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Transcriptional Regulation of Rat Microsomal Epoxide Hydrolase Gene by Imidazole Antimycotic Agents

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SUMMARY

The effects of imidazole antifungal agents, including ketoconazole, clotrimazole, miconazole, and econazole, on the expression and regulation of microsomal epoxide hydrolase (mEH) were examined in rat hepatic tissue (doses of agents, 150 mg/kg of body weight/day, orally). Immunoblot analyses revealed that administration of either ketoconazole or clotrimazole caused a ~4–5-fold increase in mEH levels, whereas either miconazole or econazole resulted in a ~7-fold increase in mEH at day 3 after treatment. RNA hybridization analyses, probed with a 1.3-kilobase mEH cDNA, revealed that administration of these imidazole antifungal agents caused substantial elevation of hepatic mEH mRNA in total RNA. Hepatic mEH mRNA levels in total RNA were elevated ~11-, 15-, and 18-fold at 12, 24, and 72 hr, respectively, after ketoconazole treatment, whereas mEH mRNA levels were increased ~14-, 19-, and 22-fold, respectively, relative to control, at the same time points after clotrimazole treatment. The rate of increase of mEH mRNA caused by miconazole was more rapid than the rates observed for the other agents examined, with a maximal increase in mRNA being noted at 12 hr after treatment. The degree of mEH mRNA increase after 3 consecutive days of miconazole treatment was appreciably less than that observed at 12 hr after a single treatment. Econazole caused a maximal increase at 24 hr and subsequent decline in mEH mRNA levels after 3 consecutive days of treatment. Elevation of mEH mRNA levels by these antimycotic agents was confirmed in poly(A)$^+$ RNA, as assessed by both Northern and slot blot hybridization analyses. Nuclear run-on analyses revealed that administration of ketoconazole, clotrimazole, or miconazole stimulated the rate of mEH gene transcription at 12 hr after treatment by 11-, 8.5-, and 9-fold, respectively, compared with control, whereas econazole resulted in a 4-fold increase in the rate of mEH gene transcription at the same time point. The transcription rates of mEH mRNA at 24 hr were significantly less than those observed at 12 hr after a single treatment with either ketoconazole, miconazole, or econazole, resulting in 6.5-, 2.5-, and 2-fold increases, respectively, relative to control. Clotrimazole, however, maintained the activated mEH transcription rate at 24 hr after treatment, exhibiting a 11-fold increase, compared with control. These results provide evidence that the imidazole antimycotic agents induce mEH and that the mEH induction involves large increases in mRNA, with transcriptional activation.

Imidazole antifungal agents, such as ketoconazole, clotrimazole, miconazole, and econazole, are effective therapeutic agents in the treatment of mycotic infections (1–3). Because of their broad spectrum, efficacy, and relatively low toxicity, compared with other classes of antimycotic agents (e.g., Amphotericin B), certain imidazole antimycotic agents (e.g., ketoconazole and miconazole) are administered systemically for systemic mycoses as well as topically for dermatophytosis, vaginal mycoses, or skin and mucous membrane infections. Imidazole antifungal agents are believed to inhibit the synthesis of ergosterol in fungi, an important component of fungal membranes that enhances the membrane stability by complexing with phospholipids (3). Thus, the mechanism of action for imidazole antimycotic agents is considered to be inhibition of the microsomal cytochrome P450-dependent 14α-demethylation of lanosterol during ergosterol biosynthesis, leading to inhibition of cell growth. In addition to the effects of these agents on fungal cytochrome P450, administration of imidazole antimycotic agents such as clotrimazole, ketoconazole, and miconazole increases microsomal cytochrome P450 levels in animals (4).

These drugs are metabolized primarily in the liver. After systemic administration, they are potentially hepatotoxic and may inhibit testosterone and corticosteroid synthesis in the body (3). The most common side effects include alterations in hepatic function, gastrointestinal reactions, and pruritus. Approximately 10% of patients have transient abnormalities in liver function and fatal hepatitis. The mechanism of liver damage induced by the antifungal agents is still unclear (4–7).

mEH is active in catalyzing the hydration of reactive epoxide intermediates that are formed by cytochromes P450. The expression of mEH is primarily associated with detoxication and with protection of the cellular macromolecules from metabolic intermediates, although mEH also provides the metabolic precursors of the vicinal diol-epoxides, which play a critical role in the carcinogenicity of several polycyclic aromatic hydrocarbons (8, 9). The specific activity and the mRNA level

ABBREVIATIONS: mEH, microsomal epoxide hydrolase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pp63, rat insulin receptor inhibitor phosphoprotein (63 kDa); SSC, standard saline citrate; Kb, kilobases.

of mEH have been reported to be inducible by phenobarbital, 3-methylcholanthrene, *N*-acetylaminofluorene, *trans*-stilbene oxide, and alkylnitrosamines (10–12). The levels of mEH protein and its mRNA have also been found to be elevated in hepatic preneoplastic nodules and neoplastic lesions (13).

Some imidazole antimycotic agents, as well as certain small molecules, including benzil and benzimidazole, have been found to stimulate mEH activity *in vitro*, resulting in ~3–7-fold activation of this enzyme (14). This significant activation of mEH by these agents is considered to be due to an interaction with the enzyme protein itself, as studied using both microsomes and reconstituted liposomes (14). Studies performed with styrene oxide as a substrate suggest that these activators of mEH bind to this enzyme at a site that is distinct from the substrate binding site. However, no information is available on the effects of imidazole antimycotic agents on the expression and regulation of this enzyme *in vivo*. In view of the chronic systemic use of ketoconazole and the broad use of other imidazole antifungal derivatives, research on the effects of these agents on mEH expression and elucidation of the molecular mechanism of mEH induction would provide valuable information.

