Inhibition of Hepatic and Cutaneous Biotransformation of Resorufin Ethers Following Intraperitoneal Administration of 1-Aminobenzotriazole

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INTRODUCTION

Cytochrome P-450 plays a major role in the detoxication and elimination of xenobiotics presented to the organism, a role attributed primarily to its action in the liver. Recently, attention has been focused on the role of biotransformation in the cutaneous absorption of compounds (1). In skin organ cultures using testosterone and benzo[*a*]pyrene, viable biotransformation capability was the major factor associated with permeation of the compounds (2). Further, it was shown that induction of cutaneous drug metabolizing enzymes increased *in vitro* permeation of benzo[*a*]pyrene severalfold (3). The role of biotransformation by cytochrome P-450 in metabolic-dependent cutaneous absorption of benzo[*a*]pyrene and testosterone is implied by the nature of the metabolic products observed. However, definitive studies establishing this role are hampered by the low activity of drug metabolizing enzymes in skin as well as equivocal inhibition studies.

An alternative approach to examine the role of biotransformation in metabolism-dependent cutaneous absorption is the use of specific inhibitors of drug biotransformation. 1-Aminobenzotriazole (ABT), a potent and highly effective inhibitor of cytochrome P-450 *in vitro* (4,5) and *in vivo* (6,7), can serve to determine the role of biotransformation in metabolism-dependent drug absorption. We used ABT to study the inhibition of skin and liver metabolism of alkyl resorufins in the mouse by P-450 isozymes. ABT was found to inhibit uniformly skin and liver metabolism of resorufin ethers in a similar fashion for both tissues.

MATERIALS AND METHODS

Chemicals. 1-Aminobenzotriazole was synthesized as described previously (8). Resorufin was obtained from Aldrich Chemical Co., Milwaukee, WI. Alkyl ethers of resorufin were obtained from Molecular Probes Inc., Eugene, OR.

Mice. Male CF-1 mice approximately 8–10 weeks of age were housed in plastic boxes with access to food and water ad libitum and maintained on a 12-hr light–dark cycle.

Experimental Procedure. ABT was administered intraperitoneally to the animals in sterile 0.85% saline in volumes ranging from 0.3 to 0.65 ml. Control animals received equivalent volumes of saline. At the required time postdosing, the animals were lightly anesthetized using CO_2 and then sacrificed by cervical dislocation 0, 0.5, 1, 4, 8, 16, 24, and 48 hr postdosing. Control animals were sacrificed at 0, 4, 8, 24, and 48 hr postdosing. At these times, the entire body skin from the region of the hindlegs to the neck was isolated, shaved, and placed on ice along with the liver. Microsomes from skin and liver were prepared as described previously (9), techniques which yielded highly active skin microsome preparations.

Determination of Microsome Protein Content and Liver Cytochrome P-450. Liver microsome protein content was determined by the method of Lowry *et al.* (10) for the purpose of quantitating the P450 content per milligram of protein of the preparation. This is our standard procedure for protein determinations on liver microsomes. Skin preparations could not be analyzed by the Lowry *et al.* method due to a combination of low protein content and interfering substances which obscured the colorimetric reaction; therefore in experiments comparing liver and skin metabolic activity, protein was quantitated by the method of Bradford (11). Determination of P-450 content of liver microsomal preparations was by the method of Estabrook *et al.* (12).

Determination of Resorufin Dealkylation Activity in Microsome Preparations. Metabolism studies were carried out in 16 × 100 glass tubes in a 1-ml reaction mixture consisting of 0.5 m*M* NADH, 0.5 m*M* NADPH, 50 m*M* K_2PO_4, 5 m*M* $MgCl_2$, 5 m*M* substrate, and approximately 0.15 or 0.30 mg of microsomal protein for liver or skin incubations, respectively. Incubations were run for 10 min in the case of liver studies and 20 min in the case of skin studies. Incubations were terminated by the addition of 0.1 ml of 1 *N* NaOH followed by 2 ml of methanol and centrifuged at 2500 rpm for 5 min. The supernatant was read at λ_{ex} 540 and λ_{em} 585 in a Hitachi 3010 fluorescence spectrophotometer. Addition of the NaOH was found to stabilize the fluorescence and to improve the reproducibility between samples. A complete reaction mixture, to which substrate was added after the reaction had been terminated, was used to blank the fluorometer. Standard curves were prepared using authentic standards of resorufin.

