from Aldrich Chemical Co. (St. Louis, MO). All other reagents were of the highest available purity from commercial sources. Male Sprague-Dawley rats weighing 175–200 g were used. These rats were bred and maintained in the Animal Resources Center at the University of Texas at Austin. They were housed in temperature (25°C)- and light cycle (12 h)-controlled rooms. Water and feed were provided ad lib.

**Preparation of Rat Liver Mitochondria**

Rat liver mitochondria were isolated according to the procedure of Chappell and Hansford (13). The final rat liver mitochondrial suspension was placed into a centrifuge tube on ice and was used 0.5–3 hours after preparation.

**Measurement of Oxidative Phosphorylation**

The respiration of the isolated rat liver mitochondria was measured polarographically with a Clark-type electrode in a 2 mL stirred vessel maintained at 30°C. The signal was recorded with a HeathKit strip Chart Recorder. The vessel contained a suspension of mitochondria (4–6 mg protein) in 0.25 M sucrose; 10 mM potassium phosphate; 5 mg/mL albumin; 5 mM MgSO$_4$, pH 7.4, in a total volume of 2.2 mL. After 4–5 minutes temperature equilibration, the endogenous rate of oxygen uptake was recorded before adding exogenous substrates in 50 μL through the capillary stem of the oxygraph vessel. The substrate stock solutions used were 0.385 M glutamate/0.115 M malate, 0.5 M succinate, 0.385 M pyruvate/0.115 M malate; 0.385 M ornithine/0.115 M malate; and 0.385 M arginine/0.115 M malate. After recording oxygen uptake for another 3–4 minutes, 0.8 μmoles ADP, pH 7.4 was added, and oxygen uptake was recorded for another 3–6 minutes. The effects of KT, MC, FL, and DAK on mitochondrial substrate- and ADP-dependent oxygen uptake were determined by adding the drugs to the oxygraph vessel to yield final concentrations above and below the 50% inhibitory concentration ($I_{50}$). Stock solutions of KT (18.8 mM) and DAK (20 mM) were prepared in 0.05 NHCl and MC (21 mM) and FL (33 mM) were prepared in dimethyl sulfoxide (DMSO). The pH was checked directly in the oxygraph chamber and adjusted to 7.4 before adding the mitochondria. The contribution of complex I and II to oxygen uptake was evaluated by adding the selective inhibitors rotenone and thenoyltrifluoroacetone. The percent inhibition of oxygen consumption was calculated from the slopes on the chart before and after the addition of KT, MC, FL, and DAK. Protein was measured by the Bio-Rad™ Protein Assay using bovine serum albumin as a standard.

**Ferricyanide Assay**

The reduction of ferricyanide by succinate catalyzed by SDH was monitored spectrophotometrically...
TABLE 1. The 50% inhibitory concentration (I_{50}’s) of state 3 respiration of various azoles (ketoconazole, deacetylated ketoconazole, miconazole, and fluconazole) with various substrates of the mitochondrial respiratory chain measured polarographically.

<table>
<thead>
<tr>
<th></th>
<th>Ketoconazole</th>
<th>Deacetyl ketoconazole</th>
<th>Miconazole</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/Malate</td>
<td>32</td>
<td>300</td>
<td>110</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>74</td>
<td>—</td>
<td>160</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ornithine/Malate</td>
<td>65</td>
<td>—</td>
<td>160</td>
<td>n.d.</td>
</tr>
<tr>
<td>Arginine/Malate</td>
<td>75</td>
<td>—</td>
<td>170</td>
<td>n.d.</td>
</tr>
<tr>
<td>Succinate</td>
<td>500</td>
<td>350</td>
<td>150</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The substrates final concentrations were 10 mM glutamate/3 mM malate; 10 mM pyruvate/3 mM; 10 mM arginine/3 mM malate; 10 mM ornithine/3 mM malate; and 12.5 mM succinate. n.d. = not detectable.

RESULTS

The mitochondrial preparations exhibited excellent respiratory control ratios, and ADP consistently stimulated glutamate/malate, pyruvate/malate, ornithine/malate, arginine/malate, and succinate oxygen uptake. The respiratory control ratios were 6.6, 5.3, 4.0, 2.0, and 2.0 for glutamate/malate, succinate, pyruvate/malate, arginine/malate, and ornithine/malate, respectively. In the presence of 0.4 mM ADP, both KT and MC inhibited the oxidation of glutamate/malate in a distinct dose-dependent manner, with KT being more potent (Table 1). The inhibitory concentration (I_{50}) was 32 and 110 μM for KT and MC, respectively. These imidazole antifungal drugs also inhibited pyruvate/malate oxidation but at somewhat higher I_{50}’s (74 μM and 160 μM for KT and MC, respectively, Table 1). On the other hand, FL up to 1 mM had essentially no detectable effect on these activities (data not shown). Whereas KT and MC also inhibited succinoxidase (Table 1), the concentrations of KT required for 50% inhibition were much higher (I_{50} = 500 μM) than with the NADH-linked substrates, MC was the more effective inhibitor. Moreover, the deacetylated metabolite of KT required higher concentrations, 300 and 350 μM, for 50% inhibition of glutamate/malate oxidase and succinate oxidase, respectively (Table 1).