The present study establishes the fact that the antimycotic agents induce mEH, the time course of mEH gene expression by each of these antimycotic agents, and the mechanism (i.e., transcriptional activation) associated with mEH gene expression. Induction of mEH protein and mRNA levels was quantified by immunoblot and RNA blot hybridization analyses, respectively. To this end, nuclear run-on analyses were performed to determine the transcription rate of mEH mRNA, using both antisense and sense mEH DNAs cloned in M13mp19 phage.

Experimental Procedures

Materials. [α-^{32}P]UTP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol), and [γ-^{32}P]ATP (3000 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Ketoconazole, clotrimazole, miconazole, and econazole were purchased from Sigma Chemical Co. (St. Louis, MO). ATP, GTP, CTP, and poly(dT)$_{16}$ were purchased from Pharmacia-LKB (Piscataway, NJ). Biotinylated goat anti-rabbit IgG, streptavidin-conjugated horseradish peroxidase, and random prime labeling and 5′-end labeling kits were purchased from BRL (Gaithersburg, MD). Rabbit anti-rat mEH antibody was kindly provided by Dr. C. B. Kasper, McArdle Laboratory for Cancer Research, University of Wisconsin (Madison, WI), and both sense and antisense pp63 DNAs cloned in M13mp19 phage were obtained from Dr. A. S. Goustin, Center for Molecular Biology, Wayne State University. Insert DNA size for antisense and sense pp63 clones was 865 base pairs, respectively.

Animals and treatment. Male Sprague-Dawley rats (150–200 g) from Harlan (Indianapolis, IN) were treated with either ketoconazole, clotrimazole, miconazole, or econazole (150 mg/kg of body weight/day, orally, for 1–3 days) and were sacrificed at 24 hr after the last treatment, except for the 12-hr time point. The antimycotic agents were suspended in 0.2% high viscosity carboxymethylcellulose (3% antimycotic agent, w/v). Control rats were treated with vehicle alone. Animals were fasted for 18 hr before sacrifice. Each data point consisted of pooled samples from groups of animals. Four to six rats were used in each treatment group.

Isolation of microsomal proteins. Hepatic microsomes prepared by differential centrifugation were washed in pyrophosphate buffer, stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol, and stored at −80° until used. Protein was assayed by the method of Lowry *et al.* (15).

SDS-PAGE. SDS-PAGE analysis was performed according to the method of Laemmli (16), using a Bio-Rad Mini-Protean II apparatus.

Immunoblot analysis. Immunoblot analysis was performed according to previously published procedures (17, 18). Microsomal proteins were separated by 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with rabbit anti-rat mEH antibody. Biotinylated goat anti-rabbit IgG was used as the secondary antibody. Immunoreactive protein was visualized by incubation with streptavidin-horseradish peroxidase, followed by addition of both 4-chloro-1-naphthol and hydrogen peroxide.

Isolation of total RNA and poly(A)$^+$ RNA. Total RNA was isolated using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction described by Cathala *et al.* (19) and Chomczynski and Sacchi (20), as modified by Puissant and Houdebine (21). One gram of hepatic tissue was homogenized in 10 ml of lysis buffer, consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol, using a Polytron homogenizer. One milliliter of 2 M sodium acetate (pH 4.0), 10 ml of water-saturated phenol, and 1 ml of chloroform were added successively to the homogenate and vortexed. After centrifugation at 10,000 × *g* for 10 min, the aqueous phase was saved and mixed with 10 ml of isopropanol, to precipitate RNA. The RNA-enriched pellet was resuspended in 2 ml of 4 M LiCl, and insoluble RNA was pelleted by centrifugation. The resulting pellet was redissolved in 4 ml of 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.5% SDS. Four milliliters of chloroform were then added and mixed. After centrifugation, the RNA in the upper phase was precipitated by addition of the same volume of isopropanol in the presence of 0.2 M sodium acetate (pH 5.0) and was resuspended in 1% diethylpyrocarbonate-treated water. Poly(A)$^+$ RNA was isolated from total RNA using an oligo(dT)-cellulose column, according to the method of Jacobson (22).

cDNA synthesis and cloning in M13mp19 phage vector. cDNA derived from hepatic poly(A)$^+$ RNA obtained from rats treated with pyrazine was amplified using a PCR technique,[1] and the amplified DNA was cloned in M13mp19 phage vector. In brief, first-strand cDNAs were synthesized using poly(A)$^+$ RNA (3.0 μg), avian myeloblastosis virus reverse transcriptase (0.5 unit/μl), random primer (50 ng/μl), 2.0 mM levels each of four nucleotide triphosphates, RNasin (0.8 unit/μl), and reverse transcriptase buffer (50 mM Tris·HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol), in a total reaction volume of 60 μl. One sixtieth of the reaction product served as template for each PCR reaction. PCR reactions consisted of 0.1 μM concentrations of both sense and antisense primers, 0.2 mM levels of each nucleotide triphosphate, 2 μl of 32.5 mM $MgCl_2$ supplement, *Thermoactinomyces aquaticus* polymerase (0.5 unit), and 2 μl of 10× PCR buffer supplied by Perkin-Elmer Cetus, in a total reaction volume of 20 μl. Gene-specific primers were carefully selected from 3′ and 5′ regions of the published cDNA sequences (23). Sense primer 5′-d(AGCAGGCACTTCTGTT)-3′ was constructed from region 108–123 in the 5′ untranslated region, and antisense primer 5′-d(CCACAGTTGGTATCC)-3′, complementary to region 1375–1390, was selected from the 3′ untranslated region of the published mEH cDNA sequence (23).