The values determined for V_{max} of skin resorufin dealkylase activities (benzyl, 25.9 > ethyl, 8.2 > pentyl, 1.0 pmol/mg/min) and liver resorufin dealkylase activities (methyl, 686 > benzyl, 196 > ethyl, 190 > pentyl, 31 pmol/mg/min) were done over a substrate concentration range of 0.039 to 20 μ*M* under conditions determined to produce linear reaction rates with respect to time and protein concentration for both types of tissue. Similar rates of metabolism for these ethers by liver and skin microsomes from female Balb/c mice have been reported (13). The experience in our laboratory has been that significant diurnal variability in P-450 levels or activity does not occur. As a result, although control animals were sacrificed at intervals spanning the entire time course,

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resulting determinations of enzyme activity were combined into a single mean value designated time zero.

RESULTS

The Effect of 1-Aminobenzotriazole on P-450 Content in Mouse Liver

Administration of 10, 50 or 200 mg/kg of 1-aminobenzotriazole to mice intraperitoneally produced a reduction in the level of P-450 per milligram protein in mouse liver (Fig. 1). The reduction was evident at 0.5 hr postdosing and was maximal between 1 and 4 hr for the 50 and 200 mg/kg dose, while the animals receiving 10 mg/kg had started to recover. All groups had returned to normal levels by 16 hr and had values equivalent to control values thereafter. The animals receiving the 200 mg/kg dose showed a tendency to overshoot control values at 48 hr.

Animals in the 0 mg/kg group were sacrificed at times 0, 8, 24, and 48 hr postdosing. The diurnal variability in levels of P-450 in untreated animals was no greater than the variability between individuals (Fig. 1).

P-450 in skin samples was not measured because of its low level.

The Effect of 1-Aminobenzotriazole on the Resorufin Dealkylation Activity of Liver and Skin Microsomes

Administration of ABT to mice inhibits the dealkylation activity of liver microsomes for the various resorufin ethers tested (Fig. 2). At 10 mg/kg, significant ($P \leq 0.05$) inhibition of metabolism was evident from 0.5 to 4 hr after dosing with the exception of benzyl resorufin, where the difference was not significant at 0.5 hr. At 50 mg/kg significant inhibition was extended from 0.5 to 24 hr with the exception of benzyl resorufin activity, which recovered after 8 hr. At 200 mg/kg inhibition was significant from 0.5 to 8 hr for all ethers, and although the benzyl activity recovered above control, it was not significant at the $P \leq 0.05$ level. The effects of the 200 and 50 mg/kg doses were similar in onset and extent of maximal inhibition; however, the 50 mg/kg dose appeared optimal for duration of inhibition. The 10 mg/kg dose revealed a lesser effect relative to maximal inhibition of biotransformation of the methyl and ethyl ethers and the shortest duration of significant inhibition.

The effect of ABT on the dealkylation activity of skin microsomes (Fig. 3) was similar to that in the liver, although dose dependency was more pronounced. At 10 mg/kg significant ($P \leq 0.05$) inhibition of metabolism occurred only at the 0.5-hr time point with respect to ethyl and benzyl resorufin, while at 50 mg/kg significant inhibition of metabolism was evident from 0.5 to 4 hr for ethyl resorufin and 0.5 to 16 hr for benzyl resorufin. Metabolism of the pentyl ether was not significantly inhibited until the 200 mg/kg dose was reached. At 200 mg/kg, significant inhibition of ethyl resorufin metabolism occurred from 0.5 to 8 hr after dosing; pentyl metabolism was inhibited from 1 to 8 hr, and benzyl metabolism at 4 and 8 hr only.