Because of these observations, other NADH oxidase-dependent substrates, arginine/malate and ornithine/malate, that enter the respiratory chain through the urea cycle were evaluated. The results with arginine/malate and ornithine/malate are shown in Table 1. Once again, KT was more potent than MC with I_{50}’s of 75 and 65 μM for arginine/malate and ornithine/malate, respectively, while the I_{50}’s for miconazole were 170 and 160 μM for arginine/malate and ornithine/malate, respectively. Thus, all of the NADH oxidase substrates were sensitive to KT but not to the extent as glutamate/malate. No uncoupling effect by KT, FL, or DAK was observed in this study. On the other hand, MC inhibited NADH oxidase and SDH equally. These data were representative of three experiments. Positive controls, such as 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, rotenone, an inhibitor of NADH oxidase, and TTFA, a potent inhibitor of SDH, demonstrated that the mitochondria reacted appropriately to the site-specific inhibitors and uncouplers (data not shown). There was minimal inhibition (1%) by DMSO on the oxidations catalyzed by mitochondria. The final concentration of DMSO with FL was 0.6% at the highest concentration and 1.0% with MC.

Because a high concentration of KT was required to inhibit SDH polarographically, additional experiments were conducted to evaluate the effect of KT on SDH activity by the ferricyanide assay. The reduction of ferricyanide by succinate catalyzed by SDH was monitored spectrophotometrically at 450 nm. KT produced a dose-dependent inhibition of SDH activity with an I_{50} of 74 μM (Figure 2). TTFA was a potent inhibitor of SDH activity, whereas rotenone had minimal effects.

DISCUSSION

In summary, ketoconazole, miconazole, and N-deacetyl ketoconazole directly inhibited both the NADH and succinate oxidase enzymes necessary for mitochondrial oxidative phosphorylation in rat liver mitochondria. NADH oxidase, complex I, appears to be the more sensitive component of the respiratory chain to the inhibitory actions of ketoconazole in mammalian mitochondria, in contradiction to a previous report that states that the most distal portion of the respiratory chain (complex IV) was the primary site of keto-
conazole inhibition on oxidative phosphorylation in fungi (9). Furthermore, succinate oxidase is also susceptible to the inhibitory effects of ketoconazole, miconazole, and N-deacetyl ketoconazole, as shown by inhibition of oxidative phosphorylation and succinate dehydrogenase activity. Thus, ketoconazole, as well as its metabolite, N-deacetyl ketoconazole, and miconazole not only inhibit the NADH-linked complex but also affect other complexes of the respiratory chain. The interesting observation that fluconazole, a triazole, did not have any detectable inhibitory effects on mitochondrial respiration may suggest that structural differences in the various azoles, particularly the imidazole ring, may result in a conformation conducive to the inhibition of mitochondrial respiration at complex I. Also, it is well known that ketoconazole is an inhibitor of cytochrome P450 in which it binds to the heme portion of the cytochrome (15,16); thus, it is not surprising that it can also inhibit cytochrome c oxidase (complex IV) in fungi (9). Furthermore, the significant differences in the potency between ketoconazole and its metabolite, N-deacetyl ketoconazole, on glutamate/malate oxidase (32 μM vs. 300 μM, respectively) not only demonstrate that an imidazole ring may be needed for the inhibition but that the lack of the acetyl group results in a 10-fold decrease in mitochondrial effects.

It has been shown that when ketoconazole is inserted into a lipid layer, the acetylated piperazine moiety is oriented toward the hydrophilic region and the dichlorophenyl substituent is found in the hydrophobic phase, thus having little effect on lipid organization (17). Such an orientation is not supposed to affect the lipid organization because the area occupied per drug molecule (30 Å²) is low. In comparison, N-deacetyl ketoconazole caused complete reversal in the insertion alignment of the molecule in artificial lipid membranes, thus having a destabilizing effect on the lipids because the inversion resulted in an increase in the area occupied per drug molecule (90 Å²) in the lipid layer (17). Miconazole was also shown to exhibit a destabilizing effect such that it maintained its dichlorophenyl groups in the hydrophobic phase, whereas the imidazole moiety was oriented in the hydrophilic phase (17). The molecular mechanism responsible for these effects has not been established. Thus, it is possible that the mitochondrial membrane, which is also composed of proteins and lipids, may undergo physical changes in the outer and/or inner membrane, rendering it permeable to protons. Because the mitochondria play a central role, not only in cellular respiration but also in maintenance of intracellular pH and ion homeostasis, the inhibitory effects of the imidazoles on mitochondrial respiration, particularly ketoconazole, may result in an early cascade of toxicological damage to the cells, resulting in cell death. Our laboratory has previously reported that ketoconazole, unlike fluconazole, produced a distinct time and dose response in cultured rat liver cells using various cytotoxic assays (7). The toxicity occurred after 2 hours with 115 μM ketoconazole and 4 hours with 90 μM ketoconazole as evaluated by lactate dehydrogenase leakage into the media of the cultured hepatocytes (7). Thus, the immediate effect in isolated mitochondria may be an early indication of a cellular target of ketoconazole, which ultimately, after several hours, results in a loss of cell viability. The lack of inhibition of mitochondrial respiration after exposure to fluconazole correlates with our earlier cytotoxicity studies in which fluconazole did not produce cytotoxic damage to the hepatocytes (7). Moreover, ketoconazole’s strong inhibition effect on the NADH oxidase in the TCA cycle can indirectly inhibit other biochemical events in the liver, in particular, urea synthesis. Ornithine and arginine are carrier compounds for urea that is formed through the “ornithine cycle” and that requires energy and utilizes ATP (18). A toxicological consequence of the inhibition of urea synthesis is increased ammonia formation that can ultimately result in hepatic coma (18,19). Interestingly, the first reported fatality of ketoconazole is a patient who died of hepatic coma (20). In summary, these mitochondrial respiration studies demonstrate that ketoconazole, miconazole, and N-deacetyl ketoconazole directly affect the mitochondria by inhibiting ADP-de-
dependent oxygen uptake at various sites in the respiratory chain that will ultimately result in a reduction of ATP formation. Thus, these events occurring within the mitochondria may be a possible explanation of the mechanism of ketoconazole’s hepatotoxicity.

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