PCR reactions were performed in a Perkin Elmer Cetus thermocycler for 30 cycles, using the following parameters: denaturing at 94° for 1 min, annealing at 50° for 1 min, and elongation at 72° for 3 min. Amplified DNA product (~1.3 kb) was analyzed on a 1% agarose gel, using a ϕX174/*Hin*cII ladder as a size marker. PCR-amplified DNA was cloned in M13mp19 phage vector. Both sense and antisense clones were successfully selected.

DNA sequence analysis. Both sense and antisense cloned mEH DNAs were analyzed by DNA sequencing, which was carried out by a modified dideoxy chain termination method (24). DNA sequence analyses revealed that the DNA sequences of sense and antisense mEH clones match the published cDNA sequences for mEH (23).

[1] S. G. Kim, G. L. Kedderis, R. Batra, and R. F. Novak. Induction of rat liver microsomal epoxide hydrolase by thiazole and pyrazine: hydrolysis of 2-cyanoethylene oxide. Manuscript in preparation.

RNA slot blot hybridization. RNA slot blot analysis was performed using a Schleicher & Schuell slot blot system (Minifold II). Total RNA or poly(A)$^+$ RNA was serially diluted in 15× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) and applied to the slots according to the manufacturer's protocols. The nitrocellulose paper was baked in a vacuum oven at 80° for 2 hr. The blot was incubated with hybridization buffer, containing 50% deionized formamide, 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin (Pentex fraction V)], 0.1% SDS, 200 μg/ml sonicated salmon sperm DNA, and 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4), at 42° for 1 hr without probe. Hybridization was performed at 42° for 18 hr with heat-denatured mEH cDNA (1.3 kb), which was random prime-labeled with [α-^{32}P] dATP. Filters were washed in 2× SSC, 0.1% SDS, for 10 min at room temperature twice and in 0.1× SSC, 0.1% SDS, for 10 min at room temperature twice. Filters were finally washed for 60 min at 60° in the solution containing 0.1× SSC and 0.1% SDS.

Northern blot hybridization. Northern blotting was carried out according to the procedures described previously (18). Briefly, poly(A)$^+$ RNA isolated from rat livers was resolved by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and was then transferred to supported nitrocellulose paper (BA-S, Schleicher & Schuell, Kenne, NH) by capillary transfer. Each membrane was baked under vacuum at 80°, prehybridized, and then incubated in a hybridization solution containing a ^{32}P-random prime-labeled cDNA probe, as described above for slot blot analysis. The same samples used for Northern blot analyses were applied to slots as well. Slot blotted membranes were hybridized with the ^{32}P-random prime-labeled cDNA probe to obtain mEH hybridization signals. The stripped membranes were hybridized with ^{32}P-end labeled poly(dT)$_{16}$ to quantitate the amount of mRNA loaded onto the slots and to ensure that equal amounts of poly(A)$^+$ RNA were loaded onto the agarose gel and transferred to the nitrocellulose paper. Films were exposed at −80° for 1–6 hr, using DuPont intensifying screens. Duplicate slot and Northern blot analyses were performed on different mRNA samples.

Isolation of nuclei. Nuclei were isolated at 4°, as described previously (25, 26). Livers were chilled in 0.25 M sucrose in TKM buffer (5 mM Tris·HCl, pH 7.5, 2.5 mM KCl, 5 mM $MgCl_2$), blotted, weighed, and minced in 2 volumes of TKM buffer containing 0.25 M sucrose. Minced tissue was disrupted in a Dounce homogenizer with a loose and a tight pestle, successively (10 strokes each). The homogenate was filtered through cheesecloth and mixed with 2 volumes of TKM buffer containing 2.3 M sucrose, to bring the sucrose concentration to 1.62 M. About 70% of the usable volume of the swing-out rotor tube (Beckman Ti40) was filled with homogenate made up to 1.62 M sucrose, and ~30% of the volume of TKM buffer containing 2.3 M sucrose was underlaid at the bottom of the tubes. The tubes were centrifuged at 60,000 × *g* for 1 hr. The supernatant was removed by pipetting, the walls of the tube were wiped with tissue paper, and the pellet of nuclei was resuspended in TKM buffer containing 40% glycerol. Isolated nuclei were aliquoted after being counted microscopically, and concentrations were normalized. Resuspended nuclei were quickly frozen in liquid nitrogen and stored at −80° until used.