DISCUSSION

The cytochrome P-450 enzyme system plays an important role in metabolism of xenobiotic as well as endogenous substances. Although the liver is the major site of the metabolism of xenobiotics, cytochrome P-450 is present in many other organs of the body. Extrahepatic cytochrome P-450, such as that in the skin, has been shown to play a considerable role in the overall interaction of organisms and the chemical environment which surrounds them (14). Various inducers or inhibitors of cytochrome P-450 have been used to study the role of these enzymes in the overall metabolic processes of the organism. ABT inactivates cytochrome P-450 by the alkylation of the prosthetic heme

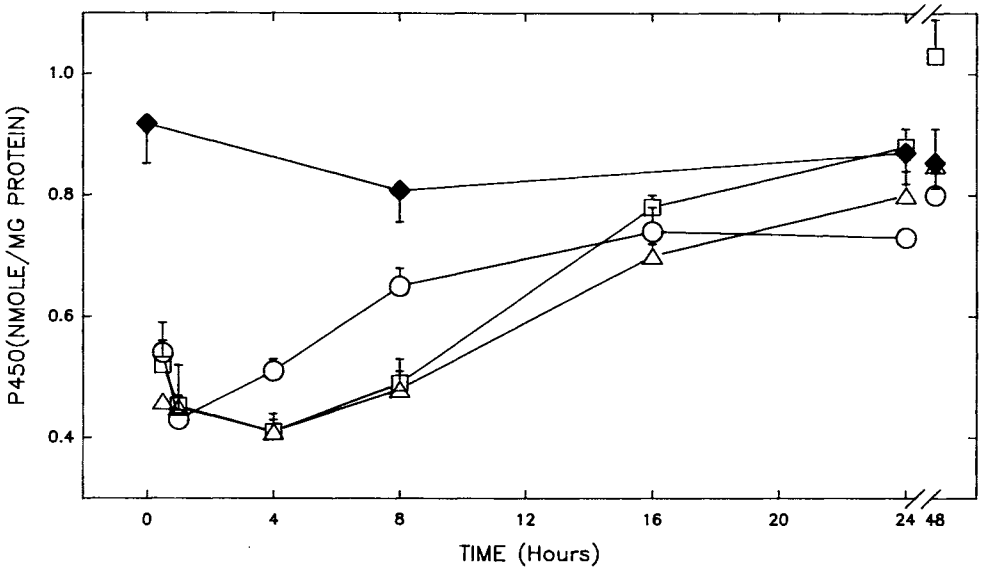

Fig. 1. The effect over 48 hr of 1-aminobenzotriazole administration on the P-450 content of mouse liver microsomes. P-450 content was measured as nanomoles per milligram protein following intraperitoneal administration of (♦) 0 mg/kg, (○) 10 mg/kg, (△) 50 mg/kg, or (□) 200 mg/kg ABT in CF-1 mice. Results are mean ± standard error ($n = 4$).

group, an activity dependent on the metabolic activation of ABT by the enzyme (7). This report demonstrates that systemic administration of ABT results in inhibition of both liver and skin metabolism through the destruction of cytochrome P-450. The effect is profound, dose dependent, time dependent, and long-lasting.

Phenoxazone and a series of its ethers have been used in the comparative study of lung, liver, and skin metabolism in Balb/c mice (13) and also to study the specificity of mixed-function oxidases in skin (15). Although the ratios of activities of the different ethers in skin and liver are similar, it is still evident that the metabolic profile is different between the tissues, reflecting differences in isozyme content. Similar results have been reported in Balb/c mice following induction studies which also indicated isozymic differences with respect to tissue type (13). Metabolism of any one ether did not appear to be preferentially inhibited, which indicates that ABT is not specific for any one isozymic form of cytochrome P-450 but will serve to inhibit the spectrum of forms present in the animal.

ABT is effective in inhibiting cytochrome P-450-dependent biotransformation in the liver and the skin of animals receiving the compound intraperitoneally and the inhibition is not confined to one isozyme. Onset of maximum inhibition is approximately 1 hr in the liver following dosing and remains so for about 8 hr, which is an excellent time frame for most experimental procedures. Similar effects occurring in the skin of the experimental animal present the opportunity to study effects of cutaneous biotransformation and its role in metabolism-dependent absorption. It may also be possible to titer the dose in such a way to maximize the liver inhibition while leaving the skin activity relatively intact. In this way, the interaction between the two metabolic systems can be elucidated. This may prove to be a useful tool in the study of biotransformation and its contribution in the overall metabolism of the organism.