Nuclear run-on transcription assay. Nuclei (5 × 10^7) were resuspended in 300 μl of 10 mM Tris·HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 80 mM KCl, 0.5 mM dithiothreitol, 20% glycerol, 1.25 mM levels of nucleotides (ATP, CTP, and GTP), and 200 μCi of [α-^{32}P] UTP (3000 Ci/mmol). Reactions were performed at 30° for 30 min, with shaking, and the nuclei were then digested with 10 μl of RNase-free DNase I (10 mg/ml) and 15 μl of 20 mM $CaCl_2$, at 26°, for 5 min. Protein in the incubation mixture was further digested with 3 μl of proteinase K (10 mg/ml), 28 μl of 10× SET (5% SDS, 50 mM EDTA, 100 mM Tris·HCl, pH 7.4), and 5 μl of yeast tRNA (10 mg/ml), at 37°, for 30 min (27). Labeled nuclear RNA was isolated by the addition of 825 μl of lysis solution, containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol, 135 μl of sodium acetate (2.0 M, pH 4.0), 1350 μl of saturated phenol, and 270 μl of chloroform/isoamyl alcohol (49:1). The mixture was incubated at room temperature for 10 min and centrifuged for 10 min. The aqueous layer was removed, combined with an equal volume of isopropanol, and incubated for 1 hr at −80°. After centrifugation, the pellet was dissolved in 300 μl of the lysis solution and reprecipitated with 300 μl of isopropanol. The pellet was dissolved in 100 μl of a solution consisting of 10 mM Tris·HCl, 1 mM EDTA, and 0.1% SDS. Approximately 2 × 10^7 cpm of ^{32}P-labeled RNA was hybridized to 3 μg of single-stranded antisense or sense mEH DNA cloned in M13mp19 phage, which was immobilized on nitrocellulose membranes. Hybridizations were carried out for 24 hr at 42°, in 5× SSPE, 0.5% SDS, 50% formamide, 1× Denhardt's solution, 0.5 mg/ml of yeast tRNA.

Filters were washed with 1× SSC, 0.1% SDS, for 20 min at 42° three times and then washed with 0.1× SSC, 0.1% SDS, for 1 hr at 60°. Both antisense and sense cloned pp63 genes were used as internal controls. Hybridization was visualized by autoradiography on Kodak X-Omat AR film, with intensifying screens.

Scanning densitometry. Scanning densitometry was performed with a Molecular Dynamics computing densitometer. The area of each slot or dot was integrated using ImageQuant software (Molecular Dynamics, version 3.0), followed by background subtraction. The quantitation of the mRNA loaded on the slot blot was accomplished by hybridization of stripped membranes with ^{32}P-end labeled poly(dT)$_{16}$, and the relative changes in mEH mRNA were determined by normalization of hybridization signals to the amount of mRNA loaded onto the slots. The quantitation of hybridization signals in the nuclear run-on assays was accomplished by normalization of mEH signal intensities to pp63 hybridization signal intensities.

Results

SDS-PAGE and immunoblot blot analyses. The SDS-PAGE of imidazole antimycotic agent-treated rat hepatic microsomes revealed an apparent increase in intensity of a band occurring in the region of mEH (data not shown). The agents used in this experiment include ketoconazole, clotrimazole, miconazole, and econazole. Because the increase in this 50-kDa protein may involve the induction of other proteins, such as cytochrome P450, a polyclonal antibody that detected mEH was used to examine the increase in mEH protein levels in rat hepatic microsomes. Immunoblot analyses revealed that administration of either ketoconazole or clotrimazole caused a ~4–5-fold increase in mEH levels, relative to control, whereas either miconazole or econazole resulted in a ~7-fold increase in mEH at day 3 after treatment (150 mg/kg of body weight/day, orally) (Fig. 1).

Time course of relative mEH mRNA increases. To determine whether mEH induction was accompanied by increases in mRNA, mEH mRNA levels were measured in total RNA isolated from hepatic tissue of vehicle-treated or ketocon-

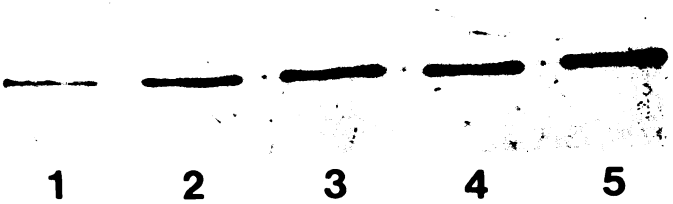

Fig. 1. Immunoblot analyses of rat hepatic microsomes with rabbit anti-rat mEH antibody. Western immunoblot analysis of hepatic microsomes isolated from vehicle-, ketoconazole-, clotrimazole-, miconazole-, or econazole-treated rats (150 mg/kg of body weight/day, orally, for 3 days) (*lanes 1* through *5*, respectively). Lanes were loaded with 0.5 μg of rat liver microsomes. This blot shows the apparent significant increases in mEH protein levels produced by treatment with each of these antimycotic agents.

azole-, clotrimazole-, miconazole-, or econazole-treated rats (150 mg/kg of body weight/day, orally, for 1 or 3 days). The increase of mEH gene expression was examined at 12, 24, and 72 hr after treatment. A single band was detected with the mEH cDNA probe in total RNA from vehicle-treated animals, as assessed by Northern blot analyses. A minor thin band of larger mRNA (~3 kb; <3% of the major band intensity) appeared along with the major mEH mRNA band in the total RNA from antimycotic-treated rats (data not shown). This minor thin band may represent mEH pre-mRNA, the level of which is elevated by treatment. However, slot blot analyses were chosen for optimal quantitation of mRNA changes in the subsequent series of experiments. In order to obtain the optimal linear range for the autoradiographic film exposure, three serially diluted RNA samples, obtained from rat hepatic tissue at the individual time points, were applied onto slots (Fig. 2A). The intensities of ~1.3-kb mEH cDNA hybridization to mEH mRNA in total RNA isolated at 12, 24, and 72 hr after antimycotic agent treatment (150 mg/kg/day, 1–3 days) were quantitated by scanning laser densitometry (Fig. 2B).

mEH mRNA levels were elevated ~11-, 15-, and 18-fold at 12, 24, and 72 hr, respectively, after ketoconazole treatment, whereas mEH mRNA levels were increased ~14-, 19-, and 22-fold, respectively, relative to control, at the same time points after clotrimazole treatment (Fig. 2B). The rate of mEH mRNA increase produced by miconazole was more rapid than those of other compounds used, with maximal increase being noted at 12 hr after treatment. The degree of mEH mRNA increase

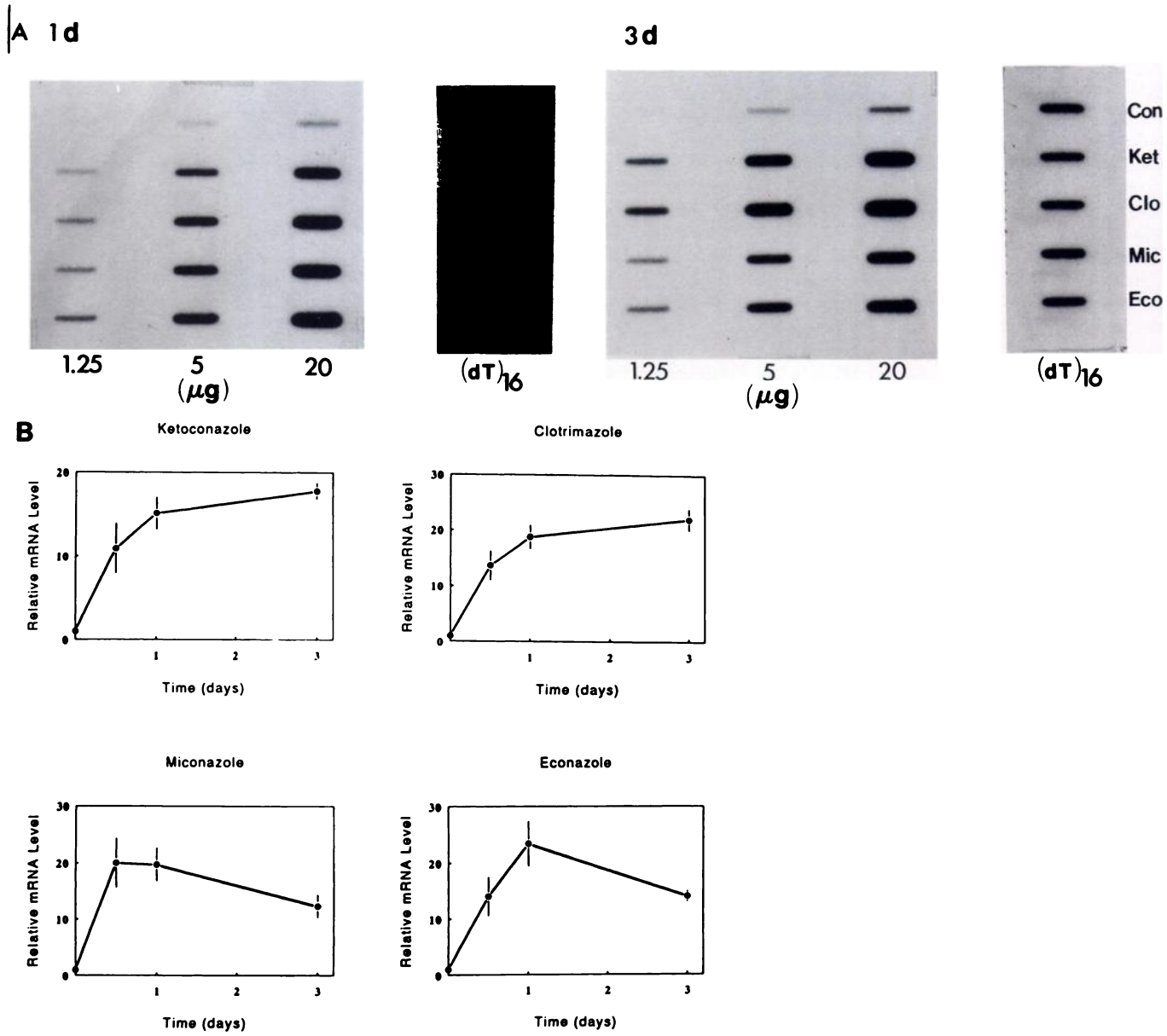

Fig. 2. Slot blot analyses of mEH mRNA in total RNA from rats after treatment with antimycotic agents (150 mg/kg of body weight/day, orally, for 1–3 days). A, Total RNA (1.25, 5.0, or 20 μg), isolated from rats at 1 day (*1 d*) or 3 days (*3 d*) after treatment with either ketoconazole, clotrimazole, miconazole, or econazole, was blotted and probed with a 1.3-kb cDNA probe for mEH. After hybridization with the mEH cDNA probe, the membrane was stripped and rehybridized with ^{32}P-labeled poly(dT)$_{16}$, for quantitation of mRNA loaded in the slots. B, Time course of relative mEH mRNA increase produced by antimycotic agents. The relative change in mEH mRNA was quantitated by densitometric scanning of slot blots. Each *point* represents the mean ± standard deviation of three determinations. *Con*, control; *Ket*, ketoconazole; *Clo*, clotrimazole; *Mic*, miconazole; *Eco*, econazole.

after 3 consecutive days of miconazole treatment was appreciably less (40%) than that observed at 12 hr after a single treatment. Econazole appears to be slower in onset of action than miconazole, resulting in maximal increase at 24 hr and a subsequent decline in the relative increase in mEH mRNA levels after 3 consecutive days of treatment (Fig. 2B). The reduced mEH mRNA increase after 3-day treatment with either miconazole or econazole, relative to that observed at early times (e.g., 12 or 24 hr) after a single treatment, might be because of multiple mechanisms active in regulating mEH mRNA levels throughout the time course (e.g., cytoplasmic mRNA stabilization occuring in concert with the initial transcriptional activation) and/or accelerated metabolism of the agents at later times.