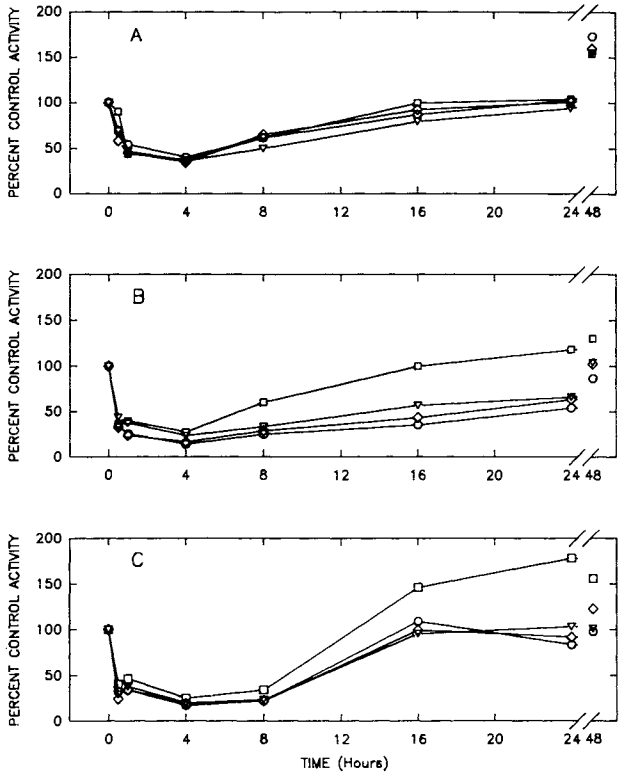

Fig. 2. Resorufin dealkylation activity of CF-1 mouse liver microsomes following administration of 1-aminobenzotriazole. The ability of mouse liver microsomes to dealkylate resorufin alkyl ethers was evaluated following intraperitoneal administration of ABT following either (A) 10 mg/kg, (B) 50 mg/kg, or (C) 200 mg/kg doses. Results are reported as the mean (n = 4) percentage of the control value (0 hr = 100%; n = 8) and analyzed using Student's t test. (◇) Methyl resorufin; (○) ethyl resorufin; (△) pentyl resorufin; (□) benzyl resorufin.

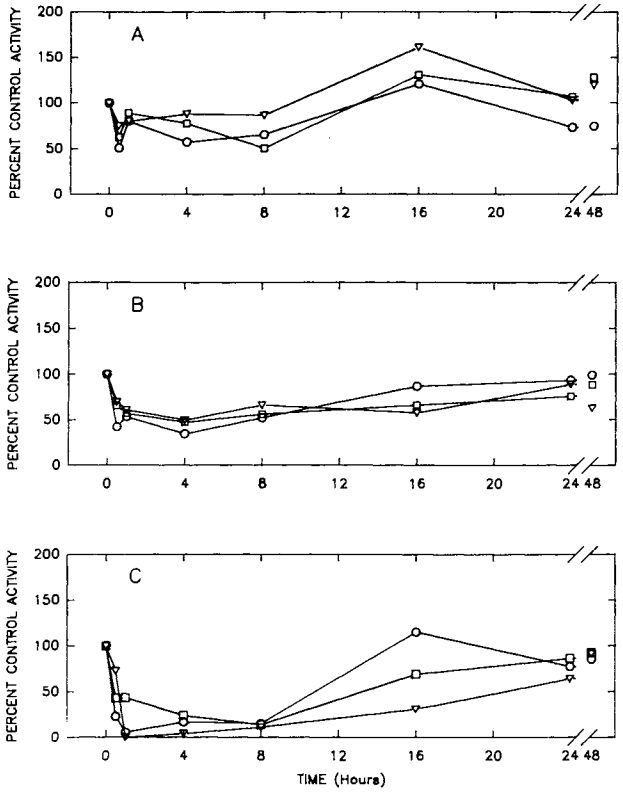

Fig. 3. Resorufin dealkylation activity of CF-1 mouse skin microsomes following administration of 1-aminobenzotriazole. The ability of mouse skin microsomes to dealkylate resorufin alkyl ethers was evaluated following intraperitoneal administration of ABT following either (A) 10 mg/kg, (B) 50 mg/kg, or (C) 200 mg/kg doses. Results are reported as the mean (n = 4) percentage of the control value (0 hr = 100%, n = 8) and analyzed using Student's t test. (○) Ethyl resorufin; (△) pentyl resorufin; (□) benzyl resorufin.

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