Enhanced expression of mEH mRNA by these antimycotic agents was confirmed in poly(A)$^+$ RNA, as assessed by both Northern and slot blot hybridization analyses. Northern blot hybridization was performed with the poly(A)$^+$ RNA isolated from rats after treatment with each of these agents. The ^{32}P-labeled mEH cDNA probe hybridized to mEH message, as evidenced by the appearance of a single band in the Northern blot analysis (~1.8 kb) (Fig. 3A). Minor smearing in the region of ~5 kb sometimes occurred with the poly(A)$^+$ RNA isolated 3 days after treatment, as shown in Fig. 3B, *lanes 2* and *3*. However, the intensity of the smeared region, when present, was <5% of the major band intensity. When poly(A)$^+$ RNA was used, the thin 3-kb band detected in total RNA disappeared, indicating that it may be nonadenylated pre-mRNA. In general, comparable relative increases in mEH mRNA levels were obtained between poly(A)$^+$ RNA and total RNA, although the fold increases obtained in poly(A)$^+$ RNA were ~10–20% less than those in total RNA. Within experimental error, there was no difference between the two RNA fractions in quantitation of mEH mRNA levels. Representative slot blots are shown in Fig. 3. The stripped membranes were reprobed with ^{32}P-end labeled poly(dT)$_{16}$ to quantitate the amount of mRNA loaded onto the slots and to ensure that equal amounts of poly(A)$^+$ RNA were loaded onto the agarose gel and transferred to the nitrocellulose paper.

Nuclear run-on analyses. In order to examine whether the enhancement of expressed levels of mEH mRNA produced by the imidazole antimycotic agents results from transcriptional activation, nuclear run-on transcription assays were initiated. The results of the mEH mRNA time course suggested that maximal increases in the rate of transcription could be obtained at 12 and/or 24 hr after drug administration. Therefore, nuclei were isolated from the livers of vehicle-treated rats and of rats 12 and 24 hr after treatment with each of the antimycotic agents (150 mg/kg, orally). Newly synthesized radiolabeled RNA was isolated and hybridized to cloned antisense or sense single-stranded mEH DNA. As internal controls, the ^{32}P-labeled transcripts synthesized in nuclei were also hybridized to rat pp63 antisense or sense DNA. The intensities of the autoradiographic signals from these hybridization experiments yielded an estimate of the relative sense and antisense transcription rates of the mEH and pp63 genes in rat hepatic tissue. In the hepatic tissue from vehicle-treated rats, the intensities of the mEH gene sense transcripts were much lower than those of pp63 counterparts (Fig. 4A, shown as mEH antisense clone). In contrast, there were marked increases in the transcription of the mEH gene in nuclei from the antimycotic agent-treated rats. Ketoconazole, clotrimazole, and miconazole stimulated the rate of mEH mRNA transcription by 11-, 8.5-, and 9-fold, respectively, compared with control, at 12 hr after treatment (Fig. 4B). These three compounds appeared to be comparable in the stimulation of mEH gene transcription rate, whereas econazole was the least active among the compounds examined, at 12 hr after treatment (4-fold increase, relative to control). The transcription rates of mEH mRNA at 24 hr were significantly less than those observed 12 hr after a single treatment with either ketoconazole, miconazole, or econazole, resulting in 6.5-, 2.5-, and 2-fold increases, respectively, relative to control.

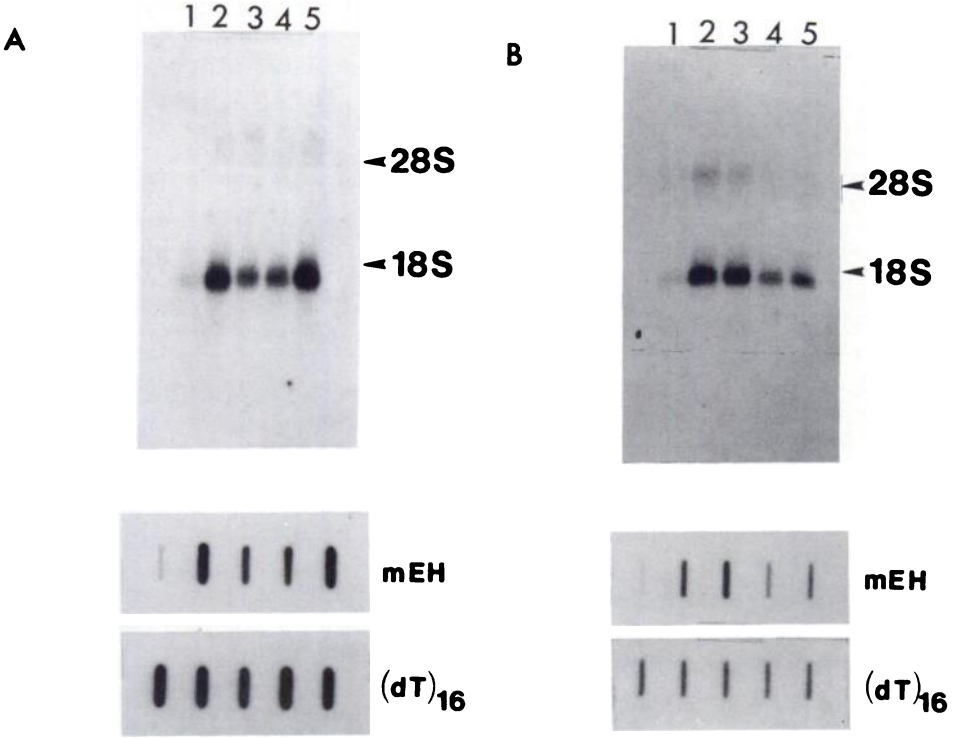

Fig. 3. Northern blot analyses to examine mEH mRNA levels in poly(A)$^+$ RNA. Two micrograms of hepatic poly(A)$^+$ RNA, isolated from rats at 1 and 3 days after treatment with either vehicle, ketoconazole, clotrimazole, miconazole, or econazole (*lanes 1* through *5*, respectively), were fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled mEH cDNA probe. A and B, 1 and 3 days after treatment, respectively. The same samples used for Northern blot analyses were applied to slots. Slot blotted membranes were hybridized with the ^{32}P-labeled cDNA probe to obtain mEH hybridization signals, and the stripped membranes were hybridized with ^{32}P-end labeled poly(dT)$_{16}$ to quantitate the amount of mRNA loaded onto the slots and to ensure that equal amounts of poly(A)$^+$ RNA were loaded onto the agarose gel and transferred to the nitrocellulose paper.

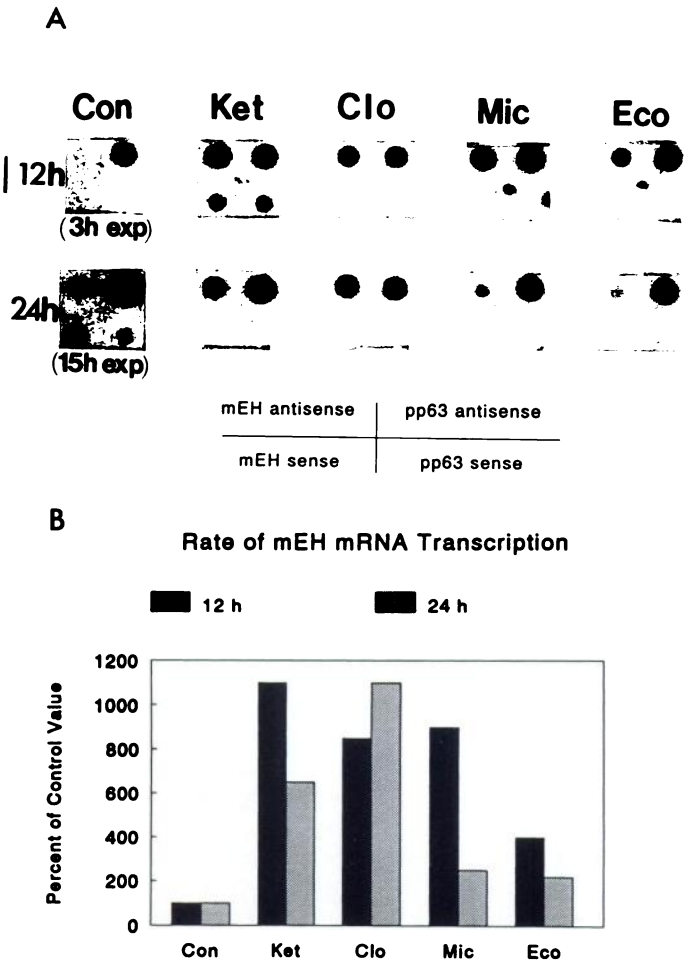

Fig. 4. Nuclear run-on analyses. Hepatic nuclei (5×10^7) isolated at 12 or 24 hr, from vehicle-treated rats and from rats treated with either ketoconazole, clotrimazole, miconazole, or econazole (150 mg/kg of body weight, orally), were incubated in the presence of [α-^{32}P]UTP. A, Newly synthesized RNA was hybridized to both antisense and sense strands of mEH DNA cloned in M13mp19 phage (*upper and lower dots on the left*, respectively). Both antisense and sense strands of pp63 DNA clones were used as internal controls (*upper and lower dots on the right*, respectively). No significant change in the transcription rates of the pp63 gene was observed after treatment with the antimycotic agents. B, Relative change in the rate of mEH mRNA transcription produced by imidazole antimycotic agents was determined from normalization of the hybridization signal to the amount of pp63 mRNA transcribed. Data represent the average of duplicate experiments. A <10% variation in integrated intensity from the two different experiments was measured. *Con*, control; *Ket*, ketoconazole; *Clo*, clotrimazole; *Mic*, miconazole; *Eco*, econazole.

Clotrimazole, however, maintained the activated mEH transcription rate at 24 hr after treatment, exhibiting a 11-fold increase, compared with control. No significant activation of the mEH gene antisense transcription was noted after treatment with these antimycotic agents (Fig. 4A). These results demonstrated that the large increase in mEH mRNA is primarily associated with transcriptional activation.

Discussion

Administration of imidazole antifungal agents, such as ketoconazole, clotrimazole, and miconazole, increases microsomal cytochrome P450 levels. Hostetler *et al.* (4) have examined the differential effects of clotrimazole, miconazole, and ketoconazole on the expression of gene subfamilies of rat hepatic cytochromes P450. Although cytochrome P4503 is the family of hepatic cytochromes P450 commonly induced by all of these agents, clotrimazole increases the accumulation of cytochrome P4503 protein to a greater extent than it increases the rate of *de novo* synthesis of mRNA, whereas econazole and ketoconazole increase cytochrome P4503 protein and mRNA to similar extents. Cytochrome P4502B is also induced by clotrimazole and miconazole, but not by ketoconazole. Cytochrome P4501A is modestly elevated by each of these drugs, whereas cytochrome P4502E1 is marginally induced by ketoconazole but not by clotrimazole or miconazole. It appears that the imidazole antimycotic agents regulate the expression of different cytochrome P450 forms by multiple mechanisms.

Ritter and Franklin (28) have shown that clotrimazole, miconazole, and tioconazole increase glutathione *S*-transferases and UDP-glucuronyl transferases, whereas these agents suppress sulfotransferase activity. Thus, imidazole antimycotic agents differentially modulate the expression of phase II drug-metabolizing enzymes.

Clotrimazole, miconazole, and econazole activate the hydrolysis of styrene oxide, which is catalyzed by mEH, in hepatic microsomes *in vitro* (3–7-fold increases) (14), suggesting that these agents interact with the enzyme protein itself. However, no information has been available on the effects of these imidazole antifungal drugs on the expression of mEH and regulation of the gene. Because the expression of cytochrome P450 gene subfamilies and phase II enzymes is differentially modulated by various imidazole antimycotic agents in rats (4, 28), the widely used antifungal agents ketoconazole, clotrimazole, miconazole, and econazole were used in this study to examine whether all of these agents increase the levels of mEH protein and mRNA to the same extent and whether a common molecular basis exists for the protein induction by each of these agents.

The present study reveals that administration of the imidazole antimycotic agents ketoconazole, clotrimazole, miconazole, and econazole induces mEH protein in rats, with large increases in mEH mRNA levels. Nuclear run-on analyses provide evidence that these large increases in mEH mRNA are primarily associated with transcriptional activation of this gene by these antimycotic agents. The activation of mEH gene transcription by these antimycotic agents is consistent with mEH gene activation by other known inducers, such as diethylnitrosamines (13). The induction of mEH by diethylnitrosamine, a hepatocarcinogen, involves no gene amplification, recombination, or mutation. The mRNA increase is partially caused by transcriptional activation, as determined by nuclear run-on analyses (13). Thus, it has been suggested that both transcriptional and post-transcriptional mechanism(s) may contribute to an increase in the mEH message (13).

The levels of mEH protein, as assessed by immunoblot analyses in the present research, at day 3 after treatment correlate well with those of mEH mRNA at 24 hr after treatment, consistent with the time course and efficacy for achieving maximal mEH mRNA levels after treatment with each of these antimycotic agents. Peak elevations for miconazole and econazole occurred 12 and 24 hr, respectively, after a single dose of treatment, whereas peak mRNA levels for ketoconazole and clotrimazole were recorded after 3 consecutive days of treatment. Thus, the elevation of mEH protein and mRNA appears to occur in parallel. Nuclear run-on analyses were used to examine the rates of mEH mRNA transcription in the nuclei

isolated from rats at 12 and 24 hr after a single dose of treatment. It is apparent from these results that the transcription rates in the nuclei were greater at 12 hr than at 24 hr for most of the agents examined. Although this leaves the possibility that the maximal transcription rate occurs earlier than 12 hr, the marked increase in the transcription rates at 12 hr correlates well with the maximal rates of increase in mEH mRNA levels (i.e., 12–24 hr). The decline of transcription rates at 24 hr for miconazole and econazole also reflects the negative rates of change in mEH mRNA levels at the time point. The slight discrepancy between increases of transcription rate and maximal mEH mRNA levels for econazole, along with the possible small differences in mEH mRNA fold increases between total and poly(A)$^+$ RNA for these antimycotic agents, may be associated with the contribution of post-transcriptional mechanism(s), such as mRNA utilization. Nonetheless, transcriptional activation appeared to be the primary regulatory mechanism for mEH induction by these antimycotic agents.

Significant sex-dependent expression of mEH by xenobiotics has been reported in rats. 2-Acetylaminofluorene increases the mEH activity toward styrene oxide in liver by 7.5-fold and glutathione *S*-transferases by 2-fold in male rats, whereas 2-acetylaminofluorene failed to induce mEH in female rats (29). Preliminary studies performed in this laboratory revealed that these antimycotic agents increase mEH mRNA levels significantly in female animals as well. Why there are such large differences between sexes in the mEH inducibility by different xenobiotics remains to be established. This may reflect differences in the metabolism (or production) of endogenous substances, including steroid hormones, or differences in metabolic patterns of phase I enzymes, such as sex-differential expression of cytochromes P450.

In summary, these results provide evidence that the imidazole antimycotic agents induce mEH protein and that the mEH induction involves large increases in mRNA, with transcriptional activation. Time-dependent mEH gene expression after treatment with these therapeutic agents could expalain, in part the physiological role of this enzyme, as well as the different pharmacological aspects of these agents. The induction of mEH by imidazole antimycotic agents may be used as an pharmacodynamic index of these compounds in cells, representing cellular uptake and response.